PHARMACODYNAMIC STUDIES

ON PROTOVERATRINE

A Thesis submitted to the University of Glasgow in candidature for the degree of Doctor of Philosophy in the Faculty of Science

by

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CONTENTS

Pharmacodynamic Studies on Protoveratrine

CHAPTER 10./

Summary/

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CHAPTER 1.

INTRODUCTION

Despite the increasingly important role of synthetic drugs in modern therapeutics, drugs of natural origin are still widely employed clinically. The last decade has witnessed the introduction of a number of new therapeutic agents derived from animal and vegetable sources, the discovery and development of which has paralleled man's ingenuity in the field of synthetic drugs.

A number of the newer naturally occurring drugs, e.g. the antibiotics and the steroid hormones are new discoveries, whilst others are older established remedies which have been used for a considerable time in folk medicine, and which have come into prominence as a result of new chemical techniques which have enabled the active principles to be isolated, purified and prepared on a commercial scale.

Striking examples of this latter group are the isolation and clinical use of the purified alkaloids of the Rauwolfia and Veratrum species. Crude extracts and preparations of the alkaloid-bearing parts of these plants have been used for many centuries, Rauwolfia in India and China and Veratrum in Europe and America. The extraction and purification of the active principles of these plant species has made possible the chemical and biological standardisation of the drugs/

drugs and paved the way for their rational clinical use.

Although the troublesome side effects sometimes associated with the administration of the Veratrum alkaloids and the introduction of more reliable ganglionic blocking agents has tended to cause a reduction in the clinical use of the purified principles of the Veratrum alkaloids, they are still regarded as therapeutically useful agents for use in the treatment of some cases of hypertension. This applies more especially to their use in the management of the hypertension which frequently occurs in the toxaemias of pregnancy.

The ancient Greek herbalists were familiar with the pharmacological action of the Veratrum alkaloids, and of their sources. The plant called by Dioscorides of Anazarba[1] Ἑλλεβορος λευκὸς, the white hellebore, is described by him in his herbal compiled in the First century A.D.

Both Hippocrates and Theophrastus mentioned the white hellebore in their writings, but failed to give a description sufficiently complete to enable the plant to be identified with any certainty. Some later writers have indeed doubted whether the Helleborus albus described by these two writers is identical with the Veratrum album found in present day Europe./

Europe.

Dioscorides, who was the first of the Greek herbalists to systematize the Greek materia medica, giving precise instructions as to the cultivation, collection and storage of medicinal herbs, gave a full account of both the pharmacology and botany of Helleborus albus. From this description and the illustration drawn by an unknown Byzantine artist (but based on the drawings of Crateuas the Greek botanist) in 512 A.D., it is almost certain that the two plant species are identical. Sibthorp[2] in the Eighteenth century found both the white and black hellebore in Greece and in the list of synonyms cited by Dioscorides in his monograph of Elleborus albus he gives Veratrum album as the name used by the Romans. The botanical description given by Dioscorides is admittedly rather vague, but the powerful emetic and sternutatory effects of the dried rhizome described so vividly by him, and so characteristic of dried extracts of Veratrum album, leave little doubt that Elleborus albus and our present day Veratrum album are the same plant.

The ancient physicians were fully aware of the potent nature of the white hellebore but this did not prevent them from administering it as the dried root indiscriminately in a wide variety of conditions. Hippocrates[3] in his Aphorisms recommended/

recommended its use in mania, melancholia, hydrops, elephantiasis, epilepsy and rabies, but he considered it to be safer if it firstly caused vomiting. He must have been aware of the toxic nature of white hellebore because he considered it to be unsafe for administration to persons of weak constitution, children, old men and those suffering from pulmonary complaints, and even in robust patients it was considered wise to add an inert diluent.

The Romans were well aware of the properties of the white hellebore which they called Veratrum album. The Roman physician and prolific writer on medical subjects, Celsus, who is believed to have lived a generation before Galen, frequently mentions the use of white hellebore in his "Treatise on Medicine". He recommends white hellebore as the drug of choice in cases where it is desired to produce vomiting, and also advocates the use as a caustic for the treatment of ulcers and in epilepsy.

It is interesting to note that both Dioscorides and Celsus counsel the use of white hellebore bruised in vinegar for external application thereby extracting the alkaloids in solution as their acetates.

During the dark ages that followed the eclipse of the Graeco-Roman civilisations no mention can be found of any medicinal/

medicinal use of Veratrum album. Goodman and Gilman[5] state that the plant was used in the Middle Ages for sorcery and in mystical rites. Of the few medical books available which were written during this period neither the translation by Heinrich of "Ein Mittelenglisches Medizenbuch" published in 1328, nor the "Science of Ciruiergie" of 1380 make any mention of either hellebore or Veratrum album.

With the advent of the Renaissance a new interest was taken in the drugs and herbs which for many centuries had been the province of wise women and charlatans. The treatment of disease began to become more rational. Woodville[6] states that the white hellebore was first cultivated in this country by the Elizabethan herbalist Gerard[7] in the Sixteenth century who describes the plant in his "Historie of Plant". Avicenna in the "Canon of Medicine" an Arabic work first published in Britain in 1523 mentions the use of hellebore as a purge but gives no details, and this reference may well refer to Helleborus niger. The iconoclastic German physician and mystic Paracelsus born in 1490, had great faith in the powers of hellebore to effect a cure in epilepsy and gout. He directed that the root be gathered in the waning of the moon when it is in the sign Libra and on a Friday.

The/

The prolific Seventeenth century writer of medical texts, Nicholas Culpeper, in his 1652 translation of the Pharmacopoeia Londinensis[8], gives white hellebore or sneezewort and numerous compounds containing the dried root. He comments very unfavourably on the use of white hellebore and its preparations, under the monograph for white hellebore he writes, "The root of white hellebore or sneezewort being grated and snuffed up the nose causeth sneezing, killeth rats and mice being mixed with their meat. It is but a scurvy, churlish medicine being taken inwardly and therefore better left alone than used". He has even more scathing criticism of the London College's instructions for making Mel Helleboratum, I quote "What a 'monstrum horrendum' horrible, terrible receipt have we got here A pound of white hellebore boyled in fourteen pints of water to seven....., purge melancholy say they:but for whom? For men or beast? For the medicine would be so strong the Devil would not take it unless it were poured down his throat with a horn".

It is obvious from Culpeper's comments that the high degree of toxicity and the uncertainty of action of this drug were well recognised but preparations of the three major Veratrum species were to remain official until 1914, during which/

which time very much more came to be learnt of their pharmacology and toxicology.

The medical writings of the Eighteenth century contain numerous references to the use of preparations of white hellebore mainly in epilepsy and other convulsive disorders and in the treatment of skin eruptions. There seems to have been little co-operation between the London and Edinburgh Royal Colleges during this period as the Edinburgh Pharmacopoeia of 1737 lists the two quite different species, Helleborus albus and Helleborus niger under one heading as hellebore root, but the London Pharmacopoeia of the same time (1746) lists the two separately. The earliest recorded clinical trial of Veratrum album was reported in 1781 by Greding[9] who gave Veratrum album root extract to twenty-eight patients suffering from mania and melancholia, the majority of these, twenty-three, received no benefit. In some cases doses of two grains caused nausea and vomiting and almost all cases showed some form of toxic reaction.

Woodville[6] (1792) states that although preparations of Veratrum have been used in a number of conditions the diseases in which its efficiency seems least equivocal are diseases of the skin. During the Eighteenth century the similarity between the American hellebore Veratrum viride and Veratrum album/

album was noticed, and Bigelow[10] gives a full account of its botany and pharmacology. He noted that the Indians of New England had a custom of electing their chiefs by the use of the roots of Veratrum viride. A portion of the root was given to each individual, and the one who resisted the emetic effect the longest was considered to be the strongest and therefore worthy of the chieftainship.

The early Nineteenth century brought a significant advance in the study of the Veratrum problem with the isolation of the purified principle veratrine by Pelletier and Caventou[11] in 1819. These workers showed that it was possible to isolate this principle from Veratrum album, Veratrum viride and also from the seeds of the South American plant Veratrum sabadilla.

The ready availability of this new drug led to a marked increase in the number of experimental studies on both animals and humans. The Pharmacopoeias of London (1824) and Edinburgh (1813) both contain tinctures of Veratrum album but Rennie in 1820 in his supplement to the Pharmacopoeias then in use, recognised the disadvantages of the powdered root and recommended the use of the newly discovered alkaloid veratrine. Magendie[12] in 1827 gave the first full account of the action of veratrine on experimental animals and he noted that it showed a general irritant effect on a large number of unrelated tissues./

tissues.

The discovery of veratrine led in 1898 to the elimination of "veratrum radix", leaving veratrine the only official preparation of Veratrum. The physicians of this era frequently noted the manifold actions which this drug exerted on the organism.

Pereira[13] in his Materia Medica (1855) cited cases in which emesis was produced by application of Veratrum preparations to the abdomen, and as suppositories, and noted that by whichever means it was introduced into the body it produced symptoms of irritation.

Many wild and unjustified claims were made for the therapeutic values of veratrine during the Nineteenth century. Turnbull[14] in his monograph published in 1834 claimed to have cured patients suffering from a wide variety of clinical conditions including heart disease, rheumatism, paralysis, dropsy and gout by the simple external application of a veratrine ointment. These claims, however, were refuted by Copland quoted by Gully[12] in his translation of Magendie, op. cit.

In the B.P. of 1867 Veratrum album rhizome was replaced by the closely allied Veratrum viride and the difference between/

between the two species was considered to be so slight, that in the United States Pharmacopoeia of 1909 they were listed under one definition.

Cutter (1862)[15] seemed to be the first to record that extracts of Veratrum species were effective in lowering the arterial blood pressure and producing bradycardia, and he advocated its use as a cardiac and arterial sedative. The value of Veratrum in the relief of puerperal eclampsia became recognised soon after Cutter's publications, and Martindale[16] (1888) mentioned the use of a tincture of Veratrum viride for this condition. The B.P. of 1898 excluded both Veratrum species but included veratrine made from Cevadilla, but in the B.P. of 1914 veratrine and preparations of veratrine were also excluded.

During the latter half of the Nineteenth century chemists succeeded in isolating a number of different alkaloids from both species of Veratrum. The B.P.C. of 1907 listed protoveratrine, jervine, pseudojervine and rubijervine as occurring in Veratrum album. A great deal of confusion, however, existed and many alkaloids were reported which were either precursors or breakdown products of existing alkaloids.

Eden[17] in 1892 described the pharmacological effects of protoveratrine/

protoveratrine but there is doubt as to the purity of the drug used. However, none of these alkaloids were proved to be in a pure state and much confusion arose during the early half of this century as to the precise chemical and pharmacological identity of many of the so-called pure alkaloids. Even in the 1948 edition of Sollman's Manual of Pharmacology[18] the pharmacological actions of Veratrum album and Veratrum viride were stated to be due to the presence of protoveratrine and all three were described under the heading Veratrum but it is now known that protoveratrine only exists in significant amounts in Veratrum album.

During the last two decades an increasingly large number of alkaloids have been isolated in a pure state and a number of preparations of both purified alkaloids and biologically standardised extracts of both Veratrum album and Veratrum viride are commercially available.

The term "Veratrum alkaloids" has been used by authors as a generic name for a number of alkaloids occurring in various species of Veratrum, a genus of the family Liliaceae. Various species of Veratrum from which the alkaloids have been isolated are listed in Table 1 page 12.

The majority of the commercially extracted alkaloids are obtained/

Table I

Botanical Sources etc. of the Veratrum Series of Drugs.

Species.	Family.	Other Names.	Habitat.
Veratrum album Linne.	Liliaceae.	White Hellebore. 'Helleborus albus' European Hellebore Sneezewort.	Central & Southern Europe. Northern Asia & Japan.
Veratrum viride Aiton.	Liliaceae.	Green Hellebore American Hellebore. Swamp Hellebore Indian Poke Itch Weed Poke root False Hellebore Devil's Bite Dick Ritter Earth Gall Bugbane Tickle Weed.	Eastern United States of America. Canada.
Veratrum sabadilla Aiton.	Liliaceae	Schoenocaulon officinale (Schacht) A. Gray Asagrosa officinalis Lindley. Spike-Flowered Asagrosa Veratrum officinale Schacht, Helonias officinalis Don.	Mexico Guatemala Venezuela
Veratrum fimbriatum Gray.	Liliaceae.		United States of America.
Veratrum eschscholtzii Gray.	Liliaceae.		United States of America.
Zygadenus venenosus wats.	Liliaceae.		United States of America.
Veratrum nigrum Linne.	Liliaceae.		Austria.

obtained from Veratrum album, Veratrum viride and Veratrum sabadilla. Veratrum album and Veratrum viride are closely related species. They are herbaceous perennials growing to a height of up to four feet. The plants have an upright rhizome and long narrow leaves. The major difference between these species is in the colour of the flowers, as their names imply Veratrum album has white flowers and Veratrum viride greenish white ones. These two species are found in the temperate latitudes.

Veratrum sabadilla is a tropical species found in Central America. It is a herb which grows to about five feet in height, having long thin leaves and grows from a bulb and not a rhizome. The flowers are yellow and produce dark brown seeds.

A large number of alkaloids have been shown to occur in Veratrum album and Veratrum viride, these are found in largest quantities in the roots and rhizome. Only a small number of alkaloids have been found in Veratrum sabadilla and these are almost entirely confined to the seeds.

The detailed chemistry of all the Veratrum alkaloids has not been fully elucidated. Over forty alkaloids have been isolated from the different Veratrum species but some

of/

of these are undoubtedly degradation products derived from the naturally occurring alkaloids. Chemically, the alkaloids fall into three well defined groups, the alkamines, which may be secondary or tertiary bases, the ester alkaloids and the glycosidic alkaloids.

The ester alkaloids consist of tertiary amines esterified with simple organic acids, they may be combined with one, two, three or four molecules of acid to give mono, di, tri or tetra esters. The mono and di esters have a much shorter duration of action and a greater stimulant action upon the Bezold-Jarisch reflex than the tri and tetra esters but the fall in blood pressure produced by both types is approximately the same. Because of this it is the tri and tetra esters and preparations containing a high proportion of these, e.g. "Veriloid", which are mainly used in therapeutics.

Protoveratrine A may be taken as an example to show the constitution of a typical ester alkaloid. The molecule is made up of a molecule of the alkamine protoverine esterified with one molecule each of (+) -α-methyl-α- hydroxybutyric acid and (-)- α-methylbutyric acid and two molecules of acetic acid[47,48] See Fig. 1, page 16. The constitution of the alkamine protoverine has not yet been elucidated but

Barton/

Barton et alia[49] suggested that the structure may be closely related to that which they have proposed for cevine which contains the unique 3,4-dihydroxy-4,∝-oxido system. Barton's suggested formula for the alkamine cevine is shown in Fig. 2.

The secondary alkamines, e.g. veratramine are combined in the plant with one molecule of D(+)-glucose to give the glycosidic alkaloids. These alkaloids have little hypotensive action and are not used therapeutically.

Clinical Uses of the Veratrum Alkaloids

Present day medical opinion is somewhat divided as to the value of preparation of the Veratrum alkaloids in clinical practice.

Pure alkaloids, e.g. protoveratrine and standardized preparations of mixtures of the alkaloids, e.g. Veriloid have been used in the treatment of hypertension resulting from a number of different aetiological factors.[19-25].

Benign essential hypertension can be controlled by a number of more suitable agents and the use of the Veratrum alkaloids in this condition is of limited value.

The treatment of cases of severe and malignant hypertension/

Protoveratrines A and B

Protoveratrine A

$C_{41}H_{63}O_{14}N$

2 molecules of acetic acid
1 molecule of (–)-α-methylbutyric acid
1 molecule of (+)-α-methyl-α-oxybutyric acid

Protoveratrine B

$C_{41}H_{63}O_{14}N$

2 molecules of acetic acid
1 molecule of (–)-α-methylbutyric acid
1 molecule of (+)-α-methyl-α-oxybutyric acid

} Protoverine (Alkamine)

Fig. 1.

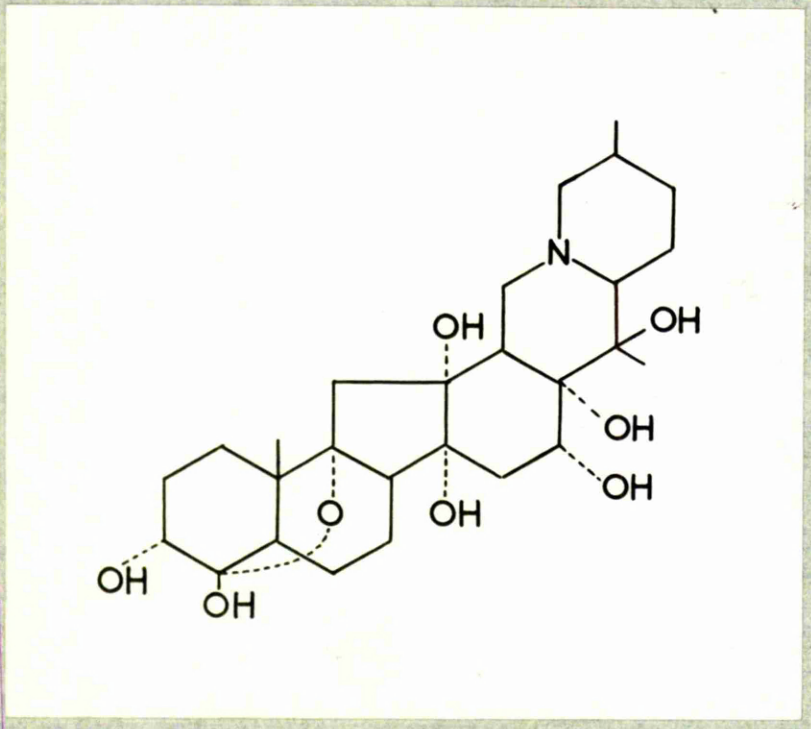


Fig. 2.

Structural formula of Cevine, an alkamine related to Protoverine.

hypertension by the Veratrum alkaloids has been widely studied. The majority of reports indicate that this drug is of definite clinical value, especially in patients where the use of ganglion blocking drugs is contraindicated. On the other hand some investigators[26, 27] claim that there is no significant difference between the results obtained by oral administration of preparations of Veratrum and a placebo.

Spuehler and Wyss[28] have reported excellent results from the oral administration of protoveratrine in patients with severe hypertension. The value of the Veratrum alkaloids in clinical practice has been critically reviewed by Hoobler and Dontas[20] and further references may be found there.

Although preparations of Veratrum are now rarely used in the treatment of essential hypertension they still find an important place in the therapy of toxaemias of pregnancy.

The term toxaemia of pregnancy is used to describe a number of related pathological conditions which may occur during pregnancy and of which the aetiology is still obscure. Some of these, for example Wernicke's encephalopathy and acute yellow atrophy appear to be deficiency diseases but the most frequently/

frequently occurring of the toxaemias is pre-eclamptic toxaemia. Pre-eclampsia is invariably accompanied by hypertension and frequently by proteinuria and oedema, other commonly occurring symptoms include headache, epigastric pain and visual disturbances which are associated with the hypertension and renal and adreno-cortical upsets, which together with the proteinuria and oedema are evidence of a disturbed salt and water balance. If pre-eclampsia is untreated it frequently develops into eclampsia, a dangerous condition which is characterised by convulsions which may be followed by coma.

The Veratrum alkaloids are only of value in the treatment of the pre-eclampsia-eclampsia syndrome, the two phases of which are in fact different stages of the same disease.

The pathogenesis of pre-eclampsia is still not fully understood, but it has long been recognised that this condition is accompanied by a marked rise in arterial blood pressure, an increase in diastolic pressure being of particular significance. Cutter[15] in 1862 showed that the reduction in arterial blood pressure following the administration of a preparation of the Veratrum alkaloids relieved the hypertensive symptoms associated with pre-eclampsia./

pre-eclampsia.

Mixtures of the Veratrum alkaloids were used with varying degrees of success for over half a century, and medical opinion as to their value was divided,[29,30,31] but the true place of the drug for the treatment of pre-eclampsia only became critically assessable with the development of a standardised and purified preparation.

Sheehan[32] has shown that some degree of generalised arteriolar spasm exists in patients with pre-eclampsia and recent work has demonstrated that a marked decrease in uterine blood flow occurs[33,34,35] which is accompanied by an increase in uterine-muscular tone[36] and a reduced placental blood flow.[33] Other workers[37,38] have been unable to demonstrate any reduction in the blood flow to the muscles and the brain but further studies are needed before any definite conclusions can be drawn.

Following the administration of standardised preparations of Veratrum viride[30,39,40] and of the pure alkaloid protoveratrine[41,42,43] significant reductions have been reported both in maternal and neonatal mortality, and in the associated symptoms of pre-eclampsia and eclampsia.

Morris[44]/

Morris[44] by measuring the rate of clearance of radioactive sodium from the myometrium has shown that a marked increase in effective uterine blood flow occurred following treatment with protoveratrine. Many of the untoward and dangerous effects upon the foetus which result from pre-eclamptic conditions are due to a decrease in placental blood flow and although no studies of the effect of the Veratrum alkaloids upon this have been published it is likely that an increase in myometrial blood flow is paralleled by an increase in placental blood flow.

Treatment with the Veratrum alkaloids promptly controls many of the symptoms associated with pre-eclampsia and it, therefore, seems possible that these conditions may be due to arterial vasoconstriction and that the relief afforded by the Veratrum alkaloids is due to their generalised hypotensive action. Van Douwdijk Bastiaanse[45] has suggested that the ischaemia of the placenta occurring in pre-eclampsia gives rise to the formation of vasoconstrictor substances, similar to those produced in the ischaemic kidney, and these in turn give rise to ischaemia of the brain, kidneys, digestive organs and retina, in addition to causing hypertension by a direct action upon the arterioles. He postulated that the symptoms mentioned/

mentioned earlier as being characteristic of pre-eclampsia may be due to ischaemia produced in these organs by this hypothetical vasoconstrictor substance. The Veratrum alkaloids may relieve the symptoms by a dual mechanism, the reduction of the primary uterine ischaemia with a corresponding reduction in the production of vasoconstrictor substance and a vasodilatation of the arterioles of the affected organs. A second hypothesis[46] is that the hypertension occurring in pre-eclampsia is the result of a disturbed salt and water balance, possibly hormonal in origin. The placenta of pre-eclamptic patients has been shown to contain an antidiuretic substance which occurs to a much lesser extent in normal patients, and this substance may be the cause of the salt-water imbalance. If this is the case the Veratrum alkaloids will only give symptomatic relief.

It may be concluded that the beneficial effects of Veratrum alkaloid therapy in the treatment of pre-eclampsia and eclampsia is due entirely to the reflex lowering of blood pressure which they induce and not to any direct effect upon the organs or tissues involved.

General Pharmacology of the Veratrum Alkaloids

A detailed review of the pharmacology of the Veratrum alkaloids would be out of place in this thesis, but because it/

it is now some twelve years since the subject was last extensively reviewed it was felt that a short account of present day views was necessary.

In 1946 Krayer and Acheson[50] published an exhaustive and critical review of the pharmacology of the Veratrum alkaloids. This work superseded previous reviews[51, 52] which had dealt with this subject to a varying extent. Since 1946 the present state of knowledge of the subject has been summarised in two reviews[53,54] and in a number of the more detailed works of reference.[19,55 to 57]

The pharmacological action of the Veratrum alkaloids in therapeutic doses is mainly upon the cardiovascular system, the respiratory system and the central nervous system. In doses above therapeutic levels the alkaloids act upon nerve fibres and skeletal and smooth muscle.

The secondary amines and their glycosides have no hypotensive action and have as yet found no clinical use. They are characterised by an effect upon the heart which is shown by very few other drugs. These alkaloids are capable of antagonising the cardioaccelerator action of both sympathomimetic amines and of sympathetic nerve stimulation. This effect is thought to be a highly selective action upon the/

the pacemaker of the heart and is not shown by the adrenergic blocking agents. The majority of the work in this particular field has been done by Krayer and his co-workers and he has used the term "antiaccelerator agents" for drugs exhibiting this particular pharmacological property. This author has recently reviewed this subject.[58]

The tertiary amines and their esters are responsible for the therapeutic value and the typical pharmacological actions of the various preparations obtained from the different Veratrum species.

The alkamines of the ester alkaloids all show closely related chemical structures and although qualitative and some minor quantitative differences have been shown to occur between the different active alkaloids,[50] these differences are of a minor nature compared to the similarities exhibited by them. These differences are possibly due to the different acid moieties and may be the result of differences in the penetration of the drug to different receptor sites.

Much of the earlier published work on the pharmacology of the Veratrum alkaloids was carried out using impure mixtures of alkaloids and many of the results are contradictory Most of the work in this thesis was carried out using either a mixture/

mixture of protoveratrines A and B in the proportion of 2:1 and was designated protoveratrine, or with pure crystalline protoveratrine A. The pharmacological differences between these two forms are very small and is quantitative rather than qualitative.[59] The pharmacology of protoveratrine may be taken as being typical of that of the other ester alkaloids.

The most marked pharmacological effect following the administration of therapeutic doses of protoveratrine is a rapid fall in arterial blood pressure accompanied by bradycardia. This has been clearly demonstrated to occur in man,[20,21,28,60,61,62] dog,[63 to 66] cat,[67 to 69] and other laboratory animals.[50,59] Quantitative differences exist between these species,[59] rodents being much more resistant to the hypotensive effect than are man, dog or cat. Protoveratrine produces a fall in blood pressure which lasts for up to two hours. This contrasts with some of the other Veratrum alkaloids, e.g. veratrine which produces a much more transient effect.

The bradycardia which accompanies the hypotension is due to increased vagal activity and may be abolished by atropine, vagal section[50,59,67] or cooling of the vagi to 4° C.[68] The bradycardia may potentiate the fall in blood pressure but this effect is not always shown and plays little part in the production/

production of the hypotensive response.

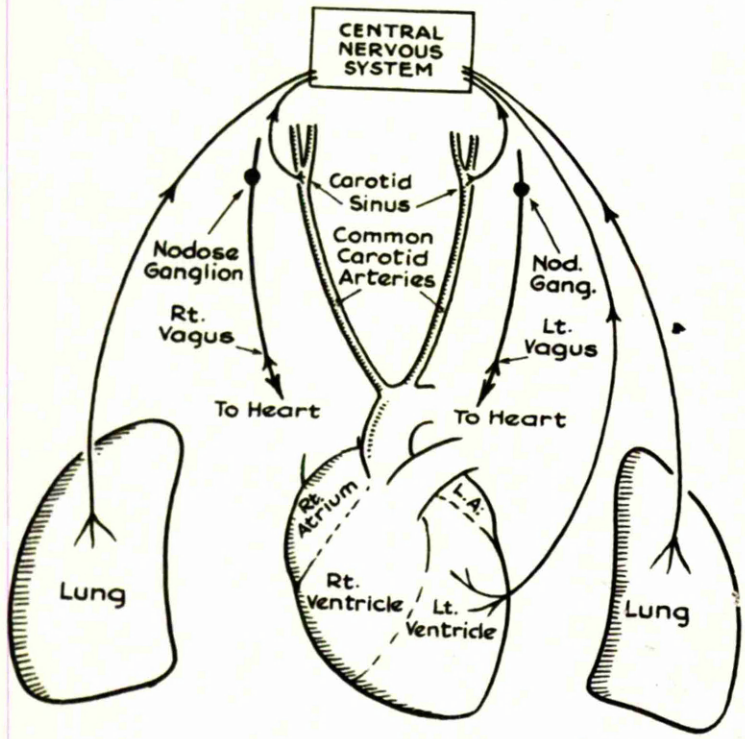
Protoveratrine has no direct peripheral vasodilator action neither does it have any sympathicolytic or ganglionic blocking action[59] but in higher doses it may cause vasoconstriction.

Part of the fall in blood pressure is undoubtedly due to the elicitation of the Bezold-Jarisch reflex.[50,53,59,69] This reflex was first demonstrated by Bezold and Hirt[70] in 1867, but their findings were published in an obscure journal and the result of their work did not receive any attention until Jarisch[71] re-discovered this effect. Initiation of this reflex results in peripheral vasodilatation and bradycardia accompanied by respiratory depression. The afferent receptors of what is now known as the Bezold-Jarisch reflex have been shown to exist in the heart and lungs. Dawes[68] has demonstrated that the major receptor area is in the region of the left coronary arterial bed, receptors also occur in the lungs, stimulation of which produces the characteristic picture of transitory apnoea and respiratory depression.[50,59,69]

The afferent fibres for the Bezold-Jarisch reflex are found in the vagi and vagal section prevents the development of the reflex, but even after vagotomy administration of protoveratrine/

protoveratrine, may still produce a fall in blood pressure,[50] mechanisms other than the Bezold-Jarisch reflex are therefore involved. These mechanisms have been studied by a number of different workers using different alkaloidal preparations. This hypotensive response to the Veratrum alkaloids in vagotomised animals has been variously attributed to depression of the central vasomotor centre,[72 to 77] to stimulation of the baroreceptors situated in the region of the carotid bifurcation, the so-called carotid sinus,[50,59,63,66,67,75,78 to 82] to stimulation of the nodose ganglion or the chemoreceptors of the carotid body.[81,83,84] See Fig. 3. p. 27.

These different suggestions are probably related to the use of differing preparations of the Veratrum alkaloids, different experimental animals used and also differences in the anaesthetic agents employed. In an attempt to rationalise these findings Wang et alia[66] carried out a large series of experiments on both cats and dogs using purified alkaloids of the Veratrum species. They concluded that in vagotomised animals the ester alkaloids, e.g. protoveratrine produce their action by causing an increase in the rate of firing of the carotid sinus baroreceptors. They could find no evidence to support any participation by the carotid body or nodose ganglion in the production of this hypotensive response. No depression of the vasomotor centre could be demonstrated with the ester alkaloids but veratrine and veratridine/



Schematic Representation of the Sites of Action of Protoveratrine.

Fig. 3.

veratridine depressed it, but this effect only played a minor role in the hypotensive response.

The hypotension following the administration of protoveratrine is probably the result of a central inhibition of the sympathetic nervous system [66] as the hypotensive response to protoveratrine is not observed in dogs which have been treated with a ganglion blocking agent [63] or a sympathicolytic drug. [26] [59] Other workers have suggested that the reflex hypotensive action of protoveratrine is due to active neurogenic dilatation.

The more recent critical work quoted above, makes it quite clear that the Veratrum alkaloids used in therapeutics for the reduction of arterial blood pressure produce hypotension by a reflex process which is initiated at sensory receptors present in the vascular bed which seem to be primarily baroreceptors.

In addition to the reduction in arterial blood pressure which follows the administration of protoveratrine other pharmacological effects have been observed. Protoveratrine has been shown [50,59] to have a direct cardiotonic action upon the failing heart. This effect is similar to that of Digitalis and this parallelism is not unexpected because both the cardiac glycosides and the Veratrum alkaloids have/

have basic steroid structures. Therapeutically effective doses of the Veratrum alkaloids occasionally give rise to cardiac irregularities similar to those seen during Digitalis therapy.[55]

The reflex vasodilatation produced by protoveratrine may be sufficient to affect renal and cerebral functioning. These effects may be manifested as oliguria if the glomerular filtration rate falls too low as a result of the reduced pressure in the renal artery. If the cerebral blood flow is decreased fainting may occur.

Effects upon respiration are rarely seen with therapeutic doses of protoveratrine. There may be some reflex inhibition of respiration due to the stimulation of afferent vagal receptors in the lungs in a similar manner to the stimulation of afferent receptors in the heart and carotid sinus.[85] Toxic doses exert a direct depressant effect upon the vasomotor centre.[5]

The greatest obstacle to the large scale clinical use of the Veratrum alkaloids in the treatment of hypertension in man is their marked tendency to induce nausea and vomiting. This effect occurs in dogs and it has been shown[83] to be due to the stimulation of afferent receptors situated within the nodose/

nodose ganglion.

The effects of protoveratrine and other Veratrum alkaloids upon isolated nerve and muscle is dealt with in the later pages of this thesis.

The Effect of Veratrum Alkaloids on Sensory Receptors

The dose of purified Veratrum alkaloids needed to produce a hypotensive response in man and experimental animals is remarkably low and a total dose of 10 ug in a 70 kg. man is frequently sufficient to elicit a marked fall in the level of the blood pressure.

There is no evidence to indicate that in therapeutically effective doses the drug has a direct action upon vascular smooth muscle, efferent nerve fibres or the central nervous system and all experimental evidence points to the drug acting upon afferent sensory receptors.[50] In all vertebrates and in many invertebrates there are found a number of different types of sensory receptors capable of responding to mechanical changes, e.g. deformation changes due to variation in pressure, chemical changes, temperature changes, changes in the intensity of the light falling upon a receptor and to pain.

The exact nature of the mechanism by which these changes are detected by sensory receptors is still unknown but there/

there is no conclusive evidence that either acetylcholine, noradrenaline or any of the other known neurohumoral agents act as a transmitter substance at sensory nerve endings and the evidence against them playing such roles is quite considerable.[88] [89] Gray is of the opinion that changes in ionic permeability of the receptor may follow the application of a stimulus and that the ionic changes produced, which mainly involve the sodium ion, give rise to a generator potential at the afferent nerve ending, which in turn sets up a conducted action potential in the afferent nerve axon.

The Veratrum alkaloids can influence both the electro: physiological state of excitable tissues and its permeability to ions and it seems highly probable that the effects of this group of drugs in producing hypotension is due to an action on sensory nerve endings involving these two factors.

The elicitation of the Bezold-Jarisch reflex accounts for a large proportion of the hypotensive action of the Veratrum alkaloids, the afferent nerve endings involved in the production of this reflex effect are situated mainly in the ventricles and the left atrium.[70,90 to 96.] In addition impulse discharge in the appropriate sensory nerve after the application of the Veratrum alkaloids to the receptor areas, has/

has been demonstrated for the carotid sinus region,[59,60,96 to 98] carotid body,[71,98 to 100] right atrium[95,101], pulmonary stretch receptors,[90,91,95,102] and gastric stretch receptors,[95] and it is possible that afferent impulses from these regions may be responsible for a small part of the reflexly induced hypotensive response to the alkaloids.

These receptors are excited physiologically by mechanical deformation of the sensory nerve ending produced by a change in pressure. A number of chemical substances including the Veratrum alkaloids can stimulate these receptors and Dawes and Comroe[87] have used the term chemoreceptor reflexes to differentiate this type from the chemoreflex which is normally initiated by alterations in the chemical composition of the bathing fluid.

The problems of directly studying the changes occurring at sensory nerve endings at the time of stimulation are very great and at the time of writing this thesis the majority of the published work refers to detailed studies made upon relatively simple receptors, e.g. the muscle spindle of the frog, the stretch receptors of the crayfish and the Pacinian corpuscles and thermal receptors of the cat.[89] Indirect studies of the effects of stimulating sensory nerve endings have been made by recording the electrical impulses in single nerve/

nerve fibres in the afferent nerves but this method can only give limited information of the changes taking place at the sensory receptor.

The Veratrum alkaloids have been shown to have a number of different effects upon sensory receptors.[90 to 92] Paintal has shown that the receptors may be first stimulated by these alkaloids which subsequently desensitize them to a stimulus which was previously sufficiently strong to excite them. This author could find no evidence to indicate that in visceral stretch receptors the alkaloids lowered the threshold of excitation to the normal stimulus, e.g. sensitized the receptor, an action which has been demonstrated for a number of volatile anaesthetics.[93, 102, 103.]

Jarisch and Zotterman[100] however, have demonstrated that the stretch receptors of frog muscle were sensitized by veratrine.[94] They have also shown that the baroreceptors of the carotid sinus were sensitized by the intracarotid injection of veratrine, to the normal physiological stimulus and to injected potassium chloride. These findings have been confirmed by Aviado et alia.[75]

Despite the work of Zottermann et alia,[94, 100] Paintal[91, 92] is of the opinion that sensitization of receptors in the cardiac/

cardiac region plays no part in the initiation of the Bezold-Jarisch reflex.

The smooth increase in the discharge frequency of the afferent nerve which accompanies the mechanical deformation of a receptor area during stimulation by the Veratrum alkaloids indicates that the alkaloids act upon the nerve ending rather than upon the nerve fibre itself.[93] The Veratrum alkaloids may act by either depolarizing the receptor and enhancing the local receptor[104] or generator[105] potential or by enhancing the negative after-potential of the receptor. As has been shown later both of these mechanisms may be explained on the basis of an alteration in the ionic permeability of the cell membrane..

Alterations in the ionic composition of the milieu of the sensory nerve endings has been shown to have a marked effect upon the activity of afferent nerves. This is especially noticeable for the Ca^{++}, excess Ca^{++} has been shown to reduce or abolish the activity in various afferent nerves following the stimulant effect of the Veratrum alkaloids,[93,94,99,100,101] and to convert the abnormal response to normal.

Potassium chloride has been shown to have a stimulant effect upon a number of sensory nerve endings,[90,94,100,106] but the evidence/

evidence for a direct relationship between the effect of K^+ and the Veratrum alkaloids upon sensory receptors is scanty. Similarly no studies have been published relating alterations in receptor activity under the influence of the Veratrum alkaloids with changes in the concentration of Na^+. Further studies in this field may give a clearer insight into the relationship between ions and the alkaloids as Straub[107,108] has demonstrated a close correlation between Na^+ fluxes and the characteristic action of the Veratrum alkaloids on nerve. Gray[87] has suggested that Na^+ plays a most important part in the generation of the receptor potential and it may be that the Veratrum alkaloids affect sensory receptors in a similar manner to that shown by Straub[111,112] to take place in nerve fibres and reviewed later in this chapter.

Theories Derived from Electrophysiological Studies

An extensive literature exists relating to the electrophysiological effects of veratrine on nerve and muscle, and only work which gives an insight into the possible mechanism of action of the Veratrum alkaloids will be considered in detail.

As the earlier studies on the effects of the Veratrum alkaloids/

alkaloids on the electrical responses of nerve and muscle were made using veratrine, the term veratrinic response has been widely applied to cover the various changes in the electrical activity which occur following treatment with any of the active Veratrum alkaloids, in particular to veratrine. This response is characterised by the production of a series of repetitive responses following a single stimulus, and the responses in both nerve and muscle show marked similarities. The effects of veratrine on nerve and muscle were first described by Prevost[109] in 1866 but a more comprehensive account was given in the following year by Bezold and Hirt.[70]

The typical myogram of a weakly veratrinised frog muscle to a brief electrical stimulus is a normal twitch contraction followed by partial relaxation, this is in turn followed by a slower secondary rise and fall. With larger doses the initial relaxation is abolished and the curve presents a smooth rise and may reach a height larger than the initial twitch. After a considerable time the myogram eventually falls slowly to the base line. This response was originally thought to be a contracture but Kuffler[110] using single fibre preparations of frog sartorius muscle showed that the response was tetanic in nature./

nature.

Veratrinic responses similar to those obtained for frog muscle have been obtained, using a wide variety of vertebrate striated muscles [112 to 114] and also in vertebrates smooth muscle. [115 to 117] Veratrinization of the heart causes a prolongation of systole and this has been taken as evidence of a similar response to that shown in skeletal muscle.[5]

Veratrine, in a higher concentration than is necessary to elicit repetitive responses in muscles, can lead to the development of repetitive responses in nerves stimulated by a single brief stimulus.[118,119] The concentrations required to produce repetitive responses in muscle and nerve fibres are higher than the blood levels reached in therapeutics: but the nerve endings in both afferent and efferent nervous systems are known to be much more sensitive to the action of drugs than either the nerves or muscles themselves, and it seems likely that the Veratrum alkaloids in therapeutic concentration act at sensory nerve endings.

Recordings of the electrical activity of nerves and muscles treated with veratrine show a marked similarity, and in muscle, parallel the mechanical changes shown in the myogram/

myogram.

The Veratrum alkaloids have a similar effect on the electrical changes occuring following stimulation in both nerve and muscle, and the differences between the two systems appear to be quantitive rather than qualitative.[114]

Although work on the effects of the Veratrum alkaloids on mechanical processes has been confined to muscle because of the technical factors involved, the majority of the bioelectrical studies have been made using nervous tissue either as nerve bundles, or more recently, as single fibre preparations.[120]

Concentrations of Veratrum alkaloids which are sufficient to lead to the development of the veratrinic response in nerve and muscle was at one time thought to have no effect on the resting potential[110,116] although one group of workers reported a rise in the resting potential cat muscle.[114] More recent evidence points to a gradual depression of the resting potential, i.e. some degree of depolarisation.[108] Straub, using frog myelinated nerve fibres has reported that veratridine in concentrations of 5 ug per ml. produced a depolarisation of 30 mV, higher concentrations produced a maximum depolarisation of 47 mV. Lorente de No[121] has shown that in long term experiments on frog/

frog nerve, concentrations of veratrine of 10 ug. per ml. can cause a partial depolarisation which has recently been shown to be accompanied by increased K^+ release and Na^+ uptake.[122]

Witt and Swaine[120] have also shown that the resting potential of frog sartorius muscle fibres is reduced from 86 mV. to 74 mV. after treatment with veratridine.

Lorente de No[121] has suggested that there are two processes involved in the maintenance of the resting potential, a fast and a slow phase, and it seems likely that the reduction in resting potential after Veratrum alkaloids may be the result of an interference with the processes which maintain this slow portion of the resting potential.

The effect of the Veratrum alkaloids on the action potentials of nerve and muscle seems to be variable, low doses have been shown to increase the magnitude of the spike potential in nerve,[118, 123] muscle,[112] and in the superior cervical ganglion,[124] but increasing the drug concentration further usually leads to a decrease in potential.[114, 118.]

In a recent publication Witt and Swaine[120] have shown that the action potential in single frog sartorius muscle fibres was/

was reduced from 119 mV to 47 mV after treatment with veratridine. As the resting membrane potential was 74 mV, this indicated that the usual reversal of the charge of the membrane which usually occurs following a stimulus did not occur. The height of the spike of a stimulated nerve progressively decreases after treatment with Veratrum alkaloids[118,120], but this is mainly due to a decrease in membrane potential with a corresponding decrease in take-off voltage. This is sufficient to account for the decrease in the magnitude of the action potential seen in the tissues studied.

The actions of the Veratrum alkaloids upon the membrane potential and upon the rising phase and magnitude of the action potential, are much less marked than those upon the falling phase of the action potential and the accompanying after potentials. It seems likely, therefore, that the very noticeable changes which occur in the second phase, namely those affecting repolarization, are those responsible for the activation and sensitization of receptors which give rise to the therapeutic effect of the drugs.

The development of the spike in a stimulated nerve or muscle is immediately followed by a second potential - the negative after potential - the electrical form of this is variable and shows some degree of independence from the spike potential./

potential. In a normal electrical recording of the action potential the two potentials occur so closely together that the recorded action potential is usually the result of the two factors.

The initial descending phase of the action potential is followed by a component of repolarisation which develops at a much slower rate. This is very sensitive to physical and chemical changes and because of the slowness of this component the magnitude of residual depolarisation of the membrane can be increased by repetitive stimulation, and substances, e.g. the Veratrum alkaloids, which can increase the effect of repetitive stimulation probably act upon this slower component of repolarisation. This component is also responsible for a small portion of the resting membrane potential and has been termed the L_1 (for labile) fraction by Lorente de No[15] and it is probably a measure of the charge separation that occurs and is maintained by a process different from any of those responsible for the development of the spike. A factor other than the negative after potential which is directly related to the L fraction, is the post-cathodal depression of excitability following a stimulus, and these factors retain definite relationships to each other under a wide variety of conditions, e.g. temperature,/

temperature, drug action, electrical polarization and ionic changes.[5]

Nerves and muscles exposed to veratrine show a marked increase in both the amplitude and length of the negative after potential. This effect was first described by Graham and Gasser.[123] The theory of the ionic basis of the action potential proposed by Hodgkin, Katz and Huxley[125] postulates that the restoration of the resting potential after the spike is due to an outward shift of potassium ions to balance the sodium ions which entered during the rising phase of the spike. Measurements using the voltage clamp technique[126] indicate that the falling phase of the action potential is accompanied by an increase in the potassium conductance of the membrane and it seems very likely that the changes in the negative after potential are the result of an interference in the normal slow repolarisation phase of the action potential. The result of this failure of repolarisation is that the excitable membrane remains in a state of total or partial depolarisation - in the case of muscle this leads to a state of prolonged contraction and can account for the characteristic myogram of the veratrinised muscle. In nerve the appearance of repetitive responses is coincident with an increase in the negative/

negative after potential but in muscle the augmented negative after potential can occur without the tetanic response.[114]

The negative after potential may be the result of changes in K^+ flux across the membrane, excess K^+ and veratrine both lower the resting potential of nerve and muscle but their effects on the negative after potential are diametrically opposite. Veratrum alkaloids increase it but excess K^+ decreases it, on the other hand it is increased by excess Ca^{++}.[13] Modifications in the K^+/Ca^{++} ratio can modify the voltage of the after potential after veratrine but do not influence its amplitude.

Using the transverse impedance technique developed by Cole and Curtis,[128] Shanes et alia[129] have shown that the initial after-impedance, i.e. a decrease in electrical conductance which normally follows the spike in nerve is abolished by treatment with either cevadine or veratridine and the increased electrical conductance conforms very closely with the negative after potential.

The occurrence of these changes in conductance are well established, but the exact mechanism is still not clearly understood. Although the increased conductance and cumulative depolarisation can be explained in terms of an increased K^+ concentration in the external medium, calculations

calculations have shown[129] that the amounts of K^+ which must be released to give an increased negative after potential similar to that obtained by veratrinization of nerve axons, would be far greater than those which have been shown to be liberated following a normal stimulus. These calculations indicate that factors other than changes in K^+ flux must be taken into account in order to explain the action of the Veratrum alkaloids on the negative after potential, Shanes et alia[129] have suggested that a decrease in the elevated chloride permeability which follows the spike may be responsible for the negative after potential.

As has been mentioned, an increase in the level of Ca^{++} in the surrounding medium can cause an increase in both the magnitude and duration of the negative after potential and it has been shown that the effects on the negative after potential are paralleled by an increase in the magnitude of the L fraction of the membrane potential.[118] Brink[130] has suggested that calcium ions have a dual action, one of which is on surface structures to change the L fraction of the membrane potential, and the other to clamp the oscillatory potential changes occurring in excitable tissues by altering the resistance to current flow and thus preventing the development of/

of rhythmic self-excitation processes.

Gordon and Welsh [131] have suggested that veratrine gives rise to repetitive responses in nerve axons following electrical stimulation by delaying the normal restorative process which restores the calcium ions to the surface complex following the breaking of the chelate linkage of ions to this complex, possibly by altering the axon surface structure.

The maintenance of the resting potential in nervous and muscular tissue and the development and conduction of the action potential in stimulated excitable tissues is dependent upon a functionally intact cellular membrane. Changes in the state of the membrane may lead to an increase or a decrease in the excitability of the tissue. Much remains to be learnt about the physico-chemical changes in the membrane accompanying changes in excitability. The simplest explanation is that the threshold of excitation is changed by an alteration of the electro-chemical properties of the membrane thereby augmenting or diminishing the passage of chemical substances or an electric current across the membrane.

Low doses of the Veratrum alkaloids first raise the electrical excitability, but as the dose is increased the degree of excitability falls, this has been demonstrated in mammalian/

mammalian nerve,[118,123,124] muscle,[70,132] and ventricular muscle.[133]

The threshold voltage required to stimulate a nerve gives some indication of its excitability. Normally a closing cathodal current will stimulate a nerve at a lower voltage than an anodal current but after exposure of mammalian nerves to veratrine this condition is reversed.[118] This effect of veratrine indicates an effect upon the normal electrical condition of the membrane.

Veratrine has little effect on the refactory period of nerve[123] but following the refactory period veratrinised nerves show a period of increased excitability. This is frequently greater and more prolonged than that exhibited by the untreated nerve.[50] Krayer and Acheson[50] consider that the repetitive response occurs in stimulated veratrinised tissues as a result of the rising phase of the negative after potential acting in an analagous fashion to the spike potential, and due to the supernormal excitability after the Veratrum alkaloids, this is sufficient to produce a conducted disturbance.

To summarise, it seems highly probable that the Veratrum alkaloids alter the normal electrophysiological properties of all excitable tissues in a similar manner. The concentrations/

concentrations necessary to produce effects on nerve and muscle are higher than those which exist following the therapeutic application of these drugs, but it is likely that the effects produced at nerve sensory endings are qualitatively similar to those produced upon other types of excitable tissue which can be more easily studied.

Modern physiological techniques have demonstrated that the electrophysiological state of normal and stimulated excitable tissue is due to an imbalance of electrical charges on either side of a selectively permeable membrance and that these charges reside on ions and changes in the distribution of these ions are responsible for changes in the bioelectrical properties of the cells. The Veratrum alkaloids are thus thought to mediate their effects by an alteration of the ionic and, therefore, electrical state of the excitable membrane.

Meier-Tripod Theory of General Receptors

Meier and his colleagues[134] have approached the study of mechanism of action from a different direction from that adopted by other workers in the field. They have demonstrated that veratrine and protoveratrine can stimulate the smooth muscle of both isolated jejunum and seminal vesicle preparations taken from the guinea pig. The resulting contraction can be reduced by drugs which are known to inhibit/

inhibit the stimulant actions of the naturally occurring agents acetylcholine, adrenaline and histamine. On guinea-pig's intestine the anticholinergic drug atropine, the antihistaminic tripelennamine and the adrenergic blocking agent phentolamine all antagonise the stimulant action of the Veratrum alkaloids, but whereas atropine is effective in low concentrations (0.1 ug. per ml.) which are similar to those required to block the effects at acetylcholine, the other two antagonists are only effective in concentrations a hundred times greater. On the other hand the isolated seminal vesicle which can be stimulated by adrenaline, acetylcholine, histamine and the Veratrum alkaloids, shows a different pattern of specificity when exposed to the blocking agents previously mentioned. The most powerful antagonist to the stimulant action of the Veratrum alkaloids on the seminal vesicle is phentolamine, with atropine and tripelennamine showing about one-thousandth of the activity of phentolamine against Veratrum-induced contractions.

In concentrations similar to those required to antagonise acetylcholine and adrenaline respectively both atropine on the intestine, and phentolamine on the seminal vesicle are active antagonists to the stimulant action of the Veratrum alkaloids. The propounders of the theory suggest that a specific influence on the stimulant effects of the alkaloids can be produced/

produced in each case only by a typical antagonist which differs from organ to organ.

On the basis of the experiments Meier et alia[135] suggest that there exists either a 'master receptor' to which the alkaloids can become attached and that this receptor is normally influenced by secondary receptors which are specific for acetylcholine, adrenaline or noradrenaline and histamine, or that the molecule of the Veratrum alkaloids can fit on to either of the two types of receptor blocked by atropine or phentolamine respectively. They thus postulate that the Veratrum alkaloids act by combining with receptors primarily concerned with the autonomic nervous system, however, in vivo the Veratrum alkaloids have little if any effect on this system in doses which are therapeutically effective. The main site of action of the alkaloids in vivo seems to be on sensory nerve endings and as yet there has been no satisfactory proof that either acetylcholine, adrenaline or noradrenaline or histamine are involved in the processes by which sensory nerve endings are stimulated.[86]

Although this theory may explain the results obtained by the simulation of isolated smooth muscle by the Veratrum alkaloids it is the opinion of the author that this theory does/

does not provide any satisfactory basis for an explanation of the effects of the Veratrum alkaloids in eliciting sensory reflexes.

Theories upon the Mode of Action of the Veratrum Alkaloids Developed from Permeability Studies.

The evidence discussed earlier in this chapter indicates that the Veratrum alkaloids have a marked effect upon the normal pattern of bio-electrical responses occurring in excitable tissues. These disturbances can be described in conventional electrical terminology and be compared to similar changes which may be produced in a model electrical system. The action of the Veratrum alkaloids upon nerve and muscle can thus be described as producing changes in resistance, altering the degree of rectification and inhibiting the damping of oscillatory processes. In normal nerve and muscle these factors have constant values which can be markedly changed following treatment of the excitable tissue with the pharmacologically active Veratrum alkaloids.

The precise pharmacological action of these drugs, however, cannot be described solely in terms of changes in the electrical properties of the veratrinized tissues. For a fuller understanding of the problem it is necessary to know by what mechanisms the drugs alter the resting electrical state/

state of nerve and muscle. Some mention has already been made of the theories put forward to explain the generation of bio-electrical potentials and it is now generally accepted that the electrical changes occurring in excitable tissues as a result of stimulation result from changes in the distribution of changed ions across the cellular membrane. To a certain degree the cell can control the ratio of intracellular to extracellular ions by alterations in the selective permeability of the membrane, but the mechanism by which this is achieved is still largely unknown.

The early membrane theory of Bernstein[136] postulated a differential permeability of the cell to the cations of Na^+ and K^+ but Hodgkin[125] and his co-workers using single nerve fibre preparations were the first to actually demonstrate changes in the relative permeability of the cell membrane to these ions.

Some of the effects of the Veratrum alkaloids on the electrical state of excitable tissues can be reproduced by changing the ionic composition of the bathing fluid. With the general acceptance of the theory of the ionic basis of electrical excitation various workers have considered the possibility that the effects of the Veratrum alkaloids on nerve/

nerve and muscle are due to alterations in the permeability of the cell membrane to ions. The ions most likely to be involved in the production and maintenance of bio-electrical potentials are the metallic cations K^+, Na^+, Ca^{++} and the anion Cl^-. A number of hypotheses have been put forward during the last twenty years in an attempt to explain the mechanism of action of the Veratrum alkaloids and the majority of these have postulated an effect upon the mechanisms responsible for the distribution of one or more of the above ions on either side of the cellular membrane. The role of each of these ions will be considered in turn and the relationship between it and the possible mechanisms of action of the active Veratrum alkaloids will be discussed.

The Potassium Ion

In 1939 Bacq[185] published evidence to show that veratrine could sensitize frog skeletal muscle to the stimulant action of K^+. This effect has since been demonstrated to occur with many of the purified alkaloids isolated from various Veratrum species[137] and can be demonstrated in other forms of excitable tissues.[5]

The majority of the intracellular K^+ is thought to be in dynamic equilibrium with the extracellular K^+ and there is a continuous movement of K^+ across the cell membrane in both directions. This equilibrium can be disturbed by alterations/

alterations in membrane permeability affecting either the inward or outward rates of transfer. This will in turn alter the ionic distribution and both the resting potential and the action potential.

As a result of his pioneer work in the field of sensitization of tissues to K^+ stimulation, Daeq[53] suggested that veratrine prevented the K^+ from reaching the membrane surface and that the ion remained in the vicinity of the cell membrane in an active state and thus gave rise to a prolonged depolarization. He put forward this theory before the full development of the ionic theory of nervous and muscular excitation, but the same situation would exist if the outward flux of K^+ was increased or the inward flux decreased, and this hypothesis may be taken as analagous to one involving a changed K^+ permeability.

Much of the earlier evidence put forward to support the theory of an action of the alkaloids on permeability was indirect; this was due to the technical difficulties involved in the measurement of cellular permeability. Mosera[139, 140] measured the degree of imbibition of toad gastrocnemius muscles immersed in Ringer's solution containing veratrine and showed that the muscles exposed to the drug gained more weight than the control muscles. He concluded that veratrine acts by influencing cellular permeability

but/

but offered no suggestions as to how the permeability was altered and in what direction. Shanes[141] on the other hand could find no significant increase in water uptake in muscles exposed to veratrine.

Adrenaline can diminish the stimulant effect of K^+ on frog muscle,[113,142] and it has also been shown to diminish the permeability of the muscle membrane to K^+.[143,144] This may be taken as indirect evidence to support the hypothesis that a change in permeability is involved, but, as Goffart and Bacq[53] suggest, the effect of adrenaline in antagonising the sensitization to K^+ occurs in less than five minutes whereas Lange[143] was unable to show any effect of adrenaline on muscle permeability in less than twenty minutes.

Further indirect evidence pointing to an effect upon permeability has been offered by various workers[141,145] who showed that some antihistaminic agents which have been shown to decrease muscular permeability to K^+ can antagonize both the sensitization of frog muscle to K^+ and the Veratrum induced Bezold-Jarisch reflex in cats.[166,167] Fleckenstein and Hardt[145] also claimed that local anaesthetics dimish membrane permeability. The can also desensitize muscle to the stimulant/

stimulant action of K^+ and can antagonize the sensitization produced by the Veratrum alkaloids.

Electrophysiological studies providing further evidence for the hypothesis that the Veratrum alkaloids produce their characteristic effects on nerve and muscle by influencing the K^+ permeability of the cell have been reviewed in the earlier pages of this thesis.

The first quantitative measurements of the relationships between K^+ and the Veratrum alkaloids were published by Shanes [122,127,148 to 151] who carried out a series of experiments in which the concentration of K^+ in the bathing fluid continuously perfusing frog nerves was measured. Nerves exposed to veratrine released significantly more K^+ into the bathing medium than did control nerves under the same conditions. In addition he showed that this increased K^+ output could be antagonised by the local anaesthetic cocaine. Shanes used the term 'unstabilizer' to describe substances which increased the permeability of the cell membrane to ions and he quoted as examples veratrine, oxygen lack, iodoacetate poisoning and calcium precipitants. Conversely substances which decreased the membrane permeability he designated 'stabilizers' and cited cocaine, antihistamines and glucose as examples. Shanes [127,151] explained the apparent increase in K^+ output as being the result/

result of an increased output of K^+ during stimulation and the failure of the nerve to re-absorb the liberated K^+ during the re-polarization phase. This would indicate that there was an increase in the permeability of the membrane ~~with respect~~ to some of the intracellular K^+ which was either accompanied or followed by a decrease in the permeability ~~with regard~~ to the extracellular K^+. In a later paper, however, Shanes [141] claimed that the excess K^+ liberated by the impulse may have been more rapidly reabsorbed in veratrinized nerves than in the control nerves.

More recently two papers have been published in which Straub [107,108] described the effects of veratridine on myelinated nerve fibres of the frog and has attempted to explain these effects as due to modifications in the ionic permeability of the nerve membrane. In his experiments Straub used a nerve which was continuously bathed with Ringer's solution and from which a continuous recording of the membrane potential was obtained. Alterations in the ionic composition of the bathing fluid and the addition of veratridine produced recordable changes in the membrane potential. Straub made no measurements of ionic concentration, he only measured differences in the membrane potential. By basing his results on the "constant field theory"/

theory" of Goldmann[152] and on formulae derived from this theory by Hodgkin and Katz,[153] Straub concluded that on frog nerve veratridine produced an increase in K^+ permeability, but he disagreed with the view held by Shanes that the depolarization produced by the Veratrum alkaloids is due primarily to an increase in the concentration of external K^+ in the immediate vicinity of the nerve membrane. Straub put forward the alternative suggestion that the electrophysiological changes seen after exposure of excitable tissues to the Veratrum alkaloids are primarily the result of alterations in the permeability of the cell membrane to the sodium ion Na^+.

The evidence cited above indicates that the Veratrum alkaloids can increase the permeability of frog nerve fibres to K^+ but further work is needed to confirm these findings and elucidate the changes in mammalian muscle and nerve.

Various authors[154 to 156] have suggested that the boundary membranes of excitable tissues have similar properties and that ions are transported across the cellular membranes by analagous processes probably involving specially differentiated carrier systems.[108] It is, therefore, to be expected that the Veratrum alkaloids would also alter the permeability to K^+ of other types of cellular membranes across which this ion is/

is known to move. In addition to nerve the tissues most widely used in the study of ionic transport are the skeletal muscles, erythrocytes and the glomerulus of the kidney.

The use of radioactive tracer techniques has made the task of studying permeability changes in living cells much less difficult than before ~~their advent~~, but at the time of writing only one paper has been published describing the use of these techniques in the study of the influence of the Veratrum alkaloids on ionic transport. In this paper Kahn and Acheson[157] describe the effect of veratridine upon the entry of radioactive potassium, $^{42}K^{+}$, into human erythrocytes. Following the earlier work of Shanes and others they propounded the working a priori hypothesis that the Veratrum alkaloids would prevent the entry of $^{42}K^{+}$ into K^{+} poor cold-stored human erythrocytes when incubated with $^{42}K^{+}$ labelled plasma. They were, however, unable to show any significant difference between the $^{42}K^{+}$ uptake of erythrocytes exposed to veratridine and that of the control cells. This was in marked contrast to the results obtained with a number of cardioactive glycosides which were shown to inhibit the uptake of $^{42}K^{+}$ by the erythrocytes from the labelled plasma.[158] Harris has studied the fluxes of $^{42}K^{+}$ and $^{24}Na^{+}$ in veratrine treated frog sartorius msucles but has been unable to demonstrate

any/

any effect of the drug on the transport of either of these two ions.

The possible effects of the Veratrum alkaloids on ionic transport in the glomerulus have not been critically investigated. It seems unlikely that studies on electrolyte exchange in the intact animal would be of great value due to the relatively large changes in blood and urine K^+ concentration produced by the liberation of antidiuretic hormone released following the administration of the Veratrum alkaloids.[159, 160]

From the work cited it may be claimed with some justification that at present the relationships between the permeability of cells to K^+ and the effects of the Veratrum alkaloids are still obscure and that much further work remains to be done before a clear picture of these complicated interrelationships emerges.

The Sodium Ion

Less work has been done on the effects of the Veratrum alkaloids on the permeability of cells to the sodium ion Na^+ than has been accomplished for K^+ but that which has been published indicates that the problem is equally complex. Ussing[161] /

Ussing[161] has suggested that the active extrusion of Na^+ is the primary process by which the potential difference across a frog's skin is maintained. It is, therefore, possible that the depolarization observed in veratrinized excitable tissue is due to an imbalance between the normally existing extra and intracellular Na^+.

It has been suggested by a number of authors[162, 163, 164] that the Na^+ and K^+ ions are distributed between a cell and its environment according to a Donnan equilibrium, but Fenn[165] has shown that Na^+ could freely cross the cell membrane and the theory in its original form was found to be untenable. The concept of active extrusion by a "sodium pump" was introduced by Dean[166] to account for this discovery, and the existence of linked carrier systems for Na^+ and K^+ has been suggested by Hodgkin and Keynes[167, 168]. On the basis of this theory alterations in the rate constants and total K^+ would result in corresponding changes in the rate of Na^+ transport and the final resting concentration of this ion. Agents which altered one of these factors for one ion, e.g. K^+, would thus be expected to give rise to changes in the rate of transport or final concentration of the other i.e. Na^+.

Recent evidence[169] has indicated that in frog muscle neither the Donnan concept nor a linked carrier system can fully/

fully explain a number of experimental findings. Shanes has shown that in frog nerve there is a relationship between K^+ release and Na^+ uptake but under conditions of anoxia the processes may be independent of each other. He has also demonstrated that a low Na^+ concentration in the bathing medium can reduce the increased K^+ output after veratrine but from his data he is unable to say whether this effect is primarily due to a change in permeability to Na^+ or to K^+.

The electrophysiological data obtained by Straub[107,108] are cited by him as evidence that the Veratrum alkaloids alter the permeability of frog nerve to Na^+. He used frog nerves which were continually washed by Ringer's solution and demonstrated that veratridine could not depolarize the nerve in an Na^+ free medium. A small degree of depolarization could be produced if the concentration of Na^+ within and without were the same, and if the Na^+ concentration in the bathing fluid was increased above that occurring under physiological conditions veratridine could produce a greater degree of polarization than was possible when the concentration of external Na^+ was the same as that existing in frog Ringer's solution.

In/

In criticising Shanes' conclusions, Straub[108], basing his explanations on Goldmann's theory[152] claimed that if the depolarization of frog nerve which develops following treatment with the Veratrum alkaloids was due to loss of some intracellular K^+, the ensuing potential change would develop slowly over a number of hours but in fact it reaches a maximum within a few minutes. Whilst agreeing with Shanes that the permeability of the nerve membrane to K^+ may be increased he claimed that the depolarization produced by the alkaloid is entirely the result of an increase in Na^+ permeability. In Shanes' experiments[122] the concentration of intracellular Na^+ was shown to be increased after veratrinization and was explained by him as due to an influx of Na^+ in compensation for the intracellular K^+ lost. Straub[108] has suggested that this increased intracellular Na^+ content is due to a greater Na^+ influx as a direct result of an increased Na^+ permeability and to the Na^+ pump being unable to compensate for this increased influx by extrusion of Na^+. He concludes that the effect of the Veratrum alkaloids in producing depolarization can be explained as being due to an inhibitory action upon the Na^+ carrying system of the nerve membrane.

The/

The Chloride Ion

The role of anions in the production and maintenance of bio-electric potentials has been studied to a much lesser extent than that of cations. The chloride ion (Cl^-) is the only anion thought to participate in the ionic changes occurring during the production of resting potential and the action potential.

The effects of the Veratrum alkaloids on the permeability of cells to Cl^- have only been reported in two papers. Shanes et alii[129] make the tentative suggestion that the negative after potential occurring in Loligo axons after treatment with the alkaloid cevadine, could be due to an increase in the permeability of the fibre to Cl^-. No analytical data was obtained in thisstudy and the authors suggestions are based upon theoretical concepts alone.

Straub[108] found a contradiction between his results using frog nerve and those of Shanes et alii[129] with Loligo axons. He was unable to demonstrate any increase in the anionic permeability of frog nerve on treatment with veratridine and he explained the negative after potential as being due to a simultaneous increase in permeability for both Na^+ and K^+.

The Calcium Ion

Transference/

Transference of the calcium ion, (Ca^{++}) across the cell membrane seems to play no part in the genesis of either the resting or action potentials of excitable tissues. The part played by this ion in the normal functioning of living cells is still largely unknown. Ringer[170] was the first to demonstrate that Ca^{++} was necessary for the normal functioning of excitable tissues.

In a recent monograph Heilbrunn[161] has considered Ca^{++} to be the most important single cation found in living cells. It is his view that the difference of potential across a resting cell membrane is due to the presence of a calcium-electrode system in the cell. He has postulated that the Ca^{++} is firmly bound to protein molecules in the cortex of the cell but exist mainly as free ions in the cell interior thus setting up a calcium electrode system; migration of the free ions from the interior to the cortex being responsible for the potential difference between the interior and exterior of the cell. In this theory the excitation of cells is thought to be due to the release of Ca^{++} from a protein bound complex at the cortex which penetrates to the interior of the cell and there acts as an enzyme activator.

Heilbrunn's theory has, however, not won general acceptance but all theories of cellular excitation agree that/

that the Ca^{++} plays an essential part in the control of cellular permeability.[130]

The relationship between the Veratrum alkaloids and Ca^{++} are as complex as those existing between these alkaloids and the monovalent cations Na^{+} and K^{+} and a considerable mass of published work on this subject has accumulated during the last two decades.

All the effects of the Veratrum alkaloids on isolated tissues are modified in some way by alterations in the Ca^{++} concentration of the bathing medium.[50] These effects can be clearly demonstrated in the isolated heart preparation upon which the Veratrum alkaloids show a cardiotonic action similar to that shown by the cardiac glycosides. In hearts which have been made hypodynamic by perfusion with Ca^{++} - free Ringer's solution the addition of the active alkaloids of the Veratrum species can restore the beat of the heart to normal.[171 to 173] The development of cardiac irregularities, however, achieved by Ca^{++} lack occurs more readily in the presence of veratrine.[173] These results indicate that Ca^{++} and the Veratrum alkaloids have opposite effects on the excitatory processes but similar effects on the processes involved in contraction.

In nerve and muscle preparations Ca^{++} markedly

antagonizes/

antagonizes the effects of veratrine, the minimal veratrinizing dose varying with the Ca^{++} content of the bathing medium.[174] Lack of Ca^{++} generally reduces the stability of cell membranes and *vice versa*.[175] Lamm demonstrated that Ca^{++} lack may produce an effect on frog muscle similar to that produced by veratrine.

In addition to the antagonism shown between Ca^{++} and the *Veratrum* alkaloids on isolated preparations of nerve and muscle the marked antagonism to the effect of the alkaloids by Ca^{++} on the intact animal has been demonstrated. The elicitation of the Bezold-Jarisch reflex by the *Veratrum* alkaloids can be prevented by pre-treatment with Ca.[101]

The findings already presented indicate that the maintenance of membrane stability is favoured by the presence of physiological concentrations of Ca^{++} and reduced by the presence of the *Veratrum* alkaloids. If the stability of the membrane is reduced depolarization occurs more readily, and as this has been shown to be a process involving changes in the permeability of the cell membrane it is quite possible that the increase in permeability of the cell to cations following its exposure to *Veratrum* alkaloids is a result of a decreased stability, and this is secondary to the effects of the alkaloids on the surface structures/

structures of the cell. Frankenhaeuser and Hodgkin[176] have suggested that changes in the Ca^{++} concentrations and changes in membrane potential, i.e. depolarization, have similar effects on the systems controlling the movements of Na^{+} and K^{+} across the membrane during the development of the spike. They have put forward evidence suggesting that depolarization increases Na^{+} permeability by displacing Ca^{++} from specific carriers or sites in the cell membrane.

A number of agents which can remove Ca^{++} from solution by forming unionized complexes can provide spontaneous autorhythmic activity in isolated arthropod nerve,[182,183] a similar condition may be seen in vertebrate nerve during low calcium tetany. Tetraethylammonium ions can produce similar responses in nerve[179] and it has been suggested by Cowan and Walter[180] that this effect is due to the competitive antagonism between the two ions at the surface of the nerve membrane. Veratrine has been shown to increase the tendency of motor axons taken from crustaceans to exhibit spontaneous activity and show repetitive discharges.[131,181,182] These phenomena are almost identical in appearance with those demonstrated as occurring in sensory nerve fibres from the cardiac-pulmonary region of mammals.[91,93.]

No evidence can be found to indicate that the Veratrum alkaloids/

alkaloids can combine chemically with Ca^{++} and due to their large molecules it seems unlikely that they compete directly with Ca^{++} at the receptor sites. Gordon and Welsh[131] have suggested that the alkaloid may become adsorbed at or near the surface of the nerve axon, thus preventing the normal reaction of Ca^{++} with the surface structures.

If this hypothesis is correct it will furnish an explanation for the observed effects of the Veratrum alkaloids upon cellular permeability and the electrical changes produced as a consequence of these.

The enhanced oscillatory behaviour which follows the exposure of arthropod axons to veratrine has been explained by Shanes[127] as being due to the increasing displacement of Ca^{++} from the membrane by successive impulses, a process which may be reversed during rest. He suggested that the slow repolarisation following veratrine is due to a delayed recalcification and consequently a prolonged disturbance of ionic permeability.

The suggestion put forward by Frankenhaeuser and Hodgkin[176] that Ca^{++} exists combined with a Na^{+} carrier system and that depolarization releases the bound Ca^{++} and/

and makes the carrier system available for Na^+ transport, fits in with the conclusions drawn by Straub[108] that the increase in Na^+ permeability is a result of alterations in the normal process of Ca^{++} binding by the membrane. Weidmann,[183] as the result of studies made on the effect of stabilizing agents on the Purkinje fibres of mammalian hearts, has suggested that Ca^{++} can alter the equilibrium state normally existing between the three states of the Na^+ carrying system envisaged by Hodgkin and Huxley.[126] In this system the Na^+ carrier exists in three forms, an active state in which the membrane permeability to Na^+ is high, a resting state in which the Na^+ permeability is low but can be raised by depolarisation, and an inactive state in which the permeability remains low. Weidmann suggested that Ca^{++} can shift the equilibrium in favour of the resting state, this effect would be to produce a decrease in excitability. Straub suggests that the Veratrum alkaloids can act by antagonising the effect of Ca^{++} and favouring the formation of the Na^+ carrier in an active state with a consequent increase in Na^+ permeability and the tendency to develop spontaneous repetitive impulses in the nerve.

The evidence reviewed here shows that although the actual mechanism by which the Veratrum alkaloids elicit their/

their characteristic effect on nerve and muscle is still not fully understood, much more is now known than when the subject was last reviewed in full.[5] As yet no work has been published on the intimate relationship between the Veratrum alkaloids and ions, at the probable site of their therapeutic actions, i.e. sensory nerve endings. An indication of the difficulties occurring in studies of these types is seen in a recent paper on the movement of Na^{+} and other ions in Pacinian corpuscles.[184]

The main effect of the Veratrum alkaloids upon isolated nerve seems to be at the point on the excitable membrane where Ca^{++} is bound. As a result of an interference in this process the permeability of the membrane to cations and possibly anions may be increased giving rise to the characteristic bio-electric changes.

This process may not, however, occur in other tissues in which ionic transport is known to take place, e.g. erythrocytes and striated muscle and caution must be observed in applying conclusions reached from the study of one excitable system to another functionally different system.

Metabolic Effects of the Veratrum Alkaloids

The electrophysiological changes brought about by the Veratrum/

Veratrum alkaloids may be due to an interference with some metabolic process of the cell which is responsible for the maintenance of normal cellular activity but at the time of writing this thesis no evidence of any enzyme inhibition or activation by any of the Veratrum alkaloids has been published.

Schmitt and Gasser[185] reported that in a veratrinized nerve in which a markedly augmented negative after potential was present nerve volleys at 2 per second led to a higher oxygen consumption than 120 nerve volleys per second in a non-veratrinized nerve.

Using slices of guinea-pig cerebral cortex Wollenberger[186] has shown that a mixture of protoveratrines A and B stimulated respiration and aerobic glycolysis and depressed anaerobic glycolysis, both of these actions could be antagonised by local anaesthetics. It is of interest to note that increasing the external K^+ concentration can also increase respiration and depress anaerobic glycolysis.

Recent studies[108,120,122] with a variety of metabolic inhibitors including 2:4-dinitrophenol, KCN, iodoacetic acid and NaCN and also the effect of anoxia have shown that the veratrine effect on nerve and muscle is not significantly affected by any of these factors.

The/

The increase in oxygen uptake by the nerve and by slices of the cerebral cortex can be explained on the basis that due to the reduction of the stability of the cell membrane by the Veratrum alkaloids previously described, the extra work must be done by the cell to maintain as nearly as possible its normal resting state and thus more energy is expended with a corresponding increase in oxygen uptake.

The experiments showing that neither an atmosphere of nitrogen nor blocking oxygen transport by sodium cyanide[120] have any effect on the response of muscle fibres to veratridine make it unlikely that the alkaloid acts by interference in oxidative metabolism.

Grupp[187] has demonstrated that the Veratrum alkaloids have no effect on acetylcholine metabolism and the results obtained by Witt and Swaine[120] with 2:4-dinitrophenol and iodoacetic acid make it highly unlikely that the alkaloids affect either lactic acid formation or the coupling of oxidative phosphorylation in muscle.

From the work described it seems unlikely that the Veratrum alkaloids exert their characteristic pharmacological actions by interference with the general metabolic functioning of the cells but more probably act by affecting a specialised system existing at cell surfaces.

From a consideration of the work reviewed in these pages it was concluded that much more must yet be done before achieving a fuller understanding of the precise mechanism by which the Veratrum alkaloids produce their characteristic pharmacological effects.

The greatest gap in our knowledge concerns the inter-relationships between the various metallic cations in particular Na^{+}, K^{+} and Ca^{++}, and the Veratrum alkaloids. From this the question arises as to whether the excitation of sensory receptors and the sensitization of striated muscle to the stimulant action of K^{+} produced by the Veratrum alkaloids, involve the same cellular mechanisms.

The investigations described in this thesis were undertaken with a view to clarifying the relationships between K^{+}, and Ca^{++} and the Veratrum alkaloids. The experiments were planned to determine if the Veratrum alkaloids altered the distribution of K^{+} between the cell and its environment in concentrations which were sufficient to sensitize muscle to the stimulant action of K^{+}, and to find out what other changes in ionic distribution occurred.

EXPERIMENTAL WORK

PART I

EXPERIMENTAL

INTRODUCTION

In accordance with current practice the full names of a number of drugs and active substances have been abbreviated. The abbreviations used are:-

1. Acetylcholine for acetylcholine bromide.
2. Atropine for atropine sulphate.
3. Adrenaline for (-)- adrenaline bitartrate.
4. Ca^{++} for the stable common calcium ion.
5. $^{45}Ca^{++}$ for the ion of the radioactive isotope of calcium used in this work.
6. 5 H.T. for 5-hydroxytryptamine creatinine sulphate.
7. K^{+} for the stable common potassium ion.
8. $^{42}K^{+}$ for the ion of the radioactive isotope of potassium used in this work.
9. Protoveratrine for the mixture of protoveratrine A and protoveratrine B as their hydrochlorides in the fixed proportion of 2 : 1.
10. Protoveratrine A for the hydrochloride of protoveratrine A.

The composition of the various physiological saline solutions and the method of preparation of the protoveratrine solutions are described in Appendix I.

In addition the following abbreviations for volume and weights of the metric system have been used: -

1. Kg. for kilogramme.
2. g. for gramme.
3. mg. for milligramme.

4./

4. ug. for microgramme.
5. ng. for nanogramme (10^{-9} gramme)
6. mEq. for milliequivalent.
7. µEq. for microequivalent.
8. mC. for millicurie.
9. µC. for microcurie.
10. °C. for degrees centigrade.
11. cm. for centimetre.
12. l. for litre.
13. ml. for millilitre.

The common chemical symbols for the elements have also been used throughout.

CHAPTER 2

EXPERIMENTS CARRIED OUT USING A PREPARATION OF ISOLATED FROG SKELETAL MUSCLE.

Bacq [188,189] in 1939 was the first to demonstrate that low concentrations of veratrine (0.5 to 1 µg. per ml.) increased the response of frog skeletal muscle to the stimulant effect of K^+. Goutier [137] using a number of purified Veratrum alkaloids has shown that they can also sensitize frog muscle to K^+.

Both striated and smooth mammalian muscle exhibit the same phenomenon [189] but the effects are more difficult to reproduce than with frog skeletal muscle. In order to obtain a clearer picture of the relationships between the K^+ and Ca^{++} in muscles, a number of experiments were performed in which the influence of varying the concentrations of both of these ions was studied.

Method

Common frogs (Rana temporaria), unselected asto age, weight or sex were used. The animals were rendered senseless by a blow on the head, rapidly decapitated and pithed. Cotton threads were attached to each end of one rectus abdominis muscle and the muscle dissected out. The muscle was suspended in an isolated tissue bath of 10 ml. capacity, by attaching one thread to a glass oxygenation tube and the other end to an isotonic frontal point writing lever, giving/

giving a magnification of 1 to 6. The load on the muscle was adjusted so that the muscle was stretched to the same extent as before dissection.

The bath was filled with frog Ringer's solution or with a modification of frog Ringer's soltuion in which the amounts of Ca^{++} and K^{+} had been either varied in order to reduce the concentrations of these ions to one half of that normally present or omitted altogether. For details of the composition of these solutions see the Appendix.

The muscle was caused to contract by adding a sufficient volume of 5 per cent w/v potassium chloride solution from a 1 ml. graduated tuberculin syringe. In order to be able to demonstrate the sensitization satisfactorily it was necessary to produce only a small contraction, the amplitude of a suitable record being between 0.5 and 1.0 cm. The sensitivity of the tissues used varied considerably but a dose of from 0.1 to 0.5 ml. of the KCl solution was found to suffice in most experiments. Some difficulty was experienced in obtaining uniform contractions but in all experiments at least two contractions of the same height were obtained before proceeding with the addition of the alkaloids.

The contraction to K^{+} developed rapidly and usually

reached/

reached a maximum within 30 seconds, and on washing out the writing point returned to the base line within 90 seconds enabling the KCl solution to be added at 3 minute intervals.

In the earlier experiments a mixture of protoveratrines A and B (henceforth referred to as protoveratrine) was used, but in later experiments pure protoveratrine A was used, both drugs were added as their hydrochlorides dissolved in distilled water. After uniform contractions had been obtained, protoveratrine was added to give a final bath concentration of between 0.1 and 1.0 µg. per ml. The drug was left in contact with the muscle for 15 minutes then without washing out, the same dose of KCl used previously was added and the contraction recorded.

Results

The muscles of "winter" frogs were more sensitive to the K^+ sensitizing action of protoveratrine than were those removed from "spring" or "summer" frogs. In the former case concentration of 0.1 µg. per ml. were used as a sensitizing dose but for the "summer" frogs it was usually necessary to increase this to 1 µg. per ml. No distinction could be made between the sensitizing effects of protoveratrine A and the mixture containing both protoveratrine A and B.

The/

The exposure of the muscles of "winter" frogs to protoveratrine A in a concentration of 1 ug. per ml. gave an average increase in the height of the response to K^+ of 1,400 per cent. Reducing the concentration of protoveratrine A to 0.1 ug. per ml. resulted in a mean percentage increase of 975 per cent.

The degree of sensitisation produced by 1 ug. per ml. of protoveratrine A in "summer" frogs was much less and the height of contraction produced by KCl solution showed an average increase of 70 per cent after 15 minutes exposure to the drug.

Reduction of the Ca^{++} content of the bathing solution

When the normal Ringer's solution was replaced by a similar solution in which the Ca^{++} content had been reduced to one half, the degree of sensitisation produced by the protoveratrine was markedly enhanced. It was approximately double that which was produced in the normal Ringer's solution but complete omission of Ca^{++} from the bathing fluid did not however lead to any further increase in the sensitisation produced by the drug.

Reduction of K^+ content of the bathing solution

The sensitisation to K^+ by protoveratrine A was generally/

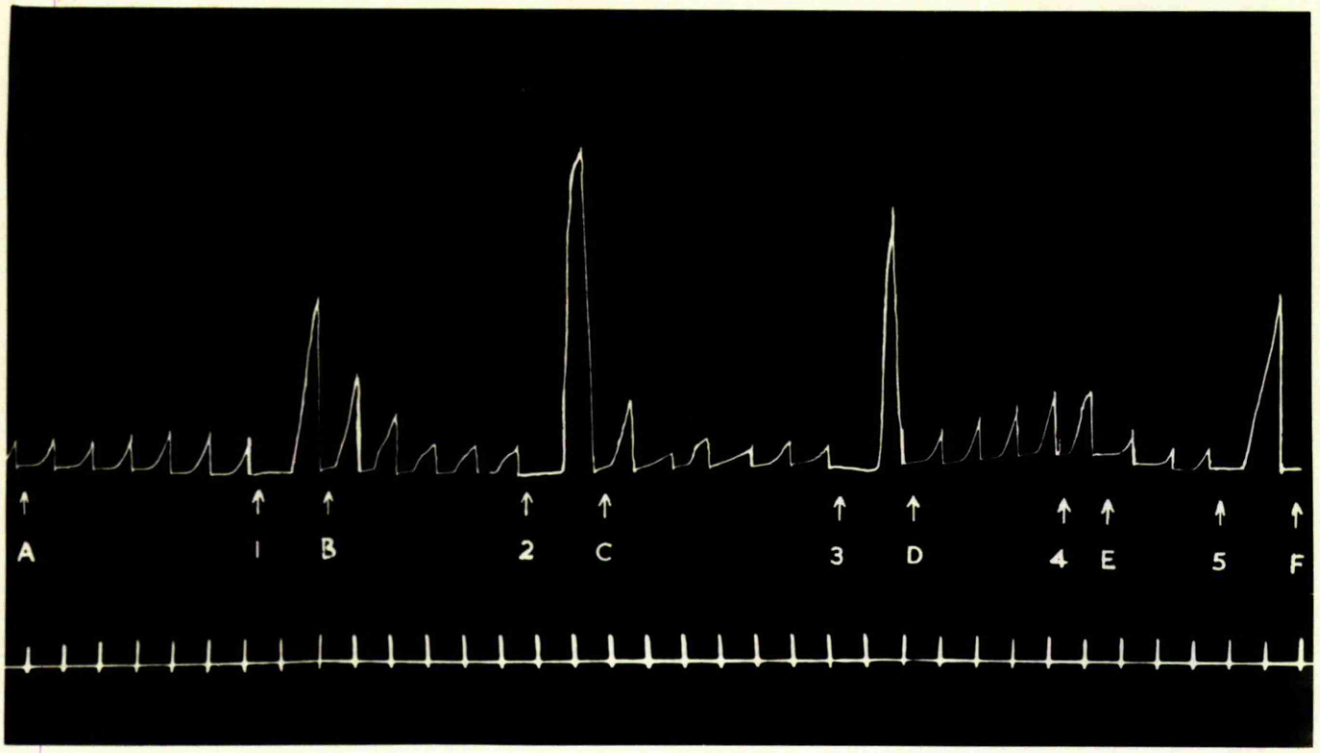


Fig. 4.

Effect of altering the ionic composition of the bathing fluid on the sensitization to K^+ of frog rectus muscle produced after exposure to protoveratrine.

At 1, 2, 3, 4 and 5, protoveratrine 0.1 ug per ml added to the bath and allowed to act for 15 minutes.

From A to B and E to F Muscle exposed to normal Ringer's solution.

From B to C Muscle exposed to Ringer's solution containing half normal Ca^{++},

From C to D Muscle exposed to Ca^{++} free Ringer's solution.

From D to E Muscle exposed to K^+ free Ringer's solution.

Time trace 3 minutes.

generally reduced by replacing the normal Ringer's solution by one containing half the normal quantity of K^+. The responses tended to be irregular and the base line frequently rose in the low K^+ solution. In K^+ free Ringer's solution the K^+ sensitising effect of protoveratrine was sometimes completely abolished.

The effects produced upon K^+ sensitisation by protoveratrine A by altering the ionic composition of the bathing fluid are shown in Fig 4.

CHAPTER 3

EXPERIMENTS USING PREPARATIONS OF ISOLATED, MAMMALIAN, SMOOTH MUSCLE.

Many of the pharmacologically active ester alkaloids of the Veratrum species have been shown to have little or no effect upon the typical pharmacological actions of acetylcholine, adrenaline, histamine and oxytocin.[50]

Since most of the previously published work on this aspect was carried out using impure alkaloids or mixtures of alkaloids, a number of preliminary experiments to ascertain whether these earlier findings could be confirmed, were carried out using a pure sample of protoveratrine A.

In addition to the physiological agents mentioned above, the effect of protoveratrine A on the stimulant action of 5 hydroxytryptamine (5 H.T.) was studied. No reports of any relationship between the Veratrum alkaloids and this interesting pharmacologically active amine have so far been published.

Protoveratrine A has a direct stimulant action upon smooth muscle in concentrations of the order of 1 ug. per ml.[59,134]. Experiments were undertaken with a view to substantiating the earlier reports which claimed that protoveratrine A showed no antagonism to the effects of the neurohumoral agents cited above.

The/

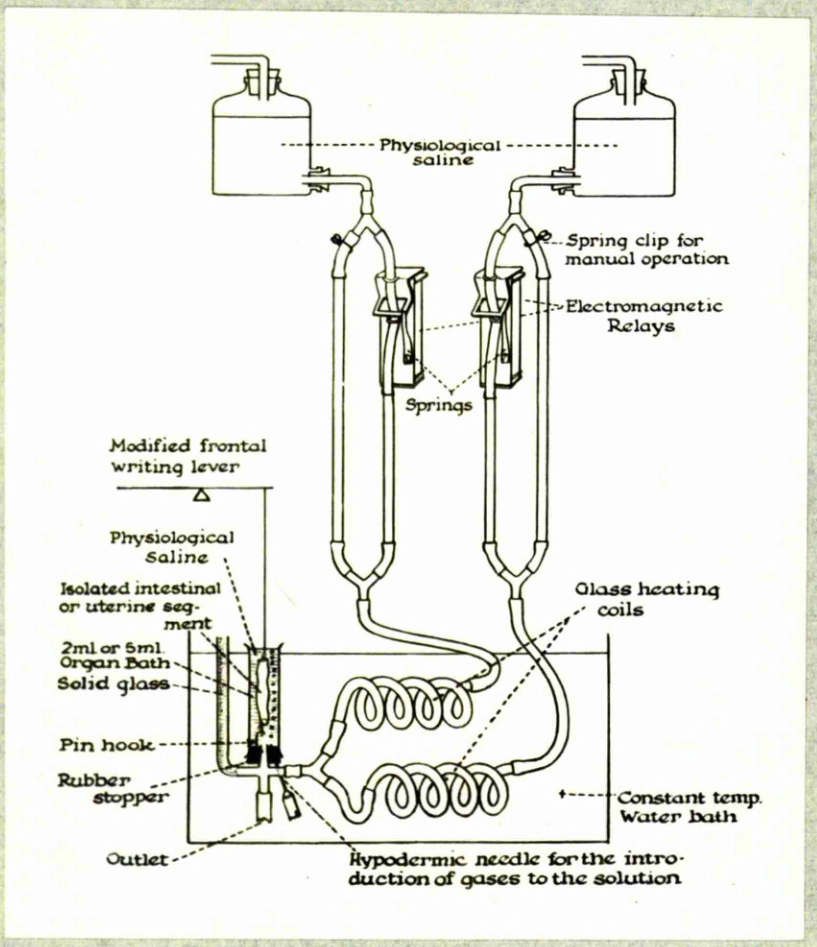


Fig. 5.

Diagram showing apparatus used for automatically adding drug solution to isolated tissue.

The drug solution is added from the right hand aspirator.

The experiments were carried out using as test objects the isolated ileum of the guinea-pig, the isolated duodenum of the rabbit and the isolated uterus of the rat.

Methods

Intestinal segments were removed from the guinea-pig or rabbit after it had been killed by a blow on the head and exsanguinated.

A segment of intestine approximately 4 cm. long was taken from the ileum proximal to the ileo-caecal junction of the guinea-pig, and from the region about 5 cm distal to the pyloric sphincter in the rabbit.

The uterine tissue was obtained from young virgin female rats which had been brought into oestrus by the subcutaneous injection of stilboestrol, 1 mg.per Kg.of body weight, administered 24 hours before the start of the experiment. One cornua of the uterus was used as the test object.

After cleaning, the tissues were set up in 2 ml. or 5 ml. organ baths by means of cotton threads attached to each end of the segments. See Fig. 5.
One end was fastened to a hook fixed to the bottom of the bath and the other to a counterbalanced frontal point writing/

writing lever having a magnification of 6 : 1. The contractions of the muscle were recorded upon a smoked surface in the conventional manner.

Contractionswere induced in the tissues by exposing them to physiological solutions containing a concentration of stimulant drug which was sufficient to cause a submaximal contraction. This concentration was determined by the manual addition of the stimulant drug in a quantity sufficient to produce the required response. The final bath concentration was calculated and a stock solution of this strength was made up in the physiological saline used.

The tissues were exposed to the drug solution and washed out automatically. The drug was added at intervals of 3 minutes and allowed to act until the maximum response had developed which was usually within 30 seconds. At the end of this period the drug solution was washed out of the bath. These additions were performed automatically and the bath was filled to overflow by both the drug containing solution and the washing solution. These processes were controlled by means of an electrically operated control system. For details see the Appendix. The organ bath and heating coils were immersed in a thermostatically controlled water bath maintained at 35°C.

The/

The bathing solution had a pH of 7.4 and the solution in the bath was continuously oxygenated and stirred by a stream of oxygen bubbles.

The physiological solutions used were Tyrode's solution for experiments carried out using the guinea-pig ileum, Locke's solution for the rabbit duodenum, and De Jalon's solution for the rat uterus. The composition of these solutions is given in the Appendix.

When a constant response to the stimulant drug had been obtained, a solution of protoveratrine A in amounts sufficient to give a final bath concentration of between 10 ug. and 1 ug. per ml. was added by hand. The solution of protoveratrine A was added from a 1 ml. tuberculin syringe 60 seconds before the addition of the stimulant drug.

Experiments were also carried out in which protoveratrine A was used to induce contractions in the guinea-pig ileum. This tissue was found to be very sensitive to the stimulant action of protoveratrine A and concentrations of 10 ng. per ml. were sometimes sufficient to produce a contraction, but the usual concentrations used were 0.1 ug. per ml. or 1 ug. per ml. which gave a response of 80 per cent of the maximal.

If additions of the drug were made at 3 minute intervals

tachyphylaxis/

tachyphylaxis rapidly developed to the stimulant actions of protoveratrine A, but reproducible responses were obtained if the time interval between successive additions was increased to 6 minutes.

A number of workers have shown that an excess of calcium ions (Ca^{++}) can antagonize many of the pharmacological actions of the Veratrum alkaloids and the evidence for this has been reviewed in the introduction to this thesis. The antagonistic action of Ca^{++} to the spasmogenic effect of the Veratrum alkaloids on smooth muscle, however, has not so far been fully investigated.

To investigate Ca^{++} antagonism to protoveratrine A-induced contractions of the guinea-pig ileum, excess Ca^{++} was added to the organ bath as a solution of calcium chloride, and the total Ca^{++} added calculated in terms of microgrammes of the ion per ml.

In some experiments the effects of adding Ca^{++} 30 seconds before the addition of the protoveratrine A were studied. In other experiments the protoveratrine A was added first and the contraction allowed to develop to a maximum, the drug was not washed out, and this led to the establishment of a plateau. Ca^{++} was then added

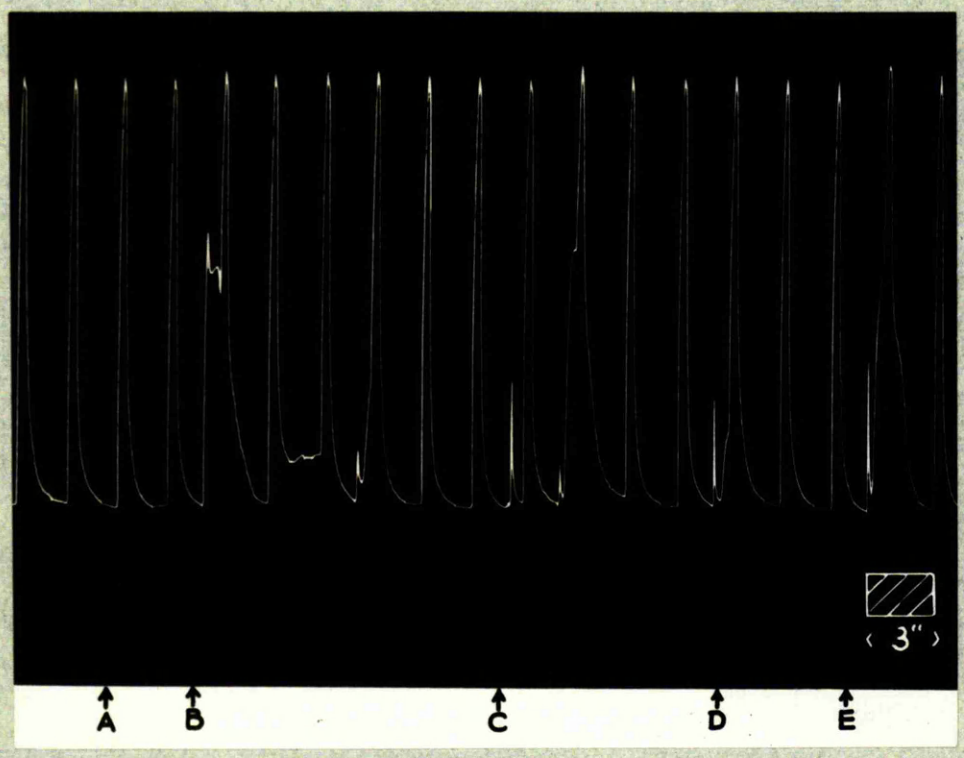


Fig. 6.

Effect of protoveratrine on histamine induced contractions of guinea-pig ileum.

At A. 0.4 ml control solution added

B. 0.1 ug per ml of protoveratrine added

C. 0.2 ug per ml of protoveratrine added

D. 0.5 ug per ml of protoveratrine added

E. 1.0 ug per ml of protoveratrine added

All contractions due to 0.1 ug. per ml. of histamine.

whilst the muscle was in a state of contraction.

Results

Experiments on the Guinea-Pig Ileum

When protoveratrine A was tested for antagonism to acetylcholine and histamine-induced contractions of the guinea-pig ileum, no evidence for any antagonistic action could be found.

Concentrations of 0.01, 0.1, 1 and 10 ug. per ml. of protoveratrine A were used. Although concentrations above 0.01 ug. per ml. frequently had a direct stimulant effect, this stimulant action was never followed by an depression of the magnitude of subsequent histamine or acetylcholine-induced contractions.

A typical trace is shown in Fig. 6.
At least five additions of protoveratrine were made at each dose level.

The physiological state of the tissue seemed to influence the effect which protoveratrine A had on 5 H.T. induced contractions, i.e. when the gut was comparatively fresh, e.g. after it had been exposed to doses of 5 H.T. for less than an hour a small dose of say, 10 ug. per ml. of protoveratrine A added 60 seconds before the addition of the/

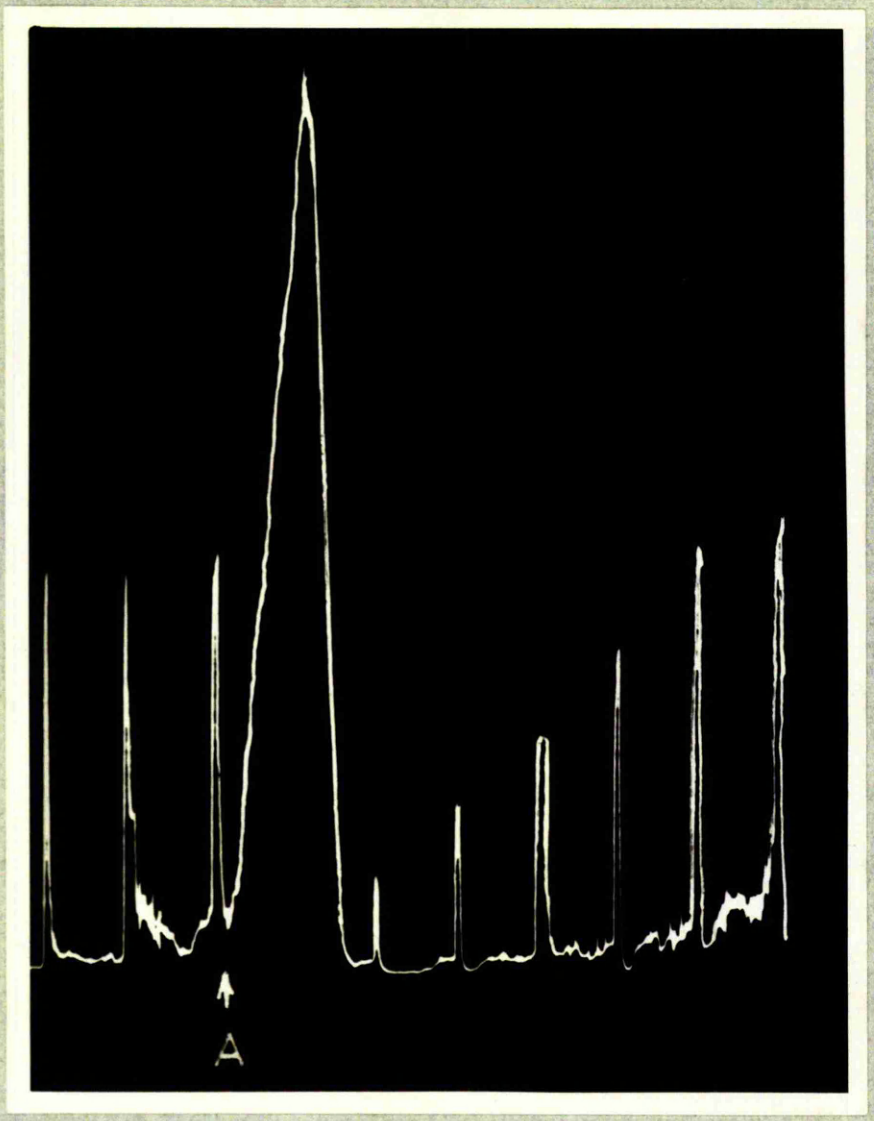


Fig. 7.

Inhibitory effect of protoveratrine A on 5 H.T. induced contractions of guinea-pig ileum.

At A. addition of 20 ng per ml of protoveratrine A.

Contractions due to 10 ng. per ml. of 5 H.T.

the 5 H.T. produced a marked contraction due to a direct action of its own. This contraction usually masked the subsequent direct stimulant action of the 5 H.T. After washout the muscle returned to its normal length but the succeeding additions of 5 H.T. gave rise to a smaller contraction than those produced before the addition of protoveratrine A. The normal control height of contraction was usually reached after about three further additions of 5 H.T. See Fig. 7.
The addition of the control solution had no effect on the response of the muscle to the stimulant action of 5 H.T.

After a period of more than an hour during which 5 H.T. was added to the muscle at regular intervals, the height of contraction usually decreased slowly but became constant again with an amplitude of contraction lower than that previously reached. When the tissue was in this condition the addition of a dose of protoveratrine A, (which had previously produced a direct effect followed by an inhibition of the 5 H.T.-induced contraction), produced a slight contraction followed by a sensitization of the muscle to the action of the 5 H.T. This direct effect was less marked than in the fresh muscle but served to potentiate/

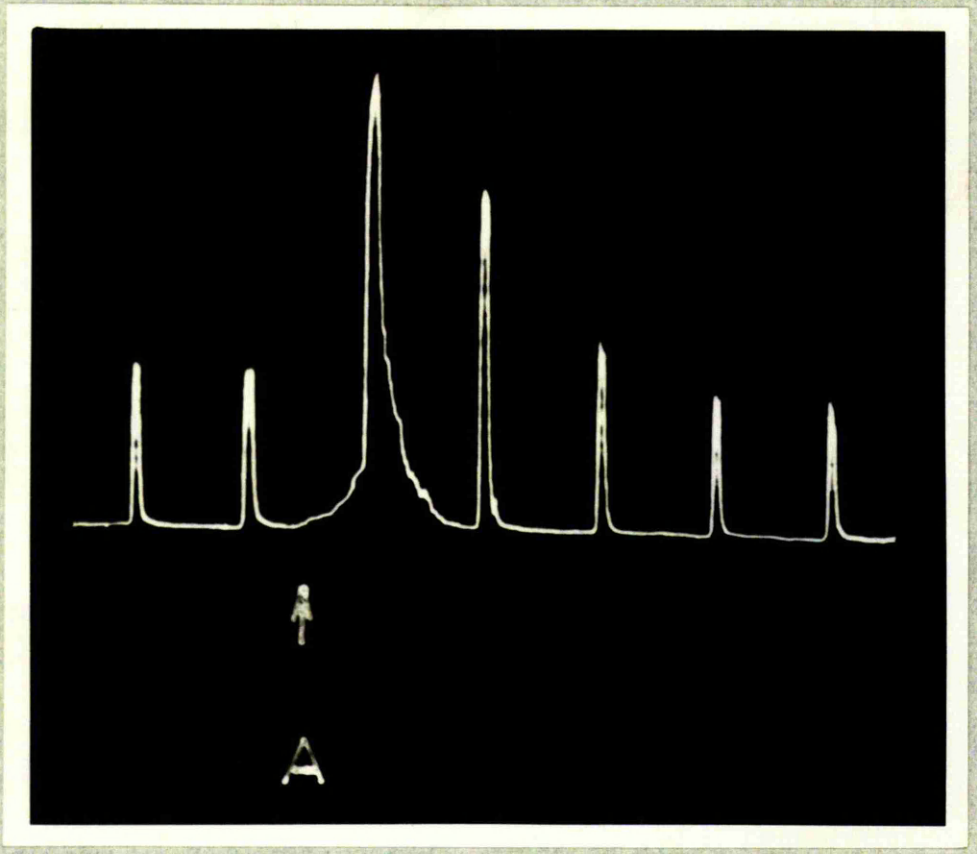


Fig. 8.

Sensitization by protoveratrine A to the stimulant action of 5 H.T. in guinea-pig ileum.

At A. addition of 10 ng protoveratrine A.

Contractions due to 10 ng. per ml. of 5 H.T.

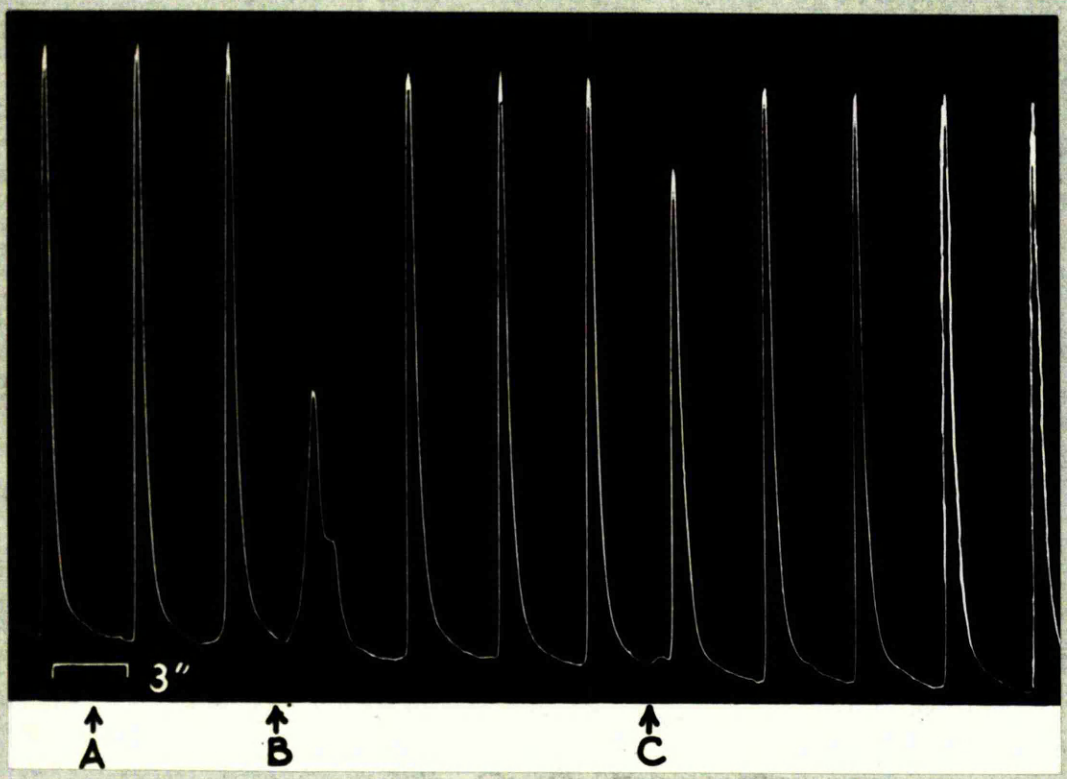


Fig. 9.

Effect of protoveratrine A on oxytocin induced contractions of the oestrus rat uterus.

At A. 0.4 ml control solution added.

At B. 1.0 µg per ml of protoveratrine A added.

At C. 0.5 µg per ml of protoveratrine A added.

All contractions due to 0.02 i.u. per ml. oxytocin.

potentiate the effect of the 5 H.T. On washout the muscle returned to the original length but the succeeding addition of 5 H.T. also gave rise to a contraction the height of which was greater than that developed before the addition of the protoveratrine A. This is clearly shown in Fig. 8.

The degree of both sensitization and inhibition shown by protoveratrine A to the stimulant action of 5 H.T. on the gut varied considerably, 10 ug.per ml.of protoveratrine A in fresh tissues gave an inhibition varying between 20 and 70 per cent with a mean value of 48 per cent. The degree of sensitization shown by semi-fatigued tissues after 10 ug. per ml.of protoveratrine A varied from 20 to 80 per cent with a mean of 24 per cent. When this dose was doubled the mean percentage increase was 48 per cent. The control solution had no effect.

Experiments on the rat uterus

Protoveratrine A in doses of up to 10 ug.per ml.had no direct effect on the rat uterus but in concentrations of 5 and 10 ug.per ml it reduced the height of oxytocin-induced contractions, see Fig. 9.

Experiments/

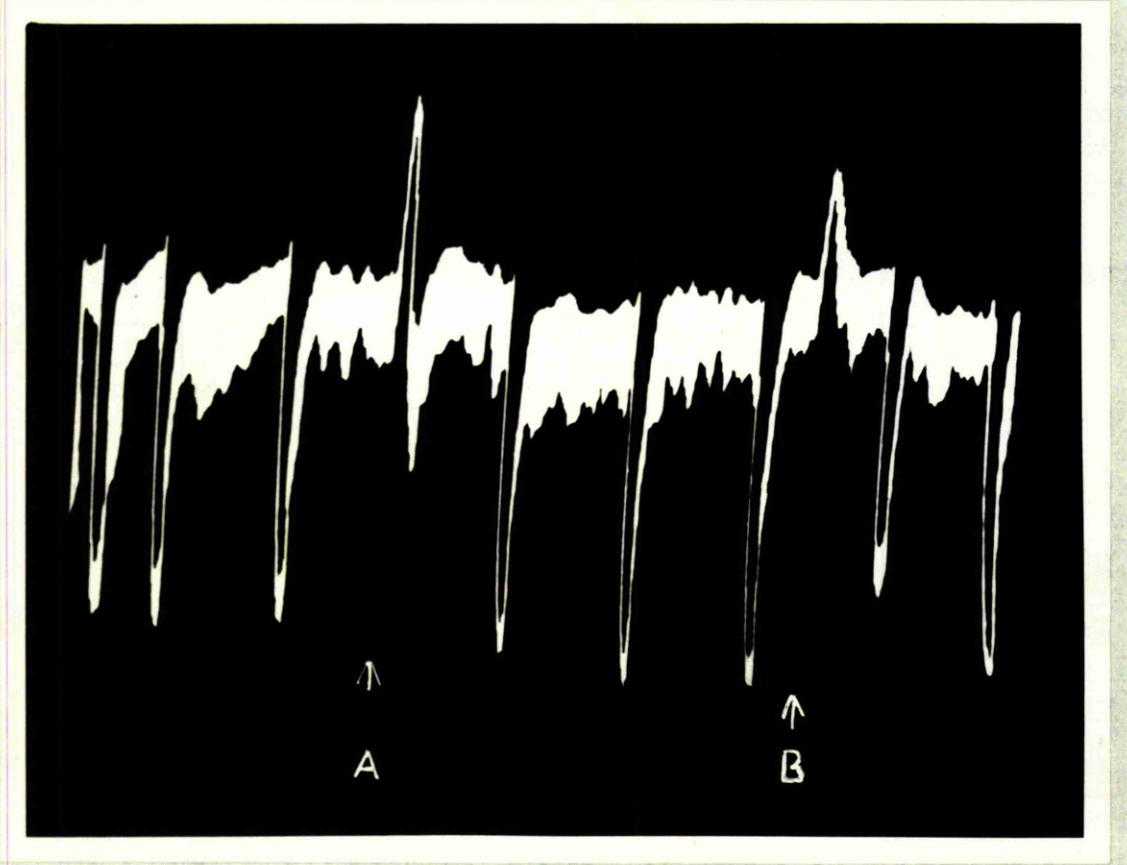


Fig. 10.

The effect of protoveratrine A on the inhibitory effect of adrenaline on isolated rabbit duodenum.

At A. addition of 0.1 ug per ml of protoveratrine A.
At B. addition of 0.2 ug per ml of protoveratrine A.

0.5 ug. per ml. of adrenaline was used to induce relaxation.

Experiments on the Rabbit Duodenum

The spontaneous contractions which occur in rabbit duodenum may be inhibited by the addition of small quantities i.e. 0.2 ug. per ml. of adrenaline. The reduction in tone of the muscle following adrenaline is rapidly abolished on washing.

It was found that the addition of 0.1 ug. per ml. of protoveratrine A prevented the development of the response to adrenaline. Larger doses gave an even greater inhibition of relaxation and also had a direct stimulant action of their own. The effect of this stimulant action was minimised by adding the protoveratrine A 90 seconds before the addition of the adrenaline, the contraction produced by protoveratrine A was usually only transient and normal tone was regained prior to the addition of adrenaline.

The effect of 0.5 and 1.0 ug. per ml. of protoveratrine A can be seen in Fig. 10. which shows a typical trace.

Three additions at each dose level were made and although the degree of inhibition varied the effects produced were qualitatively similar, the control solution had no effect.

The Effect of Ca^{++} on Protoveratrine A-Induced Contractions of the Guinea-Pig Ileum.

Protoveratrine/

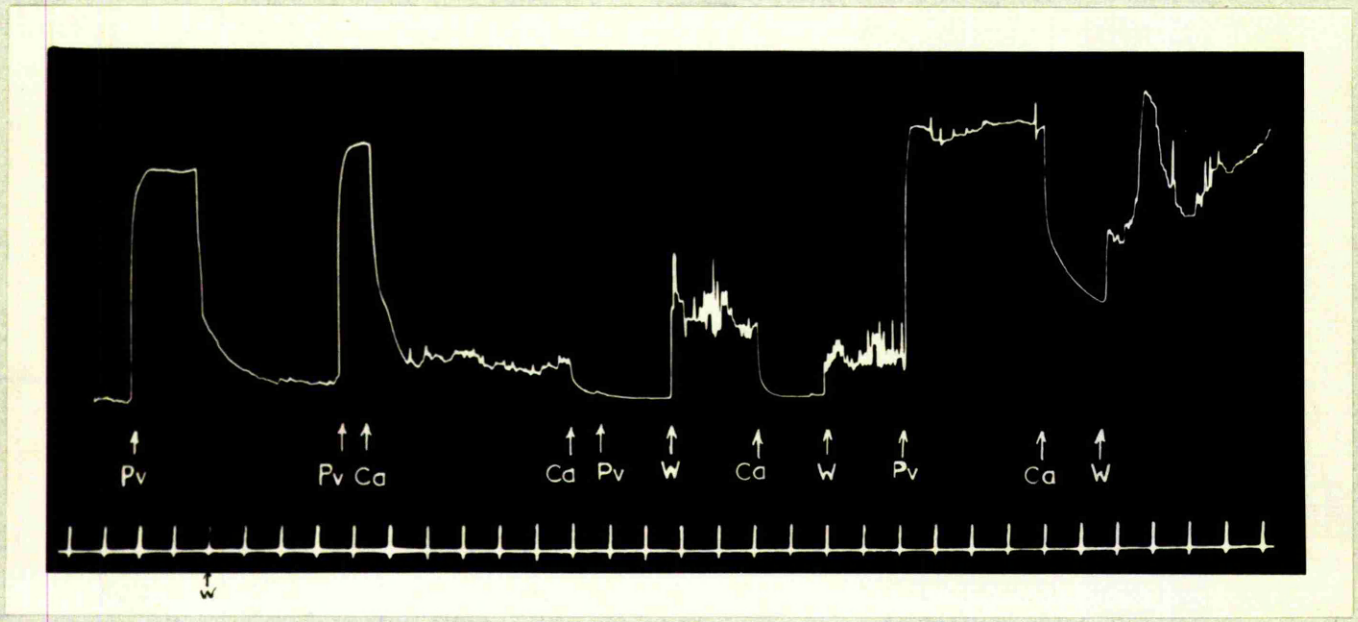


Fig. 11.

Effect of Ca^{++} on protoveratrine A induced contractions of guinea-pig ileum.

At Pv - 0.1 ug protoveratrine A added.
At Ca^{++} - 1 ug per ml of Ca^{++} added.
At W - Bath washed out twice.

Time trace at 1 minute intervals.

Protoveratrine A in a concentration of 1 ug. per ml. produced reproducible responses if the additionswere made at intervals of 6 minutes and the contractions were well maintained for a long as the drug was in contact with the tissue. The contraction could be prevented from developing if Ca^{++} in a dose of 2 ug. per ml. was added either before the addition of the protoveratrine A or by the addition of both Ca^{++} and protoveratrine A from the same syringe.

While a protoveratrine A-induced contraction was being maintained, the addition of Ca^{++} to the bath caused a rapid relaxation of the muscle and a return to the base line. Any slight spontaneous activity which was present in the muscle was invariably abolished following the addition of Ca^{++} to the bath.

It was noticed that when Ca^{++} was added to inhibit the protoveratrine A induced contractions, the subsequent washout always caused the development of a contraction of the muscle which maintained an increased tone accompanied by an increase in spontaneity. Frequent washes were needed before the muscle returned to normal.

These effects are shown in Fig 11. which is a trace obtained from a typical experiment.

CHAPTER 4

EXPERIMENTS UPON INTACT RABBITS TO DETERMINE THE EFFECT OF PROTOVERATRINE ON THE CONCENTRATION OF POTASSIUM IONS IN VENOUS PLASMA.

In the introduction to this thesis evidence has been set out which supports the view that one of the effects of the administration of protoveratrine and its congeners is to cause an increase in K^+ release from nerve. A series of experiments was planned to determine if this release was a more general phenomenon and whether it occurred in sufficiently large amounts to be measured in the blood emanating from a drug treated organ or vascular bed.

Rabbits were chosen for this study because of the ease with which multiple blood samples could be obtained, the animal could therefore be used as its own control.

Method

Rabbits of either sex weighing between 2.25 and 5 kg. were chosen, all animals were in good condition having been fed on a standard laboratory diet with the addition of fresh vegetables and water ad libitum. With one exception (rabbit number one), all the rabbits were between three months and one year old.

The rabbit was gently restrained and both marginal ear veins made visible for a distance of 2 cm. by removal of fur, the exposed region was rubbed with a little xylene followed by 70 per cent ethanol and when dry a small incision/

incision was made in the vein by means of a scalpel blade, the blood was allowed to flow freely and then two 1 ml. samples of blood were collected into a graduated centrifuge tube containing 100 i.u. of heparin.

The drug to be tested was made up in 0.9 per cent saline, and injected 10 minutes after the first sample was taken, into the marginal ear vein of the other ear. Two minutes after the injection of the drug, a further two samples of 1 ml. of blood were collected in a heparinized centrifuge tube, and two additional samples were taken after a further 20 minutes.

The blood samples were then centrifuged for ten minutes in an angled head centrifuge at a speed of 3,600 r.p.m. to separate the plasma from the cells. 0.2 ml. of plasma were removed from the centrifuged blood and diluted to 10 ml. with double glass-distilled water in a graduated, stoppered, volumetric cylinder. After shaking well, the potassium concentration in the sample was determined by comparing it with a series of standard samples on the E.E.L. Flame Photometer. For details of method used see Appendix.

The animals were given not more than one dose of alkaloid on each day. This restriction was thought necessary in/

in order to eliminate tachyphylaxis and the possibility of cumulative toxic effects.

As the main object of the study described in this thesis was to investigate certain aspects of the mechanism of the hypotensive action of the Veratrum alkaloids, it was felt to be essential to know if a hypotensive response was occurring at the dose levels used.

Accurate determination of the blood pressure in intact animals such as the rabbit presents a number of technical difficulties. In these experiments, accurate determinations were not undertaken because the only information required was whether protoveratrine reduced the blood pressure in the intact rabbit in doses of 10 ug per kg.

Estimates of the arterial blood pressure of the rabbits were made using the method first described by Grant and Rothschild[190] who state that the systolic pressure measured in the central artery of the ear can be taken as a reliable index of the systemic arterial pressure.

To measure the blood pressure in the central artery of the ear, the hair was shaved above the central artery at the base of the ear and the tips of the ears were fastened together with adhesive tape. The animal was put in a restraining box and allowed to sit quietly for about 10 minutes/

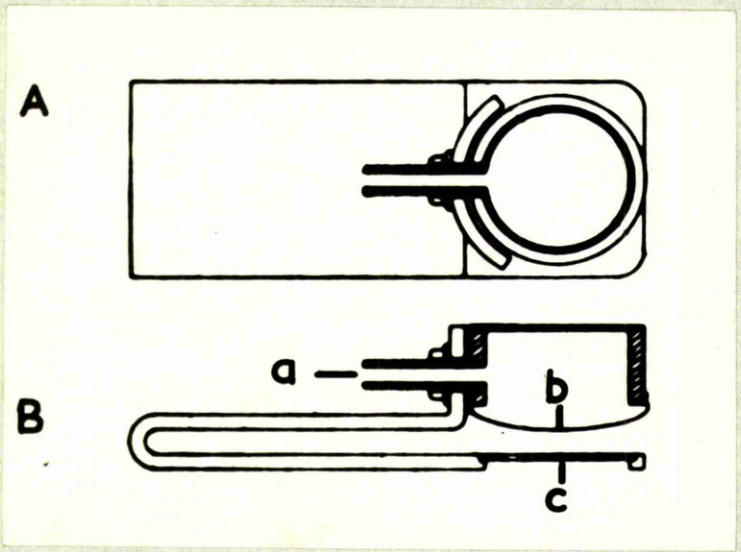


Fig. 12.

Grant's capsule used for the measurement of arterial blood pressure in intact rabbits.

A. Plan.

B. Side elevation.

a. To manometer.

b. Transparent membrane.

c. Glass window.

minutes in order to allow the blood pressure level to stabilise before taking the measurements.

The principle of this method is similar to that of the sphygmomanometer, the main difference being that the point at which the pressure prevents the blood from flowing in the artery is noted visually.

A specially designed pressure capsule, as described in the original paper, was used, see Fig. 12.
This is essentially a small cylindrical chamber which is connected to a pressure system. One end of the chamber is closed by a glass circle and the other by a transparent membrane of thin latex rubber, which when inflated is used to occlude the artery. The cylindrical chamber is mounted on one limb of the instrument, opposite this chamber on the other limb is a glass window which enables transmitted light to pass through the ear and enables the column of blood to be seen more clearly.

The pressure capsule was applied by slipping the ear between the limb of the capsule until the central artery lay directly beneath the transparent membrane. The capsule was then slowly inflated by means of a sphygmomanometer bulb until the pressure in the capsule was just sufficient to prevent the passage of blood along the artery. The pressure in/

TABLE 2

The Hypotensive Action of Protoveratrine 10 ug per Kg. in Intact Rabbits

Experiment	Mean systolic blood pressure mm Hg.		Percentage Reduction
	Before Drug	After Drug	
1	93	69	25.8
2	101	74	26.7
3	77	52	32.5
4	84	63	25.0
Mean	88.7	64.5	27.2

in the capsule which prevented the blood flow was measured by means of a mercury monometer and was taken as the systolic arterial blood pressure.

The effects of the injection of 10 ug. per kg. of protoveratrine on the blood pressure of four rabbits is shown in Table 2. Each figure is the mean of three estimations made over a period of twenty minutes following the injection of the drug. From Table 2 it may be seen that protoveratrine produced a marked fall is systolic blood pressure in doses of 10 ug. per kg. The results showed a mean fall in systolic blood pressure of 27.2. per cent.

Results

This series of experiments was carried out to determine the effect of an intravenous injection of purified Veratrum alkaloids upon the plasma K^+ levels of intact rabbits.

No reports of any similar work have been published and it was felt that these studies might indicate if any gross changes in plasma K^+ were produced by the Veratrum alkaloids. The evidence for a possible role of K^+ in the pharmacological effect of these alkaloids has been reviewed in the introductory chapter of this thesis.

The results of the twelve experiments performed have been summarised in Table 3, Each figure is the mean of

two/

The Effects of Protoveratrine on the plasma K^+ of Intact Rabbits

Experiment No.	Sex	Age in months	Weight in Kg.	Dose ug/Kg.	Mean K^+ levels of plasma mEq/l			Remarks
					Before Drug	After Drug 2 mins.	After Drug 20 mins.	
1	M	c.24	5	10	4.43	3.76	3.74	No obvious signs of discomfort noted. Some bradycardia
2	M	5	2.5	10	4.98	5.50	4.95	ditto
3	M	6	2.8	10	3.31	3.83	4.47	ditto
4	F	5	2.8	10	4.46	4.98	4.60	ditto
5	M	5	2.75	20	5.63	5.67	5.63	Slight signs of discomfort, retching.
6	M	6	4.5	20	4.60	5.88	4.75	Sneezing, loss of muscular tone, tachypnea, bradycardia.
7	M	5	3.1	20	5.10	5.26	5.36	ditto
8	M	3	2.75	20	3.58	5.88	4.60	Sneezing, muscular tremors, attempted vomiting, Periods of apnea, bradycardia.
9	M	6	2.25	20	3.66	5.26	4.60	Sneezing, retching, salivation, bradycardia.
10	F	6	3.0	20	4.56	5.45	5.05	ditto
11	M	6	2.25	30	4.34	4.60	4.35	Attempted vomiting, irregular respiration and apnea, slight convulsions, bradycardia
12	F	3	2.0	30	4.75	16.8	23.1	Spasmodic head movements, attempted vomiting, extension of neck, followed by loss of muscular tone, convulsions and death.

two determinations of the plasma K^+ concentration.

Fairly high dose levels of protoveratrine were used in order to produce readily measurable changes in the plasma K^+ level. When protoveratrine in doses of 10 ug. per kg. i.e. a dose level comparable to that used clinically in man, no marked subjective changes in the rabbit could be seen. Bradycardia was observed in two out of the four animals treated with 10 ug. per kg. of protoveratrine. In three out of the four animals the plasma K^+ level was increased in samples of blood taken two minutes after the injection of the drug. Samples taken 20 minutes later showed that the levels were at or approaching the original level in three out of the four animals.

Larger doses of protoveratrine, i.e. 20 ug. and 30 ug. per kg. produced marked signs of distress in the rabbits. The symptoms shown by each rabbit have been indicated in Table 3. The pattern was similar in all animals which received the higher doses of the drug and was characterised by a series of symptoms which were manifested as the toxic effects of the drug developed. The first symptom was restlessness which was followed by signs of definite discomfort within one minute of the injection. The animals began to salivate freely and this effect was frequently

followed/

followed by movements similar to the retching movements preceding vomiting which occur in man, dog and other animals after the administration of protoveratrine. The rabbits never actually vomited although the maximum dose used per kg. was greater than that necessary to induce emesis in man, dog and cat. It has been suggested [191] that rodents do not have the ability to vomit due to the absence or inherent defect of those areas of the brain stem reticular formation which are essential components involved in the central nervous control of the vomiting reflex. The retching movements were accompanied by tachypnea which in some cases was succeeded by periods of apnea, which was probably due to elicitation of the Bezold-Jarisch reflex. In two rabbits there was a pronounced decrease in muscular tone which prevented the animals from holding their heads erect.

Muscular tremors which were followed by slight convulsions occurred in two rabbits, one of which died later. In both of these animals the convulsions developed following a period of apnea and it seemed likely that they were a result of cerebral anoxia rather than of a direct stimulant effect upon the central nervous system.

Some degree of bradycardia was detected in all the animals treated but this could not be measured accurately due to the irregular breathing and convulsions.

The/

The toxic symptoms observed are similar to those described by Krayer and Acheson[50] as occurring in mice which had been given toxic doses of various Veratrum alkaloids.

The plasma K^+ level before the injection of the drug fell within the range given by Spector[192] i.e. 2.7 to 5.1 mEq/l in eleven out of the twelve experiments. In all cases the plasma K^+ level was significantly raised following the injection of the protoveratrine at the 20 µg. and 30 µg. per kg. level. A 'p' value between 0.02 and 0.05 was found when the differences were compared to zero by "Student's" paired 't' test. In experiment 12 the rabbit died following convulsions, the K^+ level in the plasma was very high and the values obtained in this experiment were not included in the statistical test.

These results show that the intravenous injection of protoveratrine in doses of 20 ug. or 30 ug. per kg. produces an increase in the plasma K^+ level. When all the results, excluding experiment 12, were pooled, the difference between the levels of plasma K^+ before and after protoveratrine was still found to be significant ($p = 0.05$).

Although at first sight the results of these experiments seem to support the claim of Shanes and other workers (for detailed/

detailed reference see Chapter 1 of this thesis) that the Veratrum alkaloids can give rise to an increased K^+ output from cells, no conclusions can be drawn from these experiments as a large variety of different physiological changes have been shown to cause an increase in the K^+ level of the plasma.[193]

Because of the large number of possible sources for the released K^+, it was decided to attempt a determination of possible sites for this action of the drug.

As the heart and lungs have been suggested as regions in which the afferent fibres for the Bezold-Jarisch reflex originate, see Chapter 1, it was decided to try to determine whether K^+ was released from the cardio-pulmonary region by protoveratrine.

CHAPTER 5

EXPERIMENTS ON SPINAL CATS TO DETERMINE THE EFFECT OF PROTOVERATRINE ON THE CONCENTRATION OF POTASSIUM IONS IN THE PLASMA OF BLOOD TAKEN FROM THE CARDIO-PULMONARY CIRCULATION.

The use of rabbits to continue the study of the possible source of the released K^+ demonstrated in the previous chapter was discontinued because aneasthetization and spinalization of rabbits is more difficult and results in a higher mortality than it does in cats.

Cats were therefore used in all the experiments described in this chapter. These animals are sensitive to the hypotensive effect of protoveratrine and numerous studies have been made using them.[66,67,68,69.]

Method

Healthy cats of either sex weighing between 2.5 and 3.5 kg. were used in these experiments. All the animals were fed on a normal diet of meat, fish and milk but were denied food on the day of the experiment.

The animals were anaesthetized by blowing warm ether vapour from a Wolff's bottle immersed in water at about $50^{\circ}C$, into a sealed wooden box with a glass observation panel. This method was found to give much more rapid induction of anaesthesia than the normal method in use at the time which entailed/

entailed soaking cotton wool in ether and placing it in the box with the animal. There was also very much less irritation of the respiratory tract as bronchial secretion and salivation were almost absent.

As soon as the animal lost consciousness it was removed from the anaesthetic box and tied down on to a heated operating table. The animal was kept anaesthetized by an assistant while a tracheotomy was carried out. The tracheal cannula which was then inserted was connected by means of rubber tubing to a bottle containing a mixture of air and ether vapour. Both carotid arteries were exposed, freed from connective tissue and the adjacent vago-sympathetic trunk and then tied off below the carotid bifurcation.

The animal was placed face down on the table and an incision of about 7 cm. made through the skin over the cervical region of the vertebral column. The muscles above the column were separated by blunt dissection and the spine of the second cervical vertebra exposed, this was removed using bone forceps and then the laminae were removed in a similar manner, care being taken not to sever the vertebral arteries. The spinal cord was now visible and this was sectioned with scissors. At the moment of section the tracheal cannula was connected to a Starling respiration/

respiration pump and the animal was artificially ventilated. Destruction of the central nervous system was then achieved by the passage of a probe through the foramen magnum to destroy the brain and then down through the neural canal to destroy the spinal cord. Bleeding was minimized by forcing a cone of plasticine into the neural canal and packing the surrounding space with pledgets of cotton wool soaked in very hot saline. The skin was drawn together and clipped by means of metal surgical clips and the animal was then turned over on to its back.

Heparinized glass cannulae were introduced into the left common carotid artery and into the right external jugular and left femoral veins. The cannulae in the carotid artery and femoral vein were used to obtain samples of arterial and venous blood respectively, and the cannula in the jugular vein was attached by means of rubber tubing to a burette. To replace fluid loss, 25 ml. of normal saline was slowly run into the animal from the burette. The spinalization was carried out with as little delay as possible. The time taken from anaesthetization to the completion of the spinalization never exceeded 15 minutes.

After running in the saline the animal was left for 90 minutes to allow for the elimination of the volatile anaesthetic and for the K^+ released by the trauma during the/

the operation to equilibrate with the total body potassium.

Samples of blood were obtained from the carotid artery or femoral vein by loosening a bulldog clip on the artery, allowing the first few drops of blood to run to waste and then collecting 1 ml. of blood in a graduated, heparinised centrifuge tube.

All drugs were injected through the rubber tubing attached to the cannula in the external jugular vein and were washed in with 2 ml. of 0.9 per cent sodium chloride solution from the burette.

Two samples of blood from the carotid artery and femoral vein were taken for control purposes before the administration of the drug; after the drug was injected blood samples were taken from the artery after intervals of 2 and 30 minutes. The samples were centrifuged in an angled head centrifuge at 3,000 r.p.m. for five minutes, then removed and 0.2 ml. of plasma withdrawn by means of a pipette fitted with a rubber teat, and then transferred to a 10 ml. graduated stoppered volumetric cylinder and the volume made up to 10 ml. with double distilled water. After mixing well, the concentration of potassium ions in the diluted samples was determined, using the E.E.L. flame photometer. Details of the method of determination will be found/

found in the Appendix.

Two dose levels of protoveratrine were used in these experiments and they were given as a total dose of either 10 ug or 20 ug. The number of doses was limited to not more than three in each cat so as to prevent as far as possible the development of tachyphylaxis.

Results

The K^+ concentration of arterial plasma showed a marked variation between experiments and it was also found that as the experiment progressed the K^+ concentration tended to increase. Following the 90 minute equilibration period the first sample of blood showed a mean plasma K^+ concentration of 3.66 ± 0.23 mEq/l, after 4½ hours, i.e. just prior to the completion of the experiment the K^+ concentration of the plasma had risen to 4.86 ± 1.5 mEq/l. This steady rise occurred both in animals which had been injected with protoveratrine and in animals which were untreated. These figures compare with those given by Cattel and Civin[194] which show a wide variation in K^+ levels of from 2.56 to 6.68 mEq/l.

Other workers[195,196,197] have shown that a marked rise in K^+ concentration occurred during the course of experiments involving some degree of trauma and that the K^+ level of the plasma may be increased fourfold under these/

The Effect of Protoveratrine on the Plasma K^+ level of Carotid Blood in Spinal Cats.

Sampling Time in Minutes

Experiment No.	Sex	Weight in Kg.	90	120		150		180		210		240		270	300	330		360		390
1	F	2.5				4.35		4.25	4.87	5.33										
2	F	3.5	3.58	4.35						3.84	3.80	4.20			6.30	8.40				
3	M	2.75	4.1	5.30				5.30				6.40								
4	F	2.5	4.6	4.25		5.45				4.75	4.82	5.20		5.20	6.90	6.15	6.65	7.30		
5	F	2.6	4.95	4.75		5.20				5.10	5.21	5.37		5.10						
6	F	3.0	2.76			3.50				4.20				3.40	4.20	5.0	5.75	3.9	4.3	4.6
7	M	3.25	2.98	3.23				3.33		3.33	3.92	3.94		3.46	3.90	4.8	5.24	4.08		
8	F	3.25	3.40	3.71				3.96		3.71	3.35			3.56	4.10	3.46	3.86	3.22	3.76	3.96
9	F	3.2	2.80	2.70	2.80	2.78		3.32		3.20	5.4	4.6		6.1						
10	F	2.0	3.54	3.68		3.42	4.8	3.68												
11	M	2.6	2.62	3.08				3.76		4.23		5.0	5.0	5.0	4.25					

K^+ level 1 minute after the injection of 10 ug. protoveratrine.

K^+ level 1 minute after the injection of 20 ug. protoveratrine.

All K^+ concentrations expressed as mEq. of K^+ per litre of plasma.

these circumstances.

The injection of protoveratrine into the jugular vein frequently gave rise to varying degrees of bradycardia. This was not a reflex action as the central nervous system had been destroyed, but was probably the result of a direct negative chronotropic effect on the heart.[50]

No other subjective effects following the administration of the drug were noticed in any of the experiments performed.

The results of eleven experiments are summarised in Table 4. All K^+ concentrations are expressed as mEq of K^+ per litre of plasma. The increase in plasma K^+ concentration during the course of the experiments can be seen from this table. In some experiments the rate of increase was greater than in others, a steep rise usually preceded cardiac arrest.

Following the administration of 10 ug. of protoveratrine the blood K^+ concentration rose in 6 out of 8 samplings. In 3 of these cases the K^+ level of the plasma 30 minutes later was lower than that of sample removed before the injection of the protoveratrine. This indicates that the drug may be causing some increase in the K^+ level of the plasma. When a larger dose of protoveratrine, i.e. 20 ug. was used a similar result was seen. Out of 8 experiments a rise in plasma K^+ occurred following the administration

of/

of the drug in 7 of them and in the remaining one no change was seen. In 4 of the positive experiments the K^+ level subsequently fell to below the level following drug administration.

The results obtained in this series of experiments indicated that the plasma K^+ level of the blood from the cardio-pulmonary circulation tended to rise after the injection of protoveratrine into the external jugular vein.

No information about the source of the released K^+ was obtained from these experiments. Three possibilities existed:-

(i) The drug was causing the direct liberation of K^+ from the tissues of the heart or lungs.

(ii) That the bradycardia produced by the drug led to an increased plasma K^+ by reducing the rate outflow of blood in the coronary arteries and thus allowing more K^+ to accumulate in the plasma.

(iii) The drug may give rise to the liberation of adrenaline or noradrenaline from either the adrenal glands or from chromaffin tissue in the heart itself, or from both. This may lead to alteration in the ionic equilibrium between the cells of the heart and their environment.

It was obvious from these results that more refined techniques were necessary to determine if the Veratrum alkaloids as exemplified by protoveratrine were actually causing the release of K^+ by a direct effect upon ionic transport mechanisms.

CHAPTER 6

EXPERIMENTS TO DETERMINE THE EFFECT OF PROTOVERATRINE ON THE RELEASE OF POTASSIUM IONS FROM THE SKELETAL MUSCULATURE OF CATS.

The skeletal muscles were considered to be a possible source of the release of K^+ which was demonstrated following the administration of protoveratrine. To determine if protoveratrine did release K^+ from skeletal muscle, the plasma K^+ level of venous blood was measuredafter the intra-arterial injection of protoveratrine.

Method

Spinal cats or pentobarbitone-aneasthetized cats were prepared by the methods described in Chapters 5 and 7.

Arterial blood pressure was recorded by conventional methods from left common carotid artery.

The musculature of the hind limb was taken as being a suitable area for study, it was readily accessible and the venous blood from the opposite limb could be used as a control.

The animals were stretched out on a warmed operating table and the fur over the inner aspects of both thighs was removed. Incisions were made in the skin of the thigh of both limbs, this was reflected to expose the femoral artery and vein of each hind limb.

Heparinized/

Heparinized needle-type injection cannulae were tied into the two adjacent branches of the femoral artery and vein in both limbs. These are branches of the femoral vessels and have been termed by different authors the venous and arterial rami or the muscular artery and vein. The cannulae were placed so that their tips were situated at the junction of the branch with the main vessel.

The drug was administered into the vascular bed by intra-arterial injection, this was achieved by placing a bulldog clip on the femoral artery proximal to the cannula tip and injecting 0.1 ml. of drug or control solution into the lumen of the artery, the stilette was replaced in the cannula, the bulldog clip removed and the drug distributed to the vascular bed by the arterial blood flow.

Samples of venous blood were obtained by withdrawing 0.5 ml. samples from the cannula in the venous ramus. These samples were taken before the arterial injection of the drug or control solution and then at intervals of 5, 10, 30, 60 and 120 seconds after the injection. The procedure was carried out almost simultaneously on each leg, a solution of protoveratrine was injected into one limb and a control solution into the other. Total doses of 2 and 5 µg. of protoveratrine were used, one dose at each level was given to each animal at 30 minute intervals, or/

For 2 µg. Protoveratrine

Experiment No.	Test: K^+ level of plasma mEq/l. Before drug	Test: No. of seconds after drug. 5	10	30	60	120	Control: K^+ level of plasma mEq/l. Before control	Control: No. of seconds after control. 5	10	30	60	120
1	4.21	4.23	4.20	4.26	4.18	4.21	4.19	4.22	4.17	4.22	4.20	4.18
2	3.76	3.74	3.80	3.71	3.72	3.78	3.80	3.81	3.78	3.92	3.73	3.87
3	3.98	4.10	3.89	4.00	4.01	4.00	3.96	3.96	4.00	3.89	3.94	3.96
4	3.41	3.36	3.41	3.40	3.39	3.50	3.40	3.41	3.37	3.61	3.54	3.44

For 5 µg. Protoveratrine.

1	4.23	4.24	4.19	4.26	4.20	4.20	4.21	4.22	4.19	4.29	4.18	4.20
2	3.66	3.68	3.57	3.65	3.70	3.69	3.80	3.81	3.69	3.84	3.75	3.72
3	4.12	4.12	4.20	4.15	4.08	4.10	4.00	4.20	4.22	4.16	4.06	4.04
4	3.50	3.41	3.40	3.57	3.49	3.52	3.51	3.41	3.42	3.61	3.44	3.49

The effect of protoveratrine on the K^+ level of venous plasma taken from the hind limbs of cats.

or, as in the two cases in which the dose produced a fall in arterial blood pressure, when the blood pressure returned to the control level.

The plasma K^+ levels for each sample were determined using a flame photometer, for details of the method see Appendix.

Results

The injection of the drug produced no muscular twitching in the limb and only in two cases out of eight was there any fall in arterial blood pressure. This was only slight in both cases and returned to normal after 30 minutes.

The concentrations of K^+ in the venous plasma for all the experiments performed are listed in Table 5.

The mean K^+ levels with their standard deviations for the venous plasma after the addition of 2 ug.of protoveratrine was 3.84 ± 0.31 mEq per l, the corresponding figure for the plasma from control limb was 3.86 ± 0.27 mEq per l. Similarly after 5 ug.of protoveratrine the figures were3.87 ± 0.32 for the treated and 3.89 ± 0.38 mEq per l. from the control limb.

When these means were compared statistically by the 't' test the differences were found to be not significant

i.e./

i.e. 'p' was greater than 0.9.

These results indicate that protoveratrine in the doses used does not significantly affect the concentration of K^+ in the plasma of venous blood from skeletal muscle within 120 seconds.

CHAPTER 7

EXPERIMENTS TO DETERMINE THE EFFECTS OF PROTOVERATRINE ON THE CONCENTRATION OF POTASSIUM IONS IN THE CORONARY VENOUS BLOOD OF ANAESTHETIZED CATS.

The most marked pharmacological effect following the administration of the active Veratrum alkaloids is a rapidly induced hypotension accompanied by bradycardia and transient apnoea. This reflex effect was first described by Bezold and Hirt[70] in 1867 and was rediscovered and more thoroughly investigated in 1939 by Jarisch and his co-workers.[71,198] The reflex is now known as the "Bezold-Jarisch Reflex" and will be referred to as such in the remainder of this thesis. That this effect was reflex in origin was suggested in the original paper by Bezold and Hirt and this view was confirmed by Krayer, Wood and Montes[72] who used a dog heart-lung preparation with a separate perfusion of the head and concluded that the afferent receptors for the reflex were situated in the thorax. Jarisch and Richter, and Richter and Amann[71,198] also concluded that the reflex was thoracic in origin and that the probable origin was in nerve endings of the ventricular myocardium.

More recent work has confirmed these views and shown that the reflex consists of a number of afferent components involving specialised receptors in the ventricular[68,84,91.]

and/

and atrial myocardium,[89,98,199,200] the lungs, the aortic arch[69,201] and the gastric mucosa[93] but by far the largest component arises from the region of the left ventricle which receives its blood supply from the left common coronary artery.[68] Dawes has demonstrated this fact in a number of very elegant experiments in which he injected the Veratrum alkaloid, veratridine, directly into the individual coronary arteries and recorded the effect on blood pressure, heart rate and respiration.

Amann and Jarisch[202] and Lecomte et alii[147] showed that a certain degree of cross sensitization between potassium and the Veratrum alkaloids existed in the receptors in the heart in a similar manner to the sensitization shown by Baeq and his co-workers[189,293] on the frog rectus abdominis muscle. These workers used large doses of potassium giving 20 mg. of potassium chloride intravenously and showing that this dose only elicited a reflex fall in blood pressure and reflex bradycardia if the animal was pretreated with 0.05 to 0.1 mg. per kg. of veratrine hydrochloride.[68] Dawes attempted to confirm these findings by the injection of veratridine and potassium into the coronary arteries but was unable to show any significant degree of sensitization by veratridine to the injected potassium; he did, however, demonstrate that a minimal dose of potassium chloride when injected

simultaneously/

simultaneously with veratridine into the coronary circulation caused a larger fall of blood pressure and heart rate than either the same doses of veratridine or potassium chloride given alone.

Because of these differing findings it was thought worthwhile to attempt to determine the influence of protoveratrine upon the K^+ level of the blood plasma in the coronary circulation.

METHOD

The method used to isolate the coronary vascular bed in cats was modified from that used by Dawes.[68] This method was similar to that developed by Gregg and Shipley[204] for dogs. The animals used were large cats in the weight range 3.5 to 6 kg. in good condition, having been fed on a diet of meat, fish and fresh milk. To reduce to a minimum operative trauma and to keep the reflexes as normal as possible spinalization was ruled out.

Although some barbiturates are known to depress baroreceptor reflexes, pentobarbitone sodium seemed to be inactive in this respect.[205] The effect of barbiturates on the potassium concentration of coronary blood in cats has not been studied, O'Brien et alii[206] gave a figure for the coronary/

coronary venous blood of unanesthetized dogs which did not differ from that obtained in animals anesthetized with pentobarbitone sodium.

The animals were anesthetized by intra-peritoneal injections of pentobarbitone sodium at a dose of 50 mg. per kg. When the animal was completely anaesthetized it was tied down on a warmed operating table and the fur clipped away from the left thoracic region and the ventral surface of the neck. An incision of 5 cm. long was made in the midline above the trachea. The trachea was exposed by blunt dissection. A metal tracheal cannula with a variable aperture window was inserted into the trachea and firmly tied in place. The left common carotid artery was exposed and carefully separated from the vago-sympathetic trunk and any adjoining connective tissues. Glass cannulae of the standard pattern were introduced into the left femoral artery and the right femoral vein. The blood pressure was recorded from the femoral artery and saline infusions were made into the femoral vein from a burette attached by rubber tubing to the cannula. The femoral artery was used for the recording of blood pressure because the left carotid artery was cannulated to perfuse the heart and it was felt essential to keep the head supplied with blood to maintain the activity of the cardiovascular reflexes.

Electrocardiograph/

Electrocardiograph records were taken from Lead II by subcutaneous implantation of needle electrodes and the record was displayed on an Ediswan Direct Writing Pen Recorder.

A heparinized, glass venous type cannula was tied into the left common carotid artery, the blood flow being interrupted by a rubber sheathed bulldog clip placed proximally to the cannula. After making an incision in the skin of 10 cm. along the midline of the sternum, the skin was reflected from the left thoracic region and the bleeding points were sealed off using electro-cautery. The muscular layers between the fourth and fifth ribs were separated by blunt dissection and as soon as penetration into the pleural cavity was achieved the animal was artificially ventilated through the tracheal cannula from a Starling Respiration Pump of conventional pattern. The space between the two ribs was enlarged and the ribs separated by means of self-retaining rib retractors. The internal mammary artery and the left intercostal veins of this region were tied off and the costal cartilages of the third, fourth and fifth ribs cut through with bone forceps and the ribs retracted, using the rib retractors. All bleeding points were sealed off by electro-cautery. The left subclavian artery was cleared of connective tissue from its junction with the aorta to the emergence of the internal mammary artery and a loose ligature was placed around/

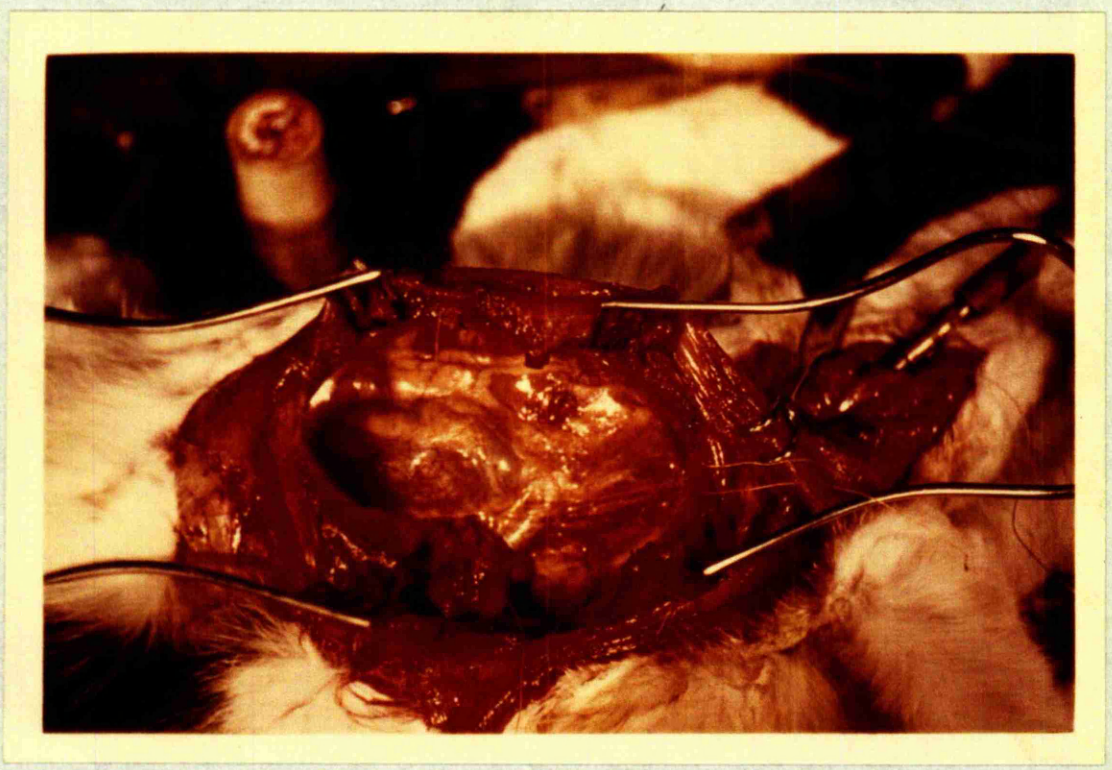


Fig. 13.

Exposure of heart and great vessels prior to cannulation of coronary artery and coronary sinus.

around it. The appearance of the preparation at this stage of the procedure is shown in Fig. 13.

The pericardium was incised and reflected back from the heart exposing the base of the great vessels and the chambers of the heart. A loose controlling ligature was passed around the pulmonary artery where it passes across the left auricular appendage. A pair of artery forceps, the jaws of which were sheathed with rubber, were clipped to the apex of the left auricular appendage which was reflected anteriorly to expose the root of the aortic arch and the origin of the left common carotid artery. In the cats used, the origin of the left common coronary artery was invariably obscured by large amounts of fatty and connective tissue and some difficulty was experienced in exposing this vessel. In the majority of animals used the left common coronary artery varied in length from 2 to 5 mm. ,but in 20 per cent of the cats both branches emerged directly from the aorta with a complete absence of the common region. This made the preparation impossible and the experiment had to be abandoned.

The common portion of the artery was carefully exposed and cleansed of adhering fatty tissue. The tips of a pair of blunt-pointed curved forceps were passed between the artery and the auricular wall and a loose ligature passed around the artery./

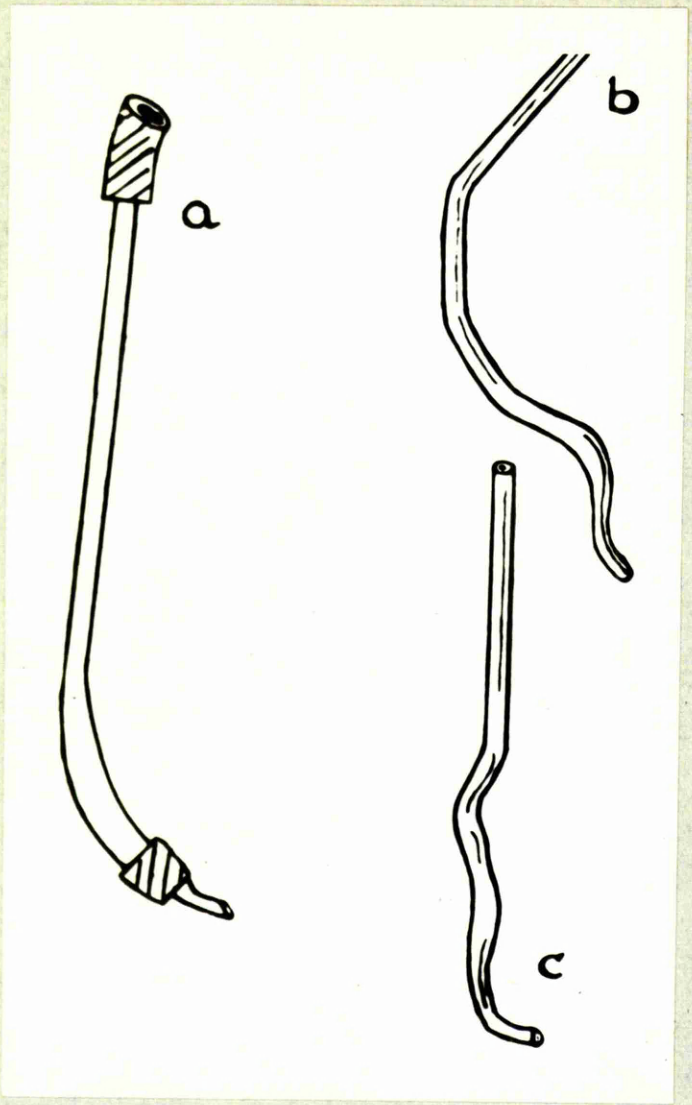


Fig. 14.

a. Coronary sinus cannula showing rubber collar near tip to occlude the coronary sinus ostia.

b. Coronary arterial cannula lateral view.

c. Coronary arterial cannula antero-posterior view.

All figures full size.

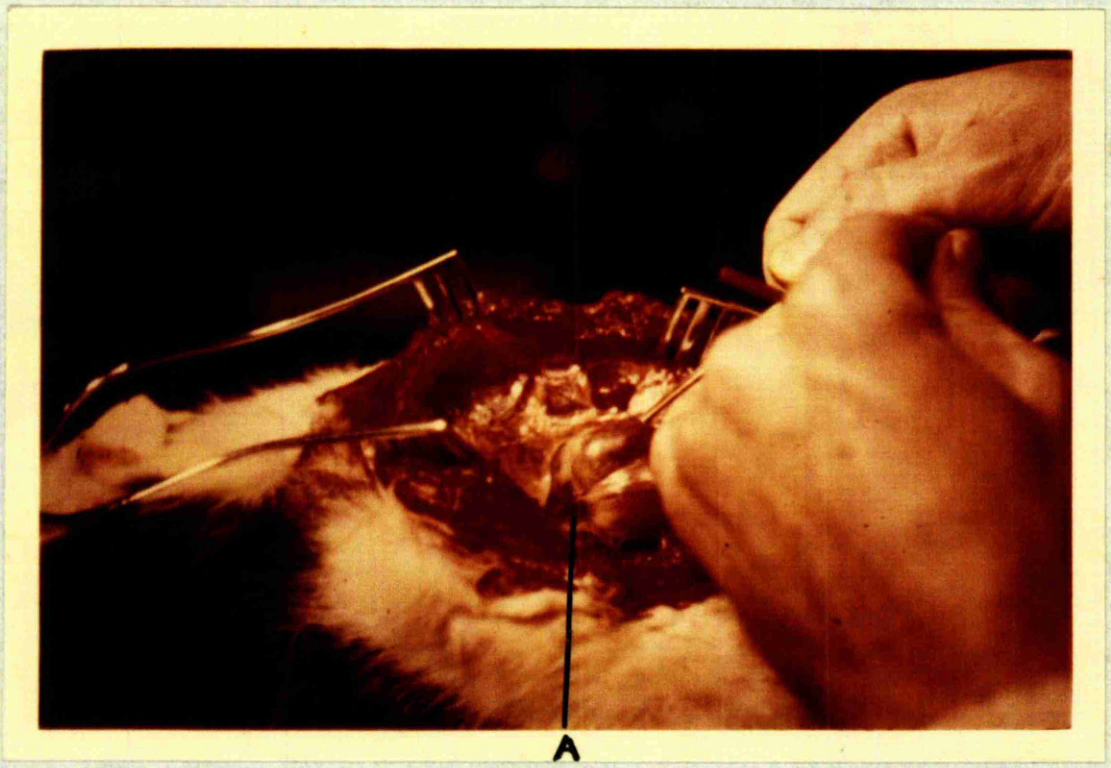


Fig. 15.

Insertion of cannula into the coronary sinus.

A - Tip of cannula in coronary sinus.

artery. By means of a fine curved needle a ligature was passed through the muscular wall of the ventricular apex and tied into place. This was done to enable the heart to be manipulated with the minimum of handling. Using this ligature the heart was reflected anteriorly exposing its dorsal surface and the larger part of the right auricle. An incision was made into the right auricular appendage and a specially designed cannula (see Fig. 14.) rapidly introduced and tied into the auricle with its tip placed within the coronary sinus. Correct positioning of this cannula was determined by examining the colour of the collected blood. Blood coming from the sinus was much darker than the blood in the auricle due to the much lower oxygen content of the coronary blood. The tip of the cannula could also be seen through the wall of the vessel, this may be seen from Fig. 15. Blood from the sinus which issued from the distal end of the cannula was collected and introduced back into the circulation through the cannula in the femoral vein.

A specially designed coronary artery cannula (see Fig.14.) similar to that employed by Dawes [68] was then introduced into the left subclavian artery and guided by touch it was pushed caudally until its tip was in the region of the lumen of the/

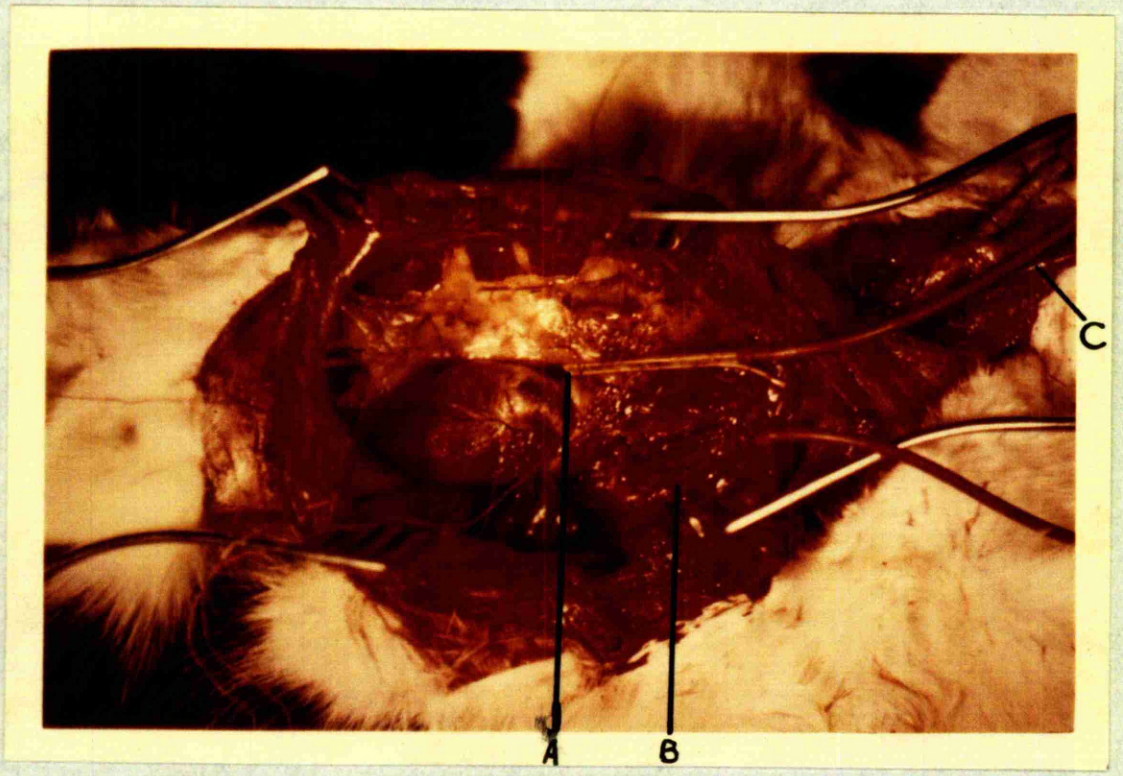


Fig. 16.

Position of cannulae during experiment.

A. Coronary sinus cannula.

B. Coronary arterial cannula passes down left subclavian artery.

C. Cannula in left common carotid artery.

the left common coronary artery. Its distal end was connected by a piece of flexible rubber tubing to the cannula in the left common carotid artery thus forming a closed circuit. See Fig.16. The bulldog clip on the carotid artery was released and when it was ensured that a clear clot-free circuit existed the tip of the subclavian artery cannula was gently introduced into the lumen of the left common coronary artery and tied into place.

Changes in the normal electrocardiographic pattern were prominent during this manoeuvre but if a good blood flow existed the normal pattern was soon resumed. The animal was heparinized before the passage of the coronary cannula by injection of 200 i.u. of heparin into the femoral vein and this was repeated at half hourly intervals following the completion of the manipulative techniques.

Before the addition of any drugs 15 ml. of 0.9 per cent saline solution was given intravenously to restore the blood volume decreased by the unavoidable bleeding. Drugs were introduced into the closed system by injecting them through the rubber tubing joining the carotid and subclavian artery. Electrocardiograph and blood pressure records were taken throughout.

Results

In this series of experiments there was a very high rate/

rate of mortality in the animals used. There were two main causes of this, the large cats which had to be used to obtain a preparation of suitable size frequently showed large deposits of fatty tissue in the region of the origin of the left common coronary artery and during the clearing of this and the subsequent exposure of the vessel and passage of the ligature, damage to the artery or to the wall of the auricle sometimes occurred, this led to fatal haemorrhage. The second cause of death was the auricular or ventricular fibrillation which often occurred whilst the two cardiac cannulae were being inserted. Thismay have been due to stimulation of the pacemaker region. In some animals the blood flow into the coronary artery *via* the cannula was insufficient to permit normal cardiac functioning due to cardiac ischaemia and led to the development of irreversible ventricular fibrillation and death. The routine administration of procaine amide in an attempt to prevent the onset of cardiac irregularities was tried, but this was discontinued because procaine is known to antagonize some of the actions of the *Veratrum* alkaloids and also because of the possibility that procaine amide acts like quinidine, [207to 209] which may produce its anti-fibrillatory effect by slowing the rate of the repolarisation process, possibly by decreasing the outward flux/

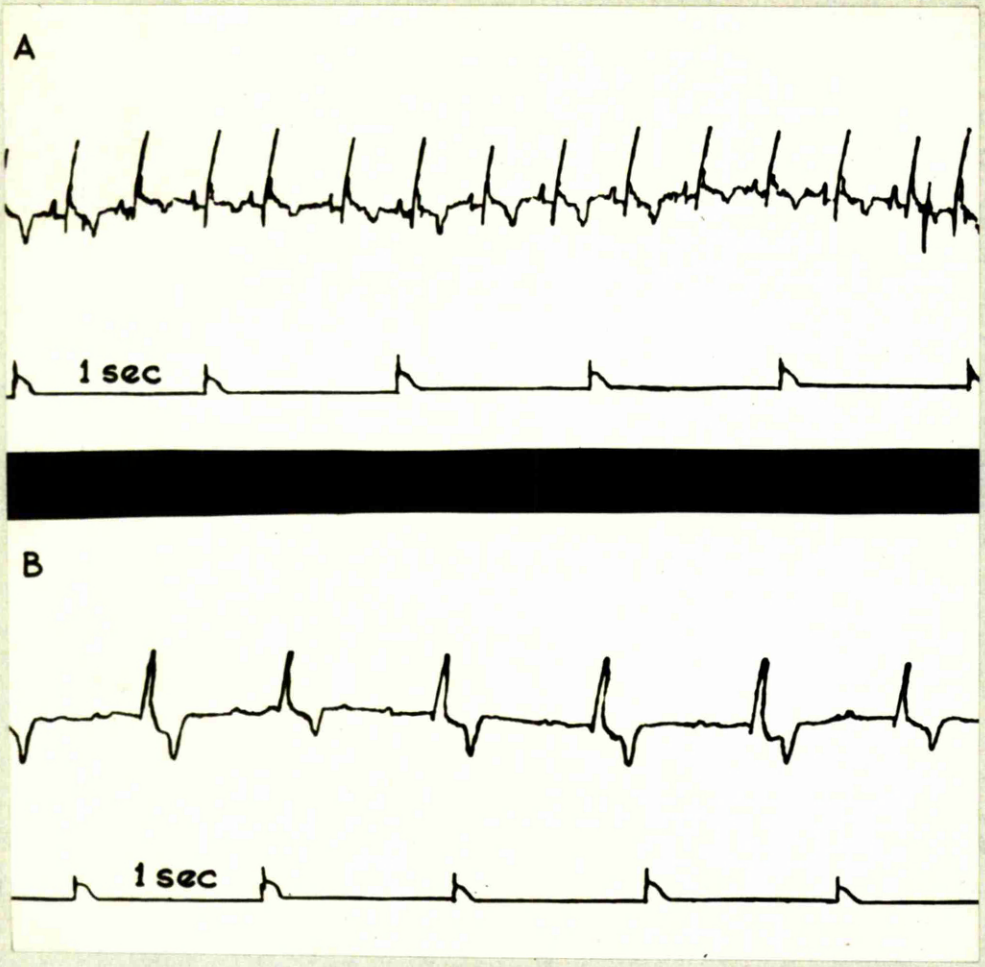


Fig. 17.

Electrocardiogram of cat, lead II, showing the bradycardia produced by the intra-coronary injection of 2 ug protoveratrine.

A. Before injection.

B. 5 seconds after injection.

210,211.

flux of K^+ which occurs during this process.

In all the successful experiments intracoronary injection of 2 ug. of protoveratrine led to a noticeable fall in arterial blood pressure and marked bradycardia. No abnormal changes could be detected in the electrocardiograms but the bradycardia following the injection of the alkaloid was readily seen, Fig. 17. The degree of bradycardia varied considerably between animals, but always occurred to some degree. The incidence of bradycardia was taken as indicating that the drug was reaching the receptor sites. Bilateral vagotomy reduced the degree of bradycardia induced by the protoveratrine.

Tachyphylaxis to the hypotensive action of protoveratrine develops rapidly, the mechanism by which this occurs is not yet known and it was, therefore, decided to leave a period of half an hour between successive additions of the drug. The first dose injected was of 2 ug. followed by a subsequent dose of 5 ug. Due to the deterioration of the preparation with time, only two doses of protoveratrine were given in each experiment.

The plasma K^+ concentration varied between cats and also tended to rise during the progress of the experiment (see also Chapter 5.)

The/

TABLE 6

The Effects of Protoveratrine on the K^+ Concentration of Blood taken from the Coronary Sinus.

Experiment No.	Total Dose of Protoveratrine	K^+ concn. in mEq/l		Percentage change
		Control.	Drug.	
1	2 ug. 2 ug. 5 ug.	1.73 1.73 4.02	1.90 1.78 5.30	+ 9.8 + 2.9 +32.8
2	5 ug.	1.54	2.19	+35.7
3	2 ug. 5 ug.	4.86 4.09	5.11 5.37	+15.8 +30.8
4	2 ug.	3.96	4.6	+16.2
5	2 ug.	2.94	3.58	+21.7
6	5 ug.	1.20	1.65	+37.5

Mean Percentage Increase in K^+ concentration after 2 ug. Protoveratrine = 13.3 $\pm$ 3.2 per cent.

Mean Percentage Increase in K^+ concentration after 5 ug. Protoveratrine = 34.2 $\pm$ 3.3 per cent.

The results obtained in six successful preparations from a total of twenty-three attempts show a marked rise in plasma K^+ concentration following the injection of protoveratrine into the coronary circulation. The results are summarised in Table 6. After the injection of 2 ug. of protoveratrine they show a mean percentage increase in plasma K^+ of 13.3 per cent with a standard error of ± 3.2 per cent and of 34.2 ± 3.3 per cent after 5 ug. of protoveratrine.

These results indicate that the K^+ concentration of the coronary blood rises after the injection of small quantities of protoveratrine into that part of the heart supplied by blood from the left coronary arteries. This region of the heart is believed to be the region from which the majority of apparent impulses of the Bezold-Jarisch reflex originate.

From the data obtained it is impossible to tell whether the K^+ is liberated by a direct action of the drug upon either the myocardium, the nerve endings in the wall of the heart, or whether the bradycardia induced by the drug led to a slower blood circulation and consequently a high plasma K^+ concentration. Bilateral vagotomy reduced but did not abolish the bradycardia by preventing the vagal inhibitory impulses from the vasomotor centre from reaching the heart. Section of the vagi could not be used as a means of eliminating/

eliminating the bradycardia because this abolished the depressor response which was the only indication by which it could be ensured that the drug was reaching the receptor areas. The use of atropine was contraindicated as it has been shown to act as an antagonist to some of the direct effects of the Veratrum alkaloids on smooth muscle.[135]

Due to the very high rate of mortality before the experiments could be finished, and the large number of complicating factors involved in experiments in which manipulation of the heart and great vessels occur, it was decided to treat the results as indicative rather than conclusive and to study the effects of protoveratrine on K^+ release on simpler tissue systems.

CHAPTER 8

THE INFLUENCE OF PROTOVERATRINE ON THE RELEASE OF POTASSIUM IONS FROM FROG SKELETAL MUSCLE.

The work already described has indicated that one of the effects of the Veratrum alkaloids may be to release K^+ into the blood stream but no conclusions were drawn as to the source of the released ions.

Bacq[202] in his early work put forward some evidence that veratrinization causes a slight increase in the K^+ content of the perfusate from perfused frogs' legs, but his experiments were not adequately controlled so that the validity of his results is questionable. Schatzmann and Witt[213] have shown that resting frog sartorius muscles bathed in a K^+-free Ringer's solution lost significantly more K^+ when exposed to strophanthin in concentrations of 10 ug. per ml. than when exposed to the control solution.

The alkamine esters of the Veratrum species and the cardioactive glycosides have a similar basic steroidal skeleton which may account for the similarity of effects of the two groups of drugs.[214] This similarity is most marked on the failing heart where both have been shown to have a positive inotropic action[72,173,215] which is accompanied by an increased performance and oxygen consumption.[50]

During the last decade a large volume of published work has appeared on the effects of the cardiac glycosides on K^+ transport/

transport in different tissues, this work has mainly been confined to cardiac muscle [216 to 220] and erythrocytes [221 to 223] but some studies have been made using frog sartorius muscles.[213,224] The general conclusion arrived at in these studies is that the cardiac glycosides cause a net loss of K^+ from the tissues due to an inhibition of the process by which K^+ is transferred into the cells.

In order to establish if in fact the effects of the active Veratrum alkaloids and the cardioactive glycosides were similar, experiments were carried out using the isolated sartorius muscle of the frog, this preparation was chosen in preference to the other methods mentioned because of the ease with which the material could be obtained and handled.

The amount of K^+ released from a muscle exposed to K^+-free Fenn-Ringer's solution is of the order of 0.1 uEq. per gm. per minute.[213] This is further reduced in normal Fenn-Ringer's solution. For details of this solution see Appendix. It was found to be impossible to detect these small changes in K^+ concentration in the presence of the normal concentration of K^+ in Fenn-Ringer's solution, i.e. 1.8 mEq per litre using the flame photometer available, and in the experiments to be described, all studies were carried out using K^+-free Fenn-Ringer's solution. Schatzmann and Witt[213] used a similar/

similar K^+-free solution and were able to demonstrate an increased K^+ loss from frog sartorius muscles following exposure to strophanthin.

A number of preliminary experiments were carried out to ascertain the effect of exposure of frog sartorius muscles to protoveratrine for short periods of time.

Methods

Method A - Preliminary Experiments

Common frogs, (Rana temporaria), unselected as to age or sex but all approximately 30 gm. in weight, were stunned by a sharp blow on the head and decapitated with a sharp pair of scissors. A probe was passed down the spinal canal to destroy the spinal cord and to prevent any reflex movements. The skin covering both thighs and the pelvic region was reflected to expose the attachments of both ends of the sartorius muscles. Each muscle was very carefully dissected free, care being taken to prevent damage to any of the muscle fibres. The distal tendons were sectioned and the muscle freed by cutting through the pelvic attachment as close to the pelvis as possible. The muscle was then rapidly weighed and it was found that with very careful dissection muscle pairs could be obtained differing in weight by less than 2 mg. Threads were fastened to the tendinous portions of the muscle and the muscle weighted to extend/

extend it to its natural length.

In the preliminary experiments each muscle of the pair was suspended in a 5 ml. capacity organ bath containing the K^+-free Fenn-Ringer's solution which was continually oxygenated and mixed by a stream of oxygen bubbles from a sintered glass distributor. Both muscles were exposed to the drug free bathing fluid for the first 60 minutes, the bathing fluid was totally removed at 20 minute intervals and replaced immediately by fresh solution. After 60 minutes it was assumed that K^+ release was occurring at a steady rate [213] and 10 ug. of protoveratrine in 0.1 ml. of Fenn-Ringer's solution was added to one muscle and 0.1 ml. of control solution added to the other; after 20 minutes exposure to the drug or the control solution, both baths were emptied and the solutions replaced by K^+-free Fenn-Ringer's solution.

The K^+ content of each sample of the bathing fluid was estimated using the flame photometer and the results expressed as uEq. per ml of K^+ after correcting for the small K^+ content of the bathing solution.

The results obtained with this method varied over quite a wide range from 0.6 to 4.3 uEq. per ml. and no significance could/

could be attached to them. To overcome this difficulty the method was modified to enable the muscle to be exposed to the action of the drug for a longer period of time.

Method B

Paired frog sartorius muscles were suspended in 5 ml. of oxygenated Fenn-Ringer's solution by means of two stainless steel hooks attached to the tendinous portions of the muscle. One hook was fastened by a thread to a wire stirrup which fitted across the top of a Pyrex glass test tube and the other consisted of a No. 18 hypodermic needle weighing 1.75 gm. which was bent into the shape of a hook. The muscles were suspended in the bathing fluid for 20 minute periods after which they were removed, shaken free from adhering moisture and re-suspended for a further period in a second tube, this procedure was repeated eight times. The control muscle was exposed to the K^+-free solution for the whole of the experiment, but the test muscle was initially exposed to the control solution for three twenty minute periods to ensure that the rate of K^+ release paralleled that of the control muscle. The five subsequent tubes contained the K^+-free solution to which had been added 2 ug. per ml. of protoveratrine.

The K^+ content of each sample was estimated by means of the flame photometer and using the technique described in the Appendix.

Results/

K Release by Frog Sartorius Muscles after Exposure to Protoveratrine

Semi-hibernatory Frogs

	Experiment No.	1		2		3		4		5		6	
		C	T	C	T	C	T	C	T	C	T	C	T
Total K^+ Output	Before Exposure	1.6	1.6	1.6	1.6	1.3	0.9	1.72	1.68	2.5	2.8	0.7	0.6
	After Exposure	1.1	1.1	1.6	1.6	1.1	1.0	2.08	2.00	1.1	1.0	0.9	0.7
	Difference	+0.5	+0.5	0	0	+0.2	-0.1	-0.36	-0.32	+1.4	+1.8	-0.2	-0.1

Active Frogs

	Experiment No.	7		8		9		10		11		12	
		C	T	C	T	C	T	C	T	C	T	C	T
Total K^+ Output	Before Exposure	4.2	2.2	1.5	0.7	3.95	3.15	2.55	2.30	3.15	3.95	2.2	2.6
	After Exposure	3.0	2.4	0.9	1.3	4.30	3.83	2.45	2.25	3.85	4.30	1.9	2.1
	Difference	+1.2	-0.2	+0.6	-0.6	-0.35	-0.70	+0.1	+0.5	-0.70	-0.35	+0.3	+0.5

C = control muscle - exposed for 20 minutes to the control solution

T = test muscle - exposed for 20 minutes to 10 ug. per ml. protoveratrine.

All concentrations given as total K^+ in uEq. in 5 ml. K^+ free Fenn-Ringer's solution. A positive difference indicates a decrease in K^+ output and a negative difference indicates an increase.

Results

Method A

Schatzmann and Witt[213] found that the amount of K^+ liberated from frog sartorius muscles bears little relation to the weight of the muscles. In these experiments care was however taken to ensure that the difference in weight between the muscles of one pair was not more than 2 mg. The relationship between the K^+ released during the twenty minute test period and the weight of the muscle was studied but the results supported the findings of Schatzmann and Witt and no correlation could be found. It was, therefore, decided to ignore the weights of the individual muscles and to compare directly the output of K^+ from individual members of a pair.

The results obtained are summarised in Table 7., all K^+ levels are expressed as total K^+ in uEq released into 5 ml. of bathing fluid. From the Table the wide variation in the total K^+ in the bathing fluid before the addition of the drug may be seen. This varied from 0.6 to 4.3 uEq. No correlation between K^+ output and the sex of the frogs could be found, but the majority of the low values were obtained from experiments carried out during March and April and the higher values came from ones performed in June. It is possible that in the spring frogs

which/

which have been hibernating all winter have a low muscle K^+ concentration or release it less readily, and that the summer frogs which were well fed have a higher muscle K^+ concentration or that it is more readily exchangeable. No estimate of the age of the frogs used was made and some of the variation may have been due to age differences.

It was expected that the total K^+ concentration in the bathing fluid during the control period would be equal or less than that of the previous sample due to the rate of K^+ release from the muscle either being constant by this time or slightly decreased. This was not found to be the case and in four out of twelve experiments the K^+ concentration during the control period was higher than that during the previous period. In one experiment there was no change, and in seven the expected decrease occurred. No explanation of this discrepancy from the expected results could be found and it was assumed to be due to biological variation.

A similar pattern was seen in the results from the muscles exposed to the protoveratrine, four showed a decrease, one no change and seven an increase in the K^+ concentration.

The figures in the Table refer to the total K^+ level measured in 20 minute samples immediately before and immediately after the addition of the drug. Each muscle was exposed to the drug once only.

The/

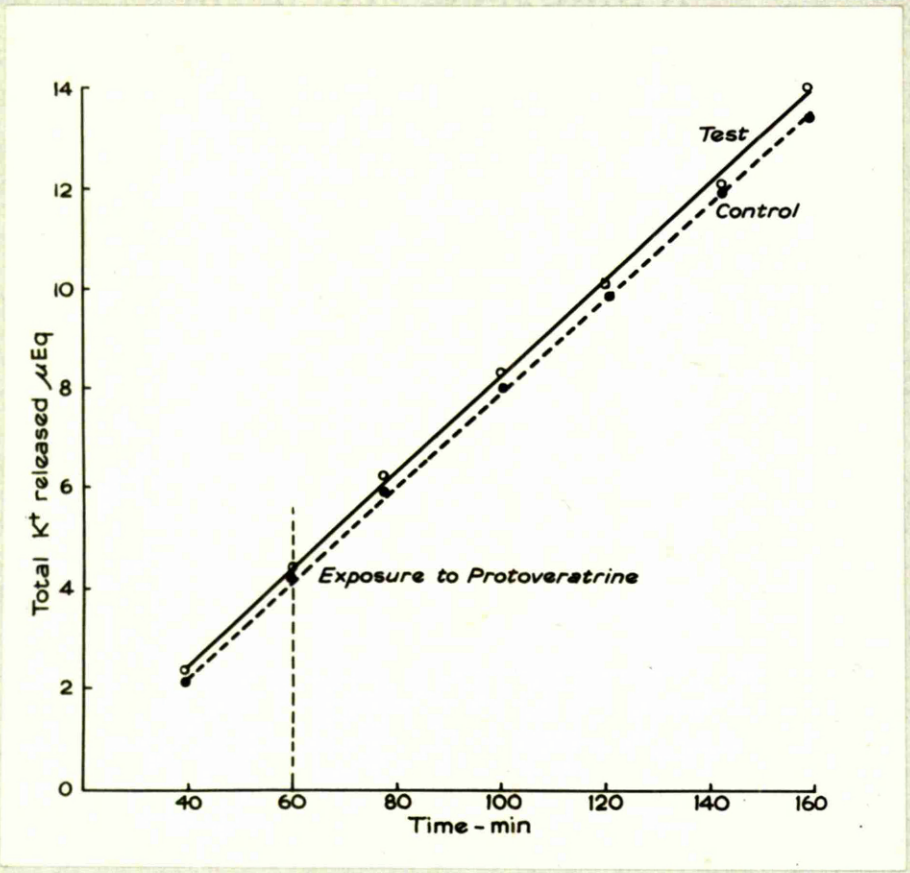


Fig. 18.

Graph showing total K^+ release from a pair of frog sartorii in K^+ free frog Ringer's solution.

The differences between the two successive samples in each group, however, were of the same order in both the muscles exposed to the drug and the corresponding control muscles.

The results expressed in the table were analysed statistically using a paired 't' test. This procedure was used to compare the differences between the K^+ concentrations of the sample before and after the addition of the drug, with those obtained from the paired control muscles. This analysis gave a 'p' value of greater than 0.9, which indicated that there was no significant difference between the amount of K^+ released into the bathing medium by the protoveratrine and by the control solution.

Method B

In the second series of experiments the drug treated muscles were exposed to the drug for a much longer period in order to determine if the drug had a delayed effect upon K^+ exchange. To obtain a clear picture, the total K^+ released was plotted against time and a typical graph obtained from one experiment is shown in Fig 18.

The results are summarised in Table 8., and like the previous experiments show a wide variation in both the total K^+ released from the muscle and in the rate of release.

In/

TABLE 8

Total Release of K^+ by Protoveratrine from the Frog Sartorius Muscle.

Test	12.70	7.62	12.07	6.98	11.21	8.89	10.09
Control	12.06	10.16	12.07	6.35	10.75	9.52	12.10
Difference (T-C)	0.64	-2.96	0	0.63	0.46	-0.63	-2.01

Test	13.98	9.27	8.25	11.57	8.90	8.56	7.30
Control	11.90	9.55	5.41	11.45	6.37	5.40	6.03
Difference (T-C)	2.08	-0.28	2.84	0.12	2.53	3.16	1.27

Test	8.57	7.61	5.40	7.94	9.34	10.73
Control	12.78	7.94	6.98	6.03	10.21	10.65
Difference (T-C)	-4.21	-0.33	-1.58	1.91	-0.87	0.08

All figures refer to uEq of K^+ released into 5 ml. of bath fluid during five 20 minute exposures to the drug or control solution.

$0.8 > p > 0.7$

In every experiment the first two samples showed a relatively high K^+ content, this was followed by a decreased rate of K^+ output which remained almost constant throughout the experiment.

Table 8 shows the mean differences for 20 experiments between the total amount of K^+ liberated from the treated and the untreated muscle during the test period.

When analysed by the paired 't' test no significant difference in the amount of K^+ released was found between the treated and the untreated muscles ($p > 0.9$).

Using the two methods described above, no significant difference between the treated and the control muscles could be demonstrated. This was in contrast to the results obtained by Schatzmann and Witt[213] who used a similar technique and were able to demonstrate a significant increase in K^+ output from frog sartorius muscles under the influence of strophanthin. These results also differ from those obtained by Shanes[122] who used isolated frog nerve and showed that veratrine caused a greater loss of K^+ from treated nerves than from the corresponding control nerves.

One marked disadvantage of this technique is that the use of a K^+ free bathing medium provides unphysiological conditions/

conditions for ionic exchange and this process is therefore liable to be disturbed. The absence of K^+ from the bathing medium would lead to a marked shift in equilibrium conditions causing an increased outward K^+ flux from the muscle. The K^+ thus released from the muscle would be replaced by an equivalent amount of Na^+ which would give rise to a changed ionic composition of the muscle fibre. Preliminary experiments described earlier, (Chapter 2.) have shown that in a K^+ free or low K^+ medium, frog muscles are much less sensitive to the K^+ - sensitizing effect of the Veratrum alkaloids. It may, therefore, be possible that the effects of the alkaloids are not manifested under these conditions.

A second possibility may be that the total amount of K^+ liberated by the drug was very small in relation to the amount released by the muscle into the K^+ free medium to maintain ionic equilibrium. Thus it may be that although the Veratrum alkaloids were causing an increased K^+ release the methods used to detect the K^+, i.e. by the flame photometer were too insensitive to detect such small differences of concentration.

To overcome the difficulties mentioned, methods were developed for studying the exchange of ions during circumstances in which no large ionic gradients existed. These methods involved the use of radioactive trace techniques and are fully described in the following sections.

EXPERIMENTAL WORK

PART 2

CHAPTER 9

THE INFLUENCE OF PROTOVERATRINE A ON THE RELEASE OF RADIOACTIVE POTASSIUM IONS FROM FROG SKELETAL MUSCLE.

These experiments were carried out with a view to overcoming the disadvantages previously mentioned as occurring in the methods described in Chapter 7.

Three different methods were used to study the efflux of radioactive potassium ions, $^{42}K^{+}$ from frog sartorius muscle.

Method A

Frogs weighing between 25 and 50 g. were used in these experiments. No distinction was made between sexes and both winter and summer frogs were used. Calculation showed that a sufficiently high concentration of $^{42}K^{+}$ could be achieved in the tissues by the injection of 1 ml. of a solution of ^{42}KCl. This solution was obtained from the A.E.R.E., Harwell as a sterile, isotonic solution containing 1.15 per cent ^{42}KCl and had a specific activity of approximately 0.15 mC. per ml. at the time of despatch. The radioactive solution was administered by injecting 1 ml. into the dorsal lymph sac.

After an equilibration period of two hours, the animals were killed and the sartorius muscles dissected free by the method/

method described previously in Chapter 8. One of the muscles of the pair was used as a test object and exposed to protoveratrine A and the other as a control.

The experimental procedure was identical with that used for the determination of $^{42}K^{+}$ output, Chapter 8, but the muscles were exposed to 10 ml. of bathing solution instead of 5 ml. and the time of exposure was reduced to 10 minutes. At the end of the experiment both muscles were dissolved in 10 ml. of concentrated nitric acid and the total activity remaining in the muscles was determined.

The radioactivity of the bathing solution, i.e. the total amount of $^{42}K^{+}$ released into the solution, was determined by counting the total volume in a Geiger-Müller liquid counter tube, type M6 or M6H. The counter tube was connected through a probe unit to an Eeko Type N530D automatic scaler. The samples were usually counted for a period of 5 minutes. After correcting for lost counts, background and decay, the counts were expressed as counts per minute.

Gillis and Lewis[225] have shown that this method can be used to study the effects of drugs on $^{42}K^{+}$ release from frog skeletal muscle.

Method/

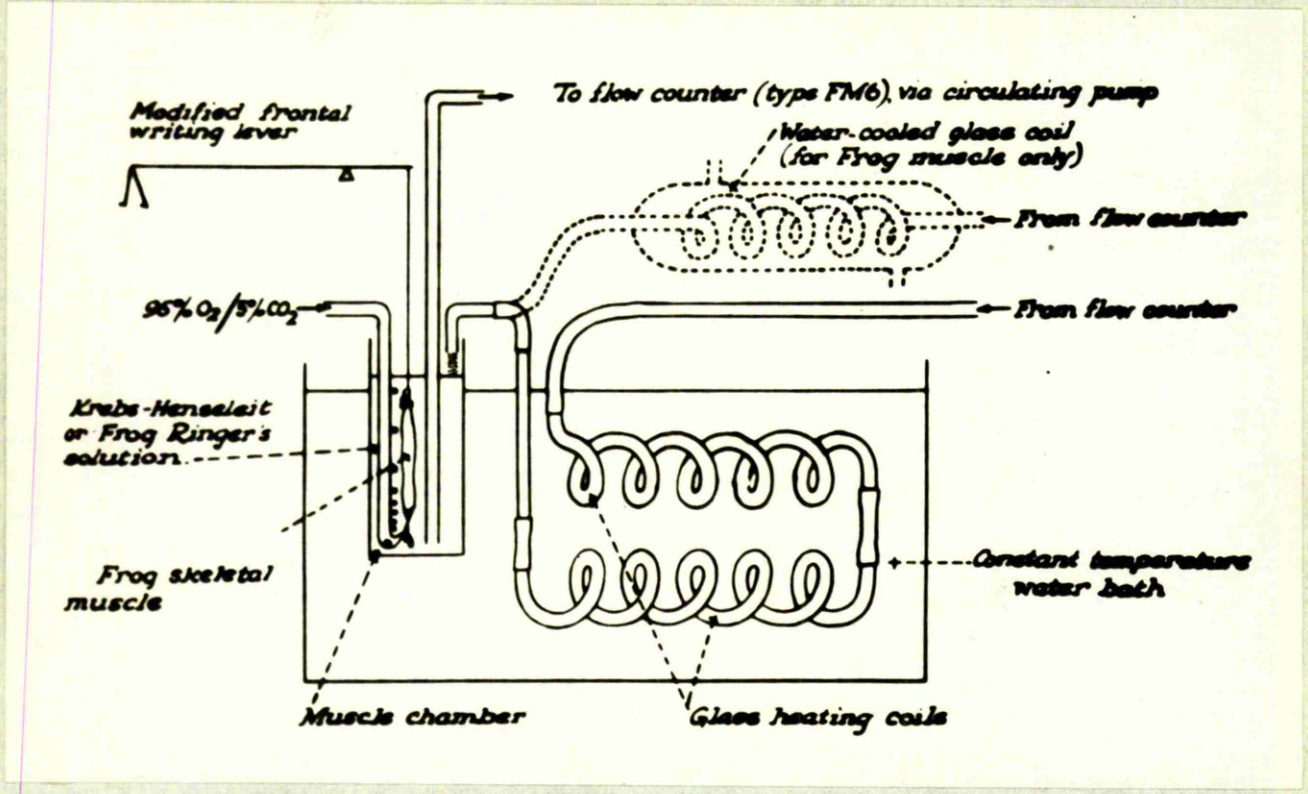


Fig. 19.

Apparatus used for the continuous measurement of the radioactivity of the bathing fluid used to perfuse frog or rat skeletal muscle.

When frog sartorii were used the cooling coil, shown by the broken line, was inserted into the system.

When rat soleus muscles were used the constant temperature water bath was used.

Method B

In an attempt to overcome the errors which occurred when Method A was used and which were due to the transference of the muscles and the consequent entrainment of a certain amount of the released $^{42}K^{+}$ and to unavoidable changes in the environment of the muscle, a method of continuous circulation of the bathing solution round the muscle was devised.

This method was developed in conjunction with Dr.C.N. Gillis and Dr. W.N. Holmes formerly of this department. In this method the muscle was exposed to a constant environment of Fenn-Ringer's solution. The $^{42}K^{+}$ released into this solution was measured continuously by circulating the solution by means of a pump around a closed circuit in which was placed a small tissue bath containing the muscle, a Geiger-Müller Type FM6 liquid flow counter and a cooling coil. The flow counter was connected to a 'Labgear' recording ratemeter which was used to give a continuous written record of the activity of the circulating fluid. A cooling coil was included in the circuit because a slight rise in the temperature of the circulating solution after its passage through the circulatory pump was noted. The circulating solution was kept at $17^{o} \pm 1^{o}C$. The arrangement of the system is shown in Fig. 19. The record obtained was a

continuous/

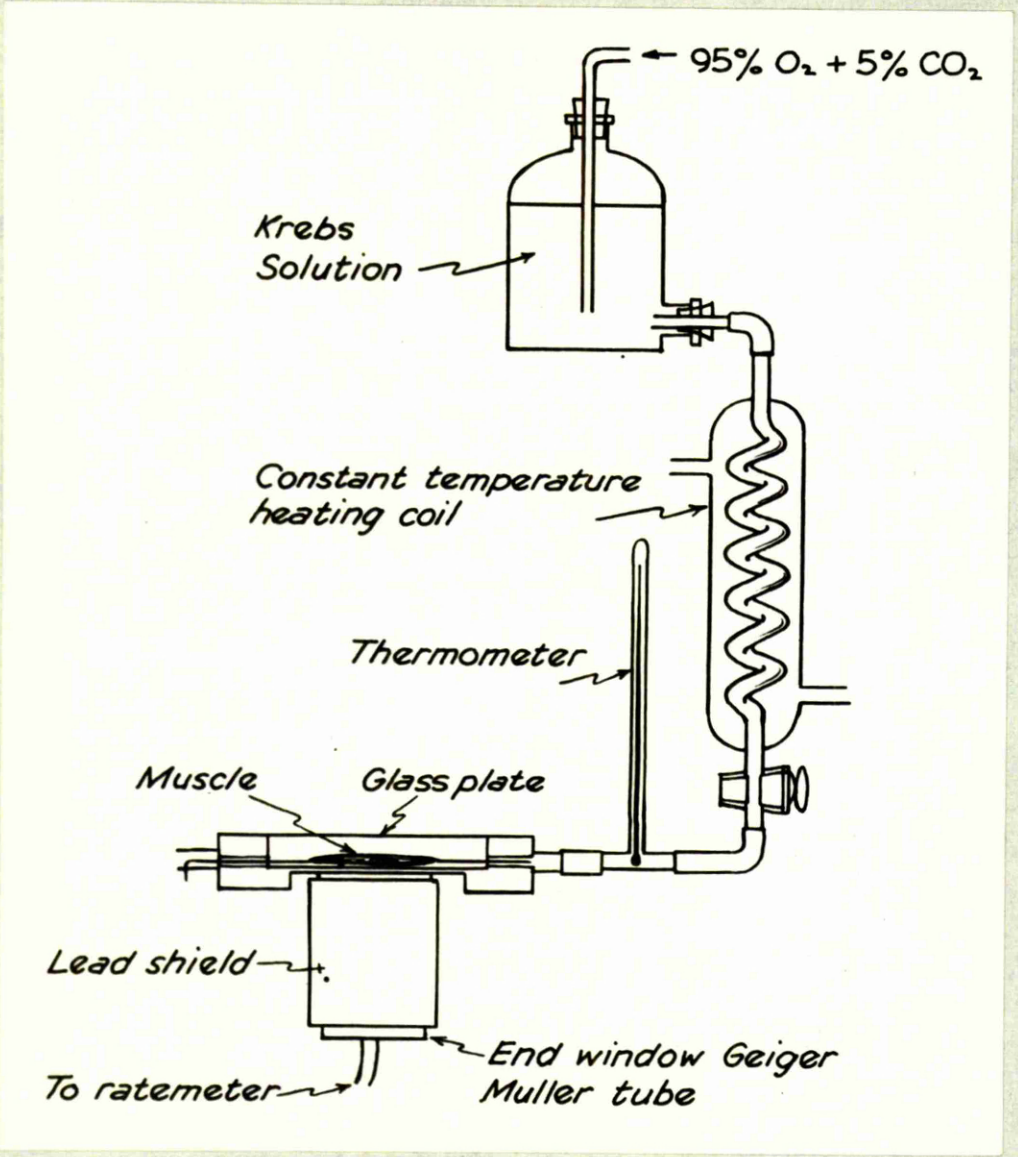


Fig. 20.

Apparatus used for the continuous measurement of the disappearance of $^{42}K^{+}$ from frog or rat skeletal muscle.

continuous measure of the $^{42}K^{+}$ released into the circulating fluid. Substances which increased the release of $^{42}K^{+}$, e.g. reserpine, from the muscle increased the slope of the curve obtained on the record and substances which depressed the $^{42}K^{+}$ release produced a decrease in the slope.[225] Drugs were introduced into the circuit by adding them to the small organ bath which contained the muscle. Any effects on muscular tone were recorded by means of an isotonic frontal point writing lever recording on a smoked surface. The total volume of the system was 100 ml. and sufficient protoveratrine A was added to give a final concentration of 1, 10 or 100 ug. per ml.

Method C

This method is a modified version of that used by Keynes.[226] It differs from the two methods previously described in that the radioactivity of the $^{42}K^{+}$ loaded muscle was measured.

Rapid washing of the muscle by non-radioactive Fenn-Ringer's solution resulted in the exchanged $^{42}K^{+}$ being washed away with a consequent decrease in the radioactivity of the muscle. This activity was measured by placing the chamber containing the muscle above a radiation detector.

The apparatus is shown in Fig.20. It consists of a perspex flow chamber into which are sealed platinum electrodes by means of which the muscle could be electrically stimulated. The bath differed from that described by Keynes in having the muscle/

muscle chamber sealed with a watertight cover. This made it possible to increase the perfusion pressure, thus enabling a rapid rate of inflow to be maintained without risk of the chamber overflowing.

The sartorius muscles of a frog were dissected free by the method described earlier (Chapter 8). Two stainless steel hooks were fastened to each end of the muscle which was then placed across two platinum wire electrodes which were raised 3 mm. above the floor of the chamber. This procedure raised the muscle above the floor of the chamber and permitted the flow of non-radioactive solution over the whole of the surface of the muscle. The threads attached to the muscle were pulled through the inflow and outflow tubes and the muscle was stretched to its normal length. The glass cover plate was then placed over the top of the chamber and secured by means of elastic bands.

The effect of protoveratrine A on the release of $^{42}K^{+}$ from the muscle was determined by adding the drug to the perfusing fluid in an amount adequate to achieve the required concentration. Experiments were done on both stimulated and non-stimulated muscles.

Non-radioactive oxygenated Fenn-Ringer's solution was run through the chamber at a constant rate of 20 ml. per minute.

The/

The disappearance of radioactivity from the muscle was measured by connecting the Geiger-Müller tube to an Ecko Type 522B ratemeter, the pulses from which were fed into a Cambridge electronic pen recorder and the rate of disappearance of radioactivity from the muscle was measured and recorded continually.

This method differed from that described by Keynes in two important points. In Keynes' experiments the muscle after being dissected free and mounted was immersed in Ringer's solution to which had been added $^{42}K^{+}$ and the muscle allowed to equilibrate for several hours. Secondly recordings of the activity were made at intervals, whereas in this method a continuous record was obtained. It was felt that a more even distribution of the $^{42}K^{+}$ within the muscle could be achieved by injecting the animal with ^{42}KCl than by soaking in radioactive Ringer's solution because the $^{42}K^{+}$ would be distributed to the muscle cells from the capillaries within the muscle rather than by diffusion through layers of muscle fibres. The use of Keynes' method is more likely to give rise to conditions in which the fibres in the outer layers of the muscle contain much more $^{42}K^{+}$ than those in the centre and would probably establish a $^{42}K^{+}$ gradient within the muscle. On the other hand distribution by the blood stream would be expected to give a more even concentration of $^{42}K^{+}$ throughout the tissue.

To/

To determine the effect of protoveratrine A on the release of $^{42}K^+$ from actively contracting muscle, the muscle was stimulated electrically by intermittent square wave pulses of 10 volts having a duration of 10 msec. Various frequencies were used but a frequency of 100 per minute was usually taken as a standard. The stimulus was applied by connecting the platinum electrodes of the bath, and which support the muscle, to the terminals of the stimulator. All muscles were made to contract isometrically.

Results

Method A

In the earlier experiments (Chapter 8) the total amount of K^+ passing into the bathing solution was measured, but with the use of $^{42}K^+$ as a tracer the total K^+ flux could only be determined with great difficulty, but it may be assumed that the $^{42}K^+$ flux is proportional to the total K^+ flux.

There were two major factors which made the determination of the total K^+ efflux difficult. The proportion of $^{42}K^+$ to K^+ in the muscles could not easily be determined before the muscles were used. This proportion differed from animal to animal depending upon the absorption of the isotope from the site of injection.

The second complicating factor was that the surface area of the individual muscles differed and the rate of exchange of/

of muscle $^{42}K^{+}$ with the K^{+} of the bathing fluid was not constant between experiments.

Because of these complicating factors, no attempt has been made to determine the total amount of $^{42}K^{+}$ exchanged or the rate constants for exchange of this ion. Errors due to these factors were reduced by using paired muscles. One muscle acting as a control for the other.

The data obtained were expressed in a graphical form by drawing a graph of the number of counts in each sample, i.e. the amount of $^{42}K^{+}$ released against time. Curves were drawn by plotting the logarithm of the total number of counts released per 10 minute period as ordinate against time as abscissa, and drawing the best curve through the points. In addition integrated curves for the data were also drawn.

Attempts were made to fit the curves to a mathematical equation of standard form, but this was found to be impossible.

The exact mechanism by which K^{+} is transferred from intracellular sites to the bathing medium is still not understood, a number of theories have been put forward to account for the known facts [138,227] but none have been found to fit all cases.

Solomon [223,228] and his co-workers have explained K^{+} exchange as involving a three compartment system and they have/

have derived a number of complex equations to fit their data but their studies have been based on simple systems, e.g. erythrocytes, and cannot be applied directly to the more complex processes of muscle ion exchange.

To eliminate the errors due to the variation in the rate of $^{42}K^{+}$ exchange, smooth curves were drawn in preference to histograms. The difference between the control and the treated muscle was determined by measuring the difference in the areas subtended by the curves for the treated and the control muscles. The difference in areas was measured with a planimeter by determining the area of the space bounded by the curves and two lines drawn parallel with the ordinate so as to cut the curves at the points where the drug was administered and where the experiment terminated.

When the total amount of $^{42}K^{+}$ released by the control muscle was greater than that for the treated muscle, the difference wastaken as being positive, when less, as negative.

A null hypothesis that the difference between the test and the control muscle was not significant, i.e. a probability 'p' of greater than 0.05 ($p > 0.05$) was proposed. To determine if this assumption was correct the differences were compared to zero by a paired 't' test and the probability that the differences were due to chance was thus determined.

The graphs drawn from the data obtained from typical experiments/

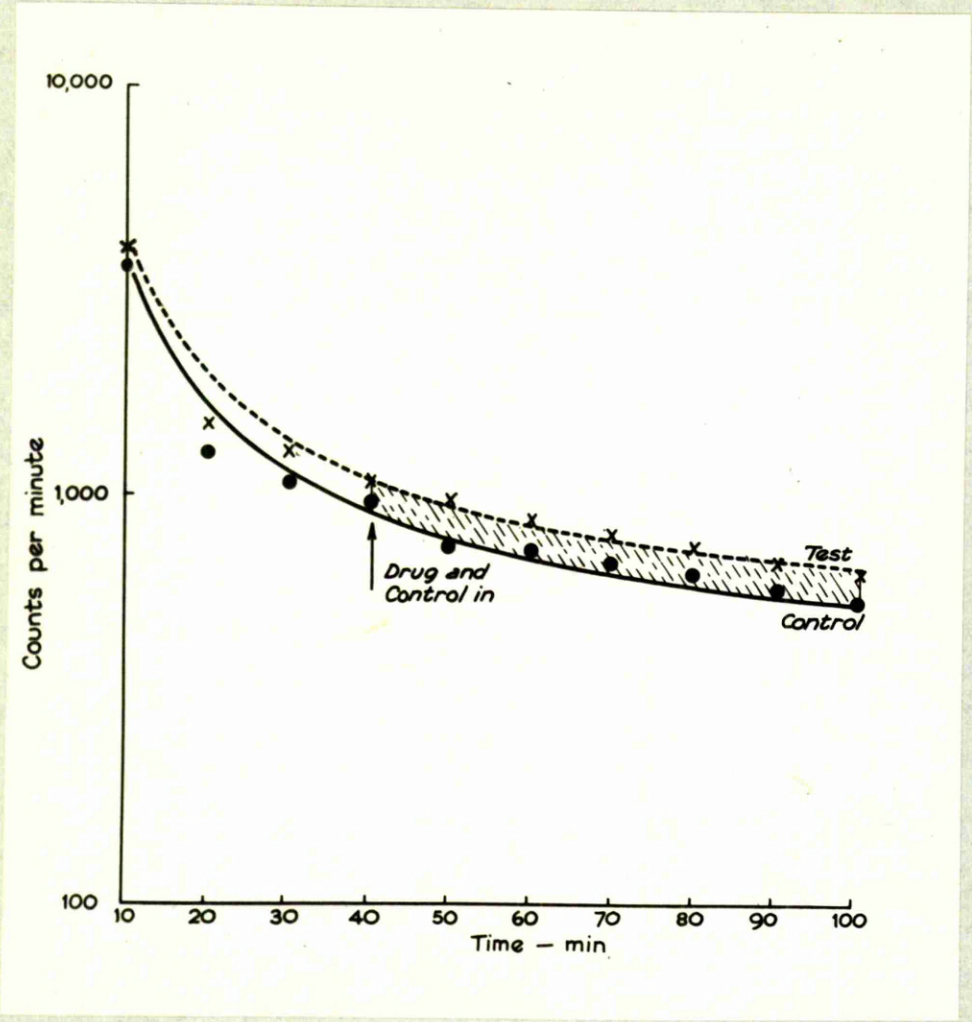


Fig. 21.

The output of $^{42}K^{+}$ from individuals of a pair of frog sartorius muscles previously loaded with $^{42}K^{+}$.

Points show the activity of each 10 ml. sample of bathing fluid.

The shaded area indicates the area measured in each experiment and which was used for the statistical analysis.

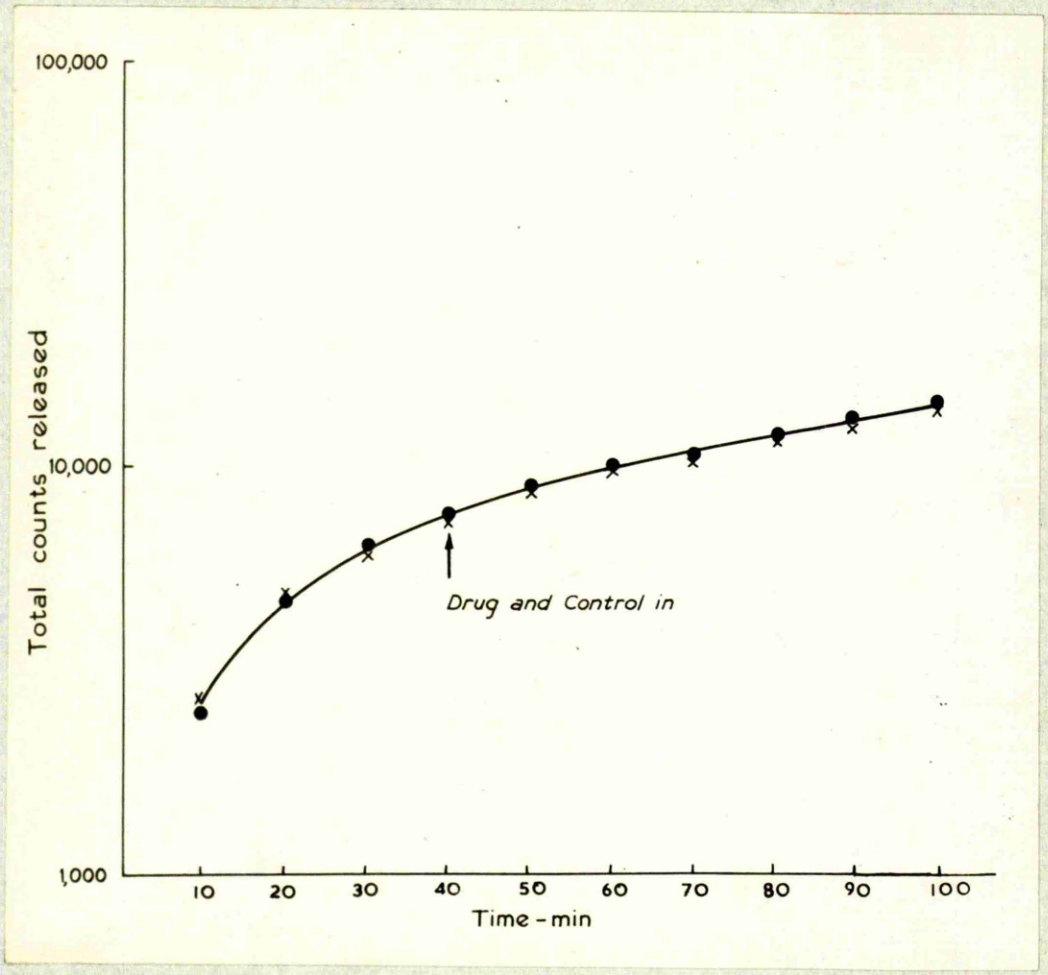


Fig. 22.

Total $^{42}K^{+}$ loss from a loaded frog sartorius muscle.

● Total activity of bathing solution from control muscle.

X Total activity of bathing solution from muscle exposed to a solution containing 10 µg per ml of protoveratrine A.

experiments are shown in Fig. 21 and Fig. 22. It may be seen from these figures that the loaded muscle loses $^{42}K^{+}$ very quickly at the start and that the gradient of the curve is at first steep. This stage is thought to represent the exchange of the extracellular $^{42}K^{+}$ with the non-radioactive K^{+} of the bathing medium. This rapid exchange is followed by a period during which the gradient of the curve is relatively constant and is much less steep. It has been suggested that this portion of the curve represents the exchange of the intracellular $^{42}K^{+}$ with the $^{39}K^{+}$ in the bathing fluid. It is this process which is most likely to be influenced by drugs as it may be that this exchange involves the utilization of energy by the cell.

The results obtained in these experiments are shown in tabular form in Table 9. This table shows the differences between the areas under the curves and also the total $^{42}K^{+}$ remaining in the treated and the control muscles at the end of the experiment.

The significance of the results when determined by means of a paired 't' test gave a value for 'p' of greater than 0.9 for the rate of $^{42}K^{+}$ released by the muscle and the difference between the amount of $^{42}K^{+}$ remaining in the muscle was not significant, $p > 0.7$. This value shows that no significant difference exists between the amounts of $^{42}K^{+}$ released by

the/

TABLE 9

$^{42}K^{+}$ Release from Frog Sartorius Muscle after Exposure to 10 ug. per ml. of Protoveratrine A.

1 Experiment No.	2 Area between curves cm^2	3 Residual $^{42}K^{+}$ in muscle: control	4 in muscle: test	5 Difference (3 - 4)
1	+ 4.4	9,721	11,127	-1,406
2	- 1.4	33,584	30,024	+3,560
3	- 9.5	6,027	7,405	-1,378
4	+ 13.0	22,992	23,836	- 844
5	- 4.1	33,665	33,937	- 272
6	+ 2.3	11,945	10,731	+1,214
7	+ 14.1	5,515	2,031	+3,484
8	- 10.0	6,416	6,255	+ 161
9	- 9.2	12,885	15,082	-2,197
p	> 0.9			> 0.7

Column 1 shows the areas beneath the curves, the sign is + when the control muscle releases more $^{42}K^{+}$ than the treated and vice versa.

Column 5 gives the difference in residual counts between the control and the test muscle.

the treated and the control muscles. These results support the conclusions reached in the earlier work that protoveratrine in concentrations which are sufficient to sensitize frog skeletal muscle to the stimulant action of K^+ do not cause any increase in the K^+ efflux from the muscle.

Method B

Gillis and Lewis[225] have shown that comparable results were obtained using both Methods A and B to measure the $^{42}K^+$ output from frog skeletal muscle.

Ten experiments were carried out in which this technique was employed and using final concentrations of 1,10 and 100 ug. per ml. of protoveratrine A. No evidence could be found to indicate that the rate of $^{42}K^+$ efflux was increased following exposure to protoveratrine A. Under the conditions of the experiment, acetylcholine and reserpine were both shown to cause an increase in the rate of $^{42}K^+$ efflux.

Method C

The rate of release of the $^{42}K^+$ from the muscle was not constant but there was a gradual decrease in the rate of release during the period of the experiment.

The slope of the curve could be measured directly on the record, this gave a measurement of the rate of release of $^{42}K^+$ from the muscle. The total amount of $^{42}K^+$ remaining in

the/

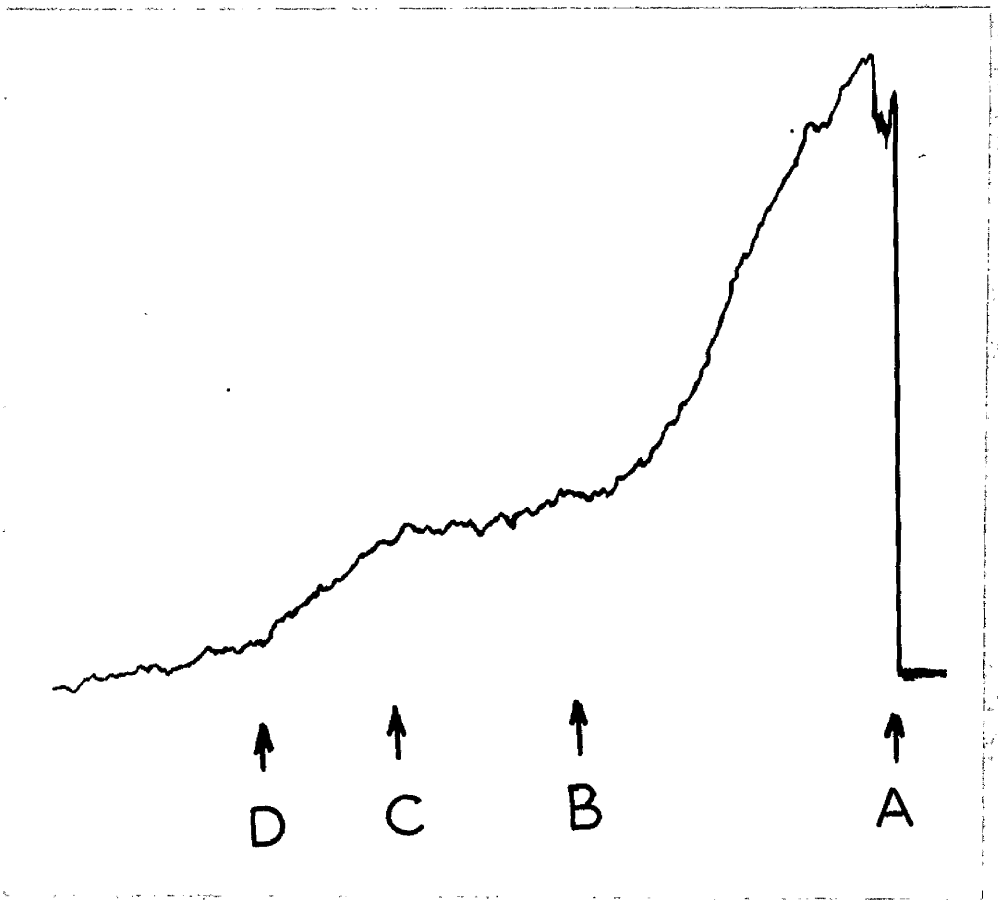


Fig. 23.

Trace showing the disappearance of $^{42}K^{+}$ from a continuously perfused frog sartorius muscle.

At A - muscle place in chamber and continually stimulated at a rate of 100 per minute.

B - muscle perfused by solution containing 10 µg per ml of protoveratrine A.

C - return to normal Penn-Ringer's solution.

C to D - rate of stimulation increased to 200 per minute.

the muscle showed a decrease when the rate of stimulation was doubled, i.e. increased from 100 to 200 stimuli per minute.

The record from a typical experiment is shown in Fig. 23. This record shows that the rate of washout of the $^{42}K^{+}$ from the muscle was increased if the rate of stimulation was doubled. In fifteen experiments carried out by this method protoveratrine A in a concentration of 10 ug. per ml. had no effect upon the release of $^{42}K^{+}$ from frog sartorius muscles whether stimulated or unstimulated.

CHAPTER 10

THE INFLUENCE OF PROTOVERATRINE A ON THE RELEASE OF RADIOACTIVE POTASSIUM IONS FROM RAT SKELETAL MUSCLE.

Most of the work done using isolated tissues as test objects has been carried out using frog skeletal muscle. It is generally believed [155,156,163] that the boundary membranes of the muscle cells show similar properties and have similar functions in all types of muscle. Some authors [219,229] have, however, reported qualitative differences between the effects of cardiac glycosides on K^+ transport in amphibian and mammalian muscles.

To determine whether the alkaloids differed in their effects upon amphibian and mammalian muscle a series of experiments was done using rat skeletal muscle. These were similar to those already carried out using frog skeletal muscle. In addition some investigations were made with the object of determining the influence of the protoveratrine A on the output of K^{42+} from the beating isolated perfused rat heart.

Rat Skeletal Muscle

The majority of experiments using frog muscle were done with the sartorius muscle, a few being carried out with the gastrocnemius muscle. The use of the sartorius muscle in the rat was impossible, however, as this muscle is absent

from/

from rodents.[230] The gastrocnemius muscle is a large thick branched muscle, this made it unsuitable for exchange studies due to (a) the large extracellular volume of the muscle and (b) the subsequent delay in ion release into the bathing medium.

The soleus muscle was chosen as being suitable for the study of ionic exchanges. This is a thin flat muscle readily accessible after reflection of the gastrocnemius muscle, it has no branches and is attached to the bones by two easily visible tendons.

Method

Laboratory bred albino rats weighing between 200g. and 300 g. unselected as to sex were used. The animals were fed on a standard laboratory diet and were between three and five months old.

After preliminary experiments to determine the optimal dose and equilibration period for the isotope, a volume of 2 ml. of isotonic $K^{42}Cl$ was chosen and a suitable equilibration period was found to be two hours. Ginsburg and Wilde[231] showed that the specific activity of rat muscle was 70 to 80 per cent of the maximum reached after intravenous injection of $K^{42}Cl$. 2 ml. of the isotope in the form of an isotonic solution containing 1.15 per cent w/v of $K^{42}Cl$ was given by intraperitoneal injection to the rats. No toxic

effects/

effects were shown at this dose level but they frequently became evident if more than 2 ml. was given at a time.

Two hours after the injection the rats were killed by a blow on the head and exsanguinated by cutting the throat. The thorax was opened and the heart rapidly dissected by cutting through the base of the great vessels and perfused in the way described in Chapter II.

To remove the sartorius muscle the skin covering the hind limb was reflected exposing the muscles of the calf. The tendo calcaneus was sectioned and the gastrocnemius muscle was reflected proximally to expose the soleus muscle. The tendinous attachments were cut through and the muscle removed. When both sartorius muscles had been removed they were rapidly weighed, attached by means of stainless steel hooks to wire stirrups, weighted with a 2 gm. weight and then suspended in 10 ml. of Krebs-Henseleit solution (for details see Appendix) in 15 ml. test tubes. The tubes were kept at 37^{o}C in a water bath and aerated and stirred by a continuous stream of a mixture of 95 per cent O_2/5 per cent CO_2. The pH of the bath fluid was 7.4. The test muscle was immersed in Krebs-Henseleit solution containing 10 ug. per ml. of protoveratrine A. The control muscle was exposed to normal Krebs-Henseleit solution throughout the experiment.

The muscles were immersed for 10 minutes, after which they/

they were removed, shaken free from excess moisture and transferred to a second tube. This procedure was repeated 8 to 10 times and was similar to that previously described for frog muscle.

The radioactivity of each tube was determined by counting all of the solution in an M6 liquid counter.

The rate and total exchange of $^{42}K^{+}$ in the treated muscle was compared with that for the control muscle by plotting graphs of the output of $^{42}K^{+}$ against time. This method was identical to that used for the study of $^{42}K^{+}$ exchange in frog muscle and has been described in the previous chapter.

A number of experiments were carried out using the method of continuous perfusion previously described in Chapter 9. and which was used for the study of $^{42}K^{+}$ efflux in frog muscle. To maintain the circulating fluid at 37°C, it was passed through glass coils immersed in a constant temperature water bath and the cooling coil was dispensed with. The small tissue bath containing the muscle was also set in the water bath. Protoveratrine A in concentrations of 1, 10 and 100 ug. per ml. was added to the circulating fluid. Krebs-Henseleit solution wasused in these experiments and the muscle was aerated with a 95 per cent O_2/5 per cent CO_2 mixture.

The effect of protoveratrine A on the release of $^{42}K^{+}$ from/

from actively contracting electrically stimulated muscles was studied using the apparatus described earlier, Chapter 9.

This was a modification of that used for frog muscle; the bathing solution was passed through a water jacketed heating coil before it entered the perspex muscle chamber. The temperature of the water in the water-jacket was adjusted so that the perfusing fluid was maintained at 37°C. Krebs-Henseleit soltuion was used as the perfusing fluid and was aerated by bubbling a rapid stream of CO_2/O_2 mixture through the solution in the reservoir.

Square wave impulses of 10 volts at a frequency of 100 per minute were used to stimulate the muscle which was made to contact isometrically.

Results

The results for the release of $^{42}K^+$ from the soleus muscles of rats which were exposed to protoveratrine A have been treated in the same manner as was used for expressing the results obtained with frog sartorius muscles under similar conditions.

The methods used were similar in both cases, the chief differences being that for the mammalian muscles the experiments were performed at 37°C and the muscles immersed in Krebs-Henseleit solution and gassed with a 95 per cent oxygen/5 per cent CO_2 mixture.

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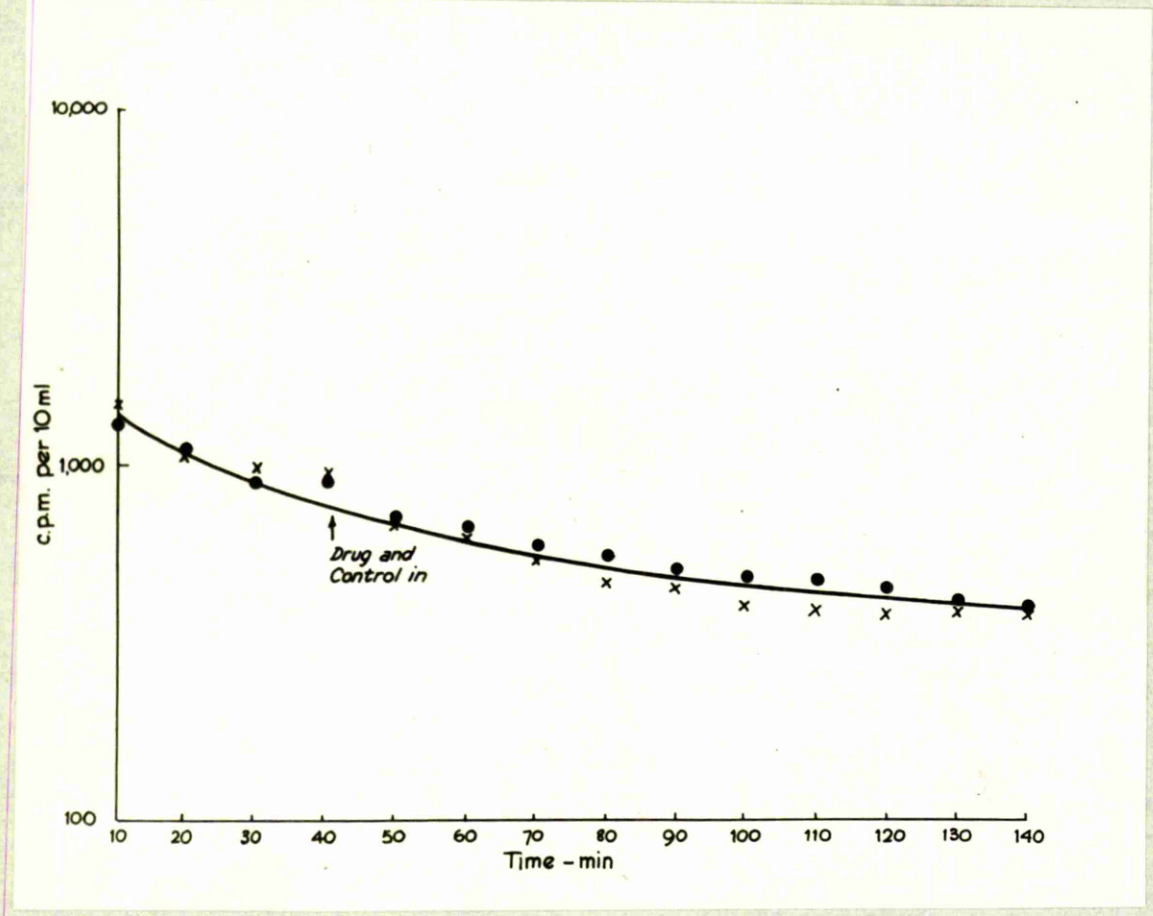

Fig. 24.

Output of $^{42}K^{+}$ from paired rat soleus muscles previously loaded with $^{42}K^{+}$.

O - Activity of bathing solution from control muscle.

X - Activity of bathing solution from muscles treated with 10 ug per ml of protoveratrine A from point indicated.

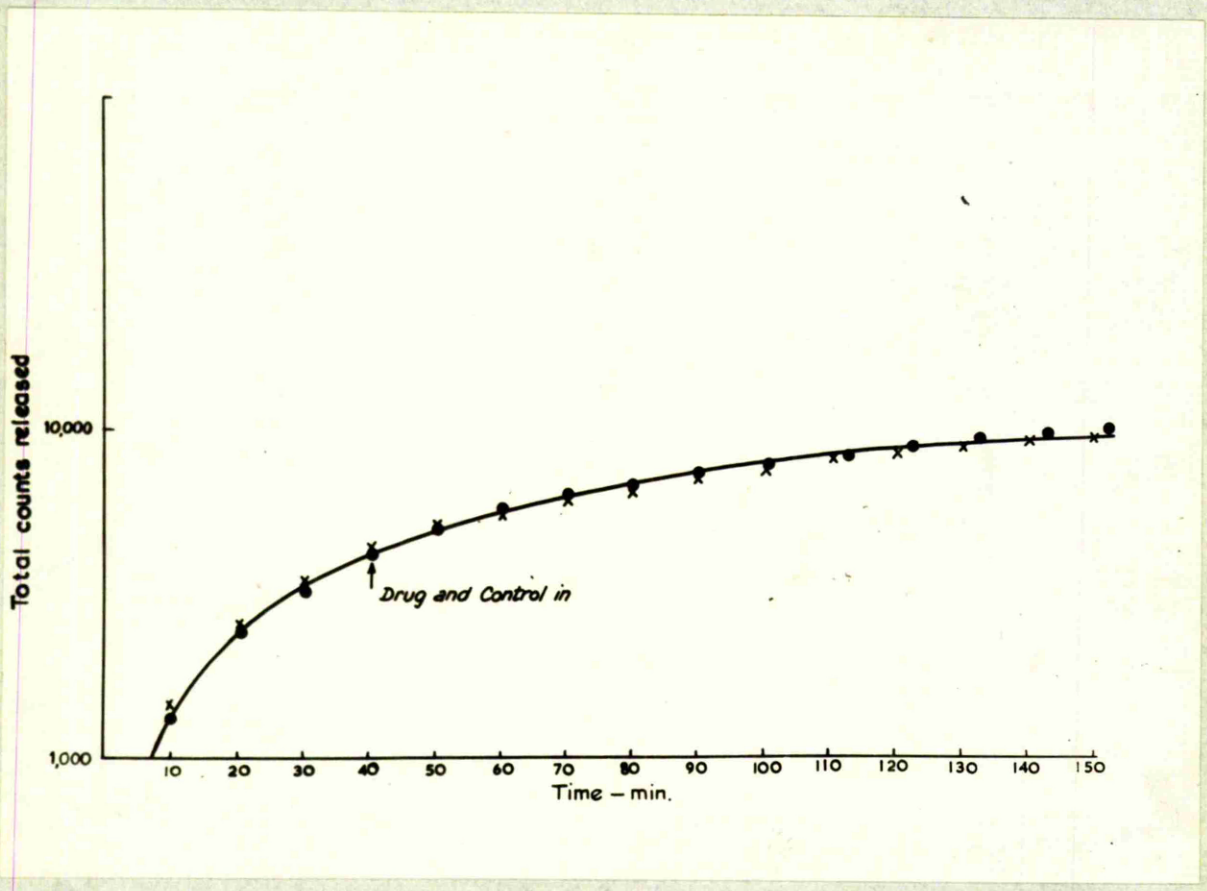

Fig. 25.

Total $^{42}K^{+}$ loss from $^{42}K^{+}$ loaded rat soleus muscles.

O - Total activity of the control solution.

X - Total activity of the solution containing 10 ug per ml protoveratrine A.

The reasons for using the method described and for the statistical procedures by which the results were interpreted are similar in both cases. The curves obtained from a typical experiment are shown in Fig 24 and Fig. 25. The first graph shows the result of plotting the logarithm of the counts obtained from each sample as ordinate against time as abscissa and the second graph is plotted from the same results and was obtained by plotting the logarithm of the total $^{42}K^{+}$ output as ordinate against the time as abscissa. This second graph is, therefore, the integrated curve obtained from the same data. Very little difference could be seen in the curves obtained, the results were analysed by measuring the areas between the curves with a planimeter. When the control muscle exchanged its $^{42}K^{+}$ at a lower rate than the treated muscle this value was given as positive. When it exchanged at a higher rate the value was taken as being negative. The differences in the areas by the curves were compared to zero using a paired 't' test. These differences are shown in tabular form in Table 10. The result of the 't' test performed with this data gave a 'p' value of between 0.7 and 0.8.

This value of 'p' is not considered to be significant. It was, therefore, concluded that protoveratrine A in concentrations of 1 ug. and 10 ug. per ml. has no significant effect upon the rate of exchange of $^{42}K^{+}$ in rat skeletal muscle./

muscle. This conclusion was supported by the results obtained from continuous perfusion experiments.

In 10 continuous circulation experiments no increase in the rate of $^{42}K^{+}$ release from rat soleus muscles could be detected using protoveratrine A in concentrations of 1, 10 and 100 ug. per ml.

Six experiments were carried out using the washout technique in stimulated rat soleus muscles but in no case could any effect be noticed following exposure to protoveratrine A in concentrations of 1 and 10 ug. per ml.

The results obtained from this series of experiments indicate that protoveratrine A in doses up to 100 ug. per ml. has no significant effect upon the release of $^{42}K^{+}$ from stimulated or unstimulated rat striated muscle.

TABLE 10

Date	Weight of muscles in mgms		Difference in the area between curves cm^2
	Test	Control	
5.2.57.	120	121	0
19.2.57.	87	88	+ 32.0
5.3.57.	118	116	0
12.3.57.	124	124	0
19.3.57.	78	78	- 4.7
26.3.57.	84	84	-42.6
2.4.57.	92	92	- 6.6
28.1.58.	86	85	+ 0.4
Mean	99	99	- 2.7

Effect of Protoveratrine A on the $^{42}K^{+}$ Efflux from Rat Skeletal Muscle.

$$e_d = \pm 7.12$$

$$t = \frac{-2.7}{\pm 7.1} = 0.379$$

$$0.8 > p > 0.7$$

Differences in the areas between the curves obtained by plotting the $^{42}K^{+}$ release from rat soleus muscles.

All test muscles exposed to 10 ug. per ml. Protoveratrine A.

For further details see text.

THE EFFECT OF PROTOVERATRINE A ON THE EFFLUX OF RADIOACTIVE POTASSIUM IONS FROM PERFUSED ISOLATED RAT HEARTS.

These studies were undertaken to determine whether there was an increased release of K^+ from isolated rat hearts following the administration of protoveratrine A. This has already been demonstrated as taking place in the heart *in situ*, (Chapter 7).

Because of possible direct drug-induced effects upon the inotropic and chronotropic mechanisms of the heart, the study of $^{42}K^+$ exchange in heart muscle is more difficult than it is in skeletal muscle. This difficulty has led various workers who have studied the effects of drugs on K^+ exchange in cardiac muscle to use electrically driven, isolated auricles[220,232] or ventricles[218] in preference to the whole heart.[216]

The earlier experiments were carried out using spontaneously beating hearts, but the variation in the heart rate following the administration of protoveratrine A and thus in the efflux of $^{42}K^+$ led to the adoption of a method in which the heart was driven electrically.

Methods

Rat hearts dissected out as described in Chapter 10 were removed and placed in a Petri dish containing oxygenated Locke's solution at 34°C, the blood was gently squeezed out and any extraneous fat and connective tissue was removed.

A/

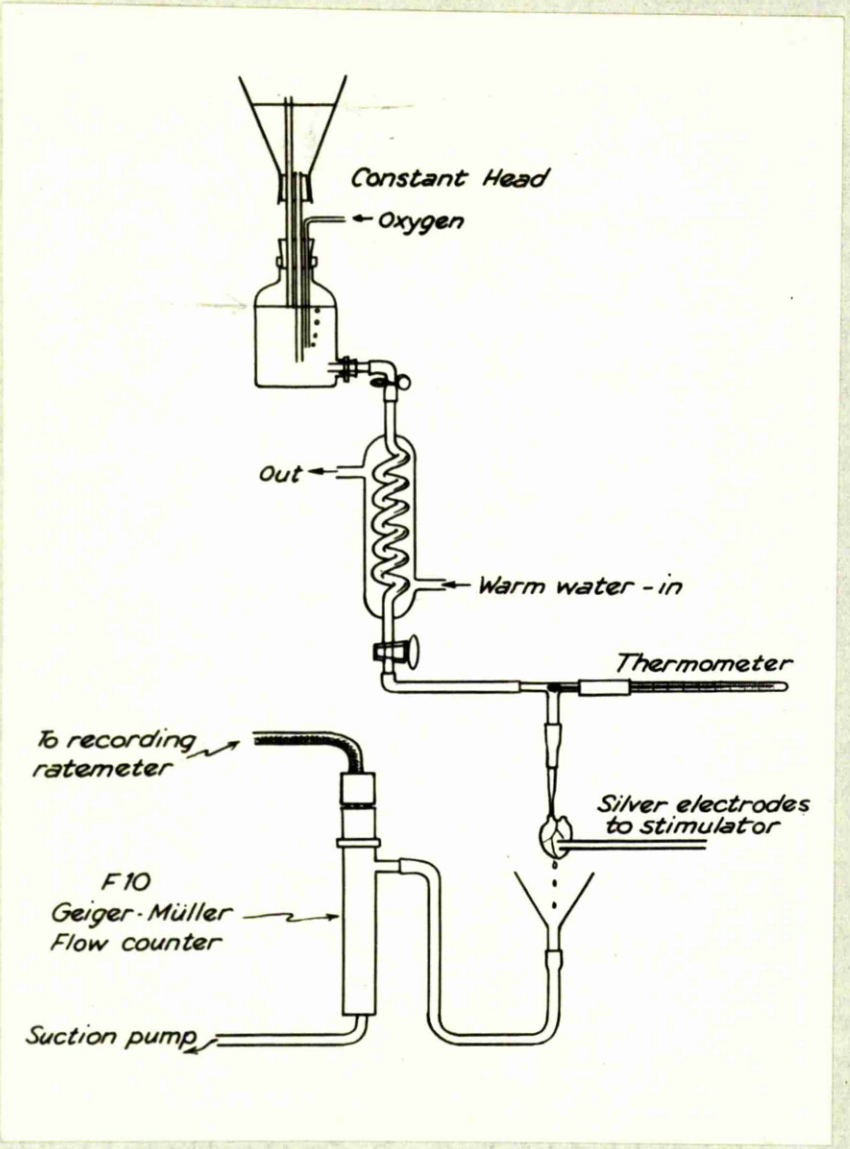

Fig. 26.

$^{42}K^{+}$ Apparatus for the continuous measurement of efflux from the electrically driven, isolated, rat heart.

A glass cannula containing a little heparin was tied into the aorta with its tip distal to the aortic valves and the heart perfused with oxygenated Locke's solution at a temperature of 34 to 35°C after the method proposed by Langendorff.[233] Perfusion by this technique has been criticised by many workers, and Wegria[234] in his review of the pharmacology of the coronary circulation gives a number of references to these published criticisms. The major objections to using this method for the accurate study of coronary flow are that a true measure of the coronary flow is not obtained if the aortic valves are incompetent and that a constant perfusion pressure is not maintained in the original Langendorff system. No control can be exerted over the first of these two factors but the second objection was overcome by using a constant head device see Fig.26. to maintain a constant perfusion pressure.

Administration of drugs was achieved by injecting them into the perfusing fluid through the rubber tubing connecting the cannula to the perfusion system.

The $^{42}K^{+}$ released into the perfusion fluid was measured by two methods. In the first of these the effluent was collected for a 3 minute period every 10 minutes throughout the period of the experiment. The volume of each sample collected was measured and made up to 10 ml. with water. The radioactivity of this solution was then counted using an M6 liquid counter connected to an automatic scaler as described/

described previously.

After about 30 minutes the heart was usually beating regularly and it was assumed that by this time the K^+ exchange would be proceeding at a constant rate and that extracellular $^{42}K^+$ had been washed out.

Protoveratrine A in a total dose of either 2 or 5 ugm. was injected through the rubber connecting tubing into the perfusion fluid 30 seconds before a sample was due to be collected. The addition of the protoveratrine A frequently led to a slight degree of bradycardia.

The volume of the effluent varied slightly during the collection period, it was usually between 2 mls and 5 mls. but no improvement in the shape of the graphs was obtained when the results were expressed as counts per unit volume and the volume was ignored when expressing the results.

Two major drawbacks in the method just described existed. One was that activity of the effluent could not be assessed during the experiment and thus it was impossible to tell when the $^{42}K^+$ flux was constant. The second objection was that the injections of protoveratrine A sometimes showed a digitalis-like cardiotonic action due to the heart being in a hypodynamic condition. This resulted in cardiac slowing which was usually accompanied by an increased cardiac output and hence a possible increase in the output of $^{42}K^+$. In an attempt to

overcome/

overcome this difficulty the heart was driven at a constant rate. This was achieved by delivering square wave impulses from a stimulator at the rate of 120 per minute. The heart was stimulated by attaching a pair of fine silver hooks to the right ventricle in the region of the A-V node. Impulses of 10 volts lasting 10 msec. were used to stimulate the heart. It was found that the heart could be kept beating for up to three hours before any irregularities in rhythm developed. When this preparation was used protoveratrine A could be added to the perfusion fluid without producing any alterations in heart rate or cardiac output.

The acquisition of a recording ratemeter made it possible to determine the activity of the effluent as it was collected. This was achieved by allowing the effluent to flow through an F10 ethylene quenched Geiger-Müller tube connected to the recording ratemeter (see Fig.26.). The amount of radioactivity was recorded on a moving chart and when a smooth curved was being traced, i.e. the $^{42}K^{+}$ of the heart was exchanging at a constant rate with the $^{39}K^{+}$ of the perfusion fluid, the drug was added and its effect upon the $^{42}K^{+}$ exchange was recorded as a change in the slope of the record.

Results

In the preliminary experiments samples of the effluent collected/

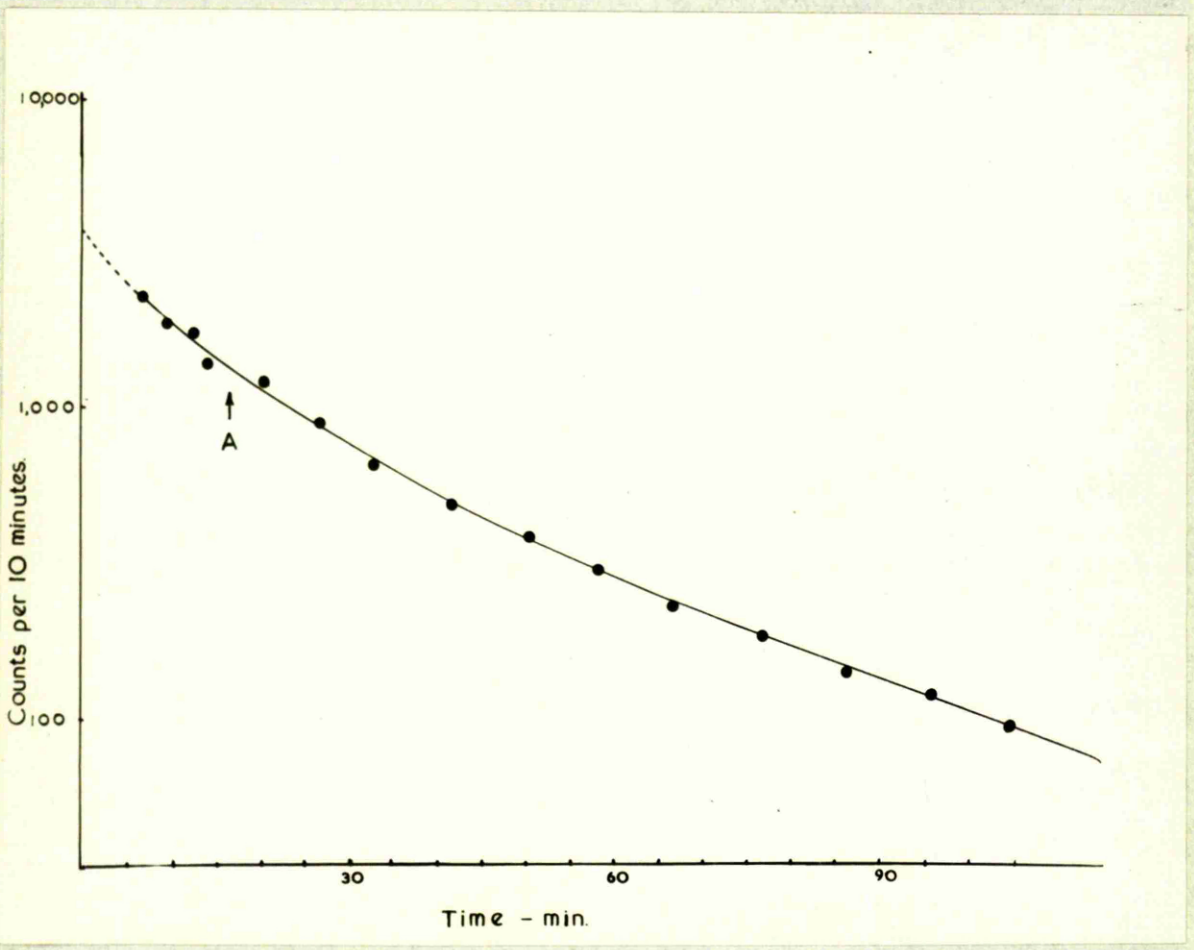

Fig. 27.

Graph showing the decline with time of the radioactivity of the effluent from a $^{42}K^{+}$ loaded, isolated, perfused rat heart.

At A 2 µg protoveratrine A was added to the perfusate.

collected at specified intervals were counted. When the logarithm of the total activity of each sample was plotted against time a smooth curve was obtained similar in shape to those obtained for rat and frog skeletal muscle.

The curve obtained from the results of a typical experiment is shown in Fig.27. From this it may be seen that the rate of exchange of $^{42}K^{+}$ occurs rapidly at first, this is represented by the initial steep portion of the curve. This is probably due to the washing out of extracellular $^{42}K^{+}$ from the heart. This steep portion is followed by a period during which the gradient of the curve is less steep and remains regular. This portion represents the exchange of intracellular $^{42}K^{+}$ with the K^{+} of the bathing solution.[10,11] In the preliminary experiments the administration of a total dose of 2 or 5 µg. of protoveratrine A sometimes led to an increase in the $^{42}K^{+}$ content of the effluent but it also frequently caused some alteration in the heart rate. The differences in radioactivity between the sample of effluent collected immediately before the administration of protoveratrine A and the sample collected whilst the drug was acting were very small and close to the limits of experimental error. They did, however, indicate that more refined methods were required if results of any significance were to be obtained.

When the hearts were kept beating at a constant rate the/

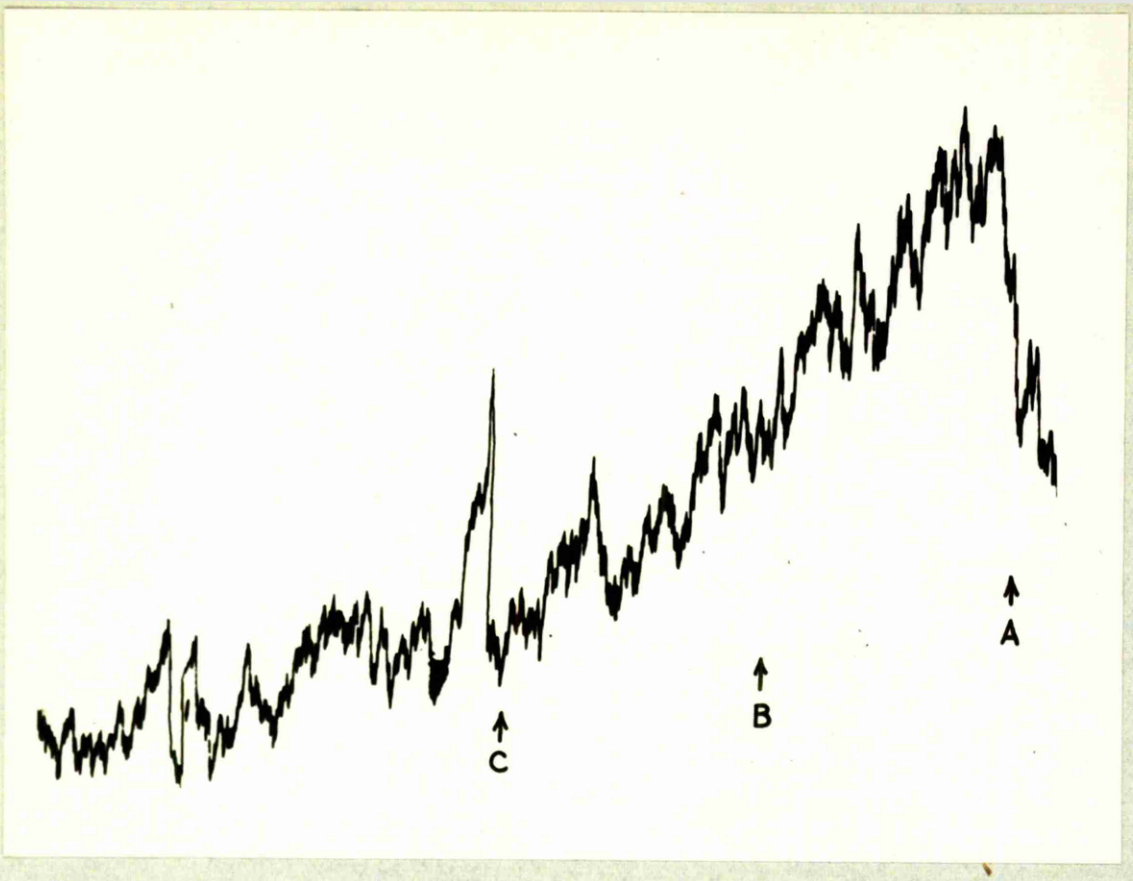

Fig. 28.

Record showing output of $^{42}K^{+}$ from driven isolated perfused rat heart.

A. Start of experiment.

B. Addition of 0.4 ml. control solution.

C. Addition of 2 µg. protoveratrine A.

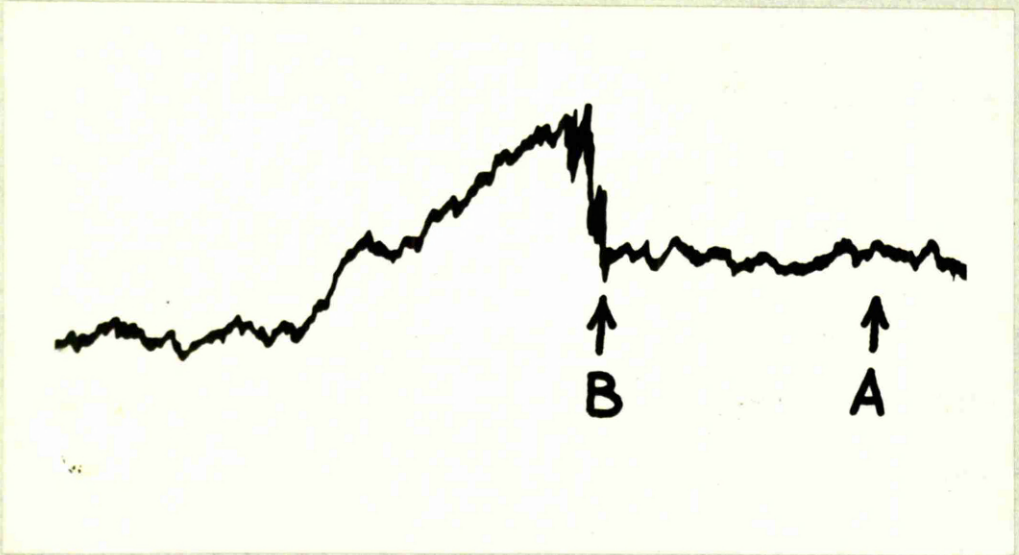

Fig. 29.

Record as above but at a later stage.

A. Addition of 0.4 ml. control solution.

B. Addition of 5 µg. protoveratrine A.

the administration of protoveratrine A to the heart in a total dose of either 2 or 5 µg. caused a consistent rise in the rate of $^{42}K^{+}$ output. This is clearly shown in Fig. 28. A steep rise in the trace occurred following the drug. This rise was not due to any change in perfusion pressure caused by the injection of the small volume 0.2 ml. drug solution because when double the volume of control solution was added no change in $^{42}K^{+}$ output occurred.

The records from two typical experiments are shown in Fig. 28 and Fig. 29. In Fig. 28. the heart loaded with $^{42}K^{+}$ was set up at A and a steady fall in the output of $^{42}K^{+}$ occurred. At B 0.4 ml. of control solution was added and this had no significant effect on the output of $^{42}K^{+}$. At C, 2 ug. of protoveratrine A was administered. This was followed by a marked rise in the level of radioactivity of the effluent. As the drug was washed out the rate of $^{42}K^{+}$ output returned to the previous level.

Fig.29. shows that effect of adding 5 ug. of protoveratrine A to the heart when the rate of exchange of $^{42}K^{+}$ had decreased and was exchanging at a constant rate. In this trace the time constant of the ratemeter was set at 80 seconds compared with a setting of 20 seconds in Fig. 28, consequently the rate of increase appears to develop and return to the base line less rapidly.

The/

The amount of $^{42}K^{+}$ released by the addition of protoveratrine varied from preparation to preparation but in all 10 experiments an increase in the rate of output of $^{42}K^{+}$ occurred. No estimates were made of either the total $^{42}K^{+}$ output or the total amount of K^{+} released during the action of the drug. An increase of up to 100 per cent in the counting rate was seen in some experiments.

Since this work was done a paper by Vick and Kahn[235] has been published; these authors have shown that the perfusion of guinea-pig hearts with a veratridine containing solution produced a greater release of K^{+} from isolated, perfused hearts than when a drug-free perfusion solution was employed.

The findings described here indicate that small total doses of protoveratrine A introduced into the coronary circulation can cause an increased rate of output of $^{42}K^{+}$ from perfused, isolated rabbit hearts which are kept beating at a constant rate.

These results support the findings obtained using mammalian hearts in situ.

CHAPTER 12

THE INFLUENCE OF PROTOVERATRINE A ON THE UPTAKE OF RADIOACTIVE POTASSIUM IONS BY FROG SKELETAL MUSCLE.

To obtain a clearer picture of the relationships existing between the mode of action of the Veratrum alkaloids and the significance of the potassium ion it was thought necessary to study the influence of a pure alkaloid on the uptake of K^{42+} by skeletal muscle from the external bathing medium. Three different methods were used in this study.

Method A

A number of preliminary experiments were conducted to determine the effect of protoveratrine A on the uptake of K^{42+} from the bathing medium. Frogs of approximately the same gross weight were killed, pithed, both gastrocnemius muscles carefully removed and each one weighed. One muscle of each pair was used as a test object and the other as its control. The muscles exposed to the drug were taken alternately from the left and right legs of successive frogs and up to eight frogs were used in each experiment. The muscles were placed into 10 ml. beakers each containing 6 ml. of Fenn-Ringer's solution, some of the K^{+} of which had been replaced by K^{42+}, (for details of the composition see the Appendix). The test solution contained 10 ugm. per ml. of protoveratrine A. The experiments were carried out at a room temperature of 18°C. and the solution had a pH. of 7.4.

The/

The test muscles and their controls were removed from the radioactive solution at successive 30 minute intervals, rapidly blotted with absorbent tissue paper and re-weighed. Each muscle was then dissolved in 8 ml. of 'Analar' concentrated nitric acid and when completely dissolved the volume was made up to 10 ml. with the acid.

The total radioactivity of the muscle was determined by counting the acid in an M6 liquid counter Geiger tube, the counts were recorded on an Ecko Type 550D automatic scaler and expressed as counts per minute.

The results showed a wide variation and the method suffered from two major disadvantages (a) the muscles were thick and the $^{42}K^{+}$ exchange only occurred slowly and (b) there was a wide variation in the weights of muscles obtained from frogs of the same gross weight. To overcome these difficulties a thinner muscle, the sartorius, was used and the $^{42}K^{+}$ uptake followed continuously in each muscle over a longer period of time. This also allowed the use of a smaller number of frogs.

Method B

Direct Determination of the Uptake of $^{42}K^{+}$ of Muscle

The paired sartorius muscles of a pithed frog were carefully exposed and dissected free, using the method of dissection previously described in Chapter 8. The muscles were/

were then rapidly weighed. It was found that by careful judgement and selection it was possible to obtain paired muscles differing in weight not more than 2 mg.

Stainless steel hypodermic needles bent into identically shaped hooks were attached to the tendons at each end of the muscle and suspended from the barrel of a 1 ml. tuberculin syringe in a vessel containing 15 ml. of Keynes's modification of frog Ringer's solution, in which some of the K^+ had been replaced by radioactive K^{42+}. The composition of this solution is based on the figures for frog plasma given by Fenn. (For details of the composition of this solution see the Appendix.) One muscle was used as a control and placed into the radioactive Fenn-Ringer's solution and the other was placed in a similar solution to which had been added sufficient protoveratrine A to give a final concentration of 10 ug. per ml.

Before exposing the muscles to the radioactive Fenn-Ringer's solution, aliquot portions of both solutions were diluted 1 in 100 and the radioactivity of each solution determined by counting 10 ml. in an M6 liquid counter. If the activities of each solution differed by more than 1 per cent they were discarded and a fresh solution prepared.

The solution was kept agitated and oxygenated by a constant stream of oxygen and was maintained at a room temperature of 16° to 18°C and at pH 6.8. The muscles were

left/

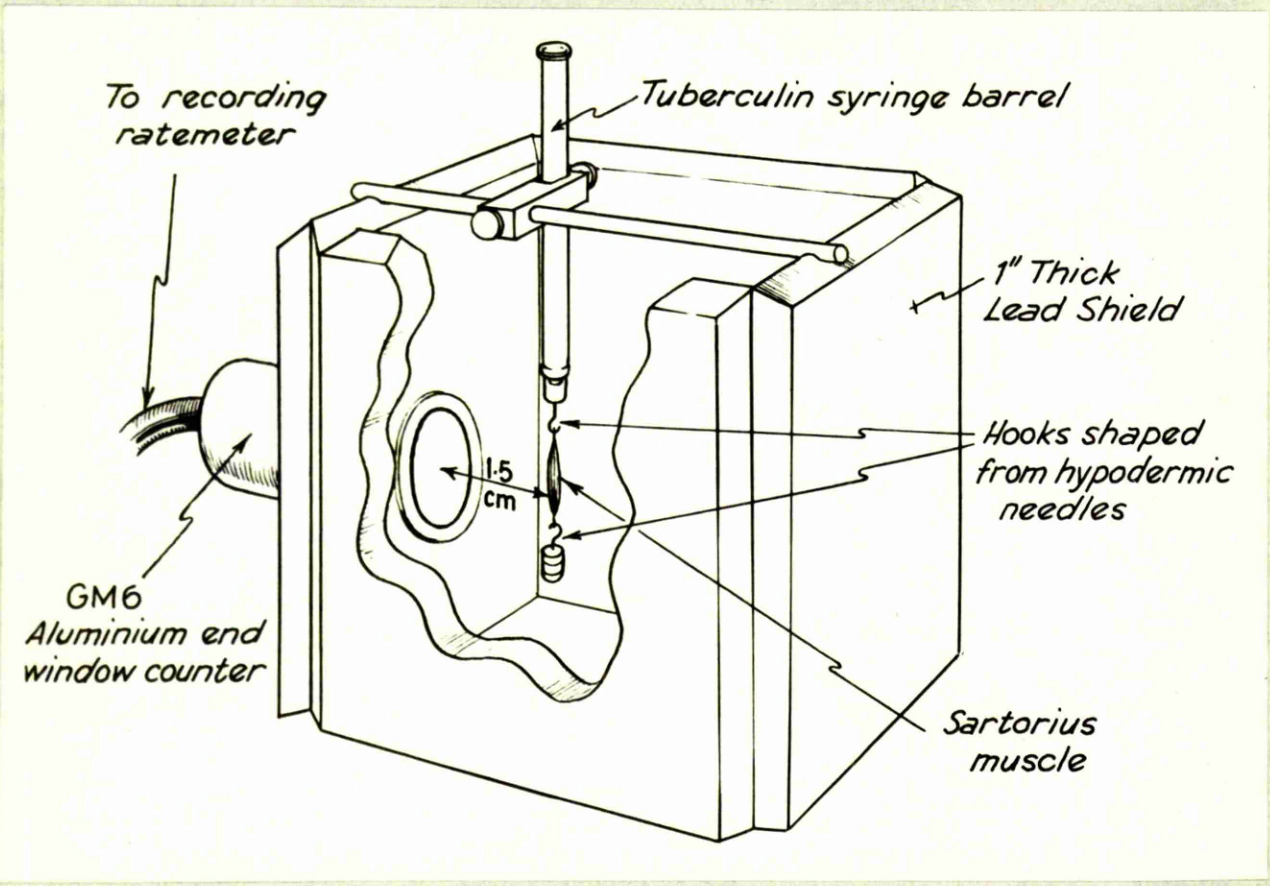

Fig. 30.

Apparatus used for the measurement of $^{42}K^{+}$ uptake by frog sartorius muscles.

left immersed for 10 minute or 20 minute periods, they were then removed from the solution and each side of the muscle was washed for 5 seconds in a stream of non-radioactive Fenn-Ringer's solution and lightly blotted with absorbent tissue paper to remove adhering moisture.

The muscle was then attached by means of the bent needles to a syringe barrel which was so arranged that the muscles were suspended 1.5 cm. from the window of GM_4 aluminium end-window Geiger counter tube with the centre of the muscle approximating to the centre of the counter window (See Fig.30.) The muscle was placed in front of the counter for 60 seconds, it was then removed, rotated through 180^0 and inverted, re-attached to the syringe and replaced before the counter for a further minute. This procedure ensured that both surfaces of the muscle were counted and eliminated as much geometrical error as possible. After counting, the muscle was returned to the radioactive solution for a further period.

The Geiger tube was connected to a Labgear Recording Ratemeter, the controls of which were adjusted to give optimal recording conditions, the degree of radioactivity being recorded by a pen on the surface of a moving paper.

The muscles were usually exposed for up to three hours, the radioactivity being measured at 20 minute intervals, but in some cases the muscle was left in the radioactive solution for

up/

up to 10 hours, after which time it was certain that equilibrium had been reached. ^{231} The total activity was measured at the end of this period by dissolving the muscle in 10 ml. of concentrated nitric acid and counting in an M6 liquid counter.

This method was also used to determine the $^{42}K^{+}$ uptake by lobster nerve, the only differences being that the lobster saline described in the appendix was used in place of the frog Ringer's soltuion, and that the hooks were attached to cotton threads used to tie off the cut ends of the nerves.

Method C - Indirect Determination of $^{42}K^{+}$ uptake.

The method described below was devised in order to avoid the continual removal of the tissue from the bathing fluid, and in the case of rat muscle, to maintain the muscle at a constant temperature.

The continuous circulation apparatus described in Chapter 9 was used and the bathing fluid was made radioactive by adding a small amount of $^{42}K^{+}$ to the circulating solution prior to the immersion of the muscle. The $^{42}K^{+}$ was added as ^{42}KCl from a syringe until maximum deviation of the pen was obtained with the ratemeter controls which were then adjusted to give maximum sensitivity. This was achieved by the addition to a circulating volume of 100 ml. of approximately 0.05 ml. of carrier free ^{42}KCl.

When/

When the counting rate became stable and had attained a mean maximum deflection (in most cases this was at 10 counts per second), the muscle was immersed in the circulating solution. Uptake of the $^{42}K^{+}$ was recorded as a decrease in the counting rate which resulted in a trace, the slope of which indicated the degree of change and an increased or decreased uptake was recorded as a change in the gradient of the recorded slope.

The method described was used with frog sartorius muscle, rat plantaris muscle and lobster nerve, but all attempts to obtain accurately reproducible effects failed. The cause of the failure seemed to be mainly due to an inability to achieve a sufficiently high specific activity in the circulating fluid because of the limitations of the counting tubes. If solutions of the activity used in the previous method were used the life of the counting tube was of only a few minutes duration. This limitation made it impossible to achieve a sufficiently high proportion of $^{42}K^{+}$ in the circulating fluid and hence the total amount of $^{42}K^{+}$ taken up by the tissue when compared with the circulating $^{42}K^{+}$ was too low to show any significant difference on the record.

Results

The results of the preliminary experiments in which Method A was used were inconclusive. The total uptake differed considerably/

The Effect of 10 ug. per ml. Protoveratrine A on the uptake of $^{42}K^{+}$ by Frog gastrocnemius muscle using Method A.

Results of Two Typical Experiments.

Experiment 1 Sample	Time of Exposure in minutes	Weight of muscles in mgm. control	test	Total counts in muscle control	test	Counts per gramme of muscle control	test
A	30	620	626	3840	4450	6193	7108
B	60	629	630	4885	5144	7766	8063
C	90	510	512	7987	7350	15660	14355
D	120	815	820	9846	13420	12080	16169
E	150	670	674	10985	11275	16395	16728
Experiment 2							
A	30	770	770	16656	16656	21631	21631
B	60	526	526	21563	20972	40994	39870
C	90	472	472	28270	44444	59894	94161
D	120	476	472	36432	49105	77186	104036
E	150	830	826	50536	68508	61181	82939
F	180	458	460	38702	51590	84134	112152
G	210	520	518	51901	85744	100194	165528
H	240	439	436	47604	65663	109183	150603

In these two experiments the treated muscle took up more $^{42}K^{+}$ than the control muscle in 10 out of the 13 exposures.

considerably between frogs and also between both muscles of a pair which were used as controls to test the validity of the method. The results from two typical experiments are shown in Table 11. From these two experiments it was seen that there was some indication that protoveratrine A caused an increased uptake of $^{42}K^{+}$ by the muscle. Six experiments were performed but because of the variability this method was abandoned in favour of method B.

The results obtained by using the second method were much more uniform and although the rate of uptake differed between frogs the results obtained were more satisfactory.

In addition to eliminating the two major disadvantages previously mentioned as associated with method A, this second method possessed the added advantage of giving a direct record. This enabled the activity of treated and the control muscle to be compared directly without the need for a decay correction to be applied.

The activity of the control and treated muscle at each counting appeared alongside each other on the record and the heights of the record for each muscle could be compared directly. Part of the record obtained from a typical experiment is shown in Fig. 31.

From this record it may be seen that the amplitude of the trace, i.e. the degree of radioactivity of the muscle was greater/

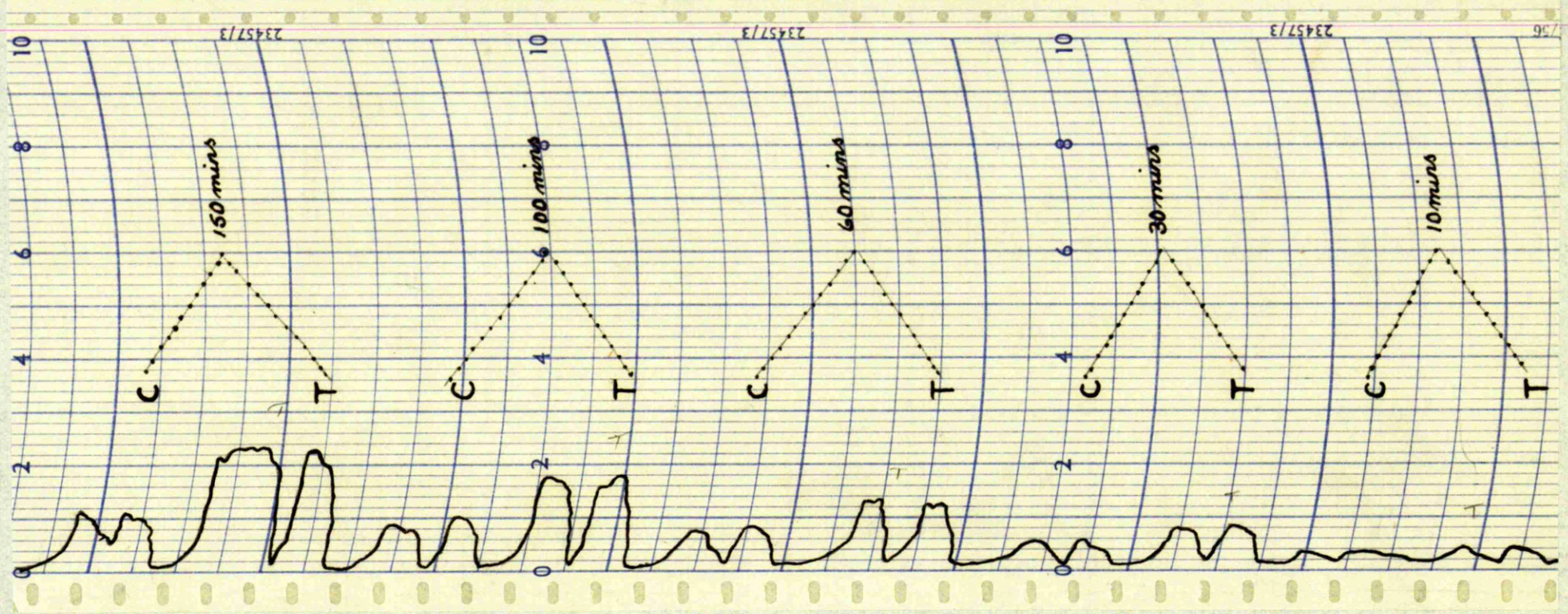

Fig. 31.

Record of $^{42}K^{+}$ uptake by frog sartorius muscles. This is a portion taken from an actual record and shows the uptake at four intervals during the experiment.

C - Activity of control muscle.

T - Activity of test muscle exposed to 10 ug per ml protoveratrine A.

Full scale deflection. 100 counts per minute.
Time constant 20 seconds.

greater for the muscle exposed to the protoveratrine A containing solution than for the muscle exposed to a control solution of identical activity. Both sides of each muscle were counted each time and the mean amplitude for the two sides taken as a measure of the radioactivity of the muscle.

The radioactivity of the treated and the control muscles were plotted against time to give an indication of both the rate of uptake and the total activity reached.

The curves obtained from a sample experiment are shown in Fig.32. From this graph it may be seen that both the rate of uptake, shown as a steeper slope, and the total uptake, i.e. the point at which the curve runs parallel with the abscissa and at which equilibrium between the $^{42}K^{+}$ of the muscle and the bathing medium was reached, are greater in the treated muscle than in the control muscle.

The differences in rate and total uptake differed between experiments, this was possibly due to a number of factors, a more detailed discussion of which is given in Chapter 8, but in every experiment performed the treated muscle took up $^{42}K^{+}$ at a higher rate than the control and also took up a greater total amount of $^{42}K^{+}$.

Attempts were made to express the form of curves obtained by mathematical equations and to compare them statistically. No standard form could be found which fitted all the curves obtained/

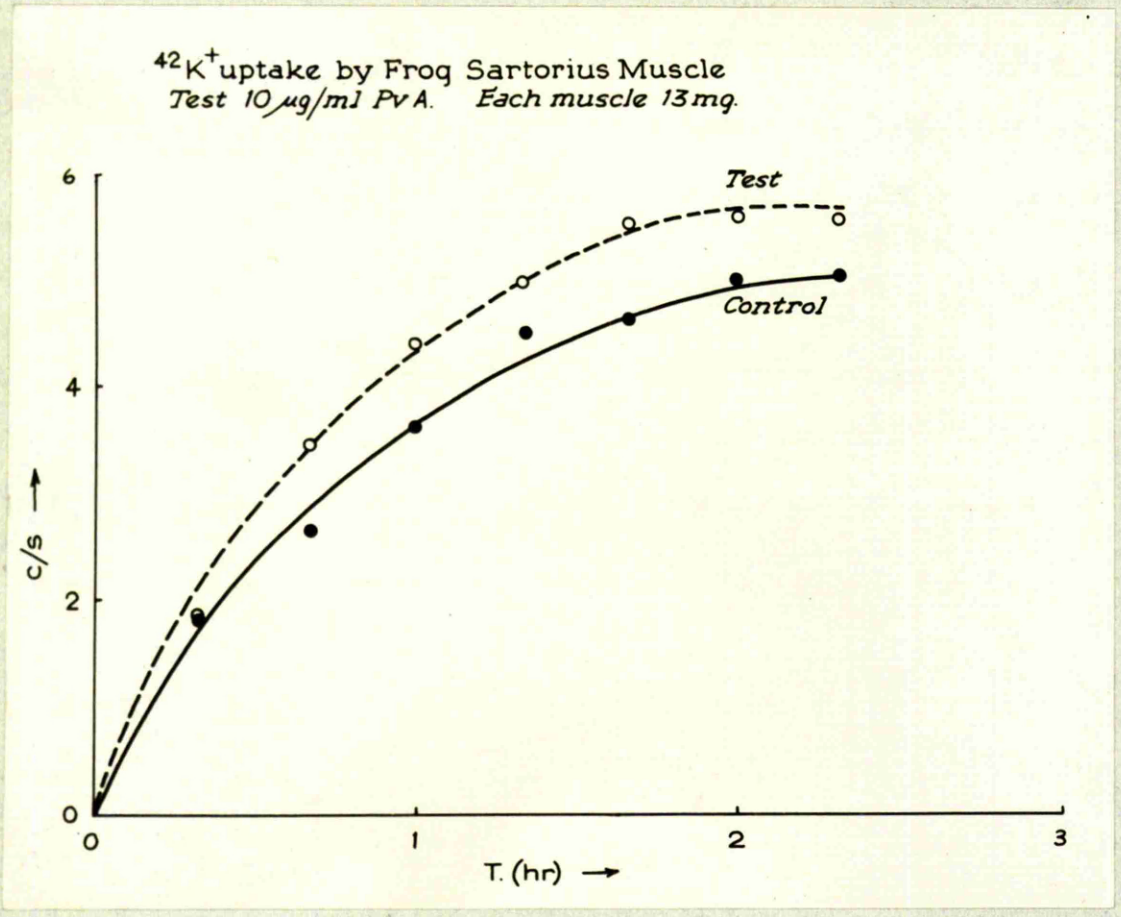

Fig. 32.

Graph showing the uptake of $^{42}K^{+}$ by frog sartorius muscle.

This is plotted from the results obtained from a typical experiment as shown in Fig. 31.

Ordinate - counts per second.

Abscissa - time in hours.

obtained in the experimental series and these attempts were not pursued further.

Ten experiments were performed with protoveratrine A at a concentration of 10 ug. per ml. and in each experiment the drug increased the rate of uptake of $^{42}K^{+}$. The probability that this increase could have occurred by chance in ten experiments out of ten can be shown to be less than 0.001. An analagous calculation has been worked out in full in Chapter 13.

A value of 'p' of less than 0.001 may be taken as highly significant and thus it was deduced from these experiments that protoveratrine A significantly increased both the rate and the total uptake of $^{42}K^{+}$ by frog sartorius muscles exposed to a bathing solution containing $^{42}K^{+}$.

Method C

No results which were of any significance were obtained using the method described, the reasons for this have been described under Method C. Lack of time prevented further modifications of this method but it is included here for completeness.

CHAPTER 13

THE INFLUENCE OF PROTOVERATRINE A ON THE RELEASE OF RADIOACTIVE CALCIUM IONS FROM FROG SKELETAL MUSCLE.

An excess of calcium ions (Ca^{++}) is known to inhibit many of the pharmacological actions of the Veratrum alkaloids; thus the hypotensive action is reduced if the animal is pretreated with an injection of a calcium salt [101]; the degree of sensitization to K^{+} shown in frog muscle is reduced or inhibited by excess Ca^{++} [53] and the effect of the Veratrum alkaloids on the electrical changes in stimulated nerve and muscle is altered [127].

Gordon and Welsh [131] suggest that veratrine acts by displacing Ca^{++} from the surface of the cell membrane, but they arrived at this conclusion as a result of studying the electrophysiological changes occurring in arthropod nerve and not by any direct measurements of Ca^{++} concentrations. Experiments were carried out to determine the validity of Gordon and Welsh's hypothesis and to find out if this held true for striated muscle.

Method

Paired frog sartorius muscles dissected by the method previously/

previously described (Chapter 8) and differing in weight by less than 2 mg. were suspended in Fenn-Ringer's solution by means of a stainless steel No.18 hypodermic needle bent into the form of a hook, and weighted with a second hook weighing 1.75 gm.

The calcium of the bathing solution was replaced by radioactive $^{45}Ca^{++}$ (for details of preparation see Appendix), the solution being stirred and oxygenated by a continuous stream of oxygen: the experiments were carried out at a room temperature of 18 to 19°C and the solution had a pH of 7.4.

The muscles were left in contact with the radioactive Fenn-Ringer's solution for 3 hours to enable the non-radioactive calcium already present in the muscle to equilibrate with the $^{45}Ca^{++}$ in the bathing medium. No accurate published information of equilibration times for $^{45}Ca^{++}$ in frog muscle could be found but Harris[236] states that from 10 to 25 per cent of the total Ca^{++} of isolated frog sartorius muscles is replaced by $^{45}Ca^{++}$ if the muscle is soaked for from 4 to 12 hours in a solution containing a proportion of $^{45}Ca^{++}$.

After/

After preliminary experiments in which muscles were exposed to a Fenn-Ringer's solution containing $^{45}Ca^{++}$ for different periods of time a 3 hour immersion time was chosen as this was found to give sufficiently high activity with a conveniently short period of exposure.

At the end of the 3 hour period the muscles were removed from the labelled Fenn-Ringer's solution and each surface of the muscle washed for 10 seconds in a continuous stream of non-radioactive Fenn-Ringer's solution, this was to wash from the surface any non-fixed $^{45}Ca^{++}$. Each muscle was then suspended in 10 ml. of oxygenated non-radioactive Fenn-Ringer's solution and left in contact for 10 minutes. The muscle was then removed, shaken to remove excess moisture and transferred to a second tube, this procedure was repeated 10 to 12 times. One of the muscles of the pair was used as a control and was exposed to normal Fenn-Ringer's solution throughout the experiment. The other muscle was exposed to 10 ug. per ml. of protoveratrine A for three immersions after being exposed for one hour to the same solution as the control. After exposure to the drug, it was returned to the drug-free solution for two further ten minute periods.

The radioactivity of the bathing solutions was determined by removing 0.5 ml. portions of the solution, adding 0.1 ml. of a 1 per cent solution of cetrimide to ensure even spreading and/

and evaporating to dryness in a flat aluminium planchette by means of heat from an infra-red lamp. When dried the mean depth of solids left on the planchette was calculated to be 0.01 mm. This was too small to make any appreciable difference to the counts and no correction for self-absorption was applied. The samples were counted using an EW3H type mica end-window counter connected through a probe unit to an automatic scaler. Three samples from each tube were taken and each one counted for 5 minutes, or where the count was very low for 1,000 seconds. After subtracting the background count the counts were expressed as mean counts per minute. No correction for decay was applied because the long half-life of $^{45}Ca^{++}$ of 164 days, made this unnecessary.

Results

A. Determination of Depth of Solid on the Planchette.

The depth of deposited solids on the planchette was determined by measuring (a) the mean radius of a number of planchettes selected at random (b) the mean weight of solids in 0.5 ml. of Fenn-Ringer's solution plus 0.1 ml. cetrimide solution. The density was taken as that of NaCl = 2.16.

Mean weight of solids (10 samples)	= 0.53 mg.
Mean radius (20 planchettes), r.	= 1.15 cm.
Density of NaCl	= 2.16.

The/

The depth of solids on the planchette was calculated from the formula

$$d = \frac{wt. \times \pi \times r^2}{\rho}$$

where d = depth ρ = density of NaCl

$$= \frac{0.93 \times 3.14 \times 1.32}{2.16 \times 1000} \text{ cm}$$

$$d = 1.02 \times 10^{-3} \text{ cm.}$$

B. Effect of Protoveratrine A on $Ca^{45}{}^{++}$ Release

The normal rate of $Ca^{45}{}^{++}$ release from frog sartorius muscles has been determined and it was found that the release from this tissue follows an exponential pattern and is similar to that shown by Harris.[256] To ensure that the rate of release in the control and the test muscle were alike the two muscles were run parallel without exposure of either to the drug for 1 hour. The test muscle was exposed to the protoveratrine A solution for three 10 minute periods then returned to normal Fenn-Ringer's solution.

In all experiments carried out exposure of the $Ca^{45}{}^{++}$ loaded muscle to 10 ug. per ml. of protoveratrine A caused the release of more $Ca^{45}{}^{++}$ into the bathing fluid than was released by the control muscle during the same period of time and exposure to normal Fenn-Ringer's solution. In seven out of then experiments the maximum $Ca^{45}{}^{++}$ release occurred during the first 10 minute period of exposure and in the remaining three/

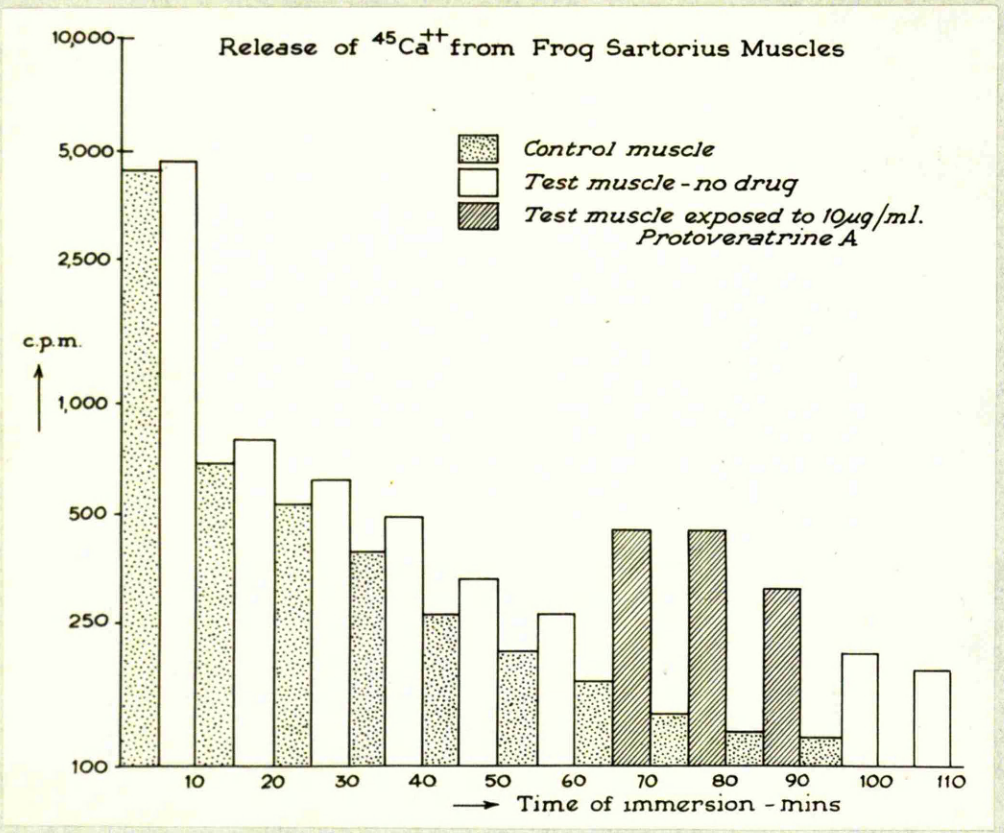
Release of $^{45}Ca^{++}$ from Frog Sartorius Muscles
Control muscle
Test muscle - no drug
Test muscle exposed to 10µg/ml. Protoveratrine A
c.p.m.
10,000
5,000
2,500
1,000
500
250
100
10
20
30
40
50
60
70
80
90
100
110
Time of immersion - mins

Fig. 33.

three, a maximum was reached during the second 10 minute period. After return to the normal bathing solution, the rate and total $^{45}Ca^{++}$ release dropped in all cases to a similar level to that shown by the control muscle.

As far as possible frogs of a similar gross weight were used, but no correlation between the body weight and weight of the sartorius muscles could be found, and the weights of muscles differed quite widely from experiment to experiment. By very careful dissection, it was possible to remove the muscles so that their weights differed by less than 2 mg. but although the muscles of a pair were usually comparable for both uptake and rate of release of $^{45}Ca^{++}$, these factors were widely different in muscles from different frogs, even in cases where the weights of the muscles were the same. Due to this lack of correlation betwe n experiments, it was found impossible to compare the results directly, using standard statistical procedures. In two experiments (Nos. 3 and 10 in Table 12) the weights of the muscles were similar, but the total counts recorded differed by a factor of four. The rate of $^{45}Ca^{++}$ release, i.e. the gradient of the curves obtained, also differed widely from one experiment to the next. Fig.33. shows the histogram obtained in a typical experiment. Attempts were made to derive equations for the curves after the methods employed by Soloway, Welsh and Solomon[237] and to derive/

TABLE 12.

^{45}Calcium Release from Frog Sartorius Muscle by Protoveratrine A. 10μg/ml

1 Expt No	2 Wt. (mg) of muscle C=Control T=Test		3 Total number of counts released during run	4 exposure to drug	5 exposure to control	6 Percentage of total released by exposure to drug	7 exposure to control	Ratio of results in column 6 to 7
1	C	80	17,085		2,361		13·82	1·27
	T	80	16,180	2,839		17·54		
2	C	36	7,104		1,552		21·84	1·38
	T	37	8,533	2,569		30·01		
3	C	24	9,918		285		2·87	2·29
	T	24	11,579	762		6·58		
4	C	40	2,044		224		1·09	1·38
	T	38	2,030	307		1·51		
5	C	40	2,436		237		0·97	1·75
	T	42	2,335	397		1·70		
6	C	54	5,289		586		1·10	1·21
	T	52	5,698	758		1·33		
7	C	78	7,099		433		6·1	2·29
	T	77	8,543	1196		14·0		
8	C	148	2,554		153		5·99	1·79
	T	148	2,644	284		10·74		
9	C	129	8,506		801		9·42	1·07
	T	130	11,067	1114		10·07		
10	C	27	2,849		161		5·65	1·64
	T	28	3,156	293		9·28		

derive a series of rate constants for the $^{45}Ca^{++}$ release, but due to the complexity of the curves it was impossible to find any standard form of equation to fit all the results.

The results were finally expressed without reference to the muscle weights at all. The total number of counts per minute released during the experiment was twenty times greater than the recorded counts, because only 0.5 ml. out of the 10 ml. of bathing fluid was estimated. The total number of counts released into the bathing medium during exposure to the drug, or during the corresponding control period, was expressed as a percentage of the total number of counts released during the run.

In every experiment the percentage of the total counts and, therefore, of $^{45}Ca^{++}$ released during exposure of the muscle to the protoveratrine A containing solution, was greater than from the control muscle during the corresponding period of time.

The results of ten experiments are summarised in Table 12, the final column shows the ratio of the two percentages and in every case this is greater than one - indicating a marked difference between the effect of the drug and of the control solution.

The/

The probability that out of ten experiments the difference between the control and the test experiment is due to chance is given by the expression [238]

$$p = (\tfrac{1}{2})^n$$

where p is the probability that the difference is due to chance

n = number of experiments.

In this case n = 10.

$$p = (\tfrac{1}{2})^{10}$$

$$= \frac{1}{1004}$$

$$p < .001$$

Therefore the probability that the difference between the control and the test experiments is due to chance is less than 0.001 and protoveratrine A has therefore been shown to have a highly significant effect on the release of $^{45}Ca^{++}$ from frog sartorius muscles.

CHAPTER 14

INFLUENCE OF PROTOVERATRINE A ON THE RELEASE OF RADIOACTIVE CALCIUM IONS FROM LOBSTER NERVE.

In the previous section it has been demonstrated that in frog sartorius muscles which have been loaded with $^{45}Ca^{++}$ more $^{45}Ca^{++}$ is released from protoveratrine A treated muscles than from control muscles.

The electrophysiological studies made by Gordon and Welsh [131,239] on veratrine treated arthropod nerve led them to conclude that the characteristic effects of veratrine were due to the displacement of Ca^{++} from the membrane surface.

The readily accessible nerves of laboratory animals are thickly myelinated and the study of ionic exchanges is difficult, but arthropod nerves have little myelin and they are more suitable for studies of this type. Much of the classic work on ionic transport was carried out using isolated giant fibres obtained from large arthropods, e.g. Loligo and Sepia. Live specimens of these were not obtainable in Glasgow and the work to be described was carried out using nerves taken from the cheliped of the common lobster, Homarus vulgaris.

The decapod crustaceans have large thinly myelinated nerve axons with a small number of fibres in each nerve bundle. The action of a number of drugs including veratrine upon the electrophysiological responses of the nerve axons of the American crayfish, Cambarus virilis, and other related species/

species has been described by Gordon and Welsh.[131] The common lobster, Homarus vulgaris, was used because of its ready availability and similarity to the species studied by Gordon and Welsh.

Method

Fresh live lobsters were obtained from a local fishmonger and used directly. The animals, which were caught locally, were in good condition and weighed between 0.7 kg. and 2.4 kg.

The larger of the two chelipeds which was usually the left one, was cut off at the coxopodite. The whole chela was placed under "lobster saline" (for details see Appendix) and the nerve to the moveable dactyl was exposed by removing with bone forceps a portion of the exoskeleton of the dactylopodite, and separating the muscle fibres by blunt dissection. The nerve was traced through the caropodite and into the meropodite by cutting through the exoskeleton and parting the muscles.

The method used for measuring the release of $^{45}Ca^{++}$ was similar to the method described by Soloway, Welsh and Solomon.[237] A portion of nerve measuring about 8 cms. in length was removed, placed into lobster saline and carefully freed from connective tissue. The nerve was then split longitudinally into two portions of equal thickness each containing about 10 axons. Fine coloured threads were attached to each end of the/

the bundles and the bundles were then transversely divided into halves. Half of each bundle was used as a control for the other half which was to be exposed to the drug. Each portion of the nerve had thus one cut end and one tied end. Tying of the cut ends has been shown to make no difference to the rate of $^{45}Ca^{++}$ release from lobster nerve.^{237}

The nerves were then suspended in lobster saline, the Ca^{++} of which had been replaced by radioactive $^{45}Ca^{++}$. This solution was stirred and oxygenated by a continuous stream ofoxygen, the solution was kept at room temperature and had a pH of 7.8. The nerves were left in this solution for 1 hour, after which they were removed and each pair washed by a large excess of non-radioactive lobster saline for 10 minutes. This was achieved by tying the threads attached to the nerves to the arms of a glass Y-piece attached to an aspirator containing the non-radioactive saline. The rate of flow of the solution was controlled by a screw clip and was adjusted to a constant rate of 120 drops per minute.

After washing, each nerve was transferred to a flat aluminium planchette, AERE Type A 36688, containing 0.5 ml. of lobster saline, the thread was removed and the nerve left in the solution for 10 minutes. After this period the nerve was then transferred to a second planchette for a further period of 10 minutes. Althgether this process was repeated ten times. The first five planchettes in the test run

contained/

contained lobster saline and the second five lobster saline to which had been added protoveratrine A. When a solution containing 100 ug./ml. of protoveratrine was used the nerve was only immersed in this solution for three 10 minute periods, i.e. in planchettes, 6 7 and 8, after which it was returned to normal lobster saline for two further periods of 10 minutes. This was done to determine the possible after effects of the drug. The control nerve was exposed to normal lobster saline throughout the experiment. At the end of the run the nerve was coiled on a tared planchette and the weight of the nerve determined by difference. All of the planchettes including the ones containing the nerve, were then dried under an infra-red lamp.

The radioactivity of each sample was determined by the method described in Chapter 13, and expressed as counts per minute after making the requisite corrections. Because of the small amount of salts deposited see Chapter 13, no correction for self-absorption was made. Each sample was counted for three periods of 5 minutes each and the mean counts per minute calculated.

Results

The effects of exposure of the $^{45}Ca^{++}$ loaded nerve to protoveratrine A at concentrations of 1, 10 and 100 ug. per ml. were studied.

No/

No method could be devised to determine the total $^{45}Ca^{++}$ uptake by the drug after immersion in the labelled lobster saline and before the start of the experiment proper. The total $^{45}Ca^{++}$ remaining in the nerve after the initial washing period was determined by adding the total counts released from the nerve to the total number of counts remaining in the nerve at the end of the experiment.

The determination of the total counts remaining in the nerve was made by coiling the nerve on a planchette, drying it and then counting. This procedure suffered from the disadvantage that no correction for self-absorption could be made because no practical means of measuring the area occupied by the nerve could be found, this was estimated to be of the order of 5 sq. mm. but varied considerably from nerve to nerve. The total $^{45}Ca^{++}$ could not be conveniently measured by the usual methods of dissolving the nerve in acid or alkali as these agents reacted with the aluminium of the planchettes. Soloway *et alii*[237] tried a number of methods without success and they finally used the method adopted here.

To reduce geometric errors each planchette was counted three times, this reduced the error as each time the orientation of the planchette on the carrier differed.

Wide variations both in the total uptake and subsequent release/

TABLE 13

The effect of 1 ug. per ml. of Protoveratrine A on the release of $^{45}Ca^{++}$ from Lobster nerve.

A

Date 1957	Percentage released by		Diff.
	Drug	Control	III-II.
I	II	III	IV
22.10.	15.80	17.61	+ 1.81
24.10.	15.71	16.61	+ 0.90
"	15.74	15.39	- 0.35
28.10.	14.89	18.76	+ 3.87
"	19.11	14.61	- 4.50
30.10.	21.79	20.94	- 0.85
"	18.71	19.26	+ 0.55
31.10.	23.20	24.40	+ 1.20
19.11.	10.53	9.95	- 0.58
Mean	17.26	17.90	+ 0.23

B

Percentage released by		Diff. VI - V.
Drug.	Control	
V	VI	VIII
9.20	11.57	+ 2.37
11.65	12.37	+ 0.62
12.37	13.37	+ 1.00
10.46	12.30	+ 1.84
11.59	10.02	- 1.57
14.01	14.59	+ 0.48
14.23	13.95	- 0.28
18.38	19.50	+ 1.12
7.41	7.10	- 0.31
12.14	12.75	+ 0.58

Table A shows that percentage of the total $^{45}Ca^{++}$ released during the run which escaped into the bathing fluid during exposure to either the drug or the control.

Table B shows that percentage of the total $^{45}Ca^{++}$ taken up by the nerve which was released during exposure to the drug or control solution.

A value in the difference column indicates a greater release of $^{45}Ca^{++}$ from the test nerve than from the control nerve and vice versa

When the 't' test applied to the means

For A $0.8 > p > 0.7$

For B $0.2 > p > 0.1$

TABLE 14

The effect of 10 ug. per ml. of Protoveratrine A on the release of $^{45}Ca^{++}$ from Lobster Nerve.

A				B		
I	II	III	IV	V	VI	VII
Date 1957	Precentage released by		Diff.	Percentage released by		Diff.
	Drug	Control	III-II	Drug	Control	VI - V
10.10.	14.72	17.44	+ 2.72	10.66	12.16	+ 1.50
"	17.79	20.44	+ 2.65	9.85	10.99	+ 1.14
15.10.	10.75	9.48	- 1.27	8.85	7.62	- 1.23
"	12.88	11.43	- 1.45	10.60	9.19	- 1.41
31.10.	16.34	18.97	+ 2.63	14.15	15.25	+ 1.10
6.11.	19.26	13.50	- 5.76	11.07	12.78	+ 1.71
"	14.59	17.71	+ 3.12	13.98	10.05	- 3.93
7.11.	20.44	23.24	+ 2.80	15.34	17.24	+ 1.90
"	16.90	7.27	- 9.63	13.13	12.75	- 0.38
12.11.	17.39	13.49	- 3.90	12.20	9.47	- 2.73
21.11.	22.22	22.83	+ 0.61	14.84	14.82	- 0.02
"	20.41	25.67	+ 5.26	13.42	16.55	+3.13
Mean	16.97	16.56	- 0.20	12.34	12.39	+ 0.06

Table details as Table 13.

For A $p > .9$

B $0.3 > p > 0.2$

TABLE 15

The effect of 100 ug. per ml. of Protoveratrine A on the release of $^{45}Ca^{++}$ from Lobster Nerve.

A				B		
I	II	III	IV	V	VI	VII
Date 1957	Percentage released by		Difference	Percentage released by		Difference
	Drug	Control	III - II	Drug	Control	VI - V
16.10	10.75	8.25	- 2.50	8.21	6.05	- 2.16
"	6.77	9.43	+ 2.66	5.02	7.27	+ 2.25
17.10.	8.00	8.22	+ 0.22	5.90	6.33	+ 0.43
"	7.61	7.06	- 0.55	6.09	5.18	- 0.91
22.10	14.47	15.62	+ 1.15	11.80	13.05	+ 1.25
14.11.	15.57	16.91	+ 1.34	9.42	10.63	+ 1.21
"	18.10	19.53	+ 1.43	11.75	11.92	+ 0.17
13.11.	14.90	12.80	- 2.10	9.76	8.69	- 1.07
Mean	12.90	12.23	+ 0.21	8.49	8.64	+ 0.15

Table details as Table 13.

For A $0.7 < p < 0.8$

For B $0.8 < p < 0.9$

release of $^{45}Ca^{++}$ occurred between the experiments and there was frequently a marked difference between the control nerve and the test nerve before the exposure to the drug. The nature of the experiment made it impossible to detect the variation between the $^{45}Ca^{++}$ content of the test and the control before the start of the experiment, because the geometrical errors involved in counting the activity of the nerve by placing the wet nerve beneath an end window counter would be much greater than the variation in the total $^{45}Ca^{++}$ uptake. To reduce these variations the total counts per minute released during exposure to the drug or the correspondin control period have been expressed as both a percentage of the total number of counts released from the nerve into the bathing solution and as a percentage of the total number of counts contained in the nerve at the start of the experiment. The latter value was determined by adding the number of counts released during the run to the number of counts remaining in the nerve.

By expressing the results as percentages, the variations between experiments were very much reduced and the differences between the control and treated nerves in different experiments were of the same order.

The results of the experiments are summarised in Table 13., Table 14 and Table 15.

The/

The figures refer to the percentage of $^{45}Ca^{++}$ released during the exposure of the nerve to protoveratrine A. The three tables show the results for protoveratrine A at concentrations of 1 ug. 10 ug and 100 ug respectively.

The differences between the treated and untreated nerves are shown in the third line. These differences were compared to zero by a paired 't' test and the probability that the differences from zero occurred by chance was determined for both methods of expressing the results.

When protoveratrine A was used in doses of 1 ug. per ml. the probability that the differences between the test and control nerves was due to change was determined from the 't' value obtained from the data by "Student's" test. When the amount of $^{45}Ca^{++}$ released was determined as a percentage of the total amount released 'p' was found to lie between 0.7 and 0.8, when the released $^{45}Ca^{++}$ was expressed as a percentage of the total taken up 'p' lay between 0.1 and 0.2.

These values for 'p' indicated that there was no significant difference from zero and that no difference could be detected between the treated and the untreated lobster nerves under the conditions of this experiment.

Similar values for 'p' were found when protoveratrine A in concentrations of 10 ug and 100 ug per ml. was used.

With concentrations of 10 ug. per ml. $p > 0.9$. When the released/

released $^{45}Ca^{++}$ during treatment was expressed as a percentage of the total amount released when expressed as total uptake $0.3 > p > 0.2$.

For protoveratrine A in concentrations of 100 ug. per ml. the repsective 'p' values were $0.8 > p > 0.7$ and $0.9 > p > 0.8$.

From these results it was concluded that under the conditions of the experiment no increased release of $^{45}Ca^{++}$ could be demonstrated in nerves exposed to concentrations of protoveratrine A in concentrations of 1 ug, 10 ug and 100 ug. per ml.

These findings were not expected, both from a consideration of the work of previous authors [131,239] and from the findings described for muscles in the earlier sections of this thesis. The possible explanation of these differences between nerve and muscle will be discussed more fully in Chapter 15.

CHAPTER 15.

DISCUSSION.

The phenomenon of sensitization to K^+ shown by many of the Veratrum alkaloids has not yet been fully explained. In their review of the subject Goffart and Bacq[53] put forward a number of hypotheses but were unable to develop any theory to account for all the known facts.

The experiments described in Chapter 2 were of only a preliminary nature but they indicate that further experiments of a simple nature, such as the ones described, may lead to a better understanding of this problem.

The degree of sensitization of frog skeletal muscle to the stimulant action of K^+ produced by protoveratrine can be varied by an alteration of the ionic composition of the bathing medium. A reduction in the Ca^{++} content of the bathing medium has been shown to potentiate the sensitizing action of protoveratrine. In a normal tissue bathed by a physiological saline solution containing a number of ions, the ions in the solution are in dynamic equilibrium with the ions either within the cell or bound at the surface. Lowering of the Ca^{++} concentration of the bathing medium would, therefore, shift the equilibrium point in favour of the solution and lead to reduction in the number of Ca^{++} bound to the cell surface.

Lowering of the external Ca^{++} concentration has been shown to result in an increase in the permeability of cell membranes to Na^+ and K^+.[130,131,156] Evidence from previously published

work/

work summarised in the introduction, and obtained from the original work described in this thesis, indicates that protoveratrine also increases the cationic permeability of certain tissues, thus it may well be that the increased sensitization to K^+ produced by protoveratrine in Ca^{++} - low and Ca^{++} - free Ringer's solution is an additive effect.

The findings described in Chapter 2 may, therefore be taken as supporting the suggestions of previous authors[52,107,131] that the action of the Veratrum alkaloids may be due to the displacement of Ca^{++} from the cell surface.

The reduction of the K^+ sensitizing effect of protoveratrine in a K^+-free bathing medium can be explained as being due to an alteration in the Ca^{++}/K^+ ratio of the bathing medium. Reduction of the K^+ concentration may therefore be looked upon as producing a relative increase in the Ca^{++} concentration which leads to a shift in the equilibrium in the opposite direction to that which occurs in the low Ca^{++} solution. This shift in equilibrium opposes the action of the protoveratrine and prevents the drug from showing its characteristic K^+-sensitizing effect by reducing the increased permeability brought about by the protoveratrine.

Previous workers have shown that mixtures or impure preparations of the Veratrum alkaloids have no antihistaminic or anticholinergic actions.[50] These findings have been confirmed/

confirmed using pure protoveratrine A, and it therefore seems most unlikely that the mediation of either the reflexly induced effects of this drug, or its direct effects on nerve and muscle, is brought about by antagonism to the actions acetylcholine or histamine.

The two effects, viz. inhibition, and later sensitization to 5-HT induced contractions of the guinea-pig ileum which have been shown to develop following exposure to low concentrations of protoveratrine have not previously been described in the literature. From a consideration of the chemical structure there seems to be no obvious relationship between either the alkamine nucleus or the acid moieties of the protoveratrine molecule, and it thus seems unlikely that the effects shown by the drug on 5-HT induced contractions are due to any form of competition for receptor sites.

A possible explanation of the inhibitory effect of protoveratrine A on 5-HT induced contractions observed in fresh gut is that the contractile state of the gut differed slightly from that which existed at a later stage in the experiment and whilst in this condition it was more sensitive to the stimulant effect of 5-HT. This decrease in sensitivity to 5-HT was frequently observed but it differed from tachyphylaxis in that constant responses to a given dose were eventually obtained. The large contraction caused by the addition of protoveratrine/

protoveratrine to the gut may have led to the exhaustion of available reserves of metabolites or to fatigue in the muscle, during this period doses of 5-HT. were less effective in producing a contraction.

It has been reported that intravenous injections of 5-HT. can elicit the Bezold-Jarish reflex[240] and stimulate carotid baroreceptors[241] In addition 5-HT has a marked stimulant effect on sensory nerve endings, especially those of pain fibres.[242] These effects are also characteristic of protoveratrine and it may be that the effects of both protoveratrine and 5-HT. are mediated *via* a common afferent nervous pathway and this effect may involve changes in ionic distribution at or across the cell membrane.

The sensitization to the stimulant action of 5-HT shown after exposure of segments of ileum to protoveratrine may be analagous to the sensitization of the frog rectus to K^+ which was discussed earlier. It may be seen from Fig. 8 that the sensitization is not a simple additive effect because the effect persisted in response to a subsequent dose of 5-HT after the protoveratrine A had been washed out and the base line had returned to its normal level.

The stimulant effects of acetylcholine are believed to involve the participation of K^+ and it may be that this ion is somehow/

somehow involved in the stimulant action of 5-HT. Thus the sensitization to 5-HT. shown by protoveratrine may be due to an effect which involves sensitization to the K^+ participating in the 5-HT response.

The depression by protoveratrine A of oxytocin-induced contractions of the isolated rat uterus was unexpected, but it must be noted that this depressant effect only occurred with relatively large doses of protoveratrine A.

The mechanism by which oxytocin induces contraction of the smooth muscle of the uterus is still unknown. The metallic cations, especially K^+, may be involved in oxytocin induced contractions as in other forms of muscle[243,244]. No evidence of any K^+ sensitizing effect could be demonstrated in this preparation.

The oxytocin molecule is an octapeptide of known structure[5] and it is conceivable that the molecule of protoveratrine, which is also a relatively large rigid molecule, may be adsorbed on to the cell surface and by direct steric hindrance prevents the molecule of oxytocin from reaching its receptor sites. The smaller molecules of histamine and acetylcholine might not be hindered to the same extent and this may account for the selective action of protoveratrine against oxytocin induced contractions.

The direct stimulant effect of protoveratrine A on the isolated/

isolated guinea-pig ileum has been described by Meier, Tripod and Brüni[134] and for other Veratrum preparations by various authors.[50,116,117] Meier et alia[134] have shown that protoveratrine A-induced contractions of the guinea-pig ileum can be antagonised by atropine, and that phentolamine antagonizes the spasmogenic effect of protoveratrine A on the isolated seminal vesicle of the guinea-pig. They have used their results as evidence in favour of their theory of master receptors (see Chapter 1), but they have made no suggestions as to how protoveratrine induces a contraction of smooth muscle.

The contraction of isolated intestinal smooth muscle is believed to be the result of a process of depolarization and it has been shown that the contraction is accompanied by an efflux of K^+.[244] This finding fits in well with the membrane theory and on the basis of this theory it can be argued that an increase in K^+ concentration at the exterior of the cell or a decrease in internal K^+ concentration will produce depolarization and contraction. The possibility, therefore, exists that the contraction produced by protoveratrine A is the result of depolarization resulting from an alteration of the distribution of ions across the cell membrane.

No measurements of the resting potential of protoveratrine treated smooth muscle cells have been made, but it has been shown by other authors that veratridine markedly reduced the resting/

resting potential of nerve and skeletal muscle. [118 to 124]

The results of the more detailed studies on the influence of protoveratrine A on the distribution of cations which have been described in part 2 of this thesis, may offer a possible explanation of both the stimulant effect of protoveratrine A and the antagonism to this effect by Ca^{++}. The results from experiments upon frog muscle (Chapters 12 and 13) show that protoveratrine A causes an increased uptake of K^{+} and an increased output of Ca^{++}. It is possible that these two effects are related and that a similar pattern exists in both skeletal and smooth muscle.

If protoveratrine A reduces the affinity of the cell surface for Ca^{++} and permits the adsorption of K^{+} to the anionic sites, the increased concentration of K^{+} at the cell surface would lead to a reduction in the potential difference across the membrane and to subsequent depolarization. [108] The addition of excess Ca^{++} may reverse this effect and it is thus possible to account for the antagonism shown between Ca^{++} and protoveratrine A on the guinea-pig ileum. [188, 189, 203, 212.]

Ever since the pioneer work of Bacq and his school it has been generally agreed by all workers who have studied the basic mechanism of action of the Veratrum alkaloids, that definite relationships exist between the pharmacological actions of these drugs and the potassium ions present in the system.

The/

The evidence pointing to this relationship has been fully summarised in the introduction to this thesis and from a consideration of the evidence it appears that the pharmacologically active Veratrum alkaloids can alter the distribution of ions between the intracellular and the extracellular phases. This balance may be upset in a similar manner in all the cells of the body affected by the drug, but no concrete evidence has yet been put forward to confirm this view.

It has been known for many years that the metallic ions K^+ and Na^+ occur in all organisms and that they are essential for the normal functioning of living cells. It may therefore be expected that any imbalance in the ratio normally existing between the concentrations of these two ions will be accompanied by alteration in the normal activity of the affected cells.

The hypotheses which favour the participation of K^+ in the actions of the Veratrum alkaloids have been deduced mainly from indirect evidence derived from the study of two different types of tissues, viz. mammalian and amphibian nervous tissue, and amphibian skeletal muscle. The evidence obtained from experiments made upon nervous tissue has either been gained from the study of electrophysiological changes produced by the drugs and ions used or by studying the reflex effects produced by them (see Chapter 1). The evidence obtained from

amphibian/

amphibian muscle (mainly of frogs) has been gained as a result of studying the sensitization of the frog muscle to K^+ by the alkaloids. Little work has been done, however, upon the electrophysiological or permeability changes produced in muscles by doses of Veratrum alkaloids in concentrations sufficient to produce their characteristic effects on contractility.

The work described in Part 1 of this thesis indicates that although protoveratrine, a typical ester alkaloid of the Veratrum species, can increase the level of K^+ in the plasma, this increase is not the result of a release of K^+ from skeletal muscle, but more probably arises from cardiac muscle. The more detailed work during which the effects of protoveratrine on the release of K^+ from frog and rat isolated skeletal muscles were studied and which has been described in Part 2 confirms these observations.

Previous workers have either shown or suggested that the Veratrum alkaloids may cause an increase in the efflux of K^+ from amphibian and mammalian nerve. The prevailing opinion of workers engaged in the study of ion exchange mechanisms is that these mechanisms are similar for all cells in which ionic exchange across the cellular membrane is involved.[125,155,156,163] Thus it was expected that results similar to those obtained by previous workers using nerves would be forthcoming/

forthcoming if skeletal muscle was used in this study. It is generally thought that the contraction of muscle fibres is accompanied by a transfer of K^+ across the cell membrane to the exterior with a corresponding influx of Na^+.

It has, however, been found that doses of protoveratrine in concentrations equal to or higher than those necessary to produce sensitization of muscle to K^+, and to initiate impulses from sensory receptors, have no effect on the efflux of K^+ from skeletal muscle of cat, rat or frog. On the other hand it has been shown that small doses of protoveratrine A can increase the efflux of K^+ from the beating heart.

These finding are supported by other workers, Kahn et alia[157] were unable to demonstrate any change in K^+ flux in erythrocytes treated with veratridine and Harris found a similar situation in frog sartorius muscle[158], but Vick and Kahn[235] have also recently demonstrated that veratridine produces an increased output of K^+ from beating hearts.

These results when taken in conjunction with those of Shanes[122] who demonstrated an increased K^+ loss from veratrinised frog nerves, show that there may be qualitative differences between the action of the Veratrum alkaloids on tissues of different types. This difference may be explained in two ways, one is that the ease of access of the drug to the cellular membranes differs between tissues and the other is

that/

that in different tissues K^+ exchange is brought about by different mechanisms.

In a recent paper by Witt and Jaeger [245] it has been shown that electrically stimulated rabbit muscles exposed to veratridine in a concentration of 0.1 ug. per ml. showed a decrease in K^+ efflux if stimulated at a rate of 1 per second, but if the rate of stimulation was doubled the veratridine treated muscles lost less K^+ than the control muscles. It would thus appear that in addition to the two factors mentioned above a third factor, i.e. the physiological state of the tissues, may influence K^+ exchange. One major criticism of the results obtained by Witt and Jaeger is that the perfusion fluid they used was K^+ free Tyrode's solution which provides unphysiological conditions for what is essentially a physiological process.

A final explanation of the effects of the Veratrum alkaloids upon K^+ exchange in any tissue must await a fuller elucidation of the mechanisms by which this ion crosses cellular membranes both from the extracellular region to the intracellular regions and vice versa.

A number of hypotheses have been proposed to explain these mechanisms and to account for some of the known facts, but so far no single hypothesis has been found which can explain all the findings. It is now thought possible that the intracellular/

intracellular cations do not exist in free solution within the cell but may be localized in specific sites on the intrafibrillar structures.[246] There is also evidence of differences in distribution of K^+ between the cytoplasm and the mitochondria which involves a further transport of ions across the mitochondrial membrane,[247] possibly by a different mechanism. A further complication is that the metallic cations may first of all be adsorbed on to surface structures before passing into the cell.[248]

Thus there are at least three possible sites at which protoveratrine may act. It may affect the adsorption of ions at the cell surface structures; they may alter the distribution of the ions within the cell, or they may disrupt or modify possible transport mechanisms.

A number of workers have claimed that the cardiac glycosides produce their characteristic pharmacological effects by influencing the ionic transport mechanisms of the cell membrane.[213,216 to 224] As the cell can concentrate ions and other molecules against both an electrochemical and a concentration gradient the concept of active transport has been widely accepted as playing an essential part in the maintenance of cellular activity. Active transport is dependent upon metabolic activity, the energy liberated being used by the cell to overcome the adverse concentration and electrochemical gradients.[138,249 to 251]

It/

It is tempting to suggest that cardiac glycosides and the chemically related Veratrum alkaloids produce their effects by interfering with the metabolic processes responsible for the liberation of energy, which is utilised by the active processes which maintain cellular homeostasis. These drugs, however, do not produce a generalized inhibition of metabolism (see Chapter 1) and their effects may be limited to a specific chain of enzymatic processes involved in ionic transport. Some enzyme inhibitors can lower the resting potential of muscle fibres,[252] but other powerful metabolic inhibitors seem to be without effect. Fluoride and 2:4 dinitrophenol (DNP) have no effect on either the resting potential[253] or Na^+ efflux of frog muscle[138]. Iodoacetate and cyanide are also without effect on Na^+ efflux.[254]

For a time it was believed that the distribution of ions between the extracellular and intracellular fluid of living cells was in accord with a Gibbs-Donnan type of distribution. When it was found that this concept was inadequate the existence of a sodium pump mechanism was postulated (for references see Chapter 1). In the light of further knowledge this concept has been further modified and a number of suggestions have been made as to the possible nature of this system. It has been suggested that in frog muscle the active extrusion of Na^+ is accompanied by a passive inward diffusion

of/

of K^+ down the electrochemical gradient.[250] Conway has suggested the existence of a redox pump mechanism[250,251] and has shown that in yeast there may be two separate carrier systems, one for Na^+ and one for K^+. The most widely accepted concept at the present time is that there exists a linked carrier system in which K^+ is actively transported into the cell by the same mechanism which is used to extrude Na^+. This system is thought to exist in both nerve and muscle.

Shaw and his colleagues[255,256] have challenged the whole concept of the sodium pump in muscle and have exhaustively analysed a mass of data obtained from frog sartorius muscles which they have used to support their claim. This work finds support from a recent paper by Harris[257] who claims that K^+ distribution in both frog sartorius muscles and erythrocytes can be explained by the physical structure of the cell.

It has been generally assumed by most workers in the field of general physiology that ionic exchange takes place by similar, if not identical mechanisms, in all tissues. The evidence for this belief is, however, scanty, probably because the complexity of the problem prevents the study over a wider range of cell types.

The studies reported in this thesis and those of other workers show that the purified Veratrum alkaloids may affect K^+ efflux in some tissues, e.g. cardiac muscle and nerve but

have/

have no effect on the efflux in other tissues, e.g. skeletal muscle and erythrocytes. Two possible explanations exist for this discrepancy, the first is that the mechanisms by which K^+ is transferred differ in the different tissues, this may be related in some way to metabolic differences between them. A second possibility is that in tissues where an effect has been shown the drug may more readily gain access to the particular sites at which the K^+ exchange is taking place.

Results obtained from the study of the cardiac glycosides on K^+ fluxes may be taken as providing some evidence that the mechanisms of K^+ transport do not differ between themselves. Strophanthin has been shown to have a similar effect on K^+ fluxes on a variety of different tissues, including resting frog skeletal muscle, erythrocytes and cardiac muscle.[138,219] This drug and related cardiac glycosides prevents both the influx of K^+ into the cells and also the efflux of Na^+.[216 to 224] It would, therefore, appear that despite some pharmacological and chemical similarities the Veratrum alkaloids have a different mode of action from that of the cardiac glycosides.

The finding that the protoveratrine treated muscles took up more $^{42}K^+$ than their controls is difficult to reconcile with any of the theories which have been proposed to explain the action of the Veratrum alkaloids (See Chapter 1).

This effect could be explained on the basis of the sodium pump /

pump theory by postulating that the drug acts by increasing the rate by which Na^+ is extruded with a concomittant increase in K^+ influx. There is, however, no evidence that this occurs, on the contrary, evidence obtained by Straub[107,108] from the study of nerve fibres indicates that the opposite condition may exist in veratrinized nerves.

McLennan[248] has suggested that K^+ may be adsorbed on to the surface of the muscle fibres as a first stage in the process whereby the ion is transferred across the fibre membrane. This adsorption process may be the formation of a complex between the K^+ and a protein. The K^+ may then be transported into the cell in this combined form.

Recent work[256,257] has shown that some portion of the intracellular K^+ is adsorbed on to a protein complex and it seems possible that this situation can occur at the cell surface.

In Chapter 13 it hasbeen demonstrated that protoveratrine A in concentrations which can promote the uptake of K^+ displaces Ca^{++} from frog sartorius muscle. It is possible that the displacement of Ca^{++} from a bound form at the cell surface frees certain anionic sites which then bind K^+ in excess of that already existing at the surface.

In order to relate the results obtained with protoveratrine on K^+ fluxes with the direct pharmacological effect described/

described in Chapters 2 and 3, it is necessary to take into account the present theories of the ionic basis of excitability. Previous attempts to explain the pharmacological actions of the Veratrum alkaloids on this basis have been described in Chapter 1.

According to the membrane theory depolarization of the cell membrane is the result of a definite alteration in the distribution across the membrane of the major ions which are normally responsible for the resting potential. From Goldmann's constant field theory [152] the resting potential E, across a cell membrane may be expressed by the formula

$$E = \frac{RT}{F} \log_e \frac{P_K (K)_i + P_{Na} (Na)_i + P_{Cl} (Cl)_i}{P_K (K)_e + P_{Na} (Na)_e + P_{Cl} (Cl)_e}$$

In this formula P_K, P_{Na}, and P_{Cl} are the permeability constants for K^+, Na^+ and Cl^- respectively, $(K)_i$, $(Na)_i$, $(Cl)_i$ are the activities of the ions within the fibres, $(K)_e$, $(Na)_e$, $(Cl)_e$ are the activities at the external surface of the fibres.

For practical purposes the activities may be taken as being equivalent to the concentrations of the ions.

Depolarization, i.e. a reduction in E, may be produced by a decrease in the values for P_K, $(K)_i$, $(Na)_i$, P_{Cl}, $(Cl)_e$, of an increase in P_{Na}, $(K)_e$, $(Cl)_i$.

Evidence cited by previous authors and summarised in

Chapter/

Chapter 1 indicates that the Veratrum alkaloids can produce depolarization of both nerve and muscle. The experiments with protoveratrine described in the experimental section of this thesis have also shown that this drug can produce a contraction of smooth muscle. This is likely to be the result of a process of depolarization.

The experimental findings reported in this thesis indicate that protoveratrine can affect the movement and distribution of K^+ in two different ways. It may increase the efflux of K^+ from the beating heart and increase the uptake of K^+ in frog muscle. The increased efflux from the heart indicates that there must be a decrease in $(K)_i$ which will produce some degree of depolarization.

It has already been suggested that the increased uptake of K^+ which has been demonstrated as occurring in frog muscle, may be due to an increase in the K^+ adsorbed on the surface of the muscles and not to an increase in $(K)_i$, which would produce hyperpolarization and not depolarisation. The adsorption on the surface would lead to an effective increase in $(K)_e$ with consequent depolarization producing in the case of smooth muscle a contraction.

Shanes[122] has suggested that the accumulation of K^+ on the surface of the fibre causes depolarization and can also account for the development of the negative after potential.

Straub/

Straub[108] has shown that the depolarization produced by veratridine persists in a nerve which is being continually bathed with Ringer's solution. He claims that the depolarization cannot be due to the presence of excess K^+ at the fibre surface because the ions would be washed away. He made no measurements, however, of ionic concentrations. From the results obtained in this study (see Chapter 12) it would appear that the ions may be strongly adsorbed at the surface and that ordinary washing would not remove them. This could, therefore, account in some measure for the depolarization known to be produced in muscle by the protoveratrine.

The increased efflux of K^+ which has been demonstrated in heart muscle but not in skeletal muscle may be due to differences in the mechanism by which ion movements in these two types of tissue are achieved. The heart is depressed by excess K^+ in contrast to skeletal muscle which is stimulated by this ion, and it may be that this is due to functional differences in the ionic transport mechanisms between cardiac and skeletal muscle.

Straub[108] suggests that veratridine actually raises the K^+ permeability in isolated frog nerve, but again this conclusion is drawn from indirect electrophysiological evidence.

Ling[258] has gone even further and challenged the basic validity/

validity of the membrane theory. He has claimed that the Nernst equation from which the E.M.F. of the cell is calculated, does not apply because the anions which the Nernst equation requires to have zero mobility have been shown to move freely across the membrane. As an alternative hypothesis Ling suggest that a system of fixed negative charges exists within the cell which selectively accumulate K^+ in preference to Na^+ because of the lower average dielectric constant and smaller hydrated size of the K^+ when compared to Na^+. This uneven distribution of ions is thought to be responsible for the differences in potential between the inside and the outside of the cell.

This alternative hypothesis of Ling's is based entirely on work carried out with frog skeletal muscle, and until further studies of a similar nature to those which have been done with frog muscle are performed on other excitable tissues, this hypothesis must be considered with reserve.

In a recent review Conway[251] has heavily criticised both Ling's experimental data and his conclusions. He claims that all the anomalies found by Ling can be adequately explained on the membrane theory.

It is difficult to explain the results obtained in this study from the standpoint of Ling's hypothesis, according to this theory protoveratrine would have to be widely distributed throughout/

throughout the cell before it had any marked effect. There is as yet no evidence that protoveratrine actually penetrates into the cell and its rapid onset of action suggest that the drug acts at the cell surface.

Much more work must be done before a critical assessment of Ling's hypothesis is made. Studies upon the effects of protoveratrine and other active steroids on the biophysical properties of cellular proteins may shed further light on possible effects of this nature.

The steroid hormones from the adrenal cortex are known to be essential for themaintenance of salt balance in the body and some evidence exists that these substances can influence the distribution of ions between the interior and exterior of the cells.[259] It has been suggested that the structure of some of the mucopolysaccharides existing at the cell surface may be altered in the presence of certain of the mineralocorticoids, and these alterations may lead to changes in the electrolyte permeability of the cell.[260] The steroidal nature of the alkamine of protoveratrine may be such that it interferes with the normal action of the mineralocorticoids. This suggestion is entirely speculative and no evidence in its support has been obtained in this thesis. A closer investigation into the relationships between these hormones and the purified Veratrum alkaloids,may, however, prove profitable.

One/

One of the principal aims of this study was an attempt to achieve a fuller understanding of the mechanisms by which the active ester alkaloids of the Veratrum species e.g. protoveratrine, stimulate afferent sensory nerve endings. It was hoped that studies of ionic movements made upon non-nervous tissue might provide information which might be useful in attempting to explain the action of these alkaloids at sensory receptors. These hopes have not been realised because it would be unwise to presume that analagous mechanisms of ionic transport exist both at sensory nerve endings and in the muscle cell when it appears that there may be differences even between two different types of muscle, i.e. between cardiac and skeletal muscle. It may be assumed, however, that depolarizatio of sensory nerve endings would occur if the concentrations of K^+ at the external surface of the nerve endings was to be increased. This assumption is supported by the work of Hodgkin and his school [125,126,153,167,168] who by the use of micro-electrodes placed within the giant axons of arthropod nerves have demonstrated that an increase in K^+_e can reduce the resting potential and give rise to depolarization.

One explanation for the elicitation of the Bezold-Jarisch reflex may be that the ionic transport mechanisms of the heart are more sensitive to the action of protoveratrine than other tissues, and that K^+ released from cardiac muscle cells in very close/

close proximity to the sensory nerve endings of cardiac afferent fibres is sufficient to produce a depolarization in the ending and initiate the Bezold-Jarisch reflex.

Intra-coronary injections of K^{+} will not normally elicit the Bezold-Jarisch reflex but if a previous dose of an active Veratrum alkaloid has been given a subsequent dose of K^{+} may elicit the reflex.[18] One explanation for this may be that the alkaloid in addition to increasing the local K^{+} concentration in the region of the nerve ending, also sensitizes the ending to the stimulant action of the ion in a similar manner to its action upon frog skeletal muscle.

The results obtained using protoveratrine, which have been described in Chapter 2 and 3 of this thesis, support the conclusions reached by previous workers who used veratrine. One of these conclusions was that many of the pharmacological effects of the Veratrum alkaloids could be antagonized by excess Ca^{++}.

A full understanding of the part played by Ca^{++} in cellular processes is still far from being achieved, much more work must be done before any of the hypotheses put forward to explain its actions can be accepted. Any attempt to explain the action of protoveratrine or the other Veratrum alkaloids on the basis of an effect upon Ca^{++} metabolism must, therefore, be of a speculative nature.

From/

From the evidence summarised in Chapter 1 it may be assumed that the Veratrum alkaloids do reduce the stability of the cell membrane, and that this effect is related to alterations in the normally existing relationships between Ca^{++} and the cell membrane.

The findings described in Chapter 13 support the hypothesis of Gordon and Welsh[131] that the Veratrum alkaloids produce their effect by a process which involves a reduction in the power of the forces which are responsible for the fixation of Ca^{++} to the membrane surface. The evidence in this study, however, was obtained from skeletal muscle and no support could be gained for this hypothesis from the results obtained with nerve. These conflicting findings may be due to the differences in the structures of these two tissues, or to the different techniques employed in these studies. One obvious difference was that the volumes of the muscles were much greater than those of the nerves and the radioactivity of the bathing fluid was therefore much higher. Differences due to the effect of the drug were thus more readily detectable when muscle was used.

The most probable explanation, however, of the difference between the amounts of Ca^{++} displaced by protoveratrine from nerve and muscle lies in the difference in structure of the two tissues.

The/

The experiments using nerves were performed on arthropod nerve with the hope that influence of the myelin sheath on ionic movements would be minimal in this tissue. At one time these nerves were thought to be unmyelinated, but more recent work has demonstrated the presence of a myelin sheath.[261] This is much thinner than the corresponding sheath of vertebrate nerve but may still be sufficient to prevent any release of Ca^{++} into the bathing fluid. It has been demonstrated that the epineurium of frog nerve[262,263] offers a considerable barrier to ionic exchange and it may be that the myelination of the lobster nerve acts in a similar manner.

Frankenhaeuser and Hodgkin[266] have suggested that in Loligo nerve axons free diffusion of K^{+} may be prevented by the presence of a second diffusion barrier. This is a membrane associated with the Schwann cells and is distinct from the cellular membrane of the nerve. The authors suggest that the negative after potential is due to the accumulation of K^{+} between these two membranes.

This diffusion barrier being associated with the Schwann cells would only be present in nerves and may well be the cause of the differences in the findings for nerve and muscle.

A third factor which may account for the discrepancy between the results obtained using nerve (Chapter 14) and those from studies with muscle, (Chapter 13), is that the muscles studied/

studied were suspended in a large volume of solution which was continually mixed by a stream of oxygen bubles. This mixing would prevent any unbound Ca^{++} from remaining in the vicinity of the muscle and the released ions would be distributed throughout the bathing fluid. In the experiments with the nerves the bathing fluid was not agitated because of the limitations imposed by the method. Thus the Ca^{++} which may have been displaced by the drug in a similar manner to that observed in muscle, amy have remained in close proximity to the nerve and not been detected.

Great care was taken in both the dissection and subsequent handling of the nerves during the experiments but no observations on the viability of the nerves were made, and it may have been that the nerves were no longer responsive to the effects of the drug. Limitations of time and equipment made it impossible to develop a method by which these difficulties could be overcome, but measurement of the conducted action potential of the nerve at the end of a run would seem to be one method of overcoming this uncertainty.

All of the hypotheses which have been summarised in Chapter 1 require that the Ca^{++} be bound to the cell surface in some way. The most likely method of which is one involving surface adsorption. The most likely site for this adsorption is the lipoprotein layer which is believed to exist at cell surfaces. /

surfaces.[155,163,264] Monne[265] has suggested that polypeptide chains of the surface proteins may be linked by the divalent Ca^{++} which links a nucleic acid residue with a phosphatide residue. The making and breaking of this link produces changes in the configuration of the protein surface which could account for the permeability changes produced by alteration of the Ca^{++} concentration.

Protoveratrine may act either directly upon the surface structure of the cell membrane to alter the protein configuration, or by steric hindrance to prevent the adsorption of the Ca^{++}. The possibility that the molecule of protoveratrine acts by forming a chelate compound with Ca^{++} was considered, but this explanation was considered highly unlikely due to the absence of a suitable lone pair of electrons in the protoveratrine molecule, this configuration being necessary for chelate formation.

Although the exact chemical nature of the protoveratrine molecule is still unknown it is believed to have an alkamine skeleton which is hydroxylated to an even greater degree than that of cevine (see Chapter 1).

From a consideration of the stereochemistry of the cevine skeleton the molecule may be visualized as having an essentially planar structure with a number of projecting hydroxyl groups. These groups form convenient points for hydrogen bonding and it

is/

is not unlikely that the molecule may be adsorbed to the cellular surface by hydrogen bonding. This binding of the molecule to a polypeptide chain of the surface protein may affect the fixation of Ca^{++} in two ways.

As a result of the hydrogen bonding the configuration of the surface proteins may be altered in such a way that the space between adjacent polypeptide chains which is normally bridged by Ca^{++} is too large or too small to allow effective binding by the Ca^{++}.

A second possibility is that the binding of the molecule of protoveratrine at the surface prevents the access of the Ca^{++} to the receptor sites on the polypeptide chains. This possibility seems the less likely of the two because of the large difference in size between the two molecules.

It has been shown both in the introductory chapter of this thesis and in Chapter 3 that the pharmacological effects of protoveratrine can be antagonized by increasing the concentration of Ca^{++} in the bathing solution. This would suggest that a dynamic equilibrium exists between the protoveratrine and Ca^{++}. This fact supports the view that both these agents act at similar sites. The reversibility of the protoveratrine effect favours the suggestion that the forces binding the molecule to the protein surface are not chemical in nature, and makes it unlikely that protein denaturation occurs.

Shanes/

Shanes[127] has suggested that veratrine delays recalcification during the repolarisation phase of the action potential in electrically stimulated nerves, and although in this work the measurements of Ca^{++} displacement were made on resting muscle it is probable that a similar mechanism is affected in both cases.

Insufficient evidence has been obtained in this study to allow any conclusions to be drawn as to the actual part played by Ca^{++} in the control of cellular permeability. The suggestion of Frankenhaueser and Hodgkin[176] that in the resting state of the membrane Ca^{++} is combined with a Na^{+} carrier system is of great interest. Straub[108] suggests that veratrine interferes with this combination and frees the carrier for Na^{+} transport.

These suggestions must still remain speculative but the evidence obtained in this study gives indirect support to these hypotheses, but it is impossible to tell from the experimental data obtained whether the Ca^{++} which has been shown to be released by protoveratrine does in fact arise from the Na^{+} transporting system or from a general source on the cell surface.

Straub[108] suggested that veratridine increase the permeability of the cell membrane of nerve to both Na^{+} and K^{+}, but/

but the findings described in this thesis for K^+ at any rate do not provide any evidence that a similar process to that occurring in nerve takes place in muscle. The possibility exists, however, that changes in Na^+ fluxes do occur without any accompanying changes in K^+ flux. From the results of a few exploratory experiments on frog muscle which were made using radioactive sodium, $^{24}Na^+$, and which have not been described in this thesis, this seems unlikely, as no increase in Na^+ permeability could be demonstrated.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS.

1. In the introductory chapter of this thesis both the past and present value of the use of the Veratrum alkaloids in therapeutics has been reviewed. A number of theories which have been put forward to explain the mode of action of the active ester alkaloids of Veratrum have been summarised and critically examined. From a consideration of the previously published work a number of experiments were planned with a view to elucidating further the relationships between the action of the purified Veratrum alkaloid protoveratrine and the metallic cations of potassium and calcium.

2. In Chapter 2 are described the results of the alteration of the ionic composition of the bathing fluid upon the sensitizing effect produced by protoveratrine upon the stimulant action of K^+ on frog skeletal muscle. The results obtained indicate that variations in the K^+/Ca^{++} ratio can affect the K^+ sensitizing power of protoveratrine.

3. The effect of protoveratrine on the responses of preparations of isolated mammalian smooth muscle to the neurohumoral agents acetylcholine, adrenaline, histamine, 5-hydroxytryptamine and oxytocin was studied. Protoveratrine did not antagonise acetylcholine or histamine in doses up to 10 ug. per ml. but this drug showed both antagonism and sensitization to the stimulant effect of 5 H.T. in small doses. These different effects may be due to differences in the state of the tissue.

Oxytocin/

Oxytocin induced contractions of the rat uterus were inhibited by 10 ug. per ml. of protoveratrine. Possible explanations of these effect have been discussed.

4. A number of experiments were carried out to determine the effect of the administration of protoveratrine on the level of K^+ in the plasma of blood taken from different anatomical areas of rabbits and cats. A rise in plasma K^+ concentration was shown to occur and it has been shown that the probable source of the released K^+ is cardiac muscle.

5. By various techniques it has been shown that protoveratrine in doses sufficiently high to produce its normal pharmacological effects, has no effect on the efflux of K^+ from amphibian or mammalian skeletal muscle in vitro or in vivo.

6. By the use of radioactive tracer techniques it has been shown that protoveratrine can cause an increase in the K^+ efflux from electrically driven, isolated, perfused mammalian hearts.

7. Exposure of amphibian skeletal muscle to protoveratrine has been shown to cause an increase in the uptake of K^+ from the bathing medium.

8. Amphibian skeletal muscles which had been previously loaded with $^{45}Ca^{++}$ and subsequently exposed to protoveratrine A were shown to release more $^{45}Ca^{++}$ into the bathing fluid than did the corresponding control muscles.

9./

9. Protoveratrine A in concentrations of up to 100 ug. per ml. had no effect on either the rate of $^{45}Ca^{++}$ release or the total release of $^{45}Ca^{++}$, from $^{45}Ca^{++}$ loaded arthropod nerve.

10. The findings described in the experimental section have been discussed in relation both to previous theories of the mode of action of the Veratrum alkaloids, and also in the light of present day knowledge of the structure of the cell and the possible processes responsible for ionic transport across the cell membrane.

An attempt has been made to explain the different effects of protoveratrine on cardiac and skeletal muscle and also between muscle and nerve, and to relate these findings to the action of the alkaloid at sensory nerve endings.

The results obtained in this study support the theory of Gordon and Welsh that the Veratrum alkaloids act by displacing Ca^{++} from the membrane and reducing the stability of the membrane with a possible increase in ionic permeability in some tissues, e.g. cardiac muscle. The suggestion is also made that the increased uptake of K^{+} demonstrated in Chapter 12 may be due to the binding of K^{+} at anionic sites normally occupied by the displaced Ca^{++}.

APPENDICES

APPENDIX I

The composition of the physiological solutions used in this work is given in Table 16.

In Chapter 2 the modified frog Ringer's solutions were made up by reducing the concentration of either $CaCl_2$ or KCl in the solution, to maintain the bathing solution at the correct tonicity the concentration of NaCl was increased by a calculated amount to compensate for the ions lost by the reduction of KCl or $CaCl_2$ concentration.

In Chapters, 12, 13 and 14 the radioactive solutions in which the tissues were bathed, were made by replacing the KCl or $CaCl_2$ by a calculated quantity of isotopically labelled solution. This amount varied with the specific activity of the isotopic solution and was calculated for each experiment.

The lobster saline solution was modified from the figures given by Pantin[267] which were based on the concentrations of ions found in lobster blood. These figures were modified at the suggestion of Robertson[268] who found some slight differences in ionic concentrations from those given by Pantin.

The solutions of protoveratrine and protoveratrine A used in the experimental work were made by diluting concentrated stock solutions of the drug with the appropriate physiological saline to be used. These stock solutions were made by dissolving the free alkaloidal base in N/100 hydrochloric acid, 1 ml. for every 1 mg. of base. This solution was then brought to pH 6.8 by the addition/

TABLE 16

Composition of Physiological Salines Used.

Salts mmol/l / Solution	NaCl	KCl	$CaCl_2$	$MgCl_2$	Na_2HPO_4	NaH_2PO_4	K_2HPO_4	$NaHCO_3$	Na_2SO_4	$MgSO_4$	Dextrose
Frog Ringer's	111.20	1.88	1.08	-	-	-	-	2.38	-	-	-
Fenn-Ringer's	111.20	2.50	1.80	-	2.50	0.50	-	-	-	-	-
Tyrode's	136.80	2.68	1.80	0.10	-	0.32	-	11.90	-	-	5.55
Locke's	153.90	5.63	2.16	-	-	-	-	5.95	-	-	5.55
Krebs-Henseleit	118.30	4.69	2.61	-	-	-	1.17	14.16	-	2.41	11.10
De Jalon's	153.90	5.63	1.44	-	-	-	-	5.95	-	-	2.77
Lobster Saline	490.30	8.75	57.6	27.4	-	-	-	2.48	31.70	-	-

addition of N/100 sodium carbonate solution. Stock solutions containing 1 mg. per ml. calculated as the free base were made up and freshly diluted as required. These stock solutions were kept in the dark at $4^{o}C$ and showed no appreciable deterioration over a period of two weeks.

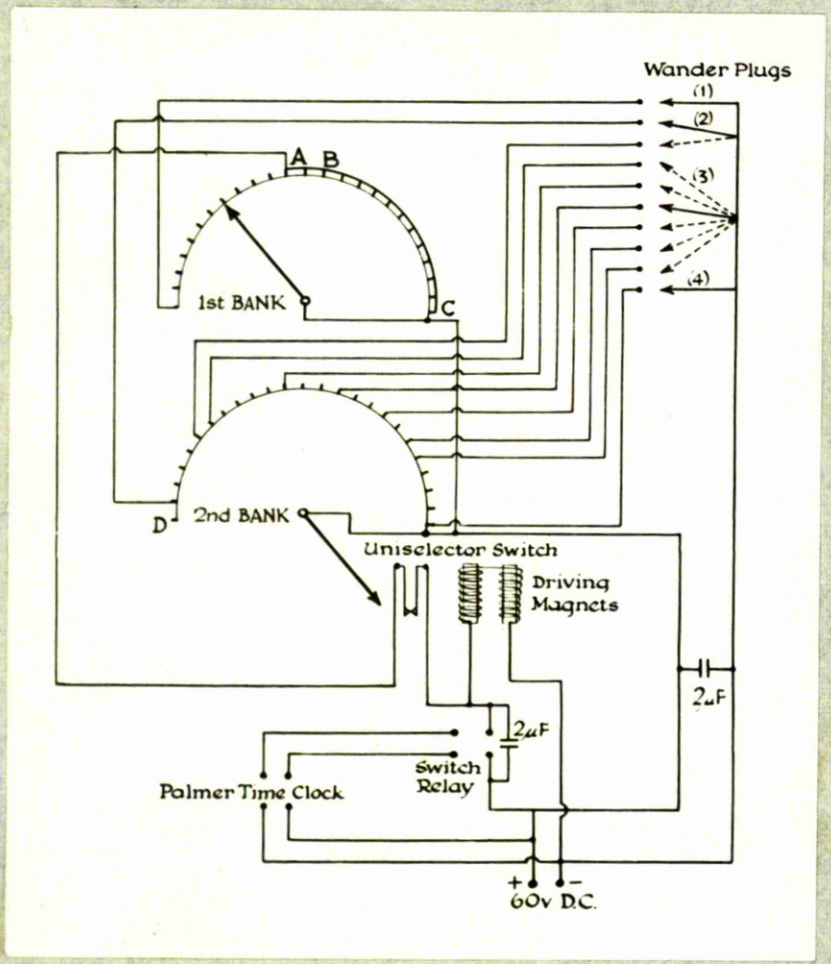

Fig. 34.

Circuit diagram of the electrical equipment used to control the semi-automatic isolated organ bath.

APPENDIX II

THE ELECTRICAL CONTROLLING MECHANISM OF THE SEMI-AUTOMATIC ISOLATED ORGAN BATH

The apparatus was developed in conjunction with Dr. C.N. Gill 269 and is modified from that described by Gaddum and Lembeck.

Post Office switch relays were modified slightly and used to control the flow of solutions into the isolated organ bath. Activation of the electromagnets by an electric current from an uniselector released the pressure on the feed tubing and allowed the solution to flow from the aspirator into the bath. The details of this mechanism can be seen in Fig.5.

A Post Office pattern uniselector was used to control the activation of the electromagnets, at predetermined intervals, the uniselector was driven by impulses from a Palmer time clock whichwere first passed through a switch relay to reduce sparking. The impulses were at 5 second intervals and thus the sweeper arms of the uniselector advanced one contact at each interval. To enable a continuous activation of the semicircular bank of contacts over the whole of the 5 minute time cycle chosen, the opposite halves of the upper and lower sweeper arms were removed.

The upper bank of 25 contacts was therefore activated for one half of a revolution of the sweeper arm and the lower bank for the second half of the revolution. To enable a full cycle of 5 minutes to be obtained 14 contacts in the upper bank were short circuited, this arrangement can be seen in the circuit diagram Fig.34. The uniselector functioned in the normal manner. When the sweeper arms made contact with the wired bank contacts, current/

current was led either to the switch relays controlling the fluid flow or to indicator lights. Each lead from the contact banks was led to a wander plug board which enabled both the time of exposure to the stimulant drug and the period of exposure to the antagonist, to be varied to suit the experiment.

Wander plug 1 was in circuit with an indicator light which lit 5 seconds before the addition of the solution containing the stimulant drug.

Wander plug 2 was in circuit with an indicator light which lit to indicate the time for the addition of the antagonist either 60 or 90 seconds before the addition of the stimulant drug.

APPENDIX III

Determination of K^+ concentrations in samples of plasma physiological salines.

All determinations were performed using the flame photometer manufactured by Evans Electroselenum Limited. This machine gave a full scale deflection with 10 parts per million (10 p.p.m.) of K^+. The air supply to the machine for the early experiments was obtained from a cylinder of compressed air fitted with a special reduction head, in the later experiments a matched air compressor pump was used. The gas used was from the normal town gas supply.

To eliminate errors due to the interference effects of other metallic ions, especially Na^+ all determinations were made by comparing the sample with a series of standard solutions in which the interfering ions were present in approximately the same concentrations as in the samples. All the standard solutions were made up with 'Analar' standard chemicals dissolved in double glass distilled water.

For the determinations of plasma K^+ a stock solution containing 50 mg. of K^+ per litre was made up using a 0.9 per cent solution of 'Analar' quality sodium chloride in double distilled water. Standard solutions of 1,2,4,6,8 and 10 p.p.m. were made by suitably diluting this stock solution. 0.2 ml. of plasma were removed from the centrifuged blood samples and diluted to 10 ml. with double distilled water. The standard solutions used for the determination of the K^+ content of the bathing fluid in the isolated muscle experiments were made by making up the stock solutions in K^+ free Fenn-Ringer's solution and diluting this/

this with double distilled water.

A calibration curve was obtained by running each of the standard solutions through the instrument and noting the scale reading for each. It was found that the instrument needed to be left for up to an hour before its operating temperature was constant and then the standardisation procedure was repeated until constant values for each standard solution were obtainted. When the readings were constant a calibration curve was drawn by plotting the scale readings against the concentration of K^+ in the standard.

The K^+ content of the plasma samples was determined by diluting 0.2 ml. of plasma to 10 ml. with double-distilled water. These were sprayed into the instrument and the scale readings noted. The samples of bathing solution were usually assayed for K^+ without any dilution but if this was necessary the samples were suitably diluted to bring the deflection within the range of the maximum sensitivity of the instrument.

The concentration of K^+ in the sample was determined by reading off the concentration equivalent to the reading from the calibration curve.

Three determinations were made on each sample and the mean value taken. At frequent intervals during the determinations the standard solutions were checked and if necessary the instrument we readjusted to give the same deflections as before.

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