INVESTIGATION OF THE CARDIAC GLYCOSIDES OF ADENIUM OBESUM (Roem & Schult) ISOLATION, IDENTIFICATION AND PHARMACOLOGICAL PROPERTIES

BY

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

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This thesis has been submitted for examination with our approval as University supervisors.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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Finally, it is perhaps worth noting here that the work for this thesis was carried out under very unfavourable conditions, at a time when the Department of Pharmacy was at its infant stage of development and hence not adequately equipped.

ABSTRACT

Adenium obesum (Roem and Schult), family apocynaceae, was chosen for investigation as a result of information obtained from indigenous people of Lunga Lunga area, along the Kenya - Tanzania border which indicated that this plant had some medicinal properties. Preliminary phytochemical screening of the material (tuber) showed that the plant had one or more compounds with α β unsaturated lactone rings, structures commonly associated with cardiac glycosides. Preliminary screening of the material was followed by a more detailed investigation, in an attempt to isolate and characterise the glycosides.

The dried powdered material was extracted with 70% ethanol, the extract detannated, and subsequently concentracted by distillation of alcohol at low temperature using a rotary evaporator. The aqueous suspension obtained was extracted with petroleum spirit (40-60°C), diethylether, chloroform and chloroform-ethanol 2:1 in that order so that fractions A, B, C and D respectively were obtained. A TLC examination of the four fractions showed that fractions, A, C and D contained relatively small amounts of glycosides which were also present in fraction B, the ether soluble fraction. Adenium obesum was found to contain four glycosides but only one has been investigated in detail in the present work.

Fraction B, which was shown to contain three glycosides was subjected to various isolation and purification procedures. These included washing with petroleum spirit 40-60^{o}C, decolourisation with activated charcoal and use of column chromatography. Whereas the decolourisation with charcoal was unsuccessful, the column chromatographic procedure removed most of the coloring matter from glycosides. However, no separation of the glycosides was achieved at this stage. The combined eluate fractions after evaporation to dryness (PE) were examined by UV and IR spectrophotometers and found to show features characteristic of cardiac glycosides.

A TLC comparison of PE with the glycosides of Adenium boehmianum Schinz showed that two of the glycosides of PE had Rf values similar to those of somalin and echujin. The third glycoside of PE had an Rf value, completely different from any of the reference compounds used. This third glycoside appeared to be the major constituent of PE.

A column chromatographic separation of the glycosides of PE was attempted. The eluate fractions were examined by TLC and those having the same compound pooled together to give three fractions DSK1, DSK2,and DSK3. After concentration to a small volume, each of the 3 fractions was examined again by TLC. Fractions DSK1 and DSK2 were shown to contain two glycosides each, but DSK3 was shown to contain a single glycoside.

An attempt to separate the glycosides of DSK1 by column chromatography resulted in the recovery of only a very small amount of one glycoside, with an Rf value very similar to that of echujin, and an absorption maximum at 218 nm, characteristic of cardiac glycosides. Fraction DSK1 was not further examined.

A preparative scale TLC separation of the glycosides of DSK2 was attempted. This procedure was found to require a large number of uniformly coated plates. One of the glycosides in DSK2 had the same Rf as that in fraction DSK3. The other one was present in very small amounts. Several attempts were made to recover adequate amount of the latter glycoside for identification purposes (IR, element analysis, molecular weight etc) but this was unsuccessful. DSK2 was set aside for future reference. The major constituent of DSK2, was in any case also the major constituent of DSK3 which was subsequently examined.

About 3.8 g of DSK3 was obtained from 4 kg of the dried tuber. The UV absorption spectrum of DSK3 showed a major absorption peak at 217 nm, with an inflexion at 211 nm and a minor absorption at 275 nm. Both the inflexion at 211 nm and the minor absorption at 275 nm were thought to be due to impurities and further purification of DSK3 was attempted.

Small samples of DSK3 were subjected to different treatments. These included passage through a chromatographic column, washing with either diethylether, water, dilute alkali or dilute acid solutions.

The column chromatographic prodecure, washing with water and dilute alkali solution did not affect the inflexion at 211 nm or the minor absorption at 275 nm.

The ether washed sample of DSK3 showed a UV absorption spectrum without the inflexion at 211 nm, but the minor absorption at 275 nm. was still evident.

The acid washed DSK3 sample showed an absorption spectrum with a considerably magnified inflexion at 211 nm. Furthermore the ether washed sample, without the inflexion at 211 nm, on washing with dilute acid solution showed a reappearance of the inflexion at 211 nm. The minor absorption at 275 nm was not affected by the acid washing.

Of all the purification procedures attempted, only the ether washing proved beneficial. The bulk of DSK3 was therefore washed with diethylether and dried.

The IR spectra of DSK3, somalin and echujin were obtained and compared. Neither of the two reference spectra was superimposable with the spectrum of DSK3.

A TLC comparison of DSK3 with the glycosides of Adenium boehmianum Schinz and also with digoxin and ouabain showed that DSK3 was not identical with any of the reference compounds used. The Rf value of DSK3 was higher than those of digoxin and ouabain. The Rf value for DSK3 was however, lower than those of the glycosides of Adenium boehmianum Schinz, under the experimental conditions.

Two sets of element analytical data were reported by two different laboratories. BMAC (England) reported carbon (C = 65.03%), Hydrogen (H = 8.53%), the rest 26.44% being oxygen as no other elements were reported. Pharm. Inst. Ethz. (Switzerland) reported C = 64.0%, H = 8.2%, N = 0.0% and O = 25%.

The molecular formula of DSK3, calculated from the element analysis results and the reported molecular weight of 739 was $C_{39}H_{65}O_{13}$.

The ^{13}C NMR spectral analysis results showed that DSK3 contained 39 carbon atoms in its molecule. This was consistent with the calculated molecular formula $C_{39}H_{65}O_{13}$.

The Beer-Lambert Law relationship was investigated, using the absorbance of the pure compound at 217 nm, the absorbances of the coloured complexes formed with alkaline dinitrobenzene and alkaline 3:5 dinitrobenzoic acid.

In all cases, Beers Law was obeyed, except for a slight deviation with the results obtained with the pure substance DSK3 at 217 nm.

The $E^{1\%}_{1cm}$ values as well as the molar extinction coefficient calculated using the results for the coloured complexes referred to above were time dependent and therefore require very accurate definitions of the experimental conditions in order to be meaningful.

In contrast, the $E^{1\%}_{1cm}$ value calculated using the absorbance at 217 nm was an independent constant. The slight deviation from Beers Law was probably due to interferences by solvent absorption and instrumental error at this low wavelength region. The equation of the line changes slightly from $Y = MX$ to $Y = MX + C$ (where C is a constant value for all absorbance readings due to interferences referred to above).

Pharmacological experiments were carried out using anaesthetised rat, anaesthetised cat and Langendorffs preparations. In all cases, DSK3 was found to have the typical cardiovascular effects of cardiac glycosides, namely a transient pressor, a positive inotropic and negative chronotropic effect on the preparations used.

CHAPTER I

INTRODUCTION

Adenium obesum (Roem & Schult) is a member of the plant family Apocynaceae. Many plant species belonging to this family are widely distributed in tropical zones, particularly in Africa and South America.

The exact details as to how and when the African peoples discovered the very toxic nature of many of these plants will probably never be known. It is now known, however, that these communities had for centuries, used extracts of these plants as arrow and ordeal poisons.

The preparation and use of arrow poisons has adequately been described by Raymond (6), Watt and Breyer-Bradwijk (17) and Maitai (20, 24)

Numerous reports have been encountered regarding the use of these plants as sources of ordeal poisons. An interesting account of the trials by ordeal using these plant extracts has been described by Taylor (21). Accused parties were given prescribed, obviously toxic doses of decoctions of these plants (mixed with other ingredients) which they were forced to take orally.

The innocent ones reportedly gulped the decoctions quickly (out of confidence) resulting frequently in the regurgitation of most of the poisons (probably due to their irritant effect on the gastric mucosa). Little of the poisonous principles were therefore absorbed under these conditions so that the innocent rarely died from their toxic effects.

The guilty, obviously afraid of death reportedly took the decoctions slowly and reluctantly. Under these conditions, vomiting rarely occured and enough of the toxic principles were absorbed, subsequently causing the death of the victims.

The rationale behind the trials by ordeal was that the guilty would die while the innocent would survive after ingesting the prescribed decoctions.

Many of the different species of apocynaceae, including those very closely related to Adenium obesum are known to contain cardiac glycosides. Bearing in mind the now known properties of cardiac glycosides, it is not surprising that these plant extracts were so effective both as arrow and ordeal poisons.

Among those species that have been investigated, many have been found to have a digitalis type of action. In many cases, the cardiac glycosides have been isolated and characterised (Table I).

Digitalis itself was introduced into medicine during the latter part of the 18th Century by William Withering.. A secret remedy for dropsy from a Shropshire woman had led Withering to the discovery of digitalis as the active ingredient among twenty or so other ingredients in the decoction (12). Withering's discovery subsequently led to the isolation and use of digoxin and digitoxin as pure cardiac glycosides in clinical situations.

Many other species have subsequently been studied, their toxic principles isolated and studied both for their chemical and pharmacological properties.

The African arrow poisons on the other hand led to the discovery of a very useful cardiac glycoside, ouabain. This drug was introduced into medicine in 1890 by Sir Thomas Fraser after a study of African arrow poisons. Ouabain is now widely accepted as the drug of choice for the initial digitalisation of patients in emergency cases of congestive heart failure. This is due to its very rapid onset of action. It is obtained from strophanthus gratus, although it has also been found to be present in Acokanthera schimperii Benth & Hook (Table I).

Thus far, the efforts of synthetic chemists have failed to produce compounds equivalent, let alone superior, to digoxin, the standard cardiac glycoside in clinical use. It is therefore apparent that man's current reliance on plant sources for cardiac glycosides may continue for some time to come.

Unfortunately, the therapeutic index of cardiac glycosides in clinical use is known to be very low. The effective dose levels are not very far from toxic dose levels and this fact posed considerable problems in the past as it still does today. Thorp et al (10) have in fact suggested that research should be directed more towards the search for cardiotonics other than cardiac glycosides.

These workers argue that it might be possible to find substances as effective but less toxic than cardiac glycosides.

This is perhaps good reasoning as far as it goes. However, as in the case with digoxin and ouabain, each of which has marked advantages over the other in different situations of clinical usage, it might be possible to find yet another cardiac glycoside from plant sources that may be a useful addition to those currently in use.

Ouabain for instance, although being the drug of choice for the initial digitalisation in emergencies as already mentioned, is unsuitable for maintenance therapy. This is mainly due to the need for very frequent medication necessary in contrast with the case of digoxin, the latter having a much longer duration of action. The point here therefore is that there is need to look for new cardiotonic substances (other than cardiac glycosides) and also possibly for new cardiac glycosides.

Adenium obesum was chosen for more detailed investigation after preliminary phytochemical screening had shown that it contained one or more compounds with the $\alpha\beta$ unsaturated lactone ring; a characteristic feature of cardiac glycosides and also because it has been used locally as an arrow poison.

It is perhaps interesting to note here that other plants now known to contain cardiac glycosides had been used by both Eastern and Western European communities mainly as cures for dropsy.

Literature survey carried out in the present work revealed no reference to the use of Adenium obesum as a dropsy cure. Information obtained from several indigenous people of Kenya who use the plant did not indicate that the plant is of any value in the treatment of dropsy.

A parallel however, was found in that whereas digitalis was employed against dropsy in Europe and Adenium obesum for syphilis in Eastern Africa the two presumed cures depended on the diuretic effects of the cardiac glycosides.

It is known that excess body fluid in dropsy cases is mostly due to congestive heart failure.

The positive inotropic effects of digitalis upon themyocardium improves the working capacity of the heart thus improving the circulation. The improved circulation results in diuresis so that excess body fluid is voided.

The use of Adenium obesum as a "cure" for syphilis although perhaps not rational in terms of antibiosis was nontheless a recognition of the diurectic properties of the cardiac glycosides contained therein.

Cardiac glycosides are thought to exert a direct effects on the kidneys to produce diuresis. The constant flushing of the urinary tract therefore probably gives some symptomatic relief to the sufferers of syphilis who use this native herb. This diuresis also probably helps to keep the numbers of the infective organism (Treponema Pallidum) in the urinary tract down. This may also have the effect of delaying somewhat the general spread and worsening of the disease state.

LITERATURE REVIEW

A review of literature on Adenium obesum revealed a considerable amount of controversy regarding the naming of this and related species.

Verdcourt (14) gives Adenium multiflorum as a synonym for Adenium obesum, thus implying that these two are the same. It was noted, however, that this author (Verdcourt) expressed some doubt as to the synonymity.

Watt and Brandwijk (17) distinguish Adenium obesum and Adenium multiflorum as two different species. These authors therefore do not agree with the synonymity suggested by Verdcourt.

The controversy is further apparent in the work of Dalziel (10). Dalziel claims that Adenium coetanum Stapf or Adenium speciosum is probably the same species referred to as Adenium obesum by Roem and Schult (10). According to Dalziel, Adenium obesum itself is an Arabian species not found in East Africa.

Watt and Brandwijk (17) claim that both species (Adenium obesum and Adenium coetanum Stapf) occur in East Africa and that they have both been used as arrow, fish and homicidal poisons.

A photograph of Adenium multiflorum presented by Watt and Brandwijk (17) was compared with the plant under investigation in the present work. It was apparent that these two plants were unlikely to be the same.

The possibility that Adenium coetanum Stapf and Adenium obesum, Roem and Schult were the same plant could not however be ruled out. Several research workers (14, 18) in fact seem to think that these two are one and the same plant.

The literature on Adenium coetanum Stapf was reviewed in view of the suggested similarity. This plant is reported to have been used in Tanganyika as an arrow as well as fish poison (17). It is also reportedly used by the Bena tribe (in Tanganyika) to mark a grave. The dry extract from a twig is said to be highly toxic, 10 mg killing a guinea pig in thirty minutes, with tonic and clonic convulsions, paralysis of the jaws and bladder. No report, however was found regarding the isolation and characterisation of cardiac glycosides from Adenium coetanum Stapf.

Adenium honghel is given as a synonym of Adenium obesum by Verdcourt (14). The literature on Adenium honghel was therefore reviewed in view of this. This plant (A. honghel) has been known to yield a glycoside, hongheloside G which has been reported to be identical with somalin (17).

The latter glycoside was isolated from Adenium boehmianum schinz by Reichstein et al (23).

In the light of these findings, Adenium obesum might be expected to contain somalin, besides possibly other cardiac glycosides. It is interesting to note here, the common occurrence of this glycoside (somalin) in different species. (1, 2, 42, 43, 44, 45, 46, 47, 60) The names of three different species of the family Apocynaceae are given in Table 2 along with glycosides isolated from them.

In view of the finding that two or more species may contain one, two or more glycosides in common, classification of these plants on the basis of glycosidal content would appear to be a difficult process.

Adenium obesum was described by Verdcourt (14). According to Verdcourt, the plant is a succulent shrub 0.3 meters tall with a very swollen lower stem.

The leaves are hairy or hairless, oblong reversed ovate or oblong, are very rounded at the apex or shortly acute from a round apex gradually narrowing to the base. They (leaves) are 3.2 - 9.5 cm. long and 0.76 - 3 cm. wide.

The flowers are in small clusters, pink or scarlet coloured with a funnel shaped tube which is very narrow at the extreme base.

They are 2.5 - 5 cm long and have a fine apex. These lobes are 1.2 - 3 cm. long and are finely hairy. The fruits consist of paired pods 15.2 - 17.8 cm. long. The seeds are oblong with a parachute at both ends and are about 7 cm. long overall.

According to Verdcourt (14) Adenium obesum is widely distributed in East Africa. It is found in Gulu (Uganda), Baralal, Marsabit, Lake Magadi and also the Kenyan Coast. The plant is also grown in Tanzania and certain parts of Kenya for ornamental purposes.

PRESENT WORK

In the present work, it was intended that after finding the number of cardiac glycosides in Adenium obesum plant material, at least one of these glycosides be isolated in pure form and identified. Identification of all glycosides in the plant would have required more time than is normally given for an MSc degree thesis. An attempt to characterise the pure glycoside from Adenium obesum formed an important part of the present work.

An exhaustive study of the pharmacological properties of the glycoside isolated from this plant (e.g. absorption, distribution, metabolism and excretion) was considered to be beyond the scope of the present work. A preliminary pharmacological screening of the isolated glycoside was, however, considered to be desirable. Of particular importance was the effect of the compound on the cardiovascular system and more specifically on the heart.

COLLECTION OF PLANT MATERIAL

The plant material for the present work was collected from Lunga Lunga, near the Kenya - Tanzania border. It fitted the description by Verdcourt (14) and was authenticated by the Botany Department of the University of Nairobi and also by a Botanist of the East African Herbarium, Nairobi. A specimen of this plant has been deposited in the Pharmacy Department, University of Nairobi. The young plant is very beautiful especially when in flower (Fig. 1). The underground stem (tuber) was found to be very large for older plants (see Figs. 2 & 3).

During the collection of plant material great care was taken to minimise injury to plant tissues. Such injury is known to cause the release of hydrolytic enzymes, which in the case of cardiac glycoside containing plants would hydrolyse off the sugar moieties. This hydrolysis posed considerable problems to earlier workers. Trim (15) had referred to the work of Stohl (1940), Stohl, Hoffmann and Kreis (1935) who noted that all cardiac glycosides isolated before 1930 were in fact artifacts due to such hydrolysis. Artifacts may also result from the sugar transferring action to certain glucosidases in the course of isolation procedures (15).

Ethyl primveroside obtained from Gautheria procumbens was shown by Rabate' (15) to be an artifact resulting from the rapid enzymatic transfer of primverose from monotripin. In order to minimise the risk of such hydrolysis or enzymatic transfer, the plant material (tuber) was quickly chopped to small pieces and dried in an oven at 60°C. At this temperature, it was hoped that any enzymes would be inactivated to a large extent. After three days in the ventilated oven, the tuber was not sufficiently dry for grinding. The material was therefore spread out on straw mats and dried in the sun for another three days.

The dry material was subsequently ground to a powdery consistency. The powder tasted very bitter and the fine dust from it evoked an allergic rhinitic condition in those present in the laboratory. Yet another surprising observation made was that when left in an open box overnight, a considerable number of house flies were found dead on top of the powder the following day. These two observations however, were not the subject of investigation in the present work.

PRELIMINARY SCREENING OF THE DRY POWDERED ADENIUM OBESUM TUBER FOR CARDIAC GLYCOSIDES

Before commencing on detailed experimental work, a preliminary investigation was carried out. The aim of this preliminary screening was to find out whether the dry powdered tuber (and hence the plant) contained cardiac glycosides.

A small amount of the dry powdered material was ground with 70% ethanol with a pestle and mortar. The detannated ethanolic extract after clarification gave a deep blue colour with alkaline 3:5 dinitrobenzene (Raymond reagent). This blue colour was found to be fairly transient.

Raymond reaction is characteristic of compounds containing α β unsaturated lactone ring systems. The presence of cardiac glycosides was therefore indicated by this reaction.

The plant material was therefore considered suitable for further detailed examination, with a view to isolating and studying the properties of cardiac glycosides present.

PREPARATION OF REAGENTS

1. 3:5 DINITROBENZENE SOLUTION (24)

A 1% solution of 3:5 dinitrobenzene in absolute ethanol was prepared by weighing the required amount of 3:5 dinitrobenzene (BDH) and dissolving it in absolute alcohol. It was found necessary to warm the solvent in order to enhance the dissolution process. The reagent was stored in amber coloured bottles.

2. 3.5 DINITROBENZOIC ACID (ALKALINE), KEDDE REAGENT (24)

This reagent was prepared by mixing equal quantities of a 2% solution of 3:5 dinitrobenzoic acid (BDH) in methanol and a 5.7% w/v aqueous solution of potassium hydroxide (KOH). Alternatively, this reagent (Kedde) was prepared by weighing 1 gm. of 3:5 dinitrobenzoic acid (BDH) and dissolving it in 100 ml. of a 0.5 N. potassium hydroxide (KOH) solution 50% methanol.

3. SODIUM HYDROXIDE SOLUTIONS (24)

These were prepared by dissolving weighed quantities of sodium hydroxide (NaOH) pellets in freshly boiled distilled water. For accurate work, the solution was standardised using standard hydrochloric acid (HCL).

4. KELLER KILIANI REAGENT (16)

A 5% solution of Ferric Sulphate ($Fe_2(SO_4)_3$ was prepared by dissolving the appropriate weight of ferric sulphate in distilled water. One volume of the resulting solution was added to 99 volumes of glacial acetic acid. This reagent was used with concentrated sulphuric acid to test for 2 - deoxy-sugars.

5. CHLORALOSE ANAESTHETIC (30)

A 1% solution of chloralose in normal saline was prepared by weighing the appropriate amount of chloralose (BDH) in normal saline. It was necessary to heat the solvent (normal saline) to about 80°C. to effect dissolution.

6. 2N SODIUM CARBONATE (SODA SOLUTION)

A weighed quantity of sodium carbonate was dissolved in freshly distilled water so that the resulting solution was 2N.

RINGER LOCKE

The following salts and sugars were weighed accurately using a Sartorius Microanalytical balance and dissolved in freshly distilled water.

TABLE 3

PREPARATION OF RINGER LOCKE SOLUTION

SALT/SUGAR	AMOUNT WEIGHED TO PREPARE 2 LITRES OF RINGER-LOCKE SOLUTION (GRAMS)
Sodium Chrolide (NaCL)	15.42
Potassium Chloride (KCL)	0.84
* Calcium Chloride ($CaCL_2.6H_2O$)	0.956
Sodium Hydrogen Phosphate (NaH_2PO_4)	0.208
Glucose or Dextrose ($C_6H_{12}O_6$)	4.0
** Sucrose ($C_{12}H_{22}O_{11}$)	9.0
Sodium Bicarbonate $NaHCO_3$	4.2

* Calcium Chloride was added after all the other solutes to avoid precipitation.

** Ringer Locke formula does not include sucrose. Sucrose however, has not been found to have any adverse effect on the formula and was therefore included.

REAGENTS/SOLVENTS USED

REAGENT/SOLVENT	GRADE	BRAND	
Absolute Alcohol	Laboratory		
Absolute Alcohol	Analar	Riedel - De Haen	
Alumina (TLC)	Type 60/E		Neutral
Alumina (Column)		BDH	Neutral
Alumina (Column)	Brockmann Grade I	Merck	Neutral
Acetone	Laboratory	M & B	
Benzene	Crystallisable	BDH	
Chloroform	Laboratory	M & B	
Chloroform	Analar	BDH	
Decolorising Chacoal		M & B	
3:5 Dinitrobenzene		BDH	
3:5 Dinitrobenzoic Acid		BDH	
Disodium Hydrogen Phosphate		M & B	
Ethyl Ether		BDH	
Ethyl Acetate		BDH	
Formamide		BDH	
Lead Acetate		M & B	
Lead Monoxide		M & B	
Methanol		BDH	
Methylene Chloride		BDH	
Ouabain		BDH	
Petroleum Ether 40-60°C		M & B	
Pyridine (absolute)		BDH	
Potassium Hydroxide		BDH	
Sodium Sulphate (anhydrous)		BDH	
Silica Gel G.		BDH	
Urethane (Ethyl Carbamate)		BDH	

INSTRUMENTS USED

1. Pye Unicam SP 800 UV Spectrophotometer.

2. Pye Unicam SP 8000 UV Spectrophotometer.

3. Pye Unicam SP 200 Grating IR Spectrophotometer.

4. Pye Unicam SP 1000 IR Spectrophotometer.

5. A Condon blood pressure manometer

6. Devices M2R and M2P blood pressure - heart rate recorder.

7. Langendorffs heart perfusion apparatus made by Scientific Research Instruments Ltd. (SRI).

8. Sartorius microanalytical balance.

9. The Ideal Respiration Pump. Model 16/24 made by G.F. Palmer (London).

10. Rotary Evaporator (Buchii).

CHAPTER II

ISOLATION OF CARDIAC GLYCOSIDE(S) FROM ADENIUM OBESUM MATERIAL

About 4Kg. of the previously dried and powdered tuberous plant material was placed in a 25 litre glass flask. Enough of 70% ethanol was then poured into the flask so that the material was completely soaked and excess solvent rose to a level slightly above that of the material.

A water cooled condenser was connected to the mouth of the flask after shaking thoroughly so that all the material was in contact with the extracting solvent. Thus extraction and further enzyme inactivation could be effected simultaneously (S.15)

The flask was then placed on a thermostatically controlled water bath which had been set at 60°C. The material was left to macerate at this temperature for three days. The water cooled condenser prevented the loss of solvent during the maceration process so that it was not necessary to top up the solvent.

After three days of maceration, the 70% ethanolic extract (yellowish brown) was separated from the ground plant material by straining through a muslin cloth and was clarified by filtration through a Whatmann No 1 filter paper.

The marc was again soaked and left in 70% ethanol at 60°C. for a further three days. The extract was again separated from the powdered material as before. This maceration process was repeatedly carried out until the remaining marc scarcely tasted bitter (almost glycoside free).

The extraction procedure was not a quantitative one. However, efforts were made to completely exhaust the glycosides from the powdered material. This was found to be very difficult under these conditions, particularly as there was no filter press or similar device available for separating the extract from the marc completely.

The clarified ethanolic extracts gave a strong Raymond reaction, were combined, and labelled OE This was set aside for subsequent investigation.

Removal of Tannins

When a small volume (5 ml) of the ethanolic extract (OE) was tested for the presence of tannins by means of 15% lead acetate solution, a yellowish precipitate was noted. Since certain cardiac glycosides are known to be very insoluble in water, it was considered necessary to show that the precipitate was not due to water insoluble glycoside or glycosides which may have precipitated out on addition of the aqueous lead acetate solution.

The control test was performed by adding to 5 ml of OE, a volume of distilled water equal to the volume of 15% lead acetate added in the previous test for tannins. No precipitate was observed on addition of water, and it was concluded that the precipitate obtained with lead acetate was due to tannins.

The whole of the ethanolic extract (OE) was therefore treated with 15% lead acetate solution until no further precipitation was noted. The nature of the precipitate made clarification by filtration very difficult. The lead acetate treated extract was therefore left undisturbed for about 48 hours and the supernatant subsequently decanted off.

The precipitate was washed several times with absolute ethanol and the ethanolic washings after clarification were combined with the detannated ethanolic supernatant and set aside for further purification.

Removal of excess lead

The detannated ethanolic extract gave a positive test for lead (a white precipitate of lead sulphate with dilute sulphuric acid).

The whole of the detannated extract was therefore treated with a strong solution of disodium hydrogen phosphate (Na_2HPO_4) until no further precipitation was noted.

The white precipitate was removed and washed in the same way as the lead-acetate-tannins precipitate.

The detannated, lead free ethanolic extract was combined with the clarified ethanolic washings and gave a strong Raymond reaction. The extract at this stage was found to be considerably less coloured than the original ethanolic extract (OE) and was set aside for further examination.

Solvent/Solvent extraction of the glycosides from an aqueous suspension

The detannated lead free ethanolic extract was freed from ethanol by means of a rotary evaporator at 60°C under vacuum. The volume of the aqueous suspension obtained was reduced from about 5 litres to 500 cc again under vacuum for convenience of handling. The suspension was placed in a 1 litre separating funnel and treated according to the method of Reichstein et al (23) as follows:

It was extracted 5 times with in each case 500 ml. of petroleum spirit (40-60°C).

The combined petroleum ether extracts were labelled FRACTION A and set aside for further examination.

The remaining aqueous suspension was again extracted 6 times with in each case 500 ml of diethyl ether. Further ether extracts gave a very weakly positive Raymond reaction. The combined ether extracts were labelled FRACTION B and set aside for subsequent examination.

The remaining watery suspension gave a positive Raymond reaction. It was extracted with Chloroform (FRACTION C) and then with Chloroform Alcohol 2:1 (FRACTION D). Fractions C and D gave positive Raymond reactions and were set aside for subsequent examination.

The remaining watery phase gave negative Raymond reaction and was not further examined.

Fractions A, B, C and D were subsequently subjected to a TLC analysis.

TLC examination of fractions A, B, C and D

Thin layer chromatographic 20 x 20 cm plates were prepared according to the method described by Stahl (25) using Silica Gel G. The plates were air dried by leaving them exposed at room temperature overnight. Several chromatographic tanks were prepared using different solvent systems (Table 4) and left to saturate for four hours.

Small volumes (about 20 ml.) of each of fractions A, B, C and D were evaporated to dryness by blowing hot air over surfaces of evaporating dishes containing these samples. The resulting residues were dissolved in small volumes of methanol (about 5 ml). The methanolic solutions were subsequently spotted onto the air dried Silica Gel G TLC plates. The spotted plates were placed in chromatographic tanks containing different solvent systems previously prepared. The original ethanolic extract (OE) was spotted on all plates for comparison purposes. The developed chromatograms were examined by spraying with Kedde reagent which produced fairly stable pink spots. The TLC results are shown in Table 4

Fraction A produced a very faint spot with an Rf value similar to that of the highest spot given by Fraction B. As this Fraction (A) also contained fatty and resinous materials, the very small amount of glycoside present was disregarded. Fraction A was therefore not further examined and was discarded.

Fraction C and D each contained one, possibly two cardiac glycosides in relatively small amounts.

The relative intensities of the pink spots produced with Kedde reagent suggested that the glycosides in Fraction C and D were present in greater amounts in Fraction B.

It was therefore considered that for the preparatory scale separation of the glycosides, Fraction B would probably yield a reasonable quantity of each glycoside. Furthermore, it was considered that most of the small quantities of glycosides in fractions C and D would probably be lost during the purification procedures.

Since only small amounts of glycosides were apparently contained in Fractions C and D, it was considered unnecessary to pool these fractions together with fraction B. In any case such procedure would probably have reintroduced into fraction B some impurities which had been extracted in Fractions C and D.

Fractions C and D were therefore not further examined and were discarded.

Fraction B appeared to contain three glycosides and attempts were subsequently made to purify this fraction.

CHAPTER III

PURIFICATION AND EXAMINATION OF FRACTION B (ETHER SOLUBLE FRACTION)

The combined ethereal extracts i.e. fraction B; (about 4 litres) were reduced to about 500 millilitres by distillation and the concentrated extracts placed in a 1 litre separating funnel. They were washed once with 100 ml. of 2N. HCl, once with 100 ml of 2N. soda solution (Na_2CO_3) and finally thoroughly rinsed with freshly distilled water. The extract was then dried over sodium sulphate and subsequently freed from ether. It was noted that the "residue" was a syrupy, viscous sticky mass, brownish in colour.

It was thought that the extract may have contained some sugars in the free form, which may have tended to caramelise on prolonged heating and thus contributing to the brownish colour.

The stickiness of the residue was considered to be possibly due in part, to the presence of waxes and resinous as well as fatty materials.

Attempts were subsequently made to purify the extract by washing with petroleum spirit (40-60°C), adsorption onto charcoal, and by column chromatography.

A. Purification by washing with petroleum spirit (40-60C)

The residue from fraction B was redissolved in methanol - water 70:30 (100 ml). The resulting mixture was found to be a milky suspension.

The suspension was placed in a separating funnel and washed 5 times with in each case 30 ml of petroleum spirit (40-60°C). The combined petroleum spirit washings were washed with 70% methanol after which they (petroleum ether washings) gave a negative Raymond reaction. The petroleum ether washings were not further examined and were thrown away.

The methanolic watery phase as combined with the 70% methanolic washings, The combined methanolic watery suspension gave a strong Raymond reaction and was set aside for further purification using charcoal.

B. Purification by Adsorption onto charcoal

The 70% methanolic solution was diluted with distilled water so that the final concentration of methanol was 10% v/v.

The mixture was placed in a round bottomed flask and refluxed with about 0.5 G of activated decolourising charcoal for half an hour.

The hot solution was filtered through a Whatmann No 1 filter paper to remove the charcoal.

The resulting clear, colourless filtrate gave a very weakly positive Raymond reaction. It was therefore apparent that most of the glycoside had been adsorbed onto the charcoal along with colouring matter.

The glycosides were recovered from the charcoal by washing with hot methanol and filtration. The washing procedure was repeated until any further methanolic washings gave a negative reaction to Raymond test for cardiac glycosides.

The mathanolic washings were combined with the 10% methanolic filtrate. The resulting solution gave a strong Raymond reaction showing that the glycosides had been recovered from the charcoal.

It was noted however, that this solution was also coloured (yellowish) showing that the colouring matter had been recovered from the charcoal along with the glycosides.

Further attempts were made to decolourise the methanolic solution of the glycosides. The methanolic watery solution from above was freed from methanol by distillation under vacuum, using a rotevaporator. The resulting aqueous suspension

was placed in a separating funnel and the glycosides extracted with ether to exhaustion. The glycoside free aqueous phase was discarded. The ether extracts were combined and dried over sodium sulphate (Na_2SO_4). They were subsequently freed from ether by distillation at 40°C.

The residue was redissolved in methanol (BDH) and the methanolic solution (100 ml) refluxed with about 0.5 g. of decolourising charcoal for 30 minutes in a round bottomed pyrex flask.

The hot methanolic solution was subsequently clarified by filtration through a Whatmann No 1 filter paper. The filtrate was found to be yellowish brown, (as before the attempted decolourisation) and gave a strong Raymond reaction. The charcoal used for this attempted decolourisation was shaken with hot methanol and the methanolic washing again filtered through a Whatmann No. 1 filter paper. The resulting filtrate gave a very weakly positive Raymond reaction, showing that very little of the glycosides had been adsorbed onto the activated charcoal.

It was therefore concluded that decolourisation of a predominantly aqueous or a methanolic solution of the glycosides was unsatisfactory and this method

was therefore abandoned.

The methanolic solution was therefore combined with the methanolic charcoal washings and set aside for further purification using column chromatography.

C. Purification by column chromatography

The methanolic solution of glycosides from the preceding section was evaporated to dryness by means of a rotary evaporator at 50°C (under vacuum).

The resulting residue was redissolved in the minimum amount of chloroform (about 10 ml).

An ordinary reflux condenser was employed as a mechanical support for the column. A "slurry" of granular Alumina (active, neutral) was made in ether (diethyl ether) and introduced into the thoroughly cleaned quickfit condenser. A small amount of glass wool was placed at the bottom of the condenser to hold the stationary phase. The column was packed according to the method described by Roberts et al (36) and the chloroformic solution of glycosides (10 ml) introduced at the top of the 50 cm. length column. The solutes (glycosides) were eluted with combinations of benzene-chloroform (see table 5) and 100 ml fractions of eluate collected after presence of glycoside in the eluate became evident (Raymond reaction).

The results of the column chromatographic purification are shown in Table 5. Various combinations of methanol - water, methanol - ether, acetone-ether, and ethylacetate-ether were used in attempts to crystallise glycosides from the different fractions obtained. As no crystals were obtained at this stage, it was considered that either the glycosides had not separated or were not sufficiently pure to crystallise out. That the glycosides had not separated was indicated by TLC results. The combined eluate fractions were evaporated to dryness and labelled PE (purified ethereal extract).

The solid phase (KBr disc) and liquid phase (nujol mull) IR spectra of PE showed the following salient features:-

a) A strong absorption peak at about 2950 cm^{-1} suggested saturated structures.

b) A strong absorption peak at about 1750 cm^{-1} suggested the presence of a ketonic group or groups.

c) A strong broad absorption peak at about 3400 cm^{-1} suggesting the presence of hydroxyl (OH) group or groups in the molecules.

All these features were consistent with previous evidence indicating that PE contained cardiac glycosides.

PE was subsequently compared with the glycosides obtained from Adenium boehmianum Schinz, as well as with digoxin and ouabain, by TLC.

Commercially coated silica Gel GF 254 20 x 20 cm plates of 0.25 mm thickness were employed. Methanolic solutions of PE, somalin, echujin, by-product - E.Acetate, digitalinum verum hexacetate, digoxin and ouabain were made and spotted onto several TLC plates described above.

The plates were subsequently placed in different chromatographic tanks. These tanks had previously been prepared with solvent systems benzene - ethanol 7.3; benzene - chloroform 7.3; benzene - methanol 7.3 and left to saturate over a period of four hours.

The developed chromatograms were examined by a UV lamp and then by spraying with Kedde reagent.

The results of the TLC comparison are shown in Table 6 .

PE was shown to be qualitatively similar to FRACTION B in terms of glycosidal content. Somalin and echujin spots appeared to have Rf values close to those of the highest and next highest PE spot respectively. None of the references used showed an Rf value similar to that of the PE spot with lowest Rf value.

CHAPTER IV

SEPARATION OF THE GLYCOSIDES OF PE BY COLUMN CHROMATOGRAPHY

TLC examinations thus far carried out were based on the adsorption/partition of the solutes onto silica gel G. To be able to select appropriate solvent systems for the column chromatographic separation of the glycosides - using alumina, it was considered necessary to carry out a preliminary TLC of PE on alumina plates.

Commercially coated alumina (Art 5713 DC Fertigplatten Aluminiumoxid 60 F254 Type E, made by Merck) 20 x 20 cm plates of 0.25 mm. thickness were employed.

PE was spotted onto different plates together with somalin, (authentic sample). The plates were developed using benzene - ethanol 7:3; benzene-methanol 9:1; benzene - methanol 8:2; and benze-chroroform 7:3.

The developed chromatograms were examined by spraying with Kedde reagent.

The results of the TLC examination of PE using alumina are shown in Table 7.

The solvent systems benzene - ethanol 7:3 and benzene-methanol 9:1 produced well resolved spots.

It was noted however, during a preliminary column chromatographic separation procedure (using alumina) that polar solvent systems (those containing fairly large proportions of either methanol or ethanol) dissolved significant amounts of adsorbent (alumina) from the column. These solvent systems were therefore not used subsequent separation procedures.

Aluminium oxide (Art 1077 Aluminiumoxide 90 active, neutral, Brockmann grade I, made by Merck) was used for packing the separation column. A specialised glass (2.5 cm. internal diameter) mechanical support with a sintered glass stationary phase support at the base was used.

The stationary phase was made into a suspension with benzene and packed according to the method described by Roberts et al (36) to a height double that of the column previously used for purifying Fraction B (100 cm). A Whatmann No. I filter paper was cut to size and placed on top of the adsorbent to minimise disturbances of the latter on addition of eluant.

The sample was dissolved in 10 ml. of chloroform and introduced by means of a pippette, at the top of the column. The column was eluted using various combinations of benzene-chloroform (see Table 8). Starting from non polar to progressively more polar combinations.

When presence of glycoside in the eluate was detected (Raymond reagent) collection of small volumes 10-50 millilitres of eluate was commenced (see Table 8). The fractions were labelled numerically according to the order of collection, and set aside.

After the 43rd fraction the column was washed with methanol, and the methanolic washing labelled MW.

The different eluate fractions were subsequently subjected to a TLC analysis using precoated alumina (Art 5713 De Fertigplatten Aluminium oxide 60 F254 Type E made by Merck) 20 x 20 cm plates of 0.25 mm thickness. Benzene-methanol 9:1 was used as the mobile phase in this experiment. Results of column chromatographic and TLC analysis are summarised in Table 8.

Fractions 1-29 were found to contain one main glycoside and a very small amount of a second glycoside. The major glycoside had an Rf value of 0.5 while the second one showed an Rf value of 0.69. These fractions were combined, labelled DSK1 and set aside for subsequent examination.

Fractions 30-35 contained two glycosides with Rf values of 0.5 and 0.25 These fractions were combined, labelled DSK2 and set aside for subsequent examination.

Fractions 36-43 contained a single glycoside with an Rf value of 0.25. These fractions were combined, labelled DSK3 and set aside for subsequent examination.

The methanolic washing of the column (MW) was found to contain a single glycoside with an Rf value of 0.25. (as for DSK3). This fraction was milky (due to alumina dissolved in methanol) and also coloured (straw). The methanol had therefore eluted out the colouring matter along with the remaining glycoside.

MW was further purified by passing through an alumina (Art 1077 Aluminium Oxide 90 active neutral Brockmann grade I by Merck) column. The sample was introduced to the column as a chloroformic solution (MW was freed from methanol and the residue redissolved in 10 ml. of analar grade chloroform). The glycoside was eluted out with chloroform (analar). Since MW had been shown to contain a single glycoside the eluate was not collected in separate fractions. The combined eluates were combined with DSK3 (since they had been shown to contain the same glycoside as DSK3).

The relative intensities of the pink spots with Kedde reagent suggested that the glycoside with an Rf value of 0.25 (using benzene-methanol 9:1 as the

mobile phase on alumina) was predominat in PE.

This was followed by the glycoside with Rf value of 0.5, the one showing an Rf value of 0.69 appearing in trace amounts only. Further attempts were made to separate the glycosides of fractions DSK1, DSK2 and DSK3 was subsequently examined.

Further separation of the glycosides of DSK1, DSK2 and examination of DSK3

DSK1

Attempts were made to separate the two glycosides contained in DSK1. A column chromatographic procedure was carried out in the same way as previously described (see "separation of the glycosides of PE"). It was hoped that the glycoside with an Rf value of 0.50 could probably be recovered in reasonable amounts, free from the glycoside with an Rf value of 0.69. The latter had been shown to occur in trace amounts only, (see "separation of the glycosides of PE by column chromatography") so that recovery of this glycoside reasonable amounts was considered extremely difficult.

DSK2

This fraction had been shown to contain two glycosides, the one with an Rf value of 0.25 (Table 3) being predominant.

The glycoside with Rf value of 0.5 occured in this fraction in trace amounts only, so that recovery of the latter on a preparatory scale from this fraction would be difficult. Attempts were however made to separate the two glycosides by preparatory TLC. Precoated alumina (Art 5713 Aluminium oxide 60 F254) 20 x 20 cm plates of 0.25 mm thickness were used. A preliminary extraction of the fluorescein incorporated plates with fairly non polar solvents (benzene-chroroform 7:3) had shown that the fluorescein did not dissolve in this solvent combination. (The extracts were examined under UV light).

It was therefore concluded that these solvents would be suitable for recovering the glycosides free from fluorescein after separation on these plates.

DSK3

This fraction had been shown to contain a single glycoside. It was evaporated to dryness by means of a rotary evaporator under vacuum at 50°C. The colourless crystalline residue was left in a dessicator overnight. The UV absorption spectrum of DSK3 was subsequently obtained using a Pye Unicam Sp 8000 spectrophotometer.

Results of attempted separation of the glycosides of DSK1, DSK2, and of the spectrophotometric examination of DSK3

During the column chromatographic separation of DSK1 glycosides, only sufficient of the glycoside with an Rf value of 0.5 (using benzene methanol 9:1 on alumina plates) was recovered for UV analysis. The UV absorption spectrum (using a Unicam Sp 8000) is shown in Fig. 4 . The glycoside with an Rf value of 0.69 (see Table 8) was not recovered at all. Fraction DSK1 was therefore not considered further.

The TLC preparatory scale separation of the glycosides of DSK2 was unsuccessful. This was probably due to the amount spotted. Since in any case the major component of DSK2 was also the major component of DSK3 and the latter was available in gramme quantities, (the dried residue weighed 3.882 g) this glycoside would be examined in DSK3. DSK2 was therefore not further examined and was set aside for future reference.

The UV absorption spectrum of DSK3 is shown in Fig. 5. The small absorption peak with a maximum at 275 nm and the inflexion at about 211 nm suggested some impurities and further attempts were made to remove these..

CHAPTER V

PURIFICATION OF DSK3

It has already been mentioned (see Chapter IV) that eluants containing large proportions of polar solvents dissolved significant amounts, of alumina. Although highly polar solvents were avoided during the column chromatographic separation of PE glycosides, the possibility that the eluants used (see Table 8) may have dissolved small amounts of alumina could not be ruled out. In further purification DSK3 prior to analysis, it was important to take this into account. It was considered that recrystallisation of DSK3 from polar solvents would leave the polar impurities (including the trace amounts of alumina possibly present) in solution while the largely non polar glycoside (DSK3) crystallised out.

Attempts to recrystallise the glycoside from methanol, ethanol as well as combination of these two solvents failed, as the glycoside was too soluble in these. Several other solvents were tried for recrystallisation. These included methanol -water 1:1, 1:2, 1:4, 1:9; methanol-ethanol - water 1:1:8, 2:2:6, 1:1:1; methanol-ether 1:1, 1:4, 1:9; ethylacetate-ether 1:1, 1:4, 1:9; acetone-ether 1:1, 1:4, 1:9. No crystals were obtained with these solvent systems.

DSK3 was therefore washed with freshly distilled water to remove any polar impurities and with cyclohexane to remove impurities less polar than the glycoside.

The possibility therefore, remained that DSK3 contained impurities with solubility properties very similar to those of the glycoside.

The nature of the impurity or impurities in DSK3 (suggested by the UV absorption spectrum (Fig 5) could not be easily ascertained. The choice of a suitable purification procedure could not therefore be rationalised. Various purification methods were nontheless attempted in the hope that some success might be achieved. These methods included column chromatography, washing with diethyl ether, washing with water, washing with dilute soda solution and finally washing with dilute acid. A small sample of DSK3 was subjected to each of these procedures in the first instance.

Alumina (Art 1077 Aluminiumoxide 90 active, neutral - Brockmann garde I) was used as the stationary phase for the column chromatographic procedure. The column was prepared as previously described and the sample, introduced as a chroroformic solution into the column was eluted with chloroform (analar grade). The eluate containing glycoside was evaporated to dryness on a water bath, and the residue examined using a UV Sp 8000 Unicam spectrophotometer.

Diethylether was used for washing a small amount of DSK3.

The ether washed sample was subsequently dried on a water bath and examined using a UV Sp 8000 Unicam spectrophotometer.

Freshly distilled water was used for washing a small amount of DSK3. The sample so treated was examined for UV asborption using a UV Sp 8000 Unicam Spectrophotometer. Soda solution 2N was used for washing a small sample of DSK3. The alkali washed sample was subsequently rinsed thoroughly with freshly distilled water prior to examination using a Unicam Sp 8000 UV spectrophotometer.

Dilute sulphuric acid (H_2SO_4) of approximately 2N. strength was used to wash a small amount of DSK3. The acid washed sample was rinsed thoroughly with freshly distilled water prior to examination using a Unicam Sp 8000 spectrophotometer.

Results of attempted purification of DSK3

Passing a sample of DSK3 through an alumina column did not make any difference to the appearance of the UV spectrum. This method did not therefore succeed in removing the impurity or impurities as the peak at 275 nm and inflexion at 211 nm were still present.

Washing a sample of DSK3 with ether made a considerable difference to the spectrum of DSK3. Whereas the small absorption peak at 275 nm was still evident with the ether washed sample, the inflexion at about 211 nm disappeared completely. It was therefore apparent that the impurity responsible for this feature had been removed by the ether. The entire UV/visible spectrum of the ether washed DSK3 is shown in Fig. 6.

Washing a sample of DSK3 with freshly distilled water did not make any difference to the appearance of the UV absorption spectrum of DSK3. Neither of the two features (inflexion at 211 nm and the small absorption peak at 275 nm) was affected significantly.

Washing a DSK3 sample with dilute soda solution (Na_2CO_3) did not affect the UV absorption spectrum. The inflexion at 211 nm and the small absorption peak at 275 nm were still evident in the spectrum of the alkali washed sample (see Fig. 6).

The UV spectrum of the acid washed sample of DSK3 showed some considerable difference. Whereas the small absorption peak at 275 nm was apparently unaffected, the inflexion at 211 nm was considerably amplified.

In order to double check this a sample of the ether washed DSK3 (which showed no inflexion at 211 nm) was washed with acid (dilute H_2SO_4) and after washing with distilled water, the UV absorption spectrum obtained. (see Fig. 6). The acid washing procedure apparently affected the sample in some way, thereby contributing to the formation of the entity (impurity) responsible for the inflexion at 211 nm.

Since none of the foregoing procedures affected the small absorption peak at 275 nm, an explanation for the presence of this feature had to be sought. It was considered a possibility that this phenomenon may have resulted from an impurity formed during prolonged heating of the material in the presence of water in the extraction purification stages.

In order to test this hypothesis a small amount of DSK3 (ether washed) was placed in an evaporating dish (porcelain). Twenty millilitres of distilled water were added and the material boiled vigoroughly in water over a heating mantle. This heating was continued for one hour, making sure that the material was all the time suspended in water to avoid charring.

The sample was then examined for absorption using a Unicam Sp 8000 spectrophotometer.

The UV spectrum of the sample subjected to the above vigorous treatment did not show any differences from that of the ether washed DSK3.

Of all the purification procedures attempted, only the one of washing DSK3 with diethylether was found to be effective, at least partially in that it removed the impurity responsible for the inflexion at 211 nm.

The bulk of DSK3 was therefore subjected to this washing. The ether washed material was dried over a water bath, and stored in a dessicator (containing self indicating silica gel) for dehydration. All attempts to remove the material responsible for the absorption peak at 275 nm had failed and no further attempts were made to remove this.

Approximately 3.852 g of pure material (DSK3) was recovered from 4 kg of the dried powdered tuber. DSK3 was subsequently analysed.

CHAPTER VI

ANALYSIS OF DSK3

The physical/chemical properties of DSK3 were investigated. This investigation entailed the determination of IR absorption characteristics; the melting point, a TLC comparison with authentic cardiac glycosides. The IR spectra of reference cardiac glycosides, somalin, echujin and ouabain were also obtained for comparison. The results of element analysis and ^{13}C NMR spectral analysis were also obtained.

The solid phase (KBr disc) IR spectrum of a thoroughly dried sample of DSK3 was obtained in a manner previously described (28). A Unicam Sp 8000 spectrophotometer was used. The spectrum obtained is shown in Fig. 7.

The melting point of DSK3 was obtained by both the cappillary method (a galenkamp melting point apparatus was used) and the hot stage microscope method. This was found to be about 138-140^{o}C. That the compound (DSK3) melted over a narrow temperature range suggested that it was reasonably pure.

Precoated alumina 20 x 20 cm plates of 0.25 mm thickness (Art 5713 DC Fertigplatten Aluminium oxide 60 F254 Type E made by Merck) were used for the TLC examination.

Authentic cardiac glycosides digoxin ouabain, somalin, echujin were used for reference purposes. Purified ethereal extract (PE) was also spotted along with DSK3 and the reference glycosides on one of the plates. Several solvent systems - benzene -ethanol 7:3; ethylacetate - ethanol 8:2; benzene - ethanol 6:4; benzene-methanol 9:1 were used for developing different TLC plates. The developed chromatograms were examined under UV light, and also by spraying with Kedde reagent.

The results of the TLC examination are presented in Table 9.

The Rf value of DSK3 was in all cases higher than those of both digoxin and ouabain, but lower than those of somalin and echujin.

The solid phase (KBr disc) IR spectra of reference cardiac glycosides somalin, echujin were obtained in the manner previously described using a Unicam Sp 3000 spectrophotometer.

The IR spectra of the reference compounds of somalin, echujin, are shown in Figs: 8 & 9 respectively.

Neither of the two spectra (of somalin and echujin) was superimposable with the spectrum of DSK3.

The IR spectrum of a polystyrene film was run using the same instrument. This reference spectrum was for finding the actual position of the peaks of the test material, since the positions of the peaks of the reference spectrum are known. The spectrum of the polystyrene film did not show any shift in expected peak positions.

The element analysis was carried out by Butterworth Microanalytical Consultancy Ltd. of England (BMAC), and also by Pharm. Inst. Eth. of Switzerland. The reported data (see Table 10) were in close agreement.

The molecular weight was determined by BMAC of England using chloroform as the solvent and was found to be 739.

The ^{13}C NMR spectral analysis was carried out by BMAC. The results are shown in Table 11. This analysis was carried out with the material dissolved in deuterochloroform ($CDCl_3$) using Tetramethylsilane (TMS) as the internal standard.

The empirical formula calculated using the element analysis was found to be C_3H_5Cl.

The molecular weight was then used to determine the molecular formula thus:-

$$(C_3H_5O_1)_n = 739$$

$$57n = 739$$

$$n = 13$$

$$(C_3H_5O_1)_{13} = 739$$

The molecular formula becomes $C_{39}H_{65}O_{13}$

Number of Carbon atoms in the DSK3 molecule from ^{13}C NMR data:

The two element analytical data independently obtained from two different sources (Table 10) indicated that the DSK3 molecule contained 39 carbon atoms. The ^{13}C NMR data was obtained for the purpose of confirming or negating the evidence, as presented by element analytical data, for the molecular formula $C_{39}H_{65}O_{13}$

The ^{13}C NMR data is shown in Table II. The peaks at 75.6424, 77.0497 and 78.4570 ppm were due to the solvent deuterochloroform ($CDCl_3$) and may be disregarded. The peak at 0.0000 ppm was due to the Internal standard tetramethylsilane (TMS) and serves only as a reference. It was evident therefore, that the relevant peaks as regards the DSK3 carbon atoms were 30 in number.

The question to be answered then was whether or not these 30 peaks were reconcilable with the 39 carbon atoms indicated by element analytical data. In the event that the answer had been in the negative, then the accuracy of the element analytical data and/or the ^{13}C NMR data would have been questionable.

It must, however, be emphasised that the close agreement of the two element analytical data from two different laboratories strongly suggested the reliability of these for molecular formula derivation.

The manner in which the 30 peaks may represent the 39 carbon atoms, and thereby confirming the molecular formula $C_{39}H_{65}O_{13}$ is suggested in the "Discussion".

CHAPTER VII

METHODS OF ESTIMATING DSK3

UV/VISIBLE SPECTROSCOPY

The suitability of UV/visible spectroscopy for the estimation of DSK3 was investigated. Methods investigated were those involving:-

A. The use of the UV absorption at 217 nm due to the α β unsaturated lactone ring.

B. The use of the visible absorption at 620 nm due to the coloured complex given by reaction of DSK3 with alkaline metadinitrobenzene (3:5 DNB).

C. The use of the visible absorption at 530 nm due to the coloured complex given by reaction of DSK3 with alkaline metadinitrobenzoic acid (3:5 DNBA).

A. Using the absorption peak at 217 nm.

The relationship between the concentration of DSK3 and the absorbance at λ max 217 nm was investigated. Approximately 3 mg of DSK3 was accurately weighed using a Sartorius microanalytical balance. The weighed quanitty was disolved in 50 ml of 95% ethanol (pronalysis) so that the resulting solution contained 0.006% w/v of DSK3.

Into each of 5 thoroughly cleaned graduated flasks with ground glass stoppers, different volumes of 0.006% w/v solution were pippetted accurately. The flasks were labelled 1, 2, 3, 4, 5 and the volume of the contents of each flask made upto 5 ml with 95% ethanol. The solutions of DSK3 thus prepared were examined for absorbance, using a Unicam Sp 8000 UV spectrophotometer against 95% ethanol in the reference cell.

A complete spectrum (UV/visible range) was obtained for each concentration of DSK3, and all the spectra were run on the same paper.

A graph of absorbance (Ordinate) against concentration of DSK3 (Abcissa) was then plotted.

The volume of 0.006% w/v solution of DSK3 added to each of the 5 flasks, the final concentration of DSK3 in each flask and absorbance at 217 nm are shown in Table 12.

The graph of absorbance versus concentration of DSK3 is shown in Fig. 11.

The graph obtained was a straight line but does not pass through the origin.

Calculation of the $E^{1\%}_{1cm}$ for DSK3

Using the data for flask number 4 (Table 12):-

A DSK3 concentration of 0.0024%

gave an absorbance of 0.63.

∴ Absorbance of a 1% solution of DSK3,

would be $\frac{0.63}{0.0024} = 262.5$

$E^{1\%}_{1cm}$ for DSK3 = 262.5

Calculation of the molar extinction coefficient (ε) of DSK3

The $E^{1\%}_{1cm}$ is related to the molar extinction coefficient (ε) by the expression

$$10\,\varepsilon = E^{1\%}_{1cm} \times \text{mol. wt. (29)}.$$

The molecular weight of DSK3 was found to be 739 (BHAC).

Thus $10\,\varepsilon = E^{1\%}_{1cm} \times \text{mol. wt.}$

$10\,\varepsilon = 262.5 \times 739$

$= 19,398.75$

$= 1.94 \times 10^4$

$\text{Log}\ \varepsilon = 4.2878$

B. Using the absorption at 620 nm (due to complex with alkaline 3.5 dinitrobenzene)

A 0.1% w/v stock solution of DSK3 in 95% ethanol was used in this experiment. Four thoroughly cleaned test tubes with ground glass stoppers were labelled 1, 2, 3 and 4. Two millilitres of freshly prepared Raymond reagent were pippetted into each of the four tubes.

This was followed by the addition of 0.25, 0.50, 0.75 and 1.0 millilitres of the 0.1. w/v of DSK3 into tubes No. 1, 2, 3 and 4 respectively. The contents of each tube were subsequently made up to 8 millilitres with 95% ethanol. All the four test tubes were placed in an ice/salt cooling mixture and left for 20 minutes for cooling and temperature equilibration.

Tube No. 1 was then removed from the cooling mixture and the volume quickly made upto 10 millilitres with 10% sodium hydroxide (freshly prepared). After shaking to ensure complete mising of tube No. 1 contents, the absorbance at 620 nm was determined using an SP 8000 Unicam spectrophotometer. A second absorbance value was read approximately one minute after the first reading.

Tubes No. 2, 3 and 4 were treated in the same way as tube No. 1 and the two absorbance readings obtained in the same way, using the same instrument. The data and results for this experiment are shown in Table 13 Graphs of absrobances (ordinate) versus the concentration of DSK3 (volumes of 0.1% w/v of DSK3) for the two sets of readings were plotted and are shown in Fig. 12

These graphs were straight lines through the origin. The graph obtained from the second set of absorbance readings had higher slope than that obtained from the first set of absorbance readings. Complete spectra (UV/visible) obtained with the contents of the four tubes showed

absorption peaks (visible region) which were not symmetrical.

The $E^{1\%}_{1cm}$ based on the results of the experiment using alkaline 3:5 dinitrobenzene.

Since the absorbance of the coloured complex faded with time, the $E^{1\%}_{1cm}$ was calculated using the two sets of absorbances. The first set of the absorbance readings had been taken immediately after the addition of 10% NaOH to the contents of each tube and shaking. The second set of readings had each been taken approximately one minute after the corresponding first readings.

Calculation using the first reading result (absorbance) for tube No. 4.

The first concentration of DSK3 in Tube No. 4 was 0.01%. Thus, a DSK3 concentration of 0.01% w/v gave an absorbance of 1.0.

∴ A 1% w/v solution of DSK3 would give an

absorbance of $\frac{1}{0.01} = 100$

$E^{1\%}_{1cm} = 100$

Using the second absorbance reading for tube No. 4

A DSK3 concentration of 0.01% w/v gave an absorbance of 1.3.

∴ A 1% w/v solution of DSK3 would give an

absorbance of : $\frac{1.3}{0.01} = 130$

$E^{1\%}_{1cm}$ calculated from the second reading = 130.

The value of $E^{1\%}_{1cm}$ calculated from the first reading of absorbance was found to be considerably different (lower) than that calculated from the absrobance reading taken about 1 minute after the first reading.

The second set of absorbance readings Table 13 ... were higher than the first set.

The molar extinction coefficient calculated from the higher value of $E^{1\%}_{1cm}$.

$$10\,\varepsilon = E^{1\%}_{1cm} \times \text{molecular weight} \qquad (20)$$

$$10\,\varepsilon = 130 \times 739$$

$$10\,\varepsilon = 9.6 \times 10^{4}$$

$$= 9.6 \times 10^{3}$$

$$\log \varepsilon = 3.9823$$

The logε value calculated from the $E^{1\%}_{1cm}$ from the first set of absorbance readings would be lower. This defference in logε values obtained at different times after mixing the reactants illustrates the need to specify the experimental conditions when giving the results (See "discussion").

Ç. Using alkaline 3:5 dinitrobenzoic acid

The same 0.1% w/v stock solution of DSK3 used in the previous experiment was used for the present determination. The experiment was carried out with the reaction mixture at room temperature.

Into each of 5 thoroughly cleaned, numbered ground glass stoppered . tubes, 4 millilitres of freshly prepared Kedde reagent was pippetted. This was followed by the addition of 5.75, 5.50, 5.25, 5.0 and 4.75 millilitres of 50% methanol into tubes 1, 2, 3, 4 and 5 respectively.

The contents of tube No. 1 were then made up to 10 millilitres with the 0.1% w/v of DSK3 solution. The mixture was thoroughly shaken to ensure complete mixing. A stop clock was used and the absorbance of the solution determined using an Sp 800 Unicam spectrophotometer. The complete spectrum (UV/visible) was run starting two minutes after the addition of the glycoside solution.

The contents of tubes No. 2, 3, 4 and 5 were treated in a similar manner and the absorption spectra obtained using the same instrument, and on the same paper. The time interval between addition of glycoside solution and the spectral scan was kept constant throughout the experiment.

A blank solution was prepared in the same manner as the mixture in tube No. 1 except that the glycoside(DSK3) was omitted. The volume of the blank was made up to 10 millilitres with 95% ethanol and the complete absorption spectrum obtained using the same spectrophotometer as before against 95% ethanol in the reference cell. This blank was necessary since the absorption spectra the different glycoside concentrations were run using 95% ethanol as the reference.

The data and results of the experiment are summarised in Table 14. A graph of absorbance(ordinate) against DSK3 concentration (abcissa) is shown in Fig. 13.

The complete absorption spectra showed symmetrical absorption peaks max 530 nm. The graph of absorbance against concentration of DSK3 showed a straight line through the origin. The calculated value of $E^{1\%}_{1cm}$ using the data obtained was found to be 91.

Molecular extinction coefficient

$10\,\varepsilon = E^{1\%}_{1cm} \times$ molecular weight (28)

$= 6.7 \times 10^3$

$\text{Log}\,\varepsilon = 3.8261.$

CHAPTER VIII

A PRELIMINARY INVESTIGATION OF THE PHARMACOLOGICAL EFFECTS OF DSK3 ON BOTH INTACT AND ISOLATED MAMMALIAN PREPARATIONS:

Three preparations were used for this investigation:

A) The anaesthetised rat preparation,

B) The anaesthetised cat preparation,

C) The isolated perfused rabbit heart preparation.

A. The anaesthetised rat preparation.

Adult rats (200-300g) were anaesthetised with a 25% w/v aqueous solution of urethane. An intraperitoneal injection of 0.3 ml/100g was given.

The trachea, common carotid artery and external jugular vein were exposed and cannulated according to the method described by Mcleod et al (30).

The arterial cannula was connected to a condon blood pressure manometer, and drug injections were given through the venous cannula.

Injections of adrenalin, 10% v/v ethanol 0.01% w/v ouabain and 0.05% w/v of DSK3 were given intravenously and the effects noted. Several rats were used for this investigation.

Typical data showing the doses given and the responses obtained are shown in Table 15

Adrenalin showed a transient pressor effect on this preparation.

The solvent (10% v/v ethanol) showed a transient blood pressure lowering effect followed by a quick recovery.

Ouabain showed an initial blood pressure fall, followed by a sustained pressor effect.

The test material (DSK3) showed a gradual but sustained rise in blood pressure in this preparation.

B. The anaesthetised cat

An adult cat (2.75 kg weight) was preanaesthetised with ether in a anaesthetising closed cage. The anaesthesia was then maintained by an intraperitoneal injection of a 1% chloralose (9) solution in normal saline at 40°C. If the anaesthetic is cooled below this temperature prior to injection, the chloralose precipitates out as it is not very soluble in normal saline.

The animal was subsequently placed on a warm operating table and the femoral artery and femoral vein cannulated by the method described by Mcleod et al (30). This cannulation was done after cannulating the trachea so that artificial respiration could be given whenever the need arose (29) The femoral artery was cannulated with a Portex red luer i.v. cannula (5FG. OD 1.63 mm of 30cm length). The femoral vein was cannulated with a green luer Portex i.v cannula (2FG. OD 0.63 mm of 30 cm length).

Heparin was injected through the venous cannula at a dose level of 1000 units/kg to minimise the risk of clotting.

The arterial cannula which had previously been filled with heparinised saline solution was connected through a 3 way tap to a strain gauge transducer which was in turn connected to a Devices M2R M2P recorder.

Balancing of the recorder

With the GAIN, OFFSET AND FINE BALANCE control all at zero reading, the electrically heated writing element of the recorder was set at the middle of the record chart using the PEN ZERO control.

Then, with the RANGE selector knob at 500 (low sensitivity) the recorder was switched from ZERO to BRIDGE and the deflection of the recorder pen adjusted by means of the COARSE BALANCE control so that no deflection occurred on switching from ZERO to BRIDGE.

This process was repeated for higher sensitivity ranges (i.e. 250, 100, 50, 10), the FINE BALANCE control being used at the high sensitivities (50-10). With this the machine was now "balanced" and ready for use.

The recorder pen was subsequently brought to rest one big square from the end of the chart (zero pressure position) by means of the PEN ZERO. The whole of the balancing process was carried out with the three way tap from the transducer vented to the AIR.

Calibration of recorder

The sensitivity range used throughout the experiment was 250.

Using the three way tap, the transducer was vented to a column of heparinised saline which was equivalent to 60 mm Hg. (hydrostatic pressure). This column was used for calibrating the recorder as follows:-

With the heparinised saline filled column vented to the transducer, and the machine in the DAMPING position, the deflection of the recorder pen was adjusted by means of the GAIN control so that the pen was deflected 3 large squares from the zero pressure position by the hydrostatic pressure.

Each big square thus represented 20 mm Hg. and the recorder was ready for use.

The balanced calibrated recorder was used for monitoring cardiovascular changes in the anaesthetised cat, after venting the transducer to the animal (using the 3-way tap) via the arterial cannula.

The arterial cannula was flushed with heparinised saline to prevent clot formation in the cannula.

Using the chart speed selector knob with the machine in the Un-damped position, it was possible to obtain the systolic and diastolic pressure as well as the heart rate. Mean pressure was obtained with the recorder in the damped position.

In all cases the blood pressure and heart rate of a stabilised preparation were recorded as normal readings prior to any drug injections.

Experiments were performed to determine a suitable dose range for demonstrating the cardiovascular effects of DSK3. The effects of a 1% v/v solution of propylene glycol (PG) in normal saline were also investigated so that the suitability of otherwise of this solvent (1% v/v PG) for making injections of DSK3 could be established Experiments were also carried out to establish the effects of a typical cardiac glycoside (digoxin), so as to establish the types of responses expected on injection of a cardiac glycoside under the experimental conditions.

Having established a suitable dose range for the test material (DSK3) and also the types of responses to be expected after injection of a typical cardiac glycoside (digoxin), experiments were carried out with the test material dissolved in 1% propylene glycol in normal saline. The volume of the intravenous injections was kept constant throughout the experiment, one millilitre being injected each time

Experiments were also carried out using anaesthetised cats with experimentally induced heart failure. The method used for depressing the heart was after La Barre *et al*, described by Cobbin *et al* (10).

This method involved the intravenous injection of a high dose of pentobarbitone sodium (in the range 30 - 50 mg/kg). This depressant was injected after the blood pressure - heart rate parameters of the preparation had stabilised. The test drug (DSK3) was subsequently injected intravenously (30 - 60 µg/kg dose range) and the cardiovascular effects noted. The test drug injections were given while the heart was still severely depressed, as was evident from the blood pressure - heart rate - beat amplitude recording.

Results:

Preliminary experiments with anaesthetised cats showed that doses of DSK3 approximately 80 µg/kg produced a transient rise in blood pressure. Dose levels approximately 150 µg/kg were too high, as arrythmias produced by such doses culminated in the death of the animal. Doses of DSK3 below 15 µg/kg produced little or no effects on preparation. Higher doses (above 15 µg/kg but well below the toxic 150 µg/kg) produced some cardiovascular responses. An appropriate dose range was found to be in the region 30 - 80 µg/kg.

Propylene glycol (1% v/v in normal saline) was found to have no demonstrable effects on the cardiovascular system of the anaesthetised cat preparation.

This solvent did not therefore significantly interfere with the results obtained with DSK3.

Experiments with digoxin showed that digoxin doses below 25 µg/kg showed little or no effects on the cardiovascular system of the anaesthetised cat preparation. Dose levels between 25 - 35 µg/kg, however, produced a significant but transient rise in blood pressure, as well as a slight reduction of the heart rate. The heart beat amplitude was also increased significantly. Digoxin dose levels above 40 µg/kg produced arrythmias, the animal dying quickly afterwards.

Typical results of several experiments using DSK3 on the anaesthetised cat preparation are shown in Table 16 and figs. 14, 15 and 16. These results showed a transient rise in blood pressure, a significant increase in heart beat amplitude and a reduction in the heart rate was preceded by an initial slight increase so that this negative chronotropic effect was somewhat delayed in this preparation (slowing was observed at least 30 minutes after the test drug injection). Typical results of several experiments with anaesthetised cats with experimentally induced heart failure are shown in Table 17 and Figs. 17-18. The severely depressed preparation showed a marked reduction of both blood pressure and heart beat amplitude. Both the blood pressure and beat amplitude showed a fairly rapid and dramatic recovery after injection of the test material (DSK3).

C. Effects of DSKC on the isolated perfused rabbit heart preparation

An isolated rabbit heart was obtained from an adult rabbit according to the method described by McLeod et al (37).

The preparation was hooked onto a perfusion apparatus and perfused with oxygenated Ringer Locke solution at 37°C as described by McLeod et al (37).

A thread was then attached to the ventricle (at the apex) by means of a hook and connected to a strain gauge transducer. The responses were recorded by Devices M2R and M2P recorder connected to the strain gauge transducer.

Heart contraction patterns showing the heart rate, beat amplitude as well as the tension developed were recorded with this set up. The recorder was, however, not calibrated to give actual tension readings so that observations made regarding the tension developed in the contracting myocardium were merely qualitative, showing an increase, a decrease or no change.

A preliminary investigation of the effect of 2% v/v propyleneglycol in this preparation did not show any demostrable effects with the amounts of this solvent injected.

The actual amount of solvent investigated in this preliminary test was double that used as solvent for the test material in subsequent examinations. propyleneglycol at the level used as DSK3 solvent (1% v/v in normal saline) was therefore deemed to be a suitable solvent for the water insoluble test compound.

Preliminary experiments showed that approximately 200 µg of DSK3 was a suitable dose for this "wash out" preparation.

Typical results of several experiments with the isolated perfused heart preparation are shown in Table 1[illegible] and Figs. 19 and 20.

Since the chart speed was known, the actual heart rate after injection of the test material could be calculated and compared to the normal reading. Any changes in the tension developed were shown by either an upward (increased tension) or downward (decreased tension) displacement of the contraction pattern.

DSK3 produced increase in heart beat amplitude, tension developed and a lowering of the heart rate.

DISCUSSION

Preliminary screening of Adenium obesum plant material for cardiac glycosides was carried out using both alkaline 3:5 dinitrobenzene (Raymond reagent) and alkaline 3:5 dinitrobenzoic acid (Kedde reagent). In both cases coloured complexes were formed (a transient blue colour with metadinitrobenzene and a fairly stable pink colour with metadinitrobenzoic acid). The reaction of cardiac glycosides with dinitro and trinitrophenyl derivatives is thought to be due to the presence of active methylene ($-CH_2$) groups at C-21. The formation of 1:1 complexes is considered likely (64, 52, 63). The solvent in which the test material was dissolved was found to be important. This was because those solvents (eg. acetone) containing ketone groups gave coloured complexes with the aromatic polynitrophenyl derivatives. The Janovsky reaction was found in literature (69) as an example of such reactions with ketones.

Cardiac glycosides undergo reactions based on three different parts of the molecule, the $\alpha\beta$ unsaturated lactone ring, the steroidal nucleus and the sugars moiety. The $\alpha\beta$ unsaturated lactone ring gives reactions with polynitrophenyl derivatives, the steroid nucleus reacts with concentrated acids and oxidising agents, while the sugar moieties react with ferric salts eg. Keller Kiliani reagent for 2 - deoxy sugars.

[illegible]

For screening purposes it was considered that free sugars present in the crude extract would make the test for cardiac glycosides based on sugar moieties unreliable.

Similarly, steroidal, non-glycosidal impurities would also probably have interfered with the reaction based on the steroid nucleus. These two reactions (based on sugar moieties and steroidal nucleus) were therefore considered unsuitable for the initial screening of the plant extract for cardiac glycosides. The reaction based on the $\alpha\beta$ unsaturated lactone ring would of course not distinguish between glycosides and aglycones, but was considered suitable for screening purposes.

Thin layer chromatographic examinations carried out suggested that Adenium obesum contained at least four cardiac glycosides.

Lead acetate used for precipitating tannins and pigments gave satisfactory results. Rowson (9) showed that two of the four stages employed by Kedde (70) were superfluous. Kedde used Ferric Chloride to precipitate tannins and removed excess Ferric Chloride by precipitation with Sodium hydroxide. According to Rowson lead acetate precipitates not only pigments but also tannins.

The solvents employed for extracting glycosides from the aqueous suspension differred in polarity.

Petroleum spirit, the least polar of the solvents used is a good solvent for fats, waxes and resins (15). The other solvents were used in the order of increasing polarity, and these were ether, chloroform and chloroform-alcohol 2:1. Reichstein et al (23) used the same solvents in the same order for extracting the glycosides of Adenium boehmianum Schinz and managed to separate most of the glycosides on the basis of their polarities. In the present study of Adenium obesum, only fractions A (petroleum ether extract) and D (chloroform extract) appeared to contain a single glycoside each. Fraction B (the ethereal extract) contained three glycosides, showing that the differences in solubilities of these three glycosides were probably not very pronounced. Alternatively, it was possible that the aqueous suspension contained trace amounts of alcohol, the latter possibly altering the solvent properties of ether. Petroleum ether used was shown to contain the least polar of the glycosides in small but significant quantities.

Dilute acid and dilute alkali were used for washing out basic and acidic impurities respectively from fraction B.

Petroleum spirit was again used to remove fats, waxes and resins which were considered to contribute to the stickiness of fraction B residue.

Decolourisation of organic compounds by boiling with activated charcoal is a classical procedure very commonly employed. In the present work, this method failed to decolorise an organic (methanolic) solution of fraction B. This was probably due to the fact that the equilibrium for the adsorption of the glycosides and the colouring matter onto charcoal, and the desorption into the methanolic phase was heavily in favour of the latter process. That both the glycosides and colouring matter were adsorbed onto the charcoal from aqueous solutions was observed, and attempts to elute the glycoside from the charcoal, free from the colouring matter failed. This method of adsorption of glycosides onto charcoal from aqueous solutions is employed for removing free sugars from glycosides. The charcoal is washed with water, which effectively removes the free unadsorbed sugars, the glycoside(s) being subsequently eluted from the charcoal by means of organic solvents (15).

The column chromatographic procedure using alumina was found to successfully decolourise the extract.

The choice of eluant was a critical factor since polar solvents were found to elute both the glycosides and colouring matter simultaneously from the column. Most of the colouring matter was, however, left adsorbed onto the column when non polar eluants were employed. That no separation of the glycosides of fraction B was achieved during the column purification procedure was attributed to the short length of the column (50 cm). For effective, separation, long columns are usually required, especially when the compounds to be separated have closely similar properties.

The IR absorption spectrum of PE (purified ethereal extract) showed absorption characteristics typical of cardiac glycosides, thus confirming the preliminary screening findings that Adenium obesum contained these compounds.

Preliminary TLC comparisons of PE with the glycosides of Adenium boehmianum Schinz (see Table 6) showed that two of the glycosides of PE (Rf values 0.61 and 0.55) had Rf values very similar to those of somalin (Rf - 0.61) and echujin (Rf = 0.56). The third glycoside of PE (Rf = 0.50) had an Rf value different from those of the reference glycosides. All the three glycosides of PE were also noted to have Rf values higher that those of ouabain and digoxin suggesting that PE glycosides were less polar than both ouabain and digoxin.

The Rf values of PE glycosides were higher on alumina than on silica Gel, (see Tables 6 and 7). This suggested that PE glycosides were more strongly held onto silica Gel surfaces than on alumina surfaces. The fact that both adsorption and partition processes may have taken place simultaneouly complicates the picture, but the observed difference noted using these two adsorbents might be explained on the basis of the greater polarity of silica gel.

When polar solvents were used to elute the solutes (glycosides) from an alumina column, little or no separation of the solutes was achieved. If an adsorption process is assumed to have the predominant one (a partition process thereby playing an insignificant role), this observation might be explained on the basis of competitive adsorption. The more polar solvents would tend to keep the solutes out of the polar adsorption sites on the adsorbent surface, so that the solutes would remain almost entirely in the mobile phase (36). Under these conditions, little or no separation would occur.

For the column chromatographic separation of PE glycosides, a stepwise (fractional) elution method was adopted. This entailed the use of a series of increasingly more polar solvent combinations as eluants, as opposed to a simple elution procedure. The latter method involves the use of a single solvent throughout the elution process.

Attempts to purify the glycoside in the eluate fraction DSK3 by recrystallisation from various solvents failed. Even though the use of polar eluants had been avoided during the separation of PE glycosides, it was considered possible that the benzene-chloroform combinations used may have dissolved trace amounts of alumina from the column. Such an impurity although present in trace amounts might affect analytical data. It was therefore important to try and remove this by other methods as recrystallisation did not succeed. Since the glycoside DSK3 was non-polar, the residue from the column was washed thoroughly with freshly distilled water in the hope that relatively more polar impurities (eg. alumina) would be removed. High Pressure Liquid Chromatography (49) and GLC (50, 57) procedures were not possible due to lack of facilities.

The UV absorption spectrum of DSK3 showed an absorption maximum at about 217nm. The absorption maximum was in the 215-220 nm range which is characteristic of compounds with the $\alpha'\beta$ unsaturated carbonyl group (28,71). This absorption spectrum (see fig. 5) showed features thought to be due to impurities. For pure cardiac glycosides, the absorption peak in the 215-220 nm region is usually symmetrical. The spectrum also usually shows no other absorption peaks in both the UV and visible region.

The inflexion at 211 nm and minor absorption at 275 nm in the spectrum of DSK3 were therefore probably due to extraneous matter. The literature survey showed that Reichstein et al (23) obtained a UV absorption spectrum of abobioside with a major absorption peak at 216 nm ($\log \varepsilon = 4.11$) and a minor absorption peak at 270 nm ($\log = 2.57$).

These workers ascribed the minor absorption at 270 nm to an impurity which they identified as a 16 - anhydro derivative. It was not clear whether the minor absorption at 275 nm in the UV spectrum of DSK3 could be explained on the same lines as that at 270 nm in the spectrum of abobioside obtained by Reichstein et al (23). It was however, noted that the ether washed sample of DSK3 no longer showed the inflexion at 211 nm.

The exact nature of this impurity could not be ascertained. Washing DSK3 with dilute acid solution, however, appeared to contribute to the formation of this impurity. It was therefore considered likely that the initial purification procedure involving the washing of the ethereal extract (Fraction B) with dilute acid (2N,HCl) may have contributed to this impurity.

That the attempted column chromatographic purification did not separate the impurity responsible for shoulder at 211 nm from the glycoside suggested that this impurity had properties closely similar to those of the glycoside. The absorption at 275 nm could not be satisfactorily explained, but may have been due to an anhydro derivative. The fact that boiling DSK3 vigorously in water for a long time did not affect this minor absorption at 275 nm indicated that this was unlikely to have been due to heating during the extraction and purification procedures.

The IR absorption spectrum of DSK3 (see fig. 7 .) showed features strongly suggestive of associated hydroxyl (OH) groups (broad peak at about 3450 cm^{-1}),(3) C - H stretch of aliphatic structures (strong absorption at about 2950 cm^{-1}). stretching vibration of an α β unsaturated ketone C = O (strong absorption peak at 1750 cm^{-1}); and an olefinic (-C = H -) stretching vibration (weak absorption at 1630 cm^{-1}). These features were consistent with those expected for a typical cardiac glycoside. The finger print region (about 1400 - 600 cm^{-1}) is usually more complicated and no attempt was made to assign the absorptions in this region. (18, 28, 33, 71)

This region is said to be characteristic of each molecular species and is commonly used for identifying compounds by matching the spectra of unknowns with those of known compounds (28).

The IR spectra of the reference glycosides, somalin and echujin were compared with that of DSK3. That neither of the reference spectra was superimposable with the DSK3 spectrum suggested that DSK3 was not identical with either somalin or echujin. It was also noted that the spectrum of somalin was better, showing sharper and better resolved peaks than the spectra of echujin, and DSK3. Since the two compounds echujin and DSK3 were reasonably pure (the former was a reference compound kindly donated by Professor Christian Tamm of Basle University and the sharp melting point of the latter 138-140°C suggested its purity) an explanation of their relatively poor spectra was called for. The molecular weights of these three compounds are somalin = 518; echujin = 842 95; DSK3 = 739. It becomes apparent that the molecular weight of somalin is considerably lower than those of echujin and DSK3. According to Dyer (28) materials of high molecular weight that contain many different funtional groups generally give poor spectra.

It is therefore possible that the relatively poor appearance of the spectra of echujin and DSK3 may be explained on the basis of their relatively high molecular weights.

A comparison of the physical/chemical characteristics of DSK3, digoxin and ouabain by TLC showed that DSK3 was less polar than both these glycosides which are in clinical use. Knowledge of the physical/chemical characteristics would enable predictions to be made regarding the absorption, distribution and metabolism of the test material. The low polarity of DSK3 suggests that this material would probably be well absorbed from the gastro intestinal tract (although absorption may be limited by the low solubility in water). Once in the plasma, DSK3 would probably be fairly widely distributed in various body "compartments" and also probably have a fairly long biological half life.

The element analytical data obtained by the two laboratories (BMAC of England and Phar Inst. Ethz of Switzerland) were in close agreement. These analytical data as well as themolecular weight result obtained by BMAC showed that DSK3 fitted the molecular formula $C_{39}H_{65}O_{13}$.

The ^{13}C NMR spectrum showed 30 peaks due to DSK3 carbon atoms. The high intensity peaks in the region 75-79 ppm were due to $CDCl_3$ Hollstein et al (65); Wandiga (79) All the 39 carbons required by the formula $C_{39}H_{65}O_{13}$ (page 49) were reflected in the 30 relevant peaks which were therefore, by implication, and as evidenced by the widely different peak integrals and hights, quantitatively non-equivalent.

On the basis of the work of Hollstein et al (65), Roberts et al (66, 67) as well as personal communication with Wandiga (79) and Kofi (80), the following interpretation was made.

Addition of all integral values (Table II) for the 30 relevant peaks gave a total of 4314. Dividing this figure by the expected 39 carbons gave an average integral value of 111. This latter figure would approximate to the theoretical integral value for single carbon resonance, assuming the absence of splitting and overlap phenomena.

It was noted, however, that integral values for single carbon resonance may be lower or higher than the theoretical figure of 111 due to the related phenomena of splitting and overlap respectively. Be that as it may, the following peaks on account of their intensity, were considered to be due to the stated No. of carbon atoms.

Delta ppm	INTEGRAL	CALCULATED NO. OF CARBONS (INTEGRAL/111)
1. 85.5522	272	2
2. 74.7629	354	3
3. 74.0592	219	2

4.	30.1983	216	2
5.	26.6214	375	3
6.	21.1681	361	3
		Total	15 Carbon Atoms

The remaining 24 ^{13}C Nm peaks were each considered to reflect single carbon resonance, so that 15 + 24= 39 gave the required no. of carbon for the molecular formula $C_{39}H_{65}O_{13}$ to hold. The assignment of particular carbon to particular resonance peaks and the elucidation of the splitting and overlap phenomena was otherwise beyond the scope of the present work.

The UV absorption maximum at about 217 nm was found to be suitable for quantitative work. The graph of absorbance at 217 nm against the concentration of DSK3 showed that for practical purposes, Beers Law was obeyed.

There was however, a small but finite intercept on the y axis probably due to instrument instability at this low wavelength. The effect of this was to increase the absorbance values by a constant value throughout. The $E^{1\%}_{1cm}$ value calculated from the data obtained at 217 nm was 262.5 (logE= 4.28). According to Higuchi et al, (72), all glycosides and aglycones possessing the characteristic $^{\alpha}C_{\beta}$ unsaturated butenolide ring absorb between 215-200 nm. and this absorption may be used for analytical purposes. Canback (68) showed that the Log E of this absorption is about 4.2 so that the value obtained for DSK3 was consistent with this literature value.

The graph of absorbance against concentration of DSK3 using the data for the colour reaction with alkaline 3.5 dinitrobenzene showed that Beers Law was obeyed.

The concentration range of DSK3 investigated was 0.0025 - 0.01% w/v. This reaction has been found by several workers among them Rowson (8), Cox et al (53), Canback (60), Raymond (73) and Hassall et al (75) to be suitable for the quantitative determination of cardiac glycosides. The results obtained in the present work confirmed the findings of the said, previous research workers. According to Rowson (8) the blue colour fades rapidly but logarithmically with time and shows an absorption maximum in the visible region at about 620 nm. On account of this instability, the $E^{1\%}_{1cm}$ and log ϵ values calculated from the data were found to be time dependent.

The experimental conditions would therefore have to be defined very precisely in order to make the results meaningful.

An investigation of the relationship between the absorbance of the coloured complex formed by the reaction between alkaline metadinitrobenzoic acid and DSK3, and the concentration of DSK3 showed that Beers Law was obeyed. (DSK3 concentration range was 0.0025 - 0.0125% w/v). This reaction is termed the "Kedde reaction" and is based on the same principle as the Raymond reaction (reaction of glycoside with alkaline metadinitrobenzene).

The Kedde reaction was described by Harkis et al (52) Pratt (76) and Tattje (77). Canback (60) described the Kedde reaction as a modification of the Raymond reaction. Rowson (8) found that the colour density increased up to 12 minutes and faded progressively upto 75 minutes. In the present study, the absorbance readings at 530 nm were obtained 2 minutes after mixing at which time in view of Rowsons findings, the maximum density had not been obtained. Since however, the object of the exercise in the present work was to merely investigate the relationship between the absorbance and the concentration of DSK3, it was not necessary to ensure that the maximum density was achieved. The critical factor in this case was the standardisation of the time interval between the mixing of the reactants and the time of taking the absorbance readings throughout the experiment. The lower value $E^{1\%}_{1cm}$ obtained from these results is evidently explained on the basis that maximum colour densities were not achieved at the time of reading the absorbance values. Corona et al (54); Rabitzch et al (55) have described other colour reactions.

The quantitation of the total glycosides of Adenium obesum and also of the pure isolated (DSK3) posed considerable problems. [Mahran et al used both colourimetric and biological methods of assay of Cardenolides of Cerbera Odollam "Gaertn" (4)]

This was mainly due to the very significant losses of material during the extraction, and purification processes involved. During a typical extraction, however, 1 kg. of the dried powder (tuber) yielded 10.6 g. of crude glycoside extract. This crude extract yielded, upon separation and purification, 0.963 g. of reasonably pure DSK3. Because of the said losses during processing, the mount of DSK3 recovered may have been less than 50% of the actual amount present in the tuber.

Intravenous injections of DSK3 produced a sustained rise in blood pressure in the anaesthetised rat. A similar response was recorded with anaesthetised cat preparations, along with increased beat amplitude (signifying a positive inotropic effect) and a negative chronotropic effect. Both the inotropic and chronotropic responses were also observed with the isolated perfused rabbit heart preparation. The beat amplitude and blood pressure of anaesthetised cats with severely depressed hearts showed a fairly rapid and remarkable recovery after intravenous injection of DSK3. No such recovery was noted when the animals with severely depressed hearts were either given control IV injections of normal saline, or left alone over a considerable period.

That DSK3 produced this recovery showed according to the findings of La Barre et el (10) that this material had cardiac stimulant activity.(22)

Kumar et al (31) reported an increase in the peripheral resistance, increased aortic pressure and decreased cardiac output, observed after IV injections of acetylstrophanthidin in anaesthetised dogs with normal hearts. Vatner et al (78) reported a transient bradycardia, and a sustained rise in arterial pressure in conscious dogs. Systemic resistances were elevated within one minute and remained elevated for 30 minutes. Anaesthetised preparations were noted by Vatner et al to show similar but more pronounced responses. These workers ascribed the pressor effect to a direct vasoconstrictor action of cardiac glycosides on blood vessels.(58) The increase in peripheral resistance has also been reported by other workers among them Higgins et al (39), Goodman and Gillman(12). The latter attributed part of the pressor effect to a central reflex action. In the present study, the effects of DSK3 on anaesthetised rats, anaesthetised cats and isolated perfused rabbit heart preparations agreed well with those reported in literature for cardiac glycosides.(40,41) These results therefore confirmed the previous screening and subsequent analytical findings that DSK3 was a digitalis type of cardiac glycoside.

CONCLUSION

The aim of the present work was (a) to find out the number of glycosides in Adenium obesum plant material, (b) to try and isolate one or more of these glycosides in pure form, (c) to attempt to characterise the pure isolates and (d) to carry out preliminary pharmacological screening experiments to confirm the cardiac stimulant action of the pure isolates.

Of the four glycosides found to be present in the plant material only one, DSK3 was isolated in pure form. A partial characterisation of DSK3 was achieved in as much as the typical cardiac glycoside structure was confirmed, and a probable molecular formula arrived at. The pharmacological screening experiments successfully demonstrated that DSK3 had cardiac stimulant properties.

The other three glycosides of Adenium obesum whose presence was shown by TLC were not isolated in pure form. Also, the complete characterisation of DSK3 was not achieved, since the molecular structure of this material was not arrived at.

Perhaps the reason why the three glycosides were not isolated was due in part to their low percentage composition in the plant material.

This problem would easily be solved by extracting larger amounts of the plant material. Another possible reason for the difficulties in isolating these three glycosides was that these glycosides (or at least some of them) occurred in small quantities in too many of the different extract fractions. Recovery from any of these fractions was made difficult by the fact that considerable losses were experienced during the separation and purification procedures. A judicious selection of the extracting solvents in order to make these more selective would probably solve this problem, thereby ensuring that each glycoside would be in one solvent or solvent combination.

The complete characterisation of DSK3 would have required detailed interpretation of the ^{13}C NMR, (61,62,63) the proton Nmr spectra and possibly a matching of the IR spectrum of DSK3 with the spectra of a number of compounds of similar molecular weights. Classical chemical procedures involving the breakdown of the molecular fragments (eg. sugars obtained by acid hydrolysis of the glycosidic ether linkages) would also probably have helped to establish the molecular structure of DSK3. The facilities and time available, however did not allow all these processes to be carried out.

All that can be said therefore from the data obtained is that DSK3 contained the typical characteristics of cardiac glycosides, i.e. an $\alpha\beta$ unsaturated lactone ring, a steroid nucleus, and possibly two sugar moieties as suggested by the molecular weight. The fact that the Keller Killani reaction was negative did not rule out the presence of sugars, as this reaction only works for 2 - deoxy sugars like digitoxose. The observed cardiac stimulant effect was further evidence for a cardiac glycoside structure. The physical/chemical characteristics of DSK3 suggested that the test material was less polar than digoxin. Since the cardiac stimulant action was shown to be qualitatively similar to that of digoxin further research work would be required to establish the toxicity, the absorption, distribution, metabolism and suitable dose levels of DSK3 vis a vis the corresponding data for digoxin. The findings of such a study would be used to determine the suitability or otherwise of this material for use in congestive heart failure, the condition for which cardiac glycosides are usually indicated.(11, 35) Subject to the comparative data for DSK3 being favourable, further work would then be necessary to establish the supply of raw material and the economics of of exploiting this drug for medical purposes.

TABLE 1

CARDIOACTIVE GLUCOSIDES IN ARROW AND ORDEAL POISONS (AFTER REICHESTEIN)

PLANT	FAMILY	GLYCOSIDE
Acokanthera Schimperii Benth & Hook	Apocynaneae	Ouabain
Acokanthera venetata G. Don.	"	Acovenoside A, B & C
Adenium Honghel ADC	"	Honghelin Honghcloside A & C Digitalinium Verum
Adenium somalense Balf.f.	"	Somalin
Apocynum camrabinum L	"	Cymarin
Cenbera odollam Gaertn	"	Gerberin
Nerium odorum Sol	"	Odoroside A-G

TABLE 1 CONT.

PLANT	FAMILY	GLYCOSIDE
Nerium leander L	"	Oleandrin
Strophanthus gratus	"	Ouabain
Strophanthus kombe' Oliv	"	Strophanthosi side
		Cymarol
Tanghinia venenifera Poir	"	Tanghinia
Thevetia peruviana Schum	"	Thevetin
Thevetia yccotli DC.	"	Thevetin
Urechites Suberecta Hull Arg	"	Urechitin
		Urechitoxin

TABLE 2 SOME SPECIES OF APOCYNACEAE FAMILY AND GLYCOSIDES ISOLATED FROM THEM:

Plant Species	Cardiac Glycosides Isolated
Adenium boehmianum Schinz	Somalin
	Echujin
	Abobioside
	Digitalinum Verum
Adenium somalease	Somalin
Adenium oleifolium Stapf	Somalin
(synonym: Adenium Lugardii)	Honghelóside A
	Echujin
	Substance A

Somalin has been reported to be present in all three species given in Table 2. Two of the three species also contain echujin besides somalin and other glycosides some of which have not been identified (e.g. Substance A).

TABLE 4

TLC EXAMINATION OF FRACTIONS A, B, C, D USING SILICA GEL G PLATES (LABORATORY MADE)

SOLVENT SYSTEM	FRACTION	NO. OF SPOTS	Rf VALUES	DEVELOPMENT DISTANCE CM
Ethylacetate - methanol formamide (85: 10:5)	A	1	0.86	15.4
	B	3	0.87, 0.74 0.32	
	C	2	0.74, 0.32	
	D	1	0.32	
Benzene:ethanol 7:3 (26)	A	1	0.95	14
	B	3	0.94, 0.79 0.45	
	C	2	0.79, 0.45	
	D	1	0.24	
Methylenechloride-methanol-formamide 80:19:1	A	1	0.96, 0.89	14.5
	B	3	0.96, 0.89 0.62	
	C	1	0.89	
	D	1	0.62	

TABLE 4 CONT..

SOLVENT SYSTEM	FRACTION	NO. OF SPOTS	RF VALUES	DEVELOPMENT DISTANCE CM
Chloroform - methanol - formamide 87:12:1	A	1	0.89	14.9
	B	3	0.89, 0.71 0.38	
	C	1	0.38, 0.71	
	D	2	0.38, 0.12	
Benzene - ethylacetate - formamide 80:19:1	A	-	-	15
	B	-	-	
	C	-	-	
	D	-	-	
Chloroform - methanol - formamide 85:15:1	OE *	4	0.89, 0.71 0.62, 0.21	14.8
	OE	4	-do-	
	OE	4	-do-	
	OE	4	-do-	

* OE was the original ethanolic extract.

TABLE 5

RESULTS OF THE PURIFICATION OF THE ETHER EXTRACT (FRACTION B) USING AN ALUMINA COLUMN

ELUANT	FRACTION NO.	VOLUME ML.	RAYMOND REACTION	NATURE OF RESIDUE
Benzene - Chloroform 9:1	1	100	positive	Straw coloured
	2	100	+	amorphous
Benzene - Chloroform 8:2	3	100	+	Straw coloured,
	4	100	+	amorphous
Benzene - Chloroform 7:3	5	100	+	Almost Clourless,
	6	100	+	amorphous
Benzene - chloroform 6:4	7	100	+	Almost colourless
	8	100	+	amorphous
Benzene - chloroform 2:8	9	100	+	-do-
	10	100	+	-do-
Chloroform	11	100	+	-do-
	12	100	+	-do-
	13	100	+	-do-
	14	100	-	

TABLE 6

TLC COMPARISON OF PE WITH AUTHENTIC GLYCOSIDES, USING PRECOATED SILICA GEL GF254 PLATES

SOLVENT SYSTEM	SAMPLE IN METHANOL	NO. OF SPOTS	Rf VALUES
Benzene - ethanol 7:3	PE	3	0.61, 0.55
			0.50
	Somalin	1	0.61
	Echujin	1	0.56
	By Product 6 Acetate	1	0.67
	Digitalinum Verum Hexacetate	1	0.66
	Digoxin	1	0.45
	Ouabain	1	0.06
Benzene - Chloroform 7:3	PE	-	-
	Somalin	-	-
	Echujin	-	-
	Digoxin	-	-

TABLE 6 CONT.

SOLVENT SYSTEM	SAMPLE IN METHANOL	NO. OF SPOTS	Rf VALUES
Benzene - methanol 7:3	PE	2	0.90, 0.84
	Somalin	1	0.89
	Echujin	1	0.86, [illegible]
	Digoxin	1	0.85
	Ouabain	1	0.21
Benzene - ethanol 7:3	PE	3	0.60, 0.54 0.40
	Somalin	1	0.63
	Echujin	1	0.61, [illegible]
	Digoxin	1	0.40

TABLE 7 TLC EXAMINATION OF PE USING ALUMINA AS THE STATIONARY PHASE

SOLVENT SYSTEM	SAMPLE IN METHANOL	NO. OF SPOTS	Rf VALUES	REMARKS
Benzene - ethanol 7:3	PE	3	0.75 0.70 0.64	
Benzene - methanol 9:1	PE	3	0.68 0.47 0.30	
Benzene - methanol 8:2	PE	2	0.62 0.56	
	Somalin	1	0.61	
Benzene - chloroform 7:3	PE	-	-	system too non-polar
	OE Somalin	-	-	

TABLE 3 THE SEPARATION OF THE GLYCOSIDES OF PE ON ALUMINA COLUMN

FRACTION	VOLUME OF EACH REACTION ML	NO. OF SPOTS	Rf VALUES USING BENZENE-METHANOL 9:1 ON ALUMINA PLATES	REMARKS
1 - 5	10	2	0.5, 0.69	Fractions 1-23 were eluted with Benzene-Chloroform 9:1
6 - 10	10	2	0.5, 0.69	
11-15	20	2	0.5, 0.69	All these fractions were clear and colourless
16-20	20	2	0.5, 0.69	
21-23	20	1	0.5, -	
24-29	30	1	0.5, -	Fraction 24-32 were eluted with Benzene-Chloroform 7:3
30-31	30	2	0.5, 0.25	All fractions were
32-37	50	2	0.5, 0.25	clear and colourless

TABLE 8 CONT.

FRACTION NO.	VOLUME OF EACH FRACTION ML	NO. OF SPOTS	RF VALUES	REMARKS
33-35	50	2	0.5, 0.25	Fractions 33-37 were eluted with benzene - chloroform 1:1
36-37	50	1	0.25	All fractions were clear and colourless
38-42	60	1	0.25	Fractions 38-43 were eluted with chloroform
43	100	1	0.25	All fractions were clear and colourless
44	200	1	0.25	Fraction 44 was the final methanolic washing of the column. This fraction was milky and straw coloured. The stationary phase was found to be glycoside free after this fraction

TABLE 9 TLC COMPARISON OF DSK3 WITH SOME GLYCOSIDES (DIGOXIN AND OUABAIN) IN CLINICAL USE

SOLVENT SYSTEM	SAMPLE IN METHANOL	NO. OF SPOTS	Rf VALUES	REMARKS
Benzene - ethanol 7:3	DSK3	1	0.55	
	Digoxin	1	0.43	
	Ouabain	1	0.06	
Ethyl acetate - ethanol 8:2	DSK3	1	0.63	
	Digoxin	1	0.16	
	Ouabain	1	0	
Benzene - ethanol 6:4	DSK3	1	0.61	
	Digoxin	1	0.38	
	Ouabain	1	0.04	

TABLE 9 CONT.

SOLVENT SYSTEM	SAMPLE IN METHANOL	NO. OF SPOTS	Rf VALUES	REMARKS
Benzene - methanol 9:1	DSK3	1	0.16	
	Digoxin	1	0.03	
	PE	3	0.16, 0.40 0.70	
	Echujin	1	0.40	
	Somalin	1	0.70	
	Ouabain	1	0	Solute did not travel from the origin

TABLE 10 ELEMENT ANALYTICAL DATA

LABORATORY	PERCENTAGE ELEMENT COMPOSITION OF DSK3								REMARKS
	C	%	H	%	N	%	O	%	
BMAC (England)	65.03	---	8.53	-	-	-	26.44	-	The percentage of oxygen was obtained by difference since no other elements were reported
Pharm. Inst. Ethz. (Switzerland)	64.0		8.2		0.0		25.0		

TABLE 11 ^{13}C NMR DATA

CURSOR	DELTA/HZ	DELTA/PPM	INTEGRAL	HEIGHT	
885	3951.3629	174.6227	156	23860	
1000	3790.7750	167.3794	28	11605	
1249	3460.3891	153.2786	93	26061	
1793	2746.5820	121.3797	50	11609	
1856	2662.9904	117.6856	82	26076	
2013	2454.6747	108.4795	56	28528	
2141	2284.8378	100.9739	140	27253	
2404	1935.8759	85.5522	272	53282	
2525	1775.3269	78.4570	859	112115	
2549	1743.4825	77.0497	936	110076	$CDCl_3$
2573	1711.6380	75.6424	878	115604	
2588	1691.7353	74.7629	354	39432	
2600	1675.8131	74.0592	219	38872	
2609	1663.8714	73.5315	112	18959	
2640	1622.7390	71.7137	141	30883	
2832	1367.9836	60.4553	108	20621	
2905	1271.1234	56.1748	48	11506	
2993	1154.3605	51.0146	104	21928	
3016	1123.8429	49.6660	44	16916	
3149	947.3717	41.8672	160	35043	
3190	906.2393	40.0494	80	21484	
3237	830.6088	36.7071	150	22940	
3252	810.7060	35.8275	134	28563	
3264	794.7838	35.1239	177	20867	
3297	750.9977	33.1888	95	17630	

TABLE 11 CONT.

CURSOR	DELTA/HZ	DELTA/PPM	INTEGRAL	HEIGHT
3348	6833.3283	30.1983	216	28896
3351	679.3478	30.0224	140	27900
3403	610.3515	26.9732	103	22852
3409	602.3904	26.6214	375	45686
3458	537.3747	23.7482	131	27483
3502	478.9932	21.1681	361	24892
3560	402.0359	17.7671	109	24128
3593	358.2498	15.8321	76	17447
3863	0.0000	0.0000	1000	170747

TABLE 12

ABSORBANCES OF DIFFERENT CONCENTRATIONS OF DSK3 AT 217 nm.

FLASK NO.	VOLUME OF STOCK SOLUTION 0.006% ETHANOL ML	VOLUME OF 95% ETHANOL	TOTAL VOLUME ML	CONCENTRATION OF DSK3 % w/v	ABSORBANCE READ AT THE ABSORPTION MAXIMUM OF 217 nm
1	0.5	4.5	5	0.0006	0.18
2	1.0	4.0	5	0.0012	0.34
3	1.5	3.5	5	0.0018	0.48
4	2.0	3.0	5	0.0024	0.63
5	2.5	2.5	5	0.0030	0.78

TABLE 12

ABSORBANCES OF THE COLOURED COMPLEX FORMED BETWEEN FIXED AMOUNTS OF ALKALINE 3,5 DNB AND DIFFERENT CONCENTRATIONS OF DSK3 (max 620 nm)

TUBE NO.	VOLUME OF 2% DNB ML	VOLUME OF 0.1% DSK3 ML	VOLUME OF 95% ETHANOL ML	VOLUME OF 10% NaOH ML	TOTAL VOLUME ML	ABSORBANCE	
						INITIAL READING	SECOND READING*
1	2	0.25	5.75	2	10	0.25	0.35
2	2	0.50	5.50	2	10	0.50	0.70
3	2	0.75	5.25	2	10	0.77	1.01
4	1	0.5	2.5	1	5	1.0	1.20

* Readings taken approximately 1 minute after initial absorbance reading.

TABLE 14

ABSORBANCES OF THE COLOURED COMPLEX FORMED BETWEEN FIXED AMOUNTS OF ALKALINE 3:5 DNBA AND DIFFERENT CONCENTRATIONS OF DSK3 (λmax 530 nm).

TUBE NO.	VOLUME OF KEDDE REAGENT	VOLUME OF 50% METHANOL	VOLUME OF 0.1% SOLUTION IN 95% ETHANOL	*ABBORBANCE READ ABOUT TWO MINUTES AFTER ADDITION OF GLYCOSIDE (DSK3)
1	4	5.75	0.25	0.24
2	4	5.50	0.50	0.46
3	4	5.25	0.75	0.66
4	4	5.0	1.0	0.91
5	4	4.75	1.25	1.12

* The entire spectra were obtained and absrobance values read from these.

TABLE 15

EFFECTS OF DSK3, ADRENALIN, OUABAIN INJECTIONS ON THE CARDIOVASCULAR SYSTEM OF AN ANAESTHETISED RAT.

INJECTION	OBSERVATIONS	REMARKS
Adrenalin in normal saline (10 ug/kg)	An immediate but transient rise in blood pressure was noted. The mean pressure rose from 5 mm Hg to about 9 mm Hg and returned to near normal level after about 2 minutes	The rise was probably due to the vasoconstrictor effect of Adrenalin
10% v/v ethanol in normal saline (1cc/kg	A very slight, insignificant fall in blood pressure was followed by an almost immediate return to normal level	ethanol probably caused transient vasodilation
Ouabain in normal saline (100 ug/kg)	A slight transient fall in blood pressure was followed by a sustained rise from 5 mm Hg normal level to about 9 mm Hg. This rise was sustained over a period of about 5 times the duration of the adrenalin effect.	pressor effect probably due to vasoconstriction

TABLE 15 CONT.

INJECTION	OBSERVATIONS	REMARKS
0.1 cc of DSK3 in 10% ethanol in normal saline 25 ug/kg	The injection produced a gradual rise in blood pressure to a new higher level (11 mm Hg) The elevated blood pressure effect was sustained lasting about 10 times longer than the effect of adrenalin	pressor effect probably due to vasoconstriction

TABLE 16

EFFECTS OF 200-250 µg OF DSK3 ON THE CARDIOVASCULAR SYSTEM OF AN ANAESTHETISED CAT

Weight of cat = 3 Kg.

INJECTION	O B S E R V A T I O N S			REMARKS
	Systolic BP mm Hg	Diastolic BP mm Hg	Heart rate beats/ Min.	
1 % PG in normal saline (1 cc injection)	80	50	144	Normal recordings
	80	50	147	No significant change from normal values.
	56	20	156	Readings taken 15 minutes after PG*
DSK3 in 1% PG in normal saline (1 cc injection 60 ug/kg	132	72	156	Over 100% rise in blood pressure recorded 2 minutes after test material. Beat amplitude also increased but the heart rate was not affected.
	48	16	160	Control readings, 70 minutes after injection of test material.

* PG = Propylene Glycol.

TABLE 16 CONT.

INJECTION	OBSERVATION			REMARKS
	Systolic BP mm Hg	Diastolic BP mm Hg	Heart rate Beats/ min.	
DSK3 in 1% PG in saline (1cc injection) 70 ug/kg	140	60	180	A dramatic rise in both systolic and diastolic pressures within 2 minutes after injection. Beat amplitude was more than doubled and the heart rate showed an initial slight increase.
	76	8	72	Fifty minutes after the injection, the blood pressure had falled considerably, and the heart rate was less than 50% of the control level prior to the injection.
	60	-	60	Readings after 55 minutes
	52		36	Readings after 70 minutes
	52		36	Readings after 90 minutes
	40		96	Readings after 2 hours
	40	4	120	Readings after 2 hours 40 minutes
DSK3 in 1% PG in normal saline (1cc n injection) 60 ug/kg	over 70	20	171	The blood pressure showed a considerable rise. The heart rate also rose considerably with evidence of arrythmias. About 10 minutes after this last injection the arrythmias became pronounced and the preparation quickly dried.

* PG = Propylene Glycol

TABLE 17

EFFECTS OF DSK3 ON THE CARDIOVASCULAR SYSTEM OF AN ANAESTHETISED CAT WITH EXPERIMENTALLY INDUCED HEART FAILURE

Weight of Animal = 3 kg.

INJECTION	O B S E R V A T I O N S				REMARKS
	BLOOD PRESSURE				
	Systolic mm Hg	Diastolic mm Hg	Mean mm Hg	Heart Rate Beats/ min.	
Pentobarbitone Sodium (IV) 30 mg/kg	160	90	120		Normal recordings
	60	10	30		Drastic fall in blood pressure. Beat amplitude also fell
DSK3 in 1% PG (IV). 30 ug/kg	120	64	90		This injection was given while the heart was still severely depressed. The blood pressure started rising immediately after the injection. Beat amplitude also showed recovery.
	140	80		126	Reading taken about 45 minutes after the previous injection. BP had stabilised.

TABLE 17 CONT.

INJECTION	OBSERVATIONS				REMARKS
	BLOOD PRESSURE				
	Diastolic mm Hg	Systolic mm Hg	Mean mm Hg	Heart Rate Beats/ min.	
Pentobabitone Sodium 40 mg/kg	Below 30	-	-		Again a drastic fall in blood pressure and beat amplitude was observed.
Test material (DSK3) 60 ug/kg	80	20		112	Readings taken 5 minutes after test material injection. BP had risen significantly. Beat amplitude was increased and the heart rate was slightly lowered
Pentobarb Sodium 50 mg/kg	Below 20	Below 20	-	84	Again a drastic fall in BP observed. Beat amplitude was significantly reduced. These effects were sustained as no "stimulant" was injected.

TABLE 18

EFFECTS OF DSK3 ON THE ISOLATED PERFUSED RABBIT HEART

INJECTION	OBSERVATIONS		REMARKS
	HEART RATE BEATS/mm	BEAT AMPLITUDE	
--	108	see fig	Normal recording
200 ug DSK3 in 1% PG	96	increased	A dramatic increase in beat amplitude was recorded. A shift of the contraction pattern towards increased tension was also evident. The heart rate decreased slightly.
	156	as normal	50 minutes after the injection the heart rate was significantly higher than the normal level. The beat amplitude had however returned to near normal.
100 ug DSK3 in 1% propylene glycol.	72	increased	Immediately after the injection the beat amplitude started rising. The contraction pattern was gradually shifted towards increased tension (upwards on the chart). The heart rate was decreased considerably (over 100%)

TABLE 18 CONT.

INJECTION	O B S E R V A T I O N S		REMARKS
	HEART RATE BEATS/mm	BEAT AMPLITUDE	
	120	Normal	10 minutes after this second dose, the beat amplitude had fallen back to normal. The heart rate had also risen.
	144	Normal	15 minutes after the 100 ug dose, the heart rate was noted to be still rising. The preparation was left for another 15 minutes and a further injection given.
100 ug	60	Increased	These readings taken 5 minutes after this injection showed a slowing of the heart as well as increased beat amplitude. An upward shift of the contraction pattern indicated increased tension developed.
	120		Normal recording
50 ug of ouabain in normal saline	36	Increased	The readings taken 10 minutes after the injection of ouabain showed a dramatic fall in the heart rate. An upward displacement of the contraction noted signifying increased tension. There was also a tendency for the beat amplitude to increase.

Fig 1

Young Adenium obesum plant in flower

Fig 2

Adenium obesum plant showing the underground stem (tuber) and some aerial parts

Fig 3

Underground stem of an older plant

(age unknown)

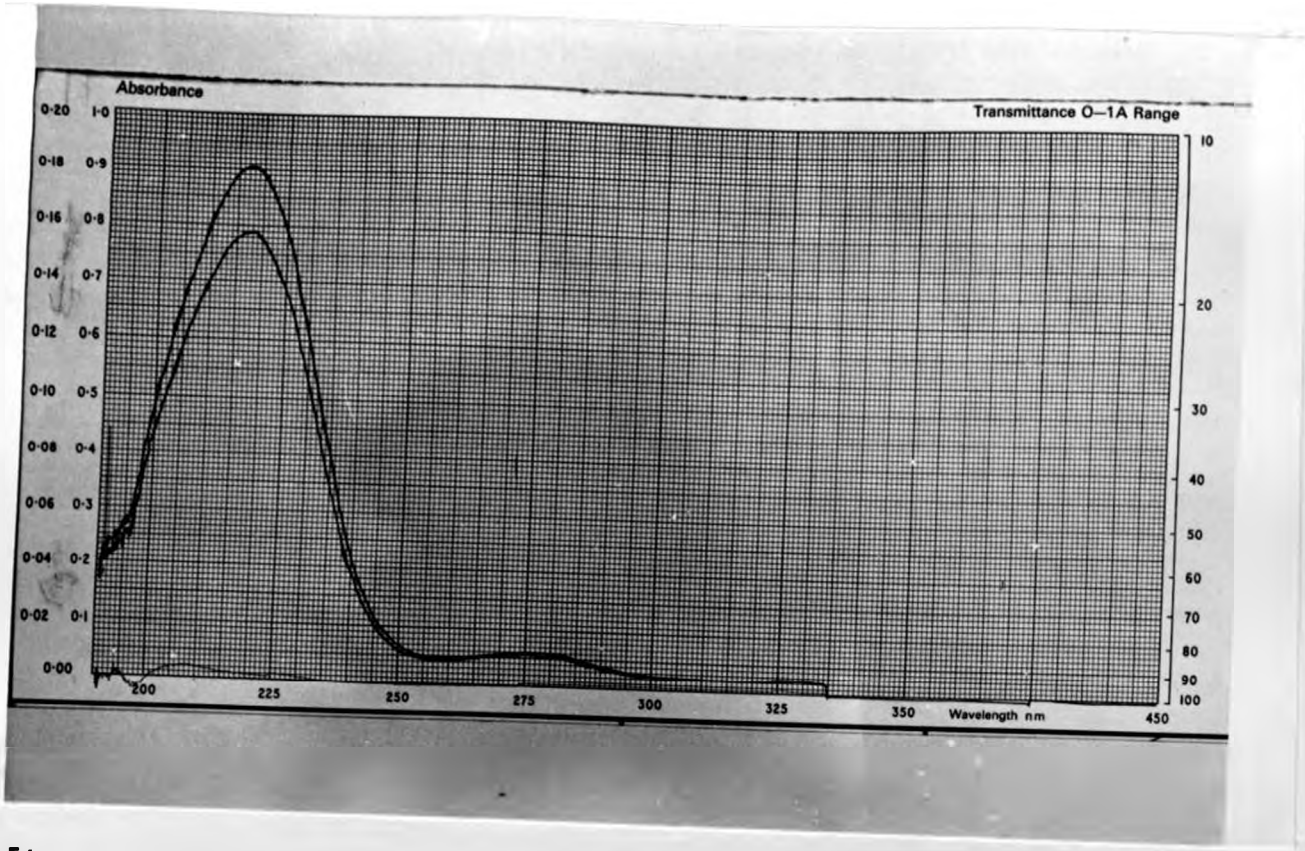


Fig 4

U/V spectrum of DSK I constituent with Rf similar to that of echujin

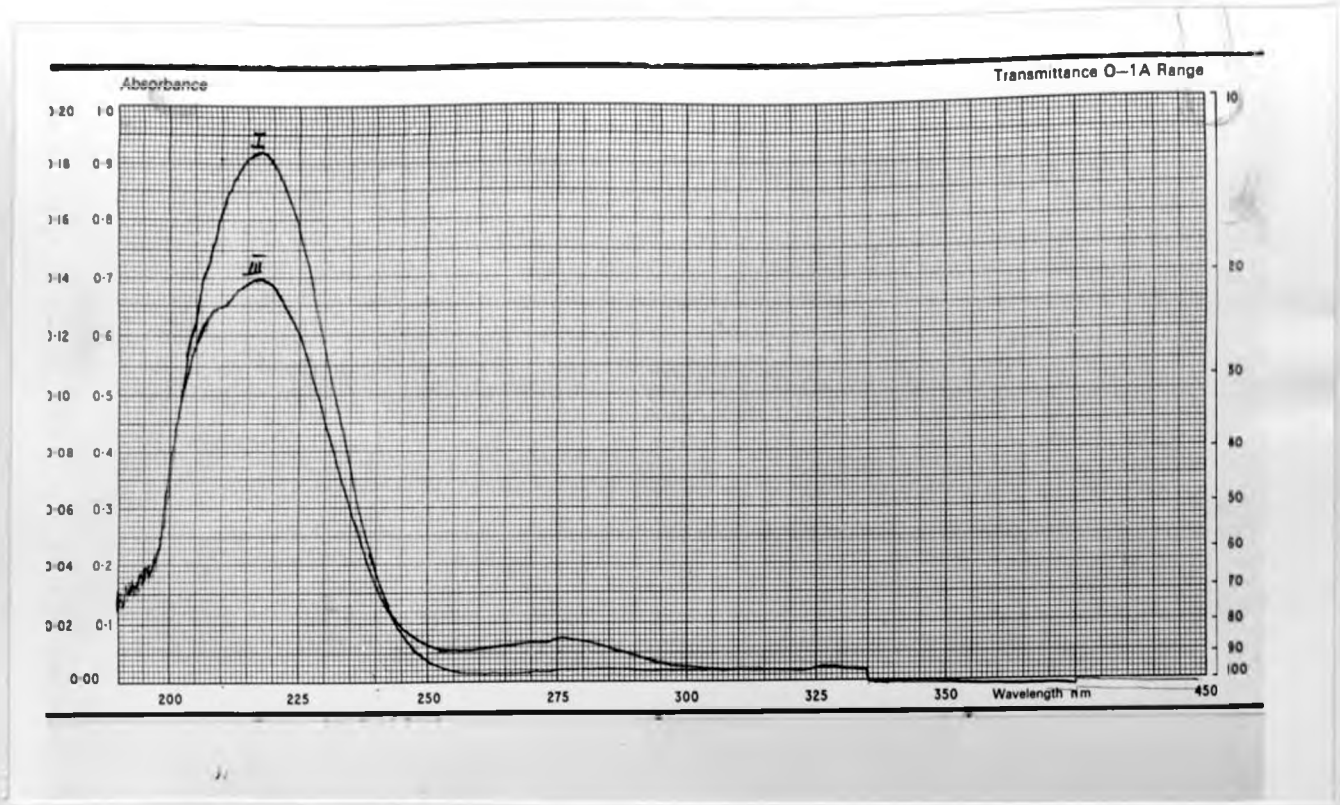


Fig 5

I ___ U/V spectrum of ouabain

III ___ U/V spectrum of DSK3 prior to final purification.

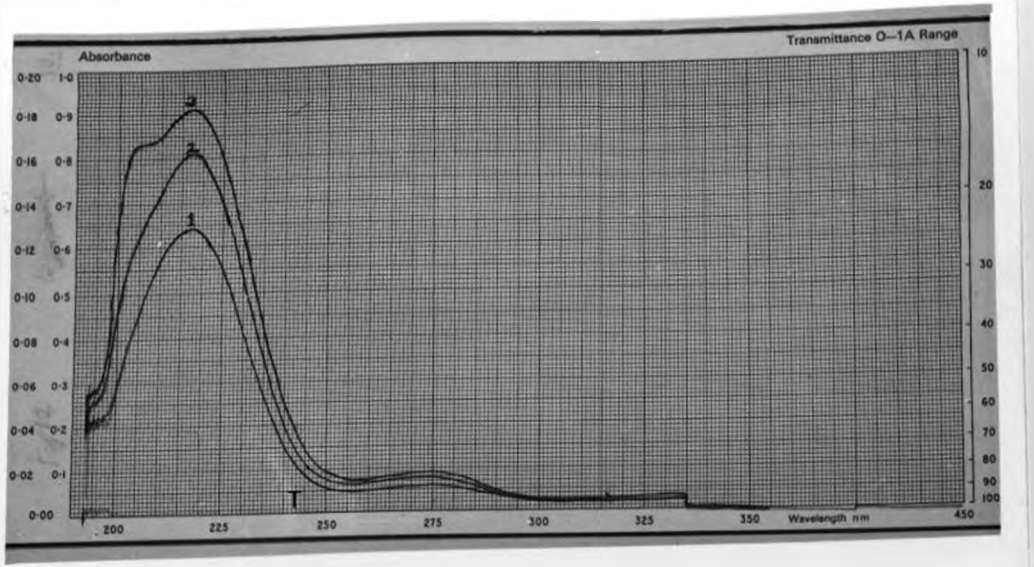


Fig 6

1.---U/V Spectrum of DSK3 (ether washed)

2.--- --as 1.---------- after washing with alkali

3.--- --as 1.---------- after washing with acid

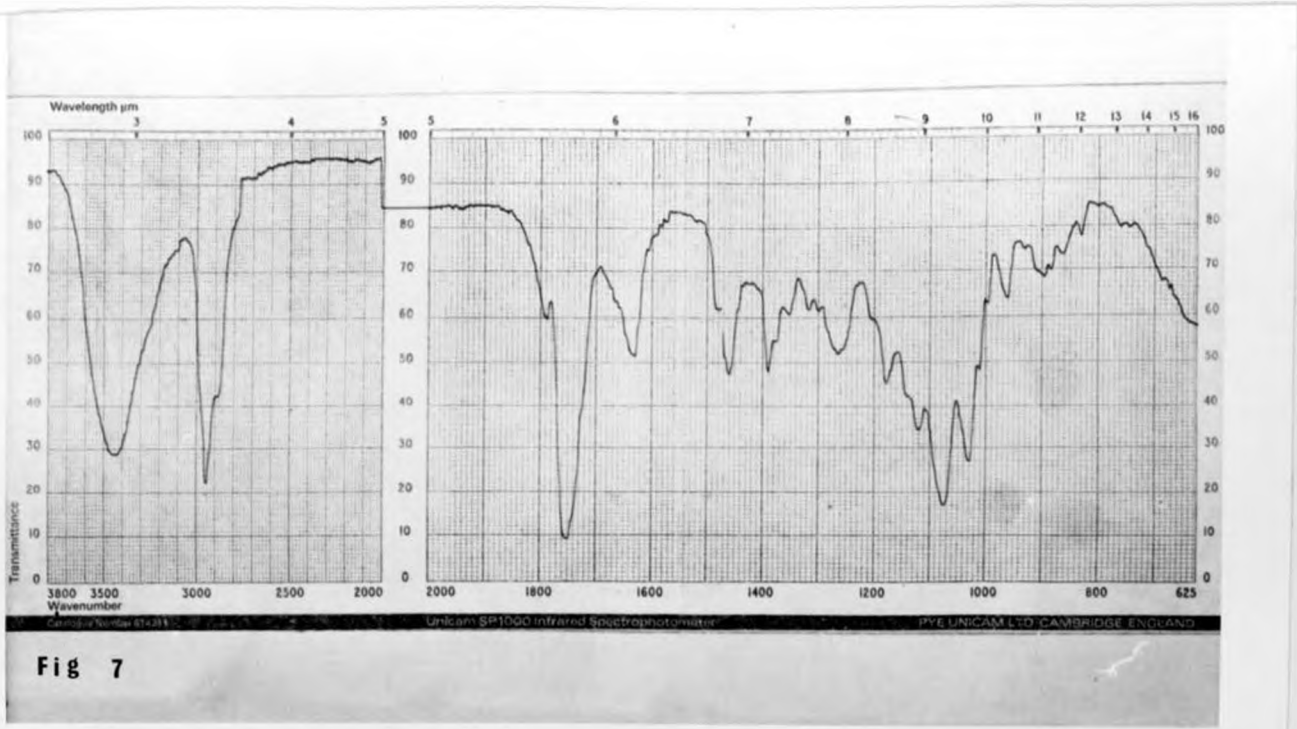


Fig 7

IR spectrum of DSK 3

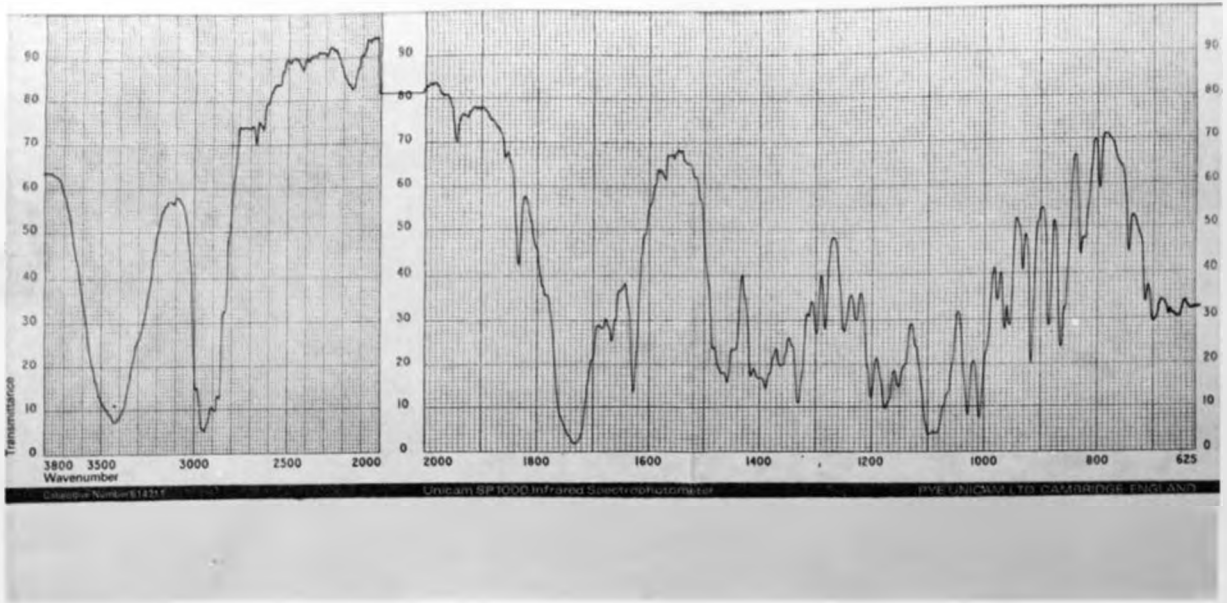


Fig 8

IR spectrum of somalin

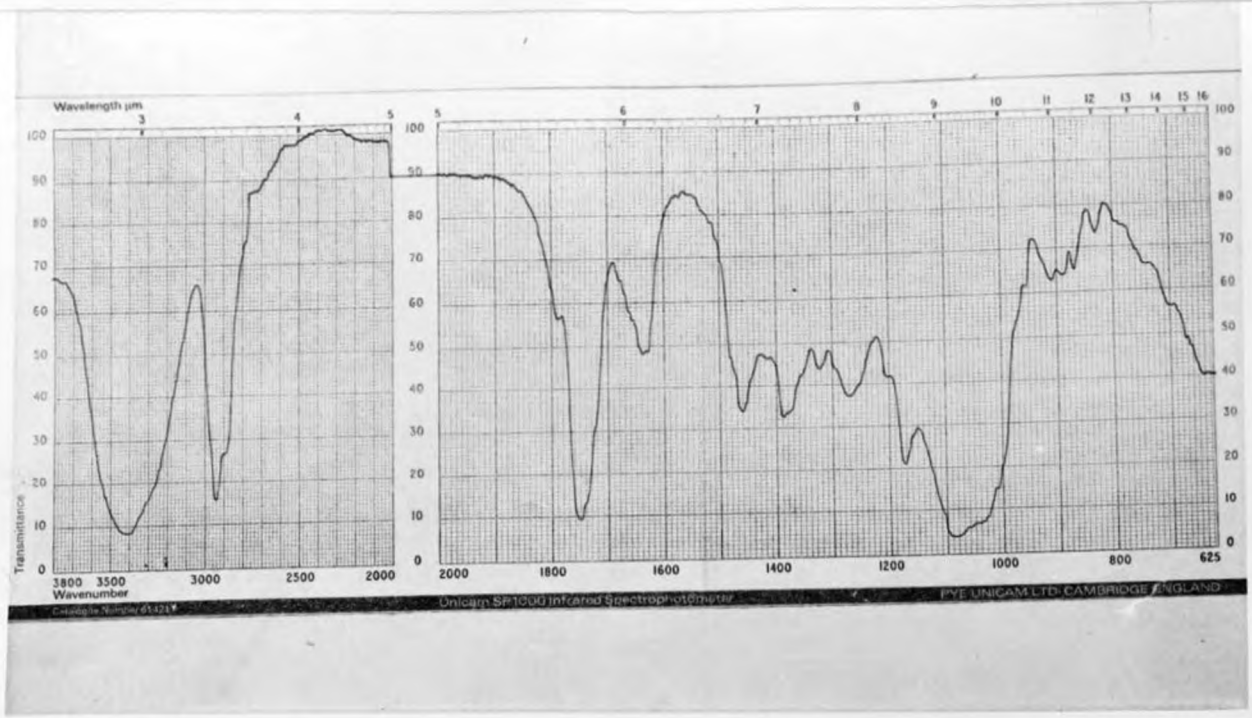


Fig 9

IR spectrum of echujin

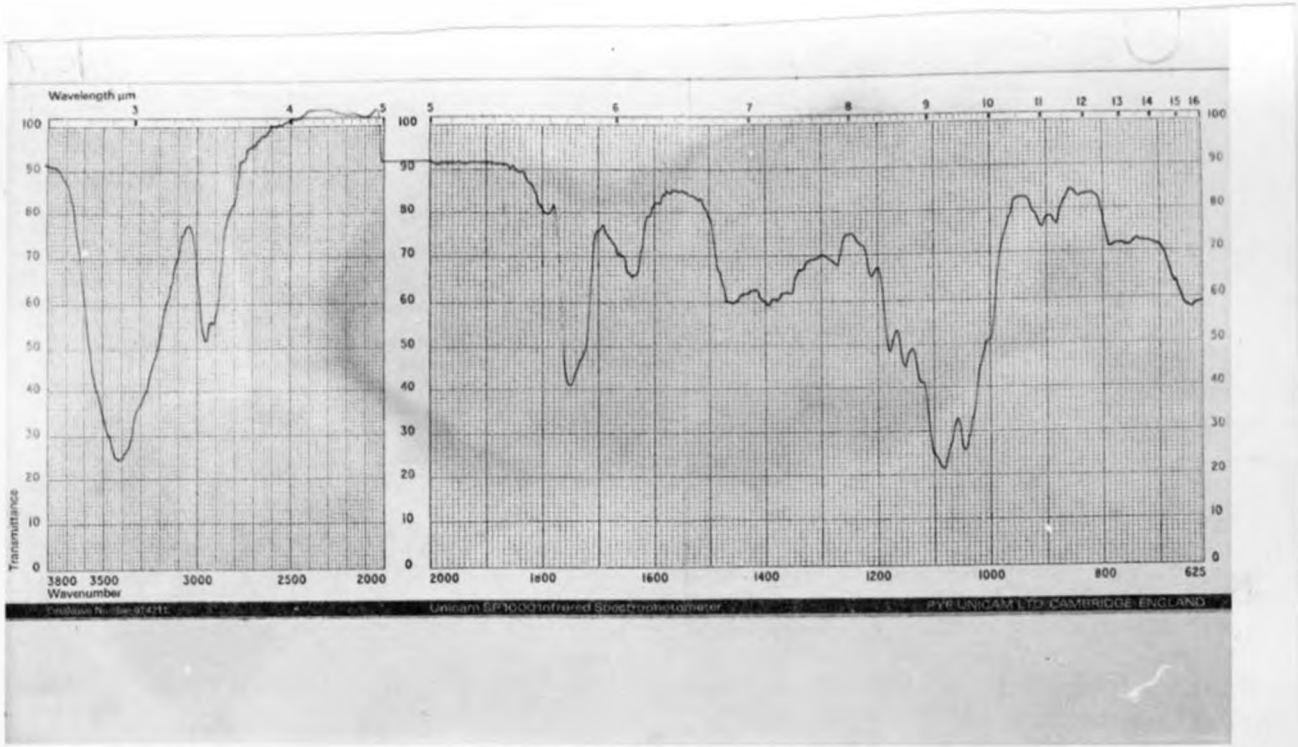


Fig 10

IR spectrum of

ouabain

GRAPH OF ABSORBANCE VERSUS CONCENTRATION OF DSK3 (USING ABSORPTION MAXIMUM AT 217 nm).

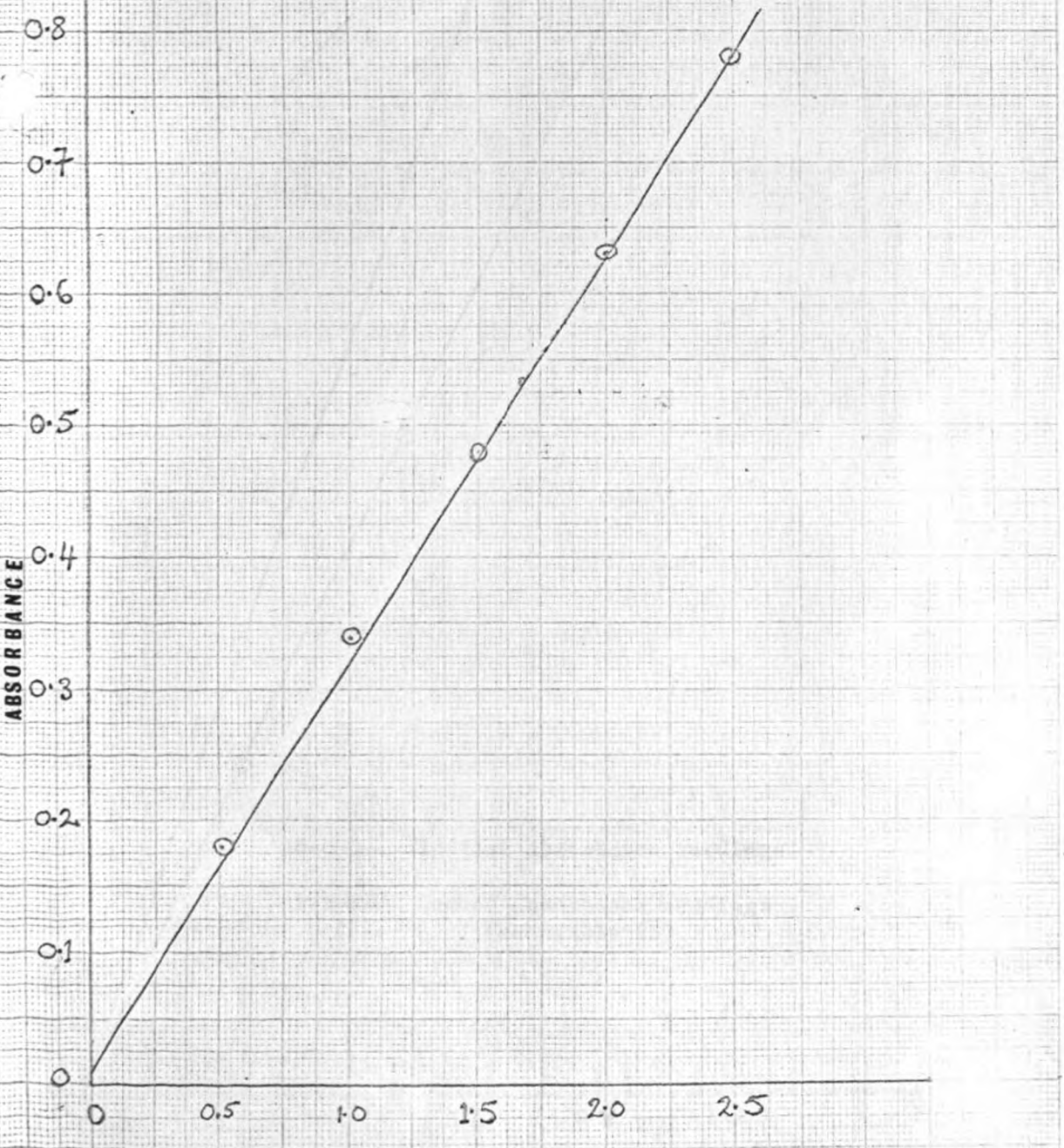
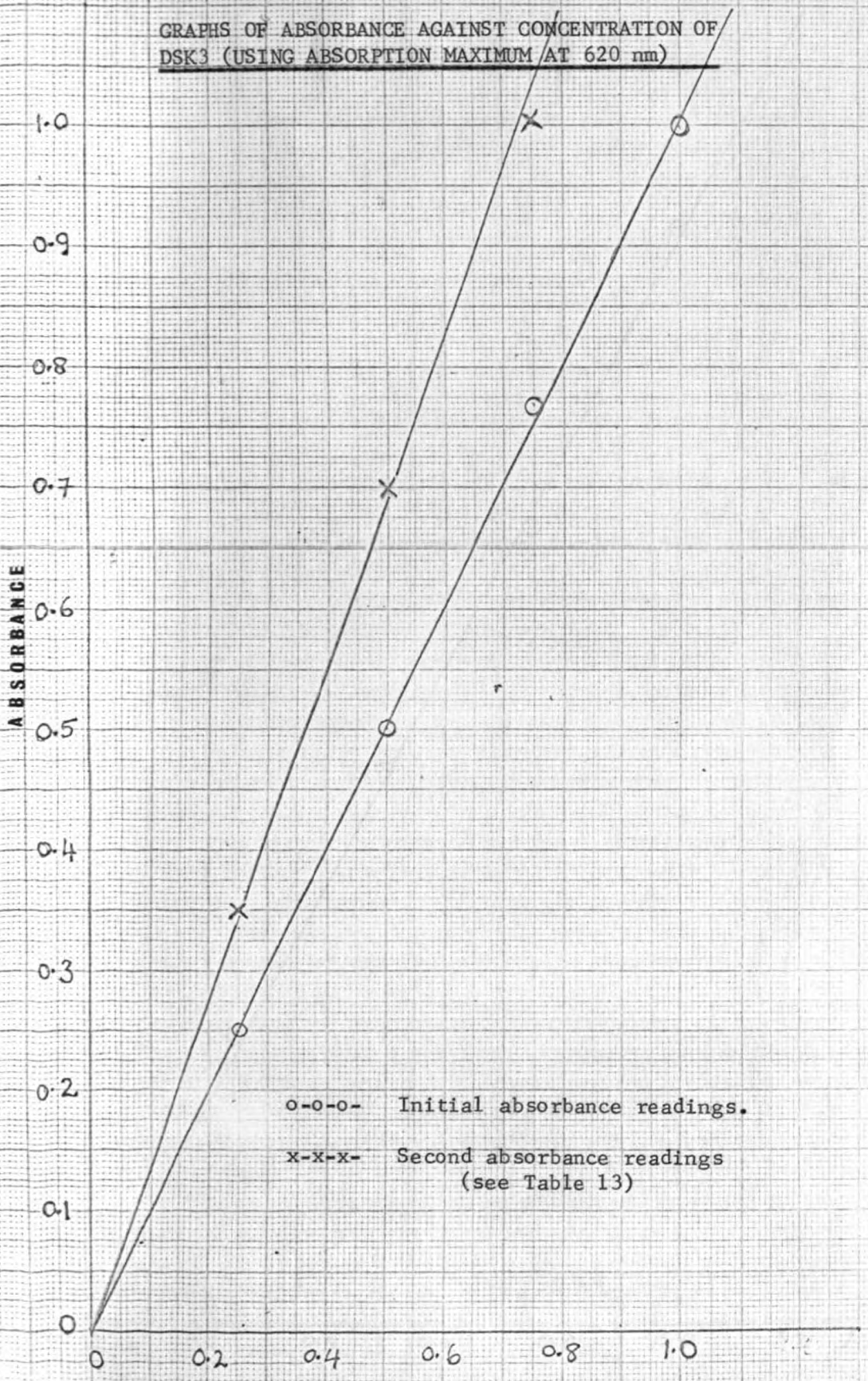


Fig 11

GRAPHS OF ABSORBANCE AGAINST CONCENTRATION OF DSK3 (USING ABSORPTION MAXIMUM AT 620 nm)



o-o-o- Initial absorbance readings.

x-x-x- Second absorbance readings (see Table 13)

Fig 12

GRAPH OF ABSORBANCE AGAINST CONCENTRATION OF DSK3
(USING ABSORPTION MAXIMUM AT 530 nm)

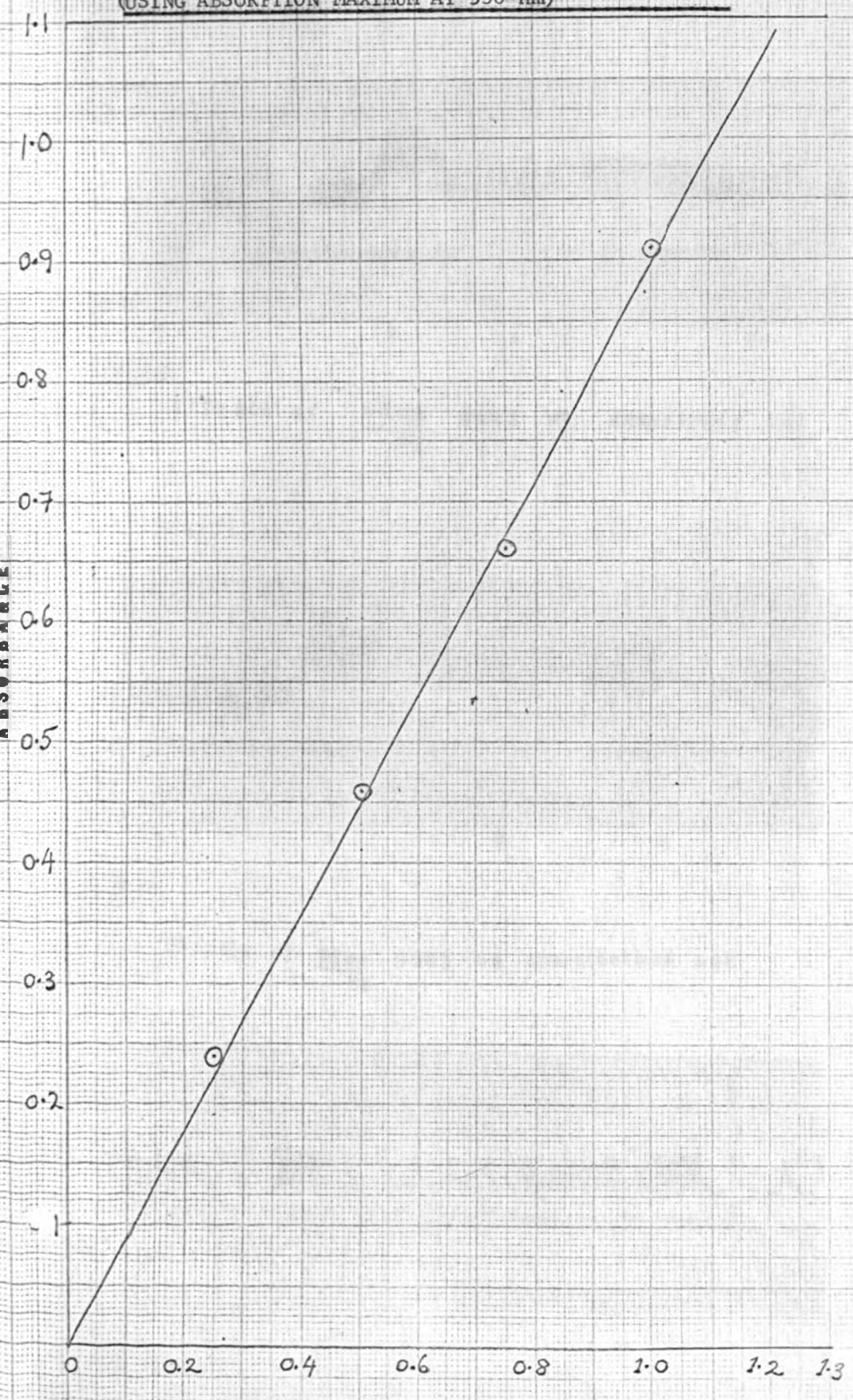


Fig 13

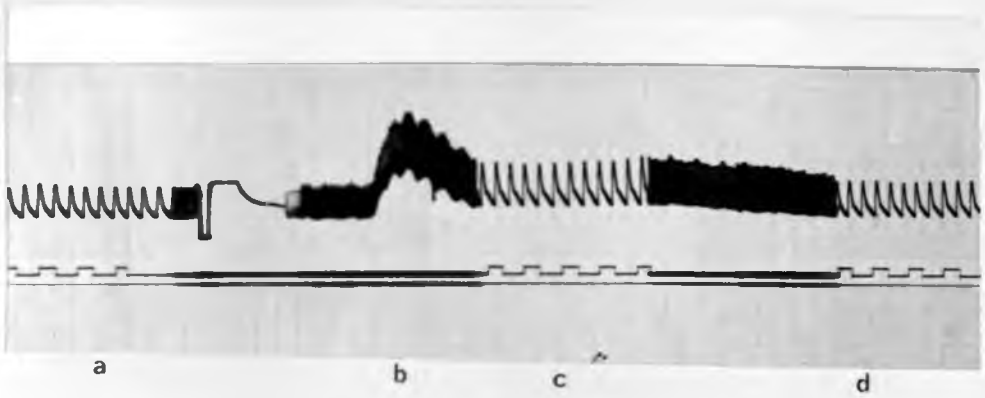


Fig 14

Effects of $\frac{60\,ug}{kg}$ DSK3 on anaestised cat

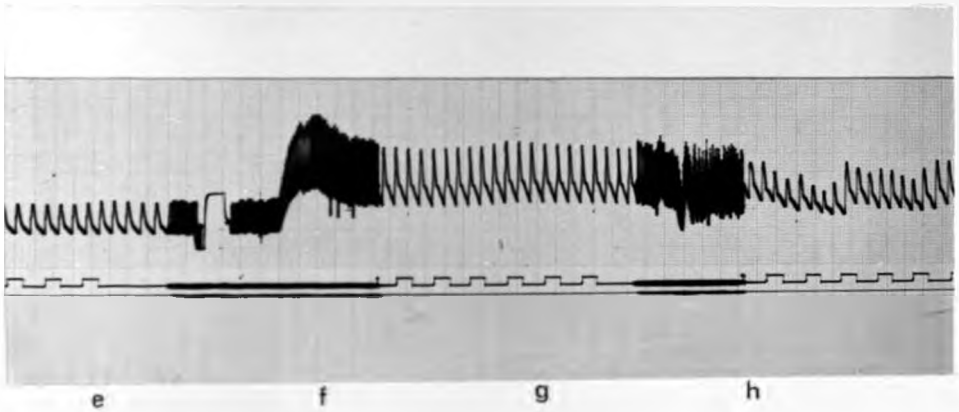


Fig 15

Effects of $\frac{70\,ug}{kg}$ DSK3 on anaesthetised cat

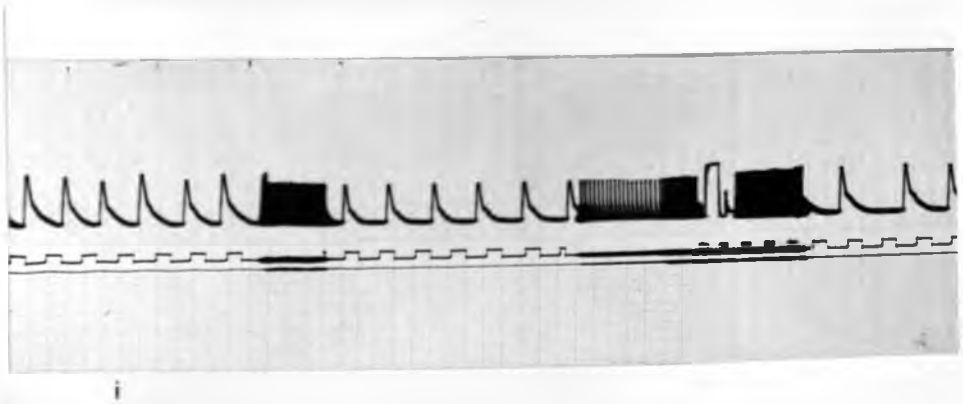


Fig 16

As fig 15

see page 130

a ..... shows normal heart rate (156/min) and normal systolic (52 mm Hg) and diastolic (20 mm Hg) blood pressure.

b..... after iv injection of 60 µg/kg of DSK3, both systolic and diastolic pressures rose dramatically to a maximum of $\frac{132 \text{ mm Hg}}{68 \text{ mm Hg}}$

c ..... shows heart rate (156/min.) and increased heart beat amplitude. Approximately 5 minutes after 60 µg/kg DSK3.

d ..... shows both the beat amplitude and blood pressure returning towards normal levels.

e ..... normal blood pressure $\frac{52 \text{ mm Hg}}{20 \text{ mm Hg}}$ heart rate (160/min.)

f ..... increased blood pressure (to a max of $\frac{140 \text{ mm Hg}}{60 \text{ mm Hg}}$) and beat amplitude following an iv dose of 70 µg/kg of DSK3.

g ..... shows dramatically increased beat amplitude and slightly raised heart rate (180/min.).

h ..... shows arrythmic activity of this high dose of DSK3.

i ..... shows lowered heart rate (60 beats/min) 1 hour after DSK3 injection.

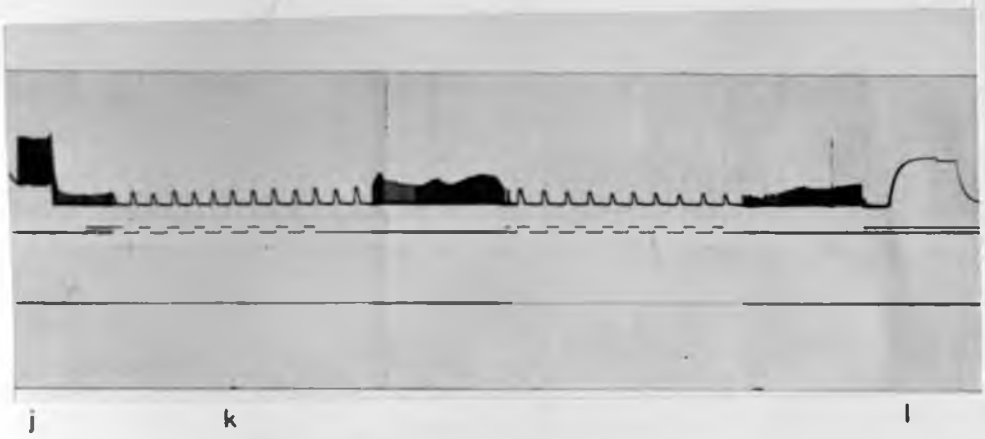


Fig 17

Anaestised cat. Heart depression with $\frac{30\,mg}{kg}$ pentobarbitone

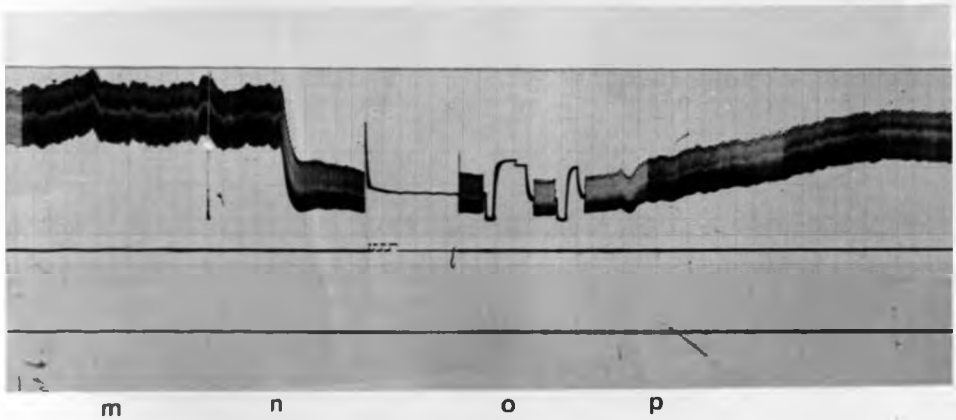


Fig 18

Effects of DSK3 on anaesthetised cat with experimentally induced heart failure.

see page 132

j ..... normal systolic (96 mm Hg) and diastolic (26 mm Hg) pressure.

k ..... blood pressure after iv injection of 30 mg/kg pentobarbitone sodium. Note drastic fall in heart beat amplitude.

l ..... strain gauge vented to the 60 mm Hg column of heparinised saline to check the calibration.

m ..... normal systolic (160 mm Hg) and diastolic (90 mm Hg) pressures.

n..... BP fall after 30 mg/kg pentobarbitione.

o ..... calibration check.

p ..... steady rise in BP and beat amplitude after 30 µg/kg DSK3 iv injection.

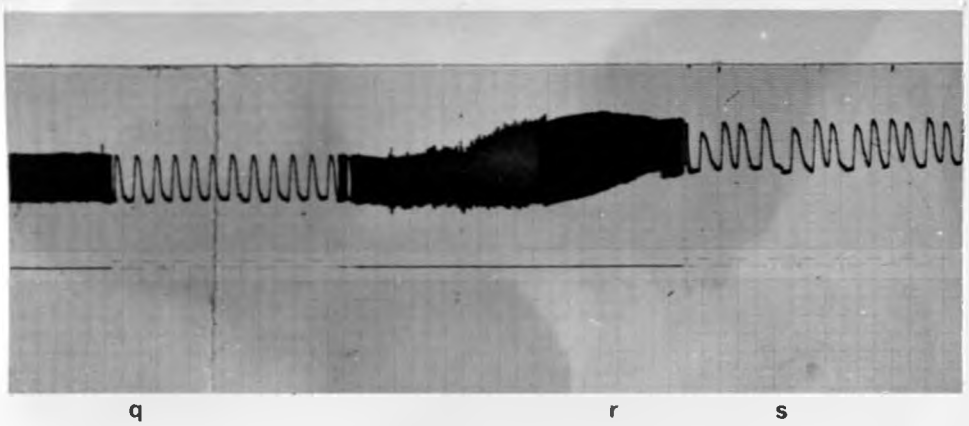


Fig 19

Effects of DSK3 on Langendorffs preparation.

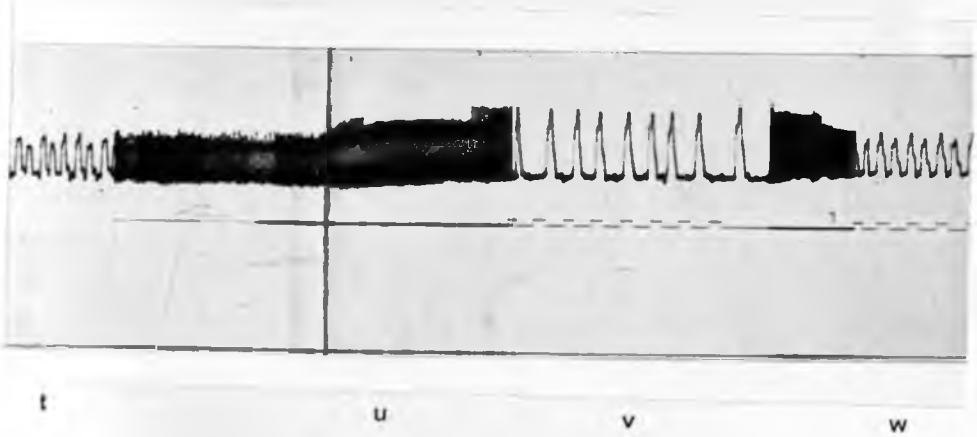


Fig 20

As fig 19

see page 134

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* A number of references, originally included, have been removed as these no longer occur in the text and were inadvertently left in the reference section. This accounts for the missing numbers in the sequence.