THE ISOLATION AND CHEMICAL INVESTIGATION OF CARDIAC GLYCOSIDES FROM AUSTRALIAN PLANTS

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A Thesis for the Degree of Doctor of Philosophy
of the University of Sydney

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Summary of Thesis Entitled:

THE ISOLATION AND CHEMICAL INVESTIGATION OF CARDIAC GLYCOSIDES FROM AUSTRALIAN PLANTS

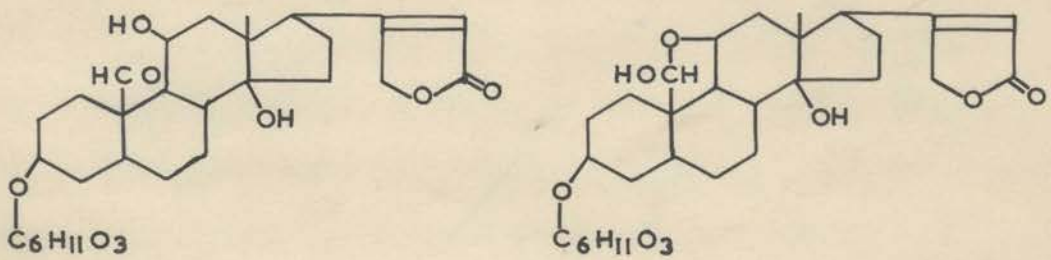
A number of plants belonging mainly to the Apocynaceae and Asclepiadaceae families have been screened for the presence of cardio-active constituents. Of those which gave positive indication of the presence of cardiac glycosides, Gomphocarpus fruticosus (R.Br.), Cerbera floribunda, Cerbera dilatata, and Cerbera manghas were selected for further investigation.

Two substances were isolated from Gomphocarpus fruticosus; Gomphoside - a pure compound, and Afroside - a mixture of two substances which could not be separated by chromatography on alumina.

Gomphoside, $C_{29}H_{44}O_8$, contains neither methoxyl nor acetyl groups, but forms a diacetate, $C_{33}H_{48}O_{10}$, which is unstable to chromium trioxide in acetic acid. Although gomphoside gives a negative Keller-Kiliani reaction, a quantitative oxidation by periodic acid indicates the presence of two free adjacent hydroxyl groups in the molecule. Hydrolysis of gomphoside gives gomphogenin, $C_{23}H_{34}O_5$, which does not give a colour reaction with tetranitromethane. The acetylation of gomphogenin yields acetyl-gomphogenin, $C_{25}H_{36}O_6$, which is unstable to chromium trioxide in acetic acid.

Gomphoside and its derivatives have ultra-violet and infra-red absorption spectra typical of those of the normal digitaloid compounds. The reactions which have been carried out on these substances indicate the presence of an hydroxyl group in gomphogenin, besides the normal hydroxyl groups at C_3 and C_{14}, which is resistant to acetylation but which can be oxidised by chromium trioxide.

Afroside can be shown to consist of a mixture of two substances by paper chromatography. Although it has not been demonstrated that these two compounds exist in equilibrium, some of the reactions which have been carried out indicate that one form may be readily converted to the other. The proposed structures of these two components of afroside are shown below:



The acetylation of the mixture of these two compounds results in a nearly theoretical yield of an homogeneous triacetate, $C_{35}H_{46}O_{12}.H_2O$. Also an attempt to hydrolyse the glycosides by hydrochloric acid in acetone gave an homogeneous compound (glycoside), $C_{29}H_{42}O_9$, which formed a triacetate and which was shown to be identical with that obtained from the acetylation of afroside.

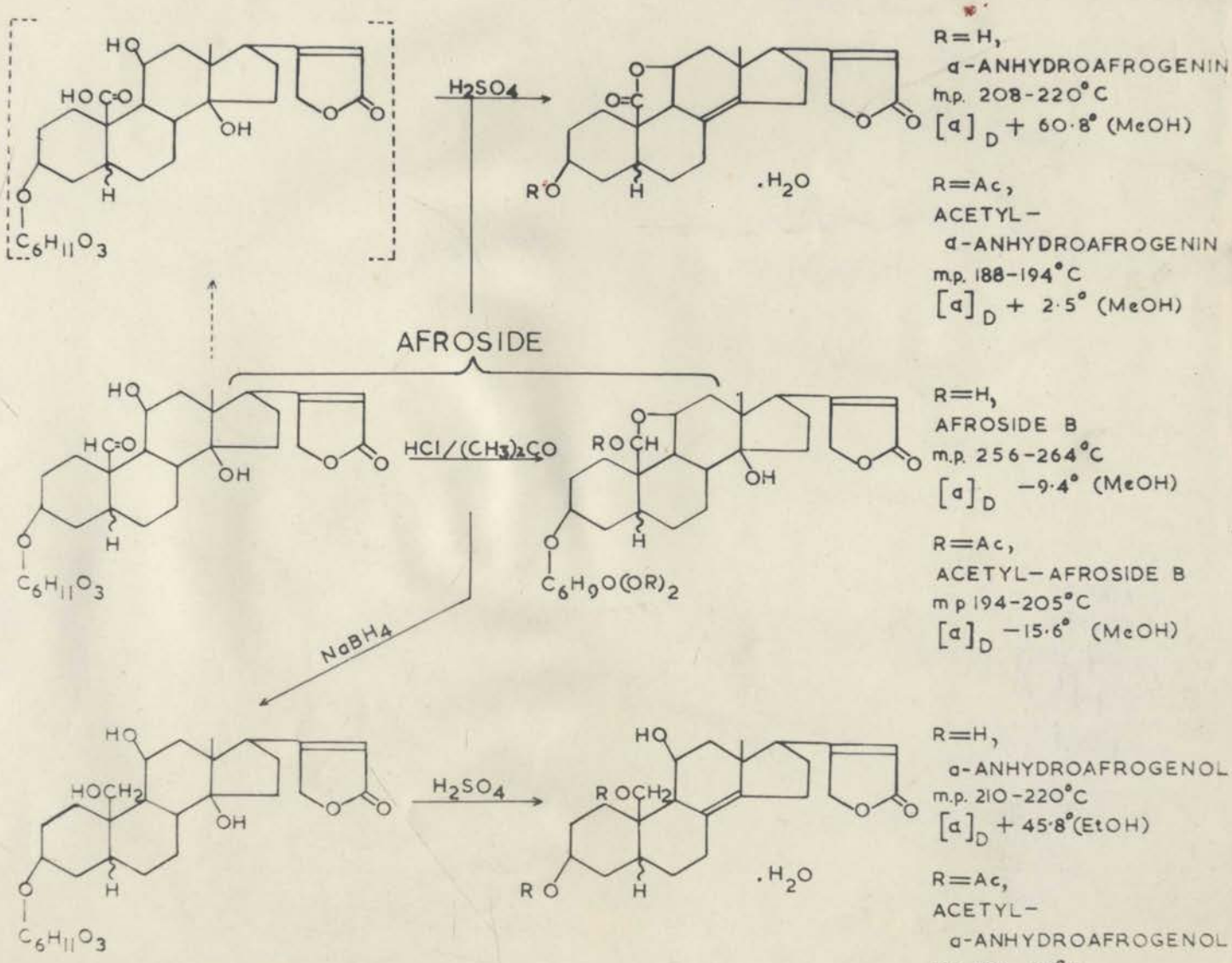
Hydrolysis of afroside gave α-anhydroafrogenin, $C_{23}H_{30-32}O_6$, which formed a monoacetate, acetyl-α-anhydroafrogenin, $C_{25}H_{34}O_8$. The infrared spectra of these two compounds showed the presence of a saturated γ-lactone in the structure of the molecule besides the normal $\Delta^{\alpha\beta}$-γ-lactone side chain.

Reduction of afroside with sodium borohydride gave Afrosidol, $C_{29}H_{44}O_9$, which on hydrolysis gave α-anhydroafrogenol, $C_{23}H_{34}O_5.H_2O$. Acetylation of α-anhydroafrogenol gave a diacetate, $C_{27}H_{38}O_8$. The infrared spectrum of α-anhydroafrogenol shows no evidence of the saturated γ-lactone which is present in α-anhydroafrogenin. These reactions and the structures proposed for the compounds which were isolated, are summarised in the attached diagram.

Cerbera floribunda contains two cardiac glycosides, which have been designated as F1 and F2. F1 is a highly polar compound which could not be obtained in a form suitable for analyses. F2 is isomeric with tanghinin, $C_{32}H_{46-48}O_{10}$, and contains one acetyl and one methoxyl group. Acetylation yields a diacetate, $C_{34}H_{48-50}O_{11}$, which was shown by infrared spectroscopy to be different from acetyl-tanghinin. Hydrolysis of F2 produces F2-genin, $C_{23}H_{34}O_5$, which would not give a crystalline acetate or benzoate.

Cerbera manghas contains two glycosides, which by paper chromatography appear to be identical with F1 and F2.

Cerbera dilatata contains four glycosides which have been designated D2, D3, D4, and D5. D2 is identical with F2 and M2, and D3 by paper chromatography appears to be monoacetyl neriifolin. D4 and D5 have not been separated from one another except on paper chromatograms, and are different from desacetyltanghinin, and acetyl tanghinin.


AFROSIDE
AFROSIDE A
H_2SO_4
$HCl/(CH_3)_2CO$
$NaBH_4$
H_2SO_4
$C_6H_{11}O_3$
$C_6H_9O(OR)_2$
.H_2O
R=H,
α-ANHYDROAFROGENIN
m.p. 208-220°C
$[\alpha]_D$ + 60·8° (MeOH)
R=Ac,
ACETYL-
α-ANHYDROAFROGENIN
m.p. 188-194°C
$[\alpha]_D$ + 2·5° (MeOH)
R=H,
AFROSIDE B
m.p. 256-264°C
$[\alpha]_D$ −9·4° (MeOH)
R=Ac,
ACETYL-AFROSIDE B
m p 194-205°C
$[\alpha]_D$ −15·6° (MeOH)
AFROSIDOL
m.p. 230-235°C
$[\alpha]_D$ +13·6° ($CHCl_3$)
R=H,
α-ANHYDROAFROGENOL
m.p. 210-220°C
$[\alpha]_D$ + 45·8° (EtOH)
R=Ac,
ACETYL-
α-ANHYDROAFROGENOL
m.p. 160-165°C

The work which has been compiled into this thesis was carried out in the Department of Pharmacology, Pharmacy Section, under the general direction of Professor R.H. Thorp, Professor of Pharmacology, and the supervision of Dr S.E. Wright, Senior Lecturer in Pharmacy.

I wish to thank those people who have assisted me in both practical and theoretical aspects of this investigation; in particular Dr A.R.H. Cole of the University of Western Australia for assistance in the interpretation of infrared spectra. Also, I wish to thank the National Health and Medical Research Council of Australia for the Provision of a maintenance grant.

[REDACTION]

Thomas R. Watson.

December, 1955.

PREFACE

The cardiac glycosides occupy a unique position in medicine, as they are the only drugs which are effective in the treatment of congestive heart failure. In view of this fact, and because of the lack of information available about the occurrence of these substances in the Australian flora, it was considered that a systematic survey of the plant families likely to contain cardiac glycosides should be undertaken. The survey has been designed in such a way that besides obtaining qualitative evidence for the presence of cardio-active constituents in the selected plants, an estimate of the glycoside content of the plant could be made. Those plants which gave evidence of yielding satisfactory amounts of cardiac glycosides could then be extracted, the pure compounds isolated, and their chemical structure and pharmacological properties investigated.

The isolation of new cardiac glycosides will, it is hoped, give more information about the relationship between chemical structure and pharmacological activity in this important group of drugs. Present knowledge about this relationship is very inexact and there is a need for close collaboration between chemists and pharmacologists to solve this problem. The availability of new sources of glycosides of known constitution in the Australian flora will also help to provide reference samples for pharmacological comparisons, and precursor substances suitable for molecular modification to assist in the study of structural and biological relationships at present being conducted in this Department.

A survey of the two plant families Apocynaceae and Asclepiadaceae, which are important sources of cardiac glycosides, has been published (Thorp and Watson, 1953). Two new cardiac glycosides have been isolated from Gomphocarpus fruticosus, namely Gomphoside and Afroside (Watson and Wright, 1954). The structure of Afroside has now been more extensively investigated, and information has been obtained about the nature of Gomphoside. In addition, several glycosides have been isolated from three species of Cerbera, and a preliminary investigation of their nature has been made.

The pharmacological properties of Afroside have been determined by separate investigators in this Department (Rand and Stafford, 1955).

The limiting factor in this work has been the supply of sufficient material with which to carry out a complete chemical identification of the new cardio-active compounds. This has been due to both the difficulty of obtaining plant supplies and to the low glycoside content of the plants.

This thesis contains in Part I, the results of the plant survey. Part II consists of a review of the recent literature relating to cardiac glycosides and derived compounds of particular significance to this study. The discussion of the results of the experimental work which has been carried out on the compounds isolated, and the details of the experiments are contained in Parts III and IV respectively.

December, 1955.

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PART I

AN INVESTIGATION OF SOME AUSTRALIAN PLANTS FOR CARDIO-ACTIVE CONSTITUENTS

INTRODUCTION

The cardio-active compounds, characterised by their powerful and specific action on the heart, occur as plant glycosides and nitrogenous venoms secreted by the toad. The glycosides consist of a steroidal genin (or aglycone) linked to one or more carbohydrate molecules, whereas the toad poisons have a steroidal genin conjugated with suberylarginine. In most cases the genin and carbohydrate constituents can be obtained in free form by simple acid or enzymatic hydrolysis of the glycoside or toad poison.

The cardiac glycosides which occur in plants may be divided roughly into two classes, namely the Digitalis - Strophanthus type and the Squill type. The former includes all those compounds which have an α-β unsaturated γ-lactone (butenolide) as the side chain, attached at C-17 in the steroid nucleus (Fig. 1), and the latter those compounds which have a six membered α-β unsaturated lactone ring containing a second conjugated double bond (pentadienolide) in that position (Fig.2)

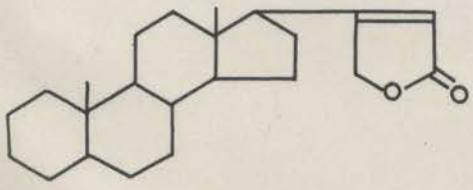


Figure 1

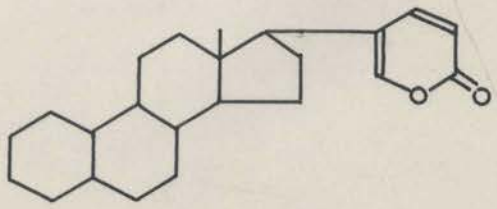


Figure 2

OCCURRENCE

The cardiac glycosides occur in a wide variety of botanical species, but the most common sources are the plants of the Apocynaceae, Asclepiadaceae, Scrophulariaceae, Liliaceae, and the Ranunculaceae. Most of the drugs used therapeutically are obtained from Digitalis lanata, Digitalis purpurea, Strophanthus kombe, and Strophanthus gratus. Comprehensive tables of the known cardiac glycosides have been given in various reviews on this subject (Reichstein, 1951; Wilson and Gisvold, 1954; McIlroy, 1951).

The squill type of glycosides is found only in the family Liliaceae, but some of the digitalis - strophanthus type glycosides have been isolated from this family, e.g. Convallatoxin (Katz, 1947; Reichstein et al, 1948, a & b) and Convalloside (Schmutz & Reichstein, 1947) from Convallaria majalis (L.).

Other botanical species in which cardio-active compounds have been found include:

Crucifereae - Cheirotoxin, Cheiroside A.

Celastraceae - Evonoside

Moroideae - α- and β- Antiarin

The cardiac genins have very little medicinal value as they are usually convulsive poisons rather than cardio-tonic compounds. However, the cardio-tonic properties of the glycosides appear to be determined by the particular structure of the genin; the carbohydrate constituent is probably important

in determining the solubility in the tissue fluids and the transport of these molecules across membranes.

When these cardio-active compounds are injected into the bloodstream of a failing heart they quickly slow the heart-beat and make the individual contractions more forceful. With prolonged administration, cardiac arrhythmias are observed with dropped heart beats, and coupling of the auricular and ventricular contractions when the heart beat is very slow. This latter gives rise to ventricular fibrillation and finally cardiac arrest.

The cardio-tonic compounds derived from plant sources are the most important, and are the only ones which will be discussed.

PLANT SURVEY

As the cardiac glycosides represent one of the few remaining groups of drugs derived solely from plant sources, they are the subject of considerable research at the present time. A preliminary survey has been made for the occurrence of these drugs in Australian plants. So far, most members of the families Apocynaceae and Asclepiadaceae which occur in Australia have been tested, together with a few plants of various other families. Of these, a number have been shown to contain cardio-active principles and most of the work embodied in this thesis is concerned with the extraction, purification, and identification of the active constituents of Gomphocarpus fruticosus (R.Br.). A portion of this work also concerns the active principles of several Cerbera species.

SCREENING OF PLANTS FOR CARDIO-ACTIVE COMPOUNDS

In order to obtain a quick evaluation of the presence of cardio-active compounds in the plants, extracts were administered by continuous intravenous injection into anaesthetised guinea pigs. This is essentially the official method of the British Pharmacopoeia (1953) for the assay of digitalis, and it provides a means of detection of almost any substance which has a direct action on the cardio-vascular system (for method see Appendix 1). A cardiac glycoside injected in this way produces the symptoms previously described and results finally in cardiac arrest in systole.

Some substances which are purely toxic to the heart cause an increase in rate together with increasingly feeble contractions. This is followed by a progressive poisoning of the heart until it fails in diastole. With toxic effects of this kind, no slowing or increase in ventricular contractile force is observed at any time during the experiment and it is reasonable to assume the absence of cardiac glycosides in such circumstances.

RESULTS

The results of the screening of the plants obtained (see Table 1) indicated a number of specimens which contained cardio-active constituents. Of these, only Gomphocarpus

fruticosus (R.Br.) [Asclepias fruticosa (L.)] and the three Cerbera species were selected for further work. Of the other samples which were shown to have cardiac activity, Carissa ovata from Australia had been investigated by Reichstein (Mohr, Schindler, and Reichstein, 1954) and Thevetia nereifolia had been extensively studied by other workers (Chen and Chen, 1934, a, b, & c.). Some Cerbera species had been investigated by various workers (Matsubara, 1937; Frerejaque, 1948; Helfenberger and Reichstein, 1952), but none of those which were tested here. Cryptostegia grandiflora (R.Br.) has also been investigated by Aebi and Reichstein (1950). The seeds of Gomphocarpus fruticosus (R.Br.), obtained from Africa were shown by Stoll et al (1949), Reichstein and co-workers (1949, a, b, & c) to contain two glycosides, - Gofruside and Frugoside - which however, are not those isolated in this work (see p.83).

Two other species of Gomphocarpus (Asclepias), namely Asclepias physocarpa and Asclepias curassavica, are known to contain cardiac glycosides (Williams, 1940; Cornforth, 1950) but have not been included in this survey.

It has been reported (Webb, 1948) that Hoya australis (R.Br.) contains an exceedingly powerful cardiac glycoside, but this was not observed in these experiments. The inclusion of fresh material (Table 1), was in fact, an effort to make sure that the drying process had not caused a loss of the toxic material expected.

In order to confirm the screening test for Gomphocarpus fruticosus, the aqueous alcoholic extract which was tested on the guinea pigs, was introduced into the perfusing solution used in the isolated rabbit heart in the Langendorff preparation (Gunn, 1913). A cannula was tied in the aorta pointing towards the heart. The warm perfusing solution was kept out of the left ventricle by the aortic valves and flowed through the coronary arteries and out through the coronary veins and the right auricle.

This preparation makes it possible to study the action of drugs on the heart muscle and the coronary vessels. (For details of the apparatus and technique, see Appendix 2). The normal amplitude of the rabbit heart was reduced by the addition of thiopentone to the perfusing solution, and then returned to an even greater amplitude than that of the normal beat by the addition of the plant extract to the perfusing solution (Fig. 3). This confirmed that Gomphocarpus fruticosus contained cardiac glycosides.

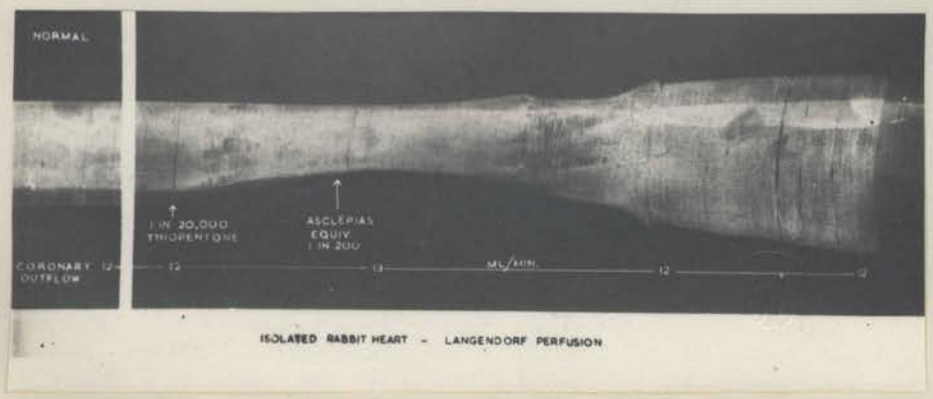


Figure 3

TABLE 1

Plant	Part of plant tested	Equiv. Wt. of dried plant per kilo of body wt. to produce toxic effect (gm.)	Remarks
APOCYNACEAE			
Alstonia constricta (F.Muell)	Bark	1.7	Heart slowed and finally stopped
Alstonia scholaris R.Br.	Bark	15.0	Non toxic
Alyxia magnifolia	Leaves & stems	12.6	Non toxic
Alyxia spicata	Leaves & stems	10.6	Non toxic
Alyxia ruscifolia	Leaves	11.3	Non toxic
Carissa ovata R.Br.	Leaves & stems	3.0	Typical cardiac glycoside action
Cerbera dilatata	Kernels	0.35	Typical cardiac glycoside action
Cerbera floribunda	Kernels	0.31	Typical cardiac glycoside action
Cerbera manghas	Kernels (of fresh fruit)	0.20	Typical cardiac glycoside action
Ervatamia orientalis R.Br.	Leaves	13.2	Non toxic
Lochnera rosea (Reichb.)	Leaves & stems	16.8	Non toxic
Ochrosia elliptica (Labill.)	Leaves	14.8	Non toxic
Parsonsia eucalypto-phylla (F.Muell.)	Leaves	14.5	Non toxic

Continued.

Plant	Part of plant tested	Equiv. Wt. of dried plant per kilo of body wt. to produce toxic effect (gm.)	Remarks
Parsonsia straminea R.Br.	Leaves & stems	6.0	Heart normal then suddenly stopped
Parsonsia latifolia	Leaves & stems	18.0	Non toxic
Thevetia nereifolia	Leaves	3.2	Typical cardiac glycoside action
Wrightia millgar	Wood	14.5	Non toxic
ASCLEPIADACEAE			
Gomphocarpus fruticosus L.	Leaves & stems	1.6	Typical cardiac glycoside action.
Asclepia fruticosa R.Br			
Cryptostegia grandiflora (R.Br.)	Leaves & stems	00.4	Extremely toxic cardiac glycoside action.
Hoya australis R.Br.	Leaves & stems	15.0	Non toxic
Hoya australis	Fresh leaves and stems	18.5	Non toxic
Hoya nicholsonia	Stems	9.0	Heart slowed, showed fibrillation before death.
Marsdenia rostrata R.Br.	Bark	6.4	Heart slowed and stopped, no fibrillation, etc.
Secamone elliptica R.Br.	Leaves & stems	16.3	Non toxic
Tylophora grandiflora	Leaves & stems	0.96	Heart stopped suddenly (insufficient sample for repetition)

Continued.

Plant	Part of plant tested	Equiv. Wt. of dried plant per kilo of body wt. to produce toxic effect (gm.)	Remarks
Araujia albens	Leaves and stems	3.1	Extremely toxic, cardiac glycoside reaction
Microstemma tuberosum	Tubers	14.6	Non toxic
EUPHORBIACEAE			
Excoecaria agallocha	Leaves and stems	1.0	Toxic, no cardiac glycoside action.
Excoecaria dallachyana	Leaves and stems	1.5	Toxic, no cardiac glycoside action.
Euphorbium peplus	Leaves	3.6	Non toxic
LILIACEAE			
Stypandra glauca	Leaves and stems	6.5	Non toxic
Cordilyne terminalis	Leaves and stems	8.0	Non toxic

P A R T II

EXTRACTION AND PURIFICATION OF CARDIAC GLYCOSIDES

METHODS OF EXTRACTION

When the cardiac glycosides, digitoxin, digoxin, and gitoxin had been isolated from Digitalis lanata and Digitalis purpurea (Stoll, 1937, review of early work; also Elderfield, 1935, and Jacobs, 1933) it was found that these pure glycosides did not possess the same physiological activity as preparations obtained from carefully dried and stored digitalis leaves. This loss of activity on the part of the pure glycosides was for a long time ascribed to the presence of inactive impurities, and it was not until Perrot and his associates (1931, a & b) pointed out the possibility of a definite chemical discrepancy between the known cardiac glycosides and the genuine compounds initially present in the plant, that a solution to this problem was found. Stoll and Kreis (1933, a, b, & c; Stoll, Kreis, and Hoffman, 1933, a & b) demonstrated that hydrolytic enzymes frequently occur in the plant along with the glycosides. Only by taking special precautions during the extraction process to inhibit the enzymes, is it possible to isolate the genuine glycosides. Thus, Stoll was able to isolate the digilanids (lanatosides) A, B, and C, and show that they are the natural precursors of digitoxin, gitoxin, and digoxin respectively. This is shown schematically in Figure 4.

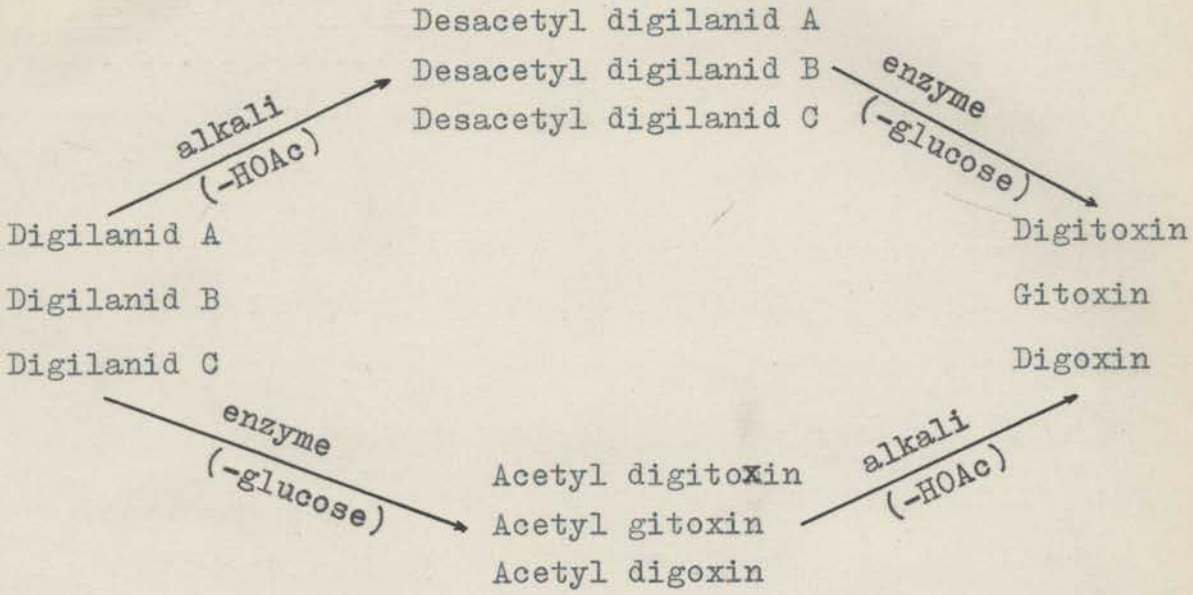


Figure 4

There are two main procedures in extracting plant material to obtain cardiac glycosides, depending on whether the primary (genuine glycosides) or the secondary glycosides (products of enzymatic hydrolysis) are required. To obtain the primary glycosides it is usually necessary to start with fresh plant material (leaves, seeds, or roots). This is ground at low temperatures with the addition of neutral salts (e.g. ammonium sulphate) which precipitate the glycosides along with the inactivated glycoside splitting enzymes. Thus the mash can be pressed out without further injuring the glycosides. The residue is then exhaustively extracted with ethyl acetate. Also, solvents such as butyl alcohol, methylethylketone, tetrahydrofuran, and methylisobutyl-ketone (Gisvold et al., 1950, a & b) may be used.

The crude glycoside pigment mixture is then recovered from the solvent. Since the glycosides are originally present as the tannoid complex, they are only sparingly soluble in ether and may be readily separated from chlorophyll, carotenoids, etc., by ether extraction. The tannins are precipitated from an aqueous alcoholic solution by lead hydroxide or basic lead acetate. The remaining glycoside solution yields on evaporation a mixture of the genuine glycosides together with some products of enzymatic hydrolysis.

In the case of dried plant material, this is usually powdered and then defatted by extraction with petroleum ether, ether, or hexane. Then the glycosides may be extracted with ethanol, and the process continued as for the extraction of the primary glycosides. The processes have been modified in many ways by other workers, but the basic principles of inactivation of the enzymes, and destruction of the tannoid complexes have been followed fairly closely.

After precipitation of the tannins, it is sometimes helpful to evaporate the aqueous alcoholic solution (under vacuum) until most of the alcohol has been removed. This aqueous phase may then be extracted in turn with petroleum ether, ether, chloroform, and chloroform - alcohol (2:1), and each of these extracts tested for the presence of cardiac glycosides. Sometimes, this process will effect a separation of the cardiac glycosides in the mixture. [This process has been used by Reichstein frequently in the

isolation of cardiac glycosides; see papers published in Helvetica Chimica Acta, from 1948 onwards, entitled "Glycosides and Aglycones"].

Occasionally the glycosidic material obtained in the extraction process is very difficult to crystallise, even after chromatography on alumina or other adsorbent or partition material. This is often the case in tri- or tetra- glycosides (i.e. those with three or four carbohydrate molecules). In these instances it is often advantageous to degrade the glycoside by enzymatic hydrolysis to the secondary or tertiary glycosides [these terms being used in the relation, digilanid A being the primary glycoside, and digitoxin (digilanid A - acetic acid, and glucose) being the secondary glycoside]. This involves the use of fresh plant material, in which the enzymes are still active, or dried material with an enzyme preparation such as β-glucosidase or some of the commercially available enzyme preparations such as Festal (Farbwerke Hoechst, Germany). The plant material is ground with water and allowed to ferment (usually at 37°C) for periods varying from a few days to weeks depending on the rate of hydrolysis by the enzyme. Addition of a small amount of toluene is necessary as a preservative. The hydrolysis products may then be extracted by the usual methods.

The isolation of cardiac glycosides is difficult owing to the low content in the plant tissues (usually 1% of dry weight), and the sensitivity of these substances to hydrolysis by enzymes, acids, or bases occurring in the plant. During the extraction processes the pH and temperature of the solvents must be carefully controlled.

The Raymond reaction (Raymond, 1938; blue colour produced when cardiac glycosides or aglycones are reacted with m-dinitrobenzene and sodium hydroxide) is useful in detecting progress of the cardiac glycosides through the extracting solvents during the extraction process. This colour reaction will detect 20 - 30 µg. cardiac glycosides in the presence of other pigments. A drop of the solution is placed on a piece of filter paper, dried, and then a drop of 10% m-dinitrobenzene in benzene applied in the same spot. When the benzene has evaporated, the spot is moistened with 20% aqueous sodium hydroxide and the blue colour develops immediately but fades after 3 - 5 minutes. This reaction is used extensively in the detection of cardiac glycosides on paper chromatograms (see section on paper chromatography, p.21).

METHODS OF PURIFICATION

The chemistry of the cardiac glycosides presents points of unusual complication and materials suitable for investigation are not easily obtained. Several physiologically active and closely related substances often occur together in the same plant. Many cardiac glycosides form mixed crystals and consequently recrystallisation is often not sufficient for the preparation of pure compounds. The "melting points" are really points of decomposition and are only roughly characteristic, affording no true indication of identity or purity. The isolation of pure cardiac glycosides requires the use of some form of adsorption or partition chromatography.

Stoll and his co-workers (Stoll and Kreis, 1933, a,b, & c; Stoll, Kreis, and Hoffman, 1933, a & b) in their pioneer work on the glycosides of Digitalis lanata, were able to separate the digilanids A, B, and C, only by a long and tedious partition of the mixed glycosides between chloroform and aqueous methanol (Stoll and Kreis, 1933, a). [This technique could be applied with the aid of a Craig-type counter current partition apparatus which would simplify the procedure to a large extent.] Alumina, and more recently partition columns have taken the place of Stoll's partition process.

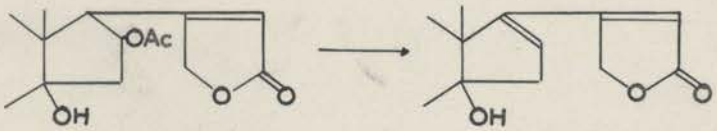
Alumina is one of the principal adsorbents used in chromatography. However, for cardiac glycoside work it must be neutral and have an activity of between II and III on the Brockman scale (Brockman and Schodder, 1941). Alkaline or highly active alumina may give rise to iso-compounds in which the normal lactone of the cardiac glycosides (and aglycones) is isomerised to the 14-β-21 epoxy-23— lactone (I — II, Fig. 5) (Reichstein and Shoppee, 1949), with consequent large losses of the active material. Alkaline alumina may also cause the de-acetylation of acetylated compounds (Abei and Reichstein, 1950; Schindler and Reichstein, 1951,a). Neutral alumina is prepared by the method outlined by Reichstein and Shoppee (1949).

I II

Figure 5

Magnesium silicate and magnesium silicate - kieselguhr (celite) mixtures have been used for the chromatography of steroids, as these adsorbents are slightly less active than alumina (Dobriner et al., 1948). Also, these mixtures do not lead to the removal of acetoxyl groups, which sometimes

occurs in the case of acetylated cardiac glycosides and aglycones, e.g. 16-acetyl glycosides and aglycones when chromatographed on alumina form the corresponding 16-anhydro compounds, III - IV (Fig. 6) (Meyer, 1946; Abei and Reichstein, 1950; Schindler and Reichstein, 1951 a & b; Rittel, Hunger, and Reichstein, 1952, a & b; Hess, Hunger, and Reichstein, 1952; Schenker, Hunger, and Reichstein, 1954, a).



III IV

Figure 6

Some cardiac glycosides, which will not separate on alumina columns (those compounds which do not have the 16-hydroxyl group) can be made to separate by acetylating the mixture and then chromatographing it on alumina. The free glycosides are recovered by mild hydrolysis with potassium bicarbonate in aqueous methanol (Rosenmund and Reichstein, 1942; Rittel and Reichstein, 1954). This technique has been used to separate two of the glycosides of Gomphocarpus fruticosus in this investigation (see p.87).

Silica gel, used purely as a support for the stationary phase in a partition column has been used satisfactorily for the separation of digitoxin and gitoxin (H. Silberman, private communication) and by Stoll and co-workers (Stoll, Angliker, Barfuss, Kussmaul, and Renz, 1951; Stoll and Kreis, 1951) on a preparative scale.

The procedure usually adopted for the separation of cardiac glycosides or aglycones on adsorption or partition chromatography columns is the continuous elution of the compounds by successively more polar solvents (Reichstein's "Liquid Chromatogram"). The fractions collected from the column are evaporated and weighed, the amount of residue then being plotted on a graph against the fraction number. In this way the separation effected may be easily determined (see p. 71). Usually the fractions obtained are not quite clear cut from one another and occasionally a component obtained from a chromatography column may consist of more than one substance which could not be separated on the column. In these cases the fractions are best identified by paper chromatography which has been used in conjunction with the weighing of the residues in this investigation.

PAPER CHROMATOGRAPHY

The application of paper chromatography to the separation and identification of cardiac glycosides followed as a natural adjunct to the application of this technique to other steroidal compounds and an extremely wide range of organic substances. It is not proposed to discuss the development of paper chromatographic techniques as there are many reviews of the subject (e.g. Cramer, 1954; Lederer and Lederer, 1954), and its use in this work was merely confined to the identification and determination of purity of the compounds isolated. The various solvent systems and techniques which were used will be described briefly here, and in more detail in the experimental section (see p.136).

Svensen and Jensen (1950), described solvent systems involving various mixtures of chloroform, methanol, and water, for the separation of the digitalis glycosides. These systems were tried for the separation of the glycosides obtained from Gomphocarpus fruticosus but the solubility of the compounds in the stationary aqueous phase was too low to effect a separation of the component glycosides. Usually only one or sometimes two spots could be detected following closely behind the solvent front. The modification of the Svensen and Jensen systems, used by Silberman and Thorp (1953) was tried also unsuccessfully.

The solubility of most cardiac glycosides in these aqueous phases is too low to allow the partition of the compounds between the organic and aqueous phases to vary to a sufficient extent to allow the separation of these compounds. Consequently in many cases the cardiac glycosides follow the solvent front very closely and no separation of component compounds is effected.

A definite advance in the separation of this type of compound arose from the work of Zaffaroni, Burton, and Keutmann (1949, a, b, & c) on the adrenocortical steroids, in which they replaced the aqueous phase by either the highly polar organic solvents formamide or propylene glycol. The mobile phases were either benzene (with formamide) or toluene (with propylene glycol). This system was adapted by Schindler and Reichstein (1951,c,) to the separation of cardiac glycosides. The solubility of the cardiac glycosides in formamide or propylene glycol is appreciably greater than in aqueous phases and hence the possibility of attaining separation by varying the composition of the mobile solvent (and hence altering the partition coefficients of the components) is greatly increased. Schindler and Reichstein (loc. cit.) used pure chloroform and chloroform - benzene mixtures to increase the rate of travel of the compounds. This system of chromatography has been used to separate many cardiac glycoside mixtures (Heftmann and Levant, 1952;

Jensen, 1953). The disadvantage of the formamide system is that highly polar glycosides (e.g. convallatoxin, ouabain) tend to travel very slowly, making identification difficult. Recently, Schenker, Hunger, and Reichstein (1954, b) have described other solvent systems which are much more effective for the separation of highly polar compounds. The stationary phase is the aqueous phase of water - n.butanol, or water - n.butanol - toluene, mixtures.

Tschesche et al. (1953) have also tried to overcome the difficulty of chromatographing highly polar compounds on paper, by reversing the nature of the stationary and mobile phases. The paper is impregnated with the organic phase of an n.octanol, pentanol, formamide and water mixture and developed with the aqueous phase of this mixture.

Apart from the method of Svensen and Jensen (1950) and the Silberman and Thorp (1953) modification, the formamide system has been almost exclusively used in this investigation, using chloroform - benzene, and chloroform - benzene - butanol (9:1 and 78:12:5 respectively) mixtures as the developing phases. Some compounds have also been chromatographed on the "reversed phase" system of Tschesche et al (1953) (see experimental section p.138 for full details).

Detection of Cardiac Glycosides on Paper Chromatograms

Of the various reagents which produce characteristic colours with the cardiac glycosides (see review by Jensen, 1953), only two have been used in this investigation:

(a) Raymond Reaction (see p.14) (Raymond, 1938; Schindler and Reichstein, 1951). When a cardiac glycoside or aglycone is treated with metadinitrobenzene and sodium hydroxide, a characteristic blue colour is produced. This reaction was found to be the most convenient in this investigation. As this colour reaction is dependent on the presence of the C 17 butenolide side chain it may be used for either glycosides or aglycones. Reichstein (Helv.Chim.Acta, 37, 680, 1954, footnote 1, p.684) favours the Kedde Reaction which involves the use of 3-5 dinitrobenzoic acid and potassium hydroxide for the detection of the spots. The colours produced by these reactions are not permanent and facsimiles of the chromatograms must be made within five minutes if a permanent record is required.

(b) Trichloracetic acid. The trichloracetic reagent, first used by Svensen and Jensen (1950), is sprayed onto the dried chromatograms which are then heated at 100-110°C for 10-15 minutes. The spots are visible sometimes in daylight, but they invariably fluoresce in ultra violet light. This reaction does not appear to be as specific as the Raymond as it can be used to detect the fully saturated dihydro-glycosides or -genins. However, its sensitivity is much greater than the Raymond reaction.

CHEMICAL CONSTITUTION OF CARDIAC GLYCOSIDES

INTRODUCTION

Chemical investigations of the cardiac glycosides date from the nineteenth century, when Homolle and Quevenne (1854) attempted to isolate the active constituents of Digitalis purpurea. Nativelle (1869), was the first to isolate a crystalline glycoside in fairly pure condition and he named it "Digitaline crystallisee". In the following years the main problem of isolation was dealt with by the German chemists, Schmiedeberg (1874, a & b), Cloetta (1920), Kiliani (1895 a, b, c, d, & e), and Kraft (1912). Windaus and Hermans (1915) suggested that the aglycones obtained by the hydrolysis of the cardiac glycosides may be related to the known steroids. In the 1920's Windaus and Stein (1928), completed their investigations on digitoxin by determining its empirical formula and its behaviour on hydrolysis. Shortly afterwards Jacobs (1935) and Tschesche (1934, a & b; 1935) proved the hypothesis suggested by Windaus, by showing that digitoxigenin and uzarigenin were related to the bile acids. Stoll et al (1933), about the same time, converted Scillaren A to allocholanic acid.

One of the most useful contributions to the understanding of the structure of the cardiac aglycones was made by G.A.R. Kon (1934, a), who suggested that on the basis of X-ray measurements by Bernal and Crowfoot (1934) these compounds were hydroxy-steroids with a special side chain structure.

Interpretations of the mass of experimental work which had accumulated by that time, in terms of the steroid structure were given by Kon (1934, b) and Jacobs & Elderfield (1935), who were able to throw much light on the numerous reactions which had been carried out by that time.

It is not attempted here to review the early work on the determination of structure of the nucleus or the side chain of the cardiac aglycones, as these aspects are fully covered in a number of excellent review articles (Jacobs, 1933; Elderfield, 1935; Stoll, 1937 and 1949; Turner, 1947; Reichstein, 1951; Heusser, 1950; Shoppee and Shoppee, 1954).

The basic structure of the cardiac aglycones, except those of the squill glycosides and hellebrigenin, is 21-hydroxynorcholenic acid lactone [cardenolide (Fig. 7)], or 21-hydroxynor-allo cholenic acid lactone [allo-cardenolide (Fig. 8)].

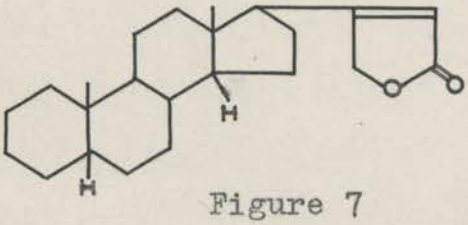


Figure 7

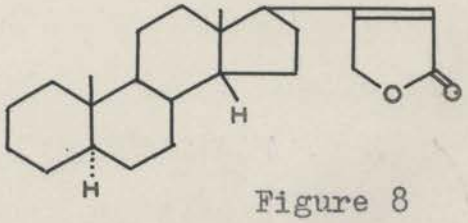


Figure 8

The normal, naturally occurring cardiac aglycones have the 17β-$\Delta^{\alpha\beta}$-γ lactone side chain, and the 14β-hydroxyl group. Thus the C/D ring junction is always cis, which is

the reverse to all the other known naturally occurring steroids. [Fieser's nomenclature 'α' and 'β' is such that α-substituents lie on the opposite side of the molecule from the C_{10} and C_{13} methyl groups, and β-substituents lie on the same side as these methyl groups.]

The orientation of the lactone ring and the 14-hydroxyl group was assumed to be cis (-β) on the basis of the formation of iso compounds by the action of alkali (Jacobs and Elderfield, 1935), and the degradation of digitoxigenin and iso-digitoxigenin to etianic acid (Jacobs and Elderfield, loc. cit.), as well as anhydrouzarigenin to allo-etianic acid (Tschesche, 1934, a). The possibility of epimerisation at C_{14} or C_{17} to form the iso-lactone was unlikely under the conditions of the reaction and hence the configuration of the C_{14} hydroxyl group was assumed to be β-. Also, it was shown that the 17-iso aglycones (allo-aglycones, 17α-lactone) did not form iso compounds. The general methods of oxidative degradation by permanganate in acetone, or ozonolysis at -80°C, which have been developed recently by Reichstein and co-workers (Meyer and Reichstein, 1947; Speiser and Reichstein, 1947, 1948; Helfenberger and Reichstein, 1948; Rauffaufand Reichstein, 1948; Buzas and Reichstein, 1948, Reichstein, 1951; Meyer, 1947), have led to a further proof of the β-configuration of the lactone side chain and the C_{14} hydroxyl group.

Ozonolysis of the $\Delta^{\alpha\beta}$-γ lactone ring (Fig. 9,V) yields a glyoxylic acid ester (Fig. 9, VI), which on saponification with $KHCO_3$ yields a ketol (Fig. 9, VII). This ketol on oxidation with CrO_3 gives a keto-lactone (Fig. 9,VIII), instead of the acid which would normally be expected.

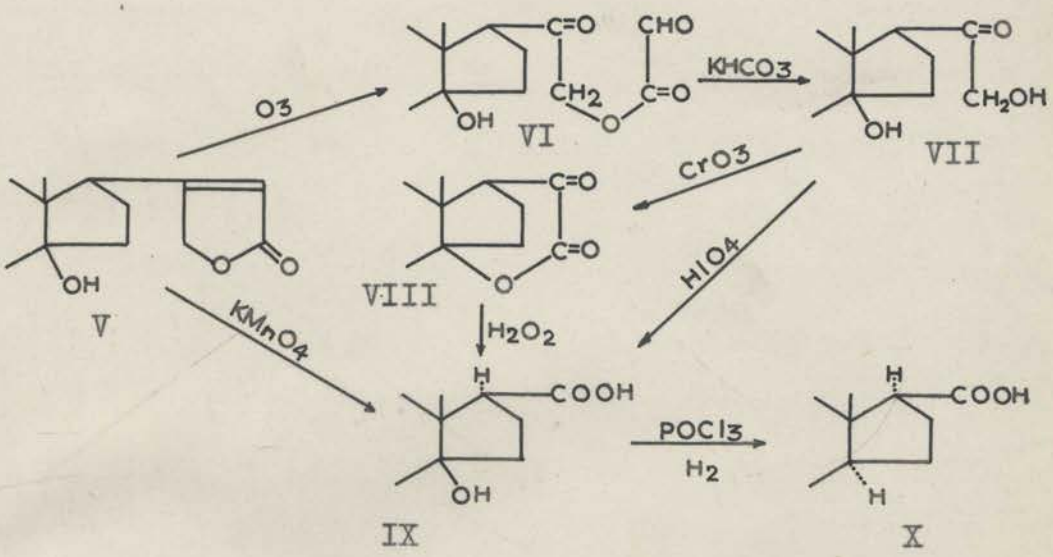
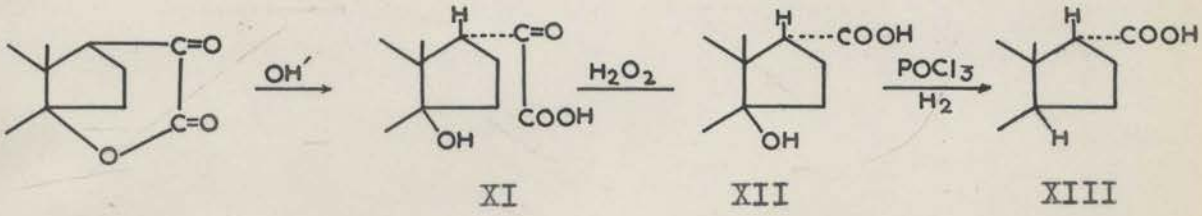


Figure 9

The formation of this keto-lactone shows that the side chain of the ketol (Fig. 9,VII) and hence also the original lactone side chain (Fig. 9,V), must be on the same side of the ring system as the C_{14} hydroxyl group. Oxidation of the keto-lactone (Fig. 9, VIII) with hydrogen peroxide yields the 14-hydroxy-17β etio acid (Fig. 9, IX), which by further degradation (involving dehydration and then hydrogenation) is obtained as the known 17-β etio acid (Fig. 9, X). The 14-hydroxy-17β etio acid (Fig. 9, X) is also obtained in poor yield by the oxidation of the original lactone (Fig. 9, V), with permanganate in acetone. Oxidation of the ketol

(Fig. 9, VII) by periodate also gives this acid.

By heating the keto-lactone with alkali, a keto-acid (Fig. 10,XI) is obtained which no longer forms a lactone. Oxidation of this acid with hydrogen peroxide gives a 14-hydroxy-17-α etio acid (Fig. 10,XII), indicating that inversion at C_{17} has occurred during these reactions. Further degradation of this acid by dehydration followed by hydrogenation yields the 17α-etio acid (Fig. 10,XIII).



XI XII XIII

Figure 10

Similar reactions have been carried out on periplogenin and 17-isoperiplogenin (alloperiplogenin). The ketol from periplogenin on oxidation with CrO_3 gave the keto-lactone which with hydrogen peroxide gave the etio acid with the 17β configuration. 17-isoperiplogenin (Fig. 11,XIV) formed the ketol (Fig.11,XV) which, with CrO_3, did not form the keto-lactone, but instead the 17-α etio acid (Fig. 11, XVI).

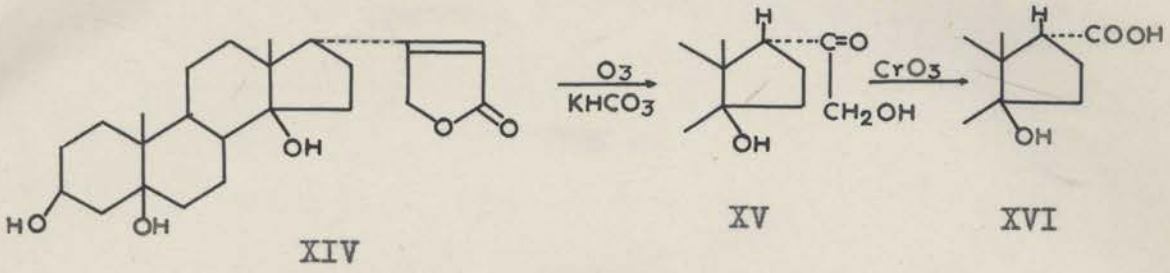


Figure 11

As no unforseen spatial rearrangement can occur in the above reactions, the 17-iso aglycones differ from the normal aglycones in having the lactone side chain in the 17-α configuration.

In the above degradations for digitoxigenin, the starting material was acetyl digitoxigenin, and the resulting etio acid was shown to be 3β acetoxy-etianic acid, thus establishing the configuration of the C_3 hydroxyl group at the same time.

The configuration of the C_3 hydroxyl group in the cardiac aglycones appears to be invariably -β. For many years it was thought that apart from urezigenin, digoxigenin was the only cardiac aglycone to have the C_3α-hydroxyl configuration. Recently Reichstein (Pataki, Meyer, and Reichstein, 1953) has shown that digoxigenin also has the

3β-hydroxyl arrangement by degrading it to the 3,12-dihydroxyetianic acid methyl ester and identifying this compound with authentic 3β,12β dihydroxyetianic acid methyl ester.

If this 3β hydroxyl configuration is invariable in the cardiac aglycones, it is a comparatively simple matter to determine the stereochemistry of the A/B ring junction from the infrared spectra (see section on infrared spectra, p. 52 ; also the section on conformation of the steroid nucleus, p. 32).

Most cardiac aglycones have the A/B ring junction in the cis form, but there are a few compounds with the A/B trans ring junction. Uzarigenin (Fig. 12, XVII) has been known for a long time and its physiological inactivity was ascribed to the trans A/B ring structure. However, Stoll et al., (1949), isolated Corotoxigenin (Fig. 12 XVIII) and showed it to belong to the 21-hydroxy nor-allocholenic acid lactone series. [The glycoside of this genin, Gofruside, and Frugoside, a glycoside of the related genin Coroglaucigenin (Keller and Reichstein, 1949; Hunger and Reichstein, 1952) have been shown by Chen (loc. cit.) to have physiological activity comparable with most other cardiac glycosides.]

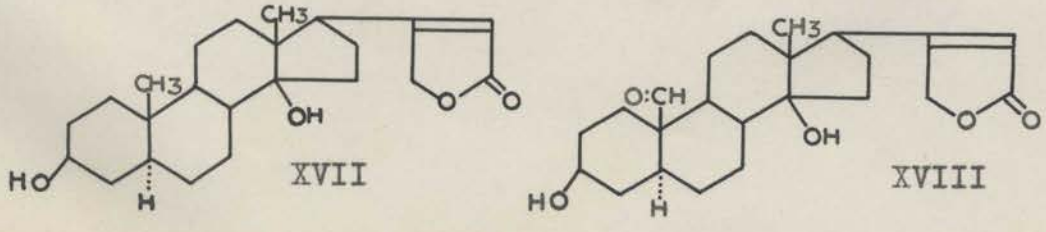
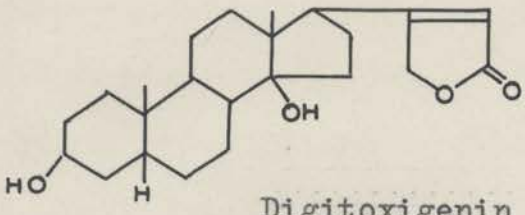
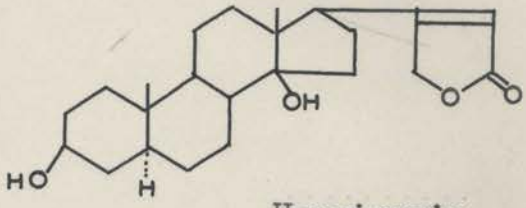


Figure 12

Other functional groups which are found in the cardiac aglycones are shown in the following table.



Digitoxigenin



Uzarigenin

Digoxigenin	= 12β-hydroxy-digitoxigenin
Gitoxigenin	= 16β-hydroxy-digitoxigenin
Strophanthidin	= 19-oxo-5-β-hydroxy-digitoxigenin
Strophanthidol	= 5β-19-hydroxy-digitoxigenin
Periplogenin	= 5β-hydroxy-digitoxigenin
Oleandrigenin	= 16β-acetoxy-digitoxigenin
Sarmentogenin	= 11α-hydroxy-digitoxigenin
Sarmutogenin	= 11α-hydroxy, 12-keto-digitoxigenin
Caudogenin	= 12α-hydroxy, 11-keto-digitoxigenin
Decogenin	= 11, 12 diketo-digitoxigenin
Ouabagenin	= 1, 5, 12, 19 tetra hydroxy-digitoxigenin
Corotoxigenin	= 19-oxo-uzarigenin
Coroglaucigenin	= 19-hydroxy-uzarigenin
Urezigenin	= 3-epi-uzarigenin

Recently Reichstein and co-workers (Hegedus, Tamm, and Reichstein, 1953, 1955; Buzas, von Euw and Reichetsin, 1950; see also Taylor, 1952, 1953) have isolated a number of compounds which are unique in the cardiac aglycones in that they have the α-ketol structure at positions C_{11} and C_{12}, viz., Saverogenin, Inertogenin, Leptogenin. The exact configuration of the hydroxyl groups at C_{11} or C_{12} in these compounds has been established but there is still one hydroxyl group as yet unplaced in the nucleus.

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CONFORMATION* OF THE STEROID NUCLEUS

Only in the past decade has it become firmly established by physical methods (e.g. Infrared and Raman spectra, electron diffraction, and thermodynamics) that the chair conformation of cyclohexane is more stable than the boat form, although the energy difference between these two forms is so low that it prevents the separation of the two isomers. By calculation of the energy differences between the various conformational isomers, it has been suggested (Barton, 1953 and references cited therein) that the most stable conformation of a fused cyclohexane system is that which has the maximum number of chairs.

In the chair conformation of cyclohexane two types of geometrically distinct carbon-hydrogen bonds can be distinguished. Those bonds which are perpendicular to the plane of the molecule are known as axial (a), as in Figure 13A. [Barton (1953) refers to these bonds as polar but the term 'axial' was introduced to prevent confusion of 'polar' with the meaning of 'polarity'.] Those bonds which lie approximately in the plane of the molecule are known as equatorial (e), as in Figure 13B.

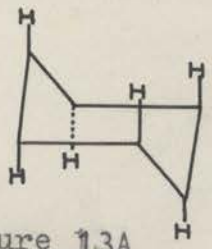

Figure 13A

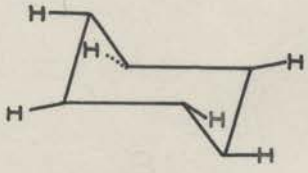

Figure 13B

* For the definition of "Conformation" see Barton, 1953, J. Chem. Soc., 1027, Footnote 1.

In the steroid nucleus (and cyclohexane rings generally), the following properties of the substituents have been noted:-

(a) an equatorial substituent is in general thermodynamically more stable than an axial substituent.

(b) ionic elimination reactions involving trans substituents on adjacent carbon atoms proceed more readily between axial bonded substituents, as the four centres involved in the reaction lie in one plane.

(c) a substituent linked to the nucleus by an equatorial bond is less sterically hindered than the same substituent linked to the same carbon atom by an axial bond, e.g. 11α-OH (e) is less hindered than the 11β-OH (a).

The energy differences involved in the different geometrical conformations are small (5-10 Kcals) compared with the activation energies of most chemical reactions. Consequently it is impossible to distinguish the separate conformations of the nucleus by the usual chemical methods employed to separate stereoisomers. However, as the thermodynamically most stable conformation of a fused cyclohexane ring system is that which contains the greatest number of chair forms (Barton, loc. cit.) the two series of steroids, A/B cis and A/B trans, are depicted below (Figs. 14 and 15) in which each cyclohexane ring is in the chair form.

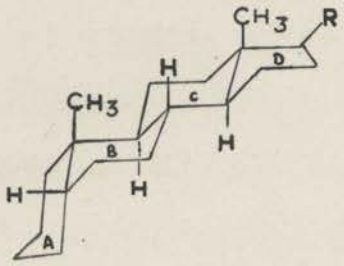

Figure 14

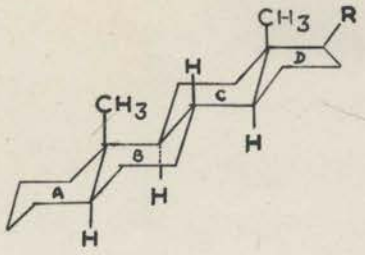

Figure 15

The cardiac glycosides are unique among the steroids in that they have a cis junction of the C and D rings and a β-hydroxyl group at C_{14}. This hydroxyl group is equatorial as the axial substituent is α. The conformations of the cis A/B and trans A/B series of cardiac genins are depicted below (Figs. 16 and 17).

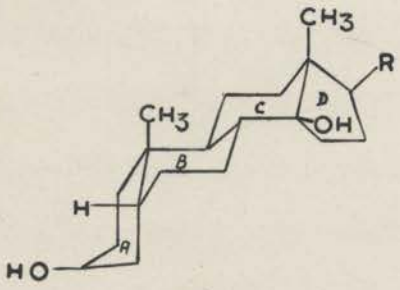

Figure 16

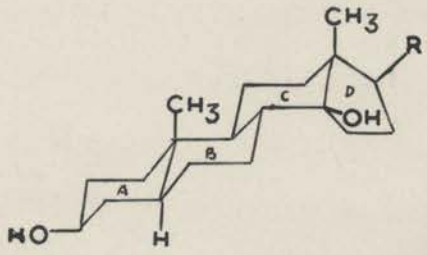

Figure 17

All the known naturally occurring cardiac genins, with the exception of urezigenin (Tschesche and Brathge, 1952) have a β-configuration of the C_3 hydroxyl group. In the

A/B cis series of steroids, the β-hydroxyl at C_3 is an axial substituent, whereas in the A/B trans series, the C_3 β-hydroxyl group is equatorial. As the majority of cardiac genins belongs to the A/B cis series, this means that the more usual arrangement of the C_3 hydroxyl group is axial. Urezigenin (3-epi uzarigenin) is unusual in that although it belongs to the trans A/B series, the C_3-hydroxyl group is axial.

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REACTIVITY OF FUNCTIONAL GROUPS

The reactivity of the functional groups in the steroid nucleus is largely determined by the degree of steric hindrance affecting the functional group and the nature of the reaction involved.

Steric hindrance by the angular methyl groups or the side chain, which are all β-orientated, would be expected to be more effective for β-orientated substituents as compared with the same α-orientated substituents, and the difference in reaction rates are frequently used to deduce configuration.

Configuration at C_3

The C_3-hydroxyl group in all the natural cardiac genins is β-orientated and is free from hindrance from the angular methyl group at C_{10} in both cis and trans A/B series. In those compounds which have a β-orientated hydroxyl group at C_5 (e.g. periplogenin) the reactivity of the C_3-hydroxyl group does not appear to be affected by the steric hindrance of the hydroxyl group at C_5. It may be noted here that the formation of an insoluble digitonide, by reaction of 3β-hydroxy steroids with digitonin is no longer accepted as proof of the 3β-configuration,as many exceptions to this rule are known, e.g. the cardiac genins, digitoxigenin, gitoxigenin, sarmentogenin, and strophanthidin with known 3β-hydroxyl groups are not precipitated by digitonin.

Configuration at C_5

The only cardiac genins which have a C_5-hydroxyl group belong to the A/B cis series in which case the hydroxyl group is β- and axial, with respect to ring A, e.g. strophanthidin and periplogenin. In both of these compounds the angular substituent at C_{10} (aldehyde group in strophanthidin and methyl group in periplogenin) appears to completely hinder the C_5-hydroxyl group. Consequently this hydroxyl group cannot be esterified.

Configuration at C_{14}

The 14β hydroxyl group of the cardiac genins is completely hindered by the C_{13} β angular methyl group and the C_{17} β lactone side chain. Consequently this hydroxyl, like that at C_5, is unreactive to esterification reactions. This hydroxyl group is involved in the irreversible isomerisation of these compounds to the iso-genins (see p.16).

The reaction involved in the elimination of the 14β-hydroxyl group in the formation of the anhydrocardiac genins appears to be of the type normally associated with bimolecular reactions (replacement with inversion and then trans elimination). Consequently the β-anhydrogenins are the only product of dehydration with phosphorus oxychloride and pyridine, the intermediate phosphorus ester undergoing trans elimination. Dehydration of the genins with mineral acids gives a mixture of the α- and β- anhydrogenins, although from stereochemical considerations the α-anhydro compounds would be expected to be formed preferentially.

Configuration at C_{11} and C_{12}

A number of cardiac genins have been isolated which contain hydroxyl groups at C_{11}, e.g. sarmentogenin, and C_{12}, e.g. digoxigenin, as well as a few which contain an α-ketol structure in these positions, e.g. intermedioside, inertoside, and leptoside (Hegedus, Tamm, and Reichstein, 1955). The 11β-hydroxyl group is particularly unreactive as it has the axial configuration and is greatly hindered by both angular methyl groups. This hydroxyl group can be esterified only under extreme conditions but will undergo ionic dehydration (11β-OH / 9α-H:trans) to $\Delta^{9.11}$ steroids with great ease. This hindrance of the 11β-hydroxyl group extends to 11-keto groups which resist Wolff-Kischner reduction but they can be reduced to 11β-hydroxy steroids by catalytic hydrogenation (Reichstein and von Euw, 1947) or by reduction with sodium borohydride (Fieser and Heymann, 1951), lithium aluminium hydride (Rosenkranz et al, 1952, a & b; Bernstein et al., 1953), or lithium borohydride (Tishler et al., 1951).

The 11α hydroxyl group (as in sarmentogenin) is equatorial and is much less hindered than the 11β hydroxyl group. This group is easily esterified and resists ionic elimination (11α-OH/9α-H: cis).

Compounds containing the equatorial 12-β hydroxyl group are readily esterified in keeping with the hypothesis that the equatorial are less hindered than the axial substituents.

The 12α-hydroxy steroids behave in a similar manner to the 12β-hydroxy compounds as, having the axial configuration, they are free from the hindering effects of the β-orientated angular substituents.

C_{11-12} α-Ketols

Leptogenin and inertogenin which have been shown to contain the 11-keto-12β hydroxyl and 11-keto-12α-hydroxyl-structures respectively, both form diacetates (3β- 12β-diacetyl leptogenin and 3β- 12α- diacetyl inertogenin) on acetylation under mild conditions (Hegedus, Tamm, and Reichstein, 1955). In leptogenin the 12β hydroxyl group is equatorial and is apparently not sufficiently hindered by the angular methyl group at C_{13} to prevent the formation of the corresponding acetate. Inertogenin has the 12α-axial configuration of the hydroxyl group, which is not sterically hindered to any extent by other substituent groups. Saverogenin, with the 11 α-hydroxy-12-keto structure also readily forms a diacetate (3β-11α-diacetyl saverogenin). The 11α-hydroxyl group is equatorial and is not sterically hindered. Sarmutogenin (Richter, Schindler, and Reichstein, 1954), which has a similar 11-12 ketol structure to saverogenin also forms the 3β-11α diacetyl sarmutogenin without difficulty.

Apparently the only other isomer of the 11-12 ketol structure, namely the 11β hydroxy-12 keto compound is not known but it can be predicted on the basis of the 11β-hydroxyl group being axial and by analogy with other 11β-

hydroxy compounds, that the hydroxyl group in this isomer would be very difficult to esterify.

Configuration at C_{16}

The C_{16} hydroxyl group of gitoxigenin has the β configuration (Moore, 1954). This group is readily acetylated, but the 16-β acetoxyl group is easily removed (e.g. by chromatography of the compound on alumina). Naturally occurring glycosides of periplogenin contain the 16β-acetyl group. This hydroxyl group is also involved in isomerisation reactions with the $\Delta^{\alpha\beta}$-γ- lactone ring of the cardiac genins.

Configuration at C_{19}

Those cardiac genins which have a β-orientated aldehyde group at C_{10} belong to either the A/B cis or A/B trans series. The aldehyde group of compounds belonging to the former series is reactive to the usual carbonyl reagents, e.g. strophanthidin forms an oxime (Jacobs and Collins, 1925), whereas in those genins containing this substituent in the A/B trans series the reaction with carbonyl reagents proceeds more slowly. [e.g., In an attempt to form an oxime of the glycoside gofruside, Reichstein (Hunger and Reichstein, 1952) isolated a compound which contained nitrogen but which could not be identified definitely as an oxime.] However, the primary alcohols corresponding to these aldehydes behave normally and can be esterified. The C_{19} aldehydes of the trans A/B series

genins undergo spontaneous oxidation to the corresponding acid (cf. Gofruside, Hunger and Reichstein, 1952; Christyside, Schindler and Reichstein, 1953), whereas those of the cis A/B series are stable, but can be easily oxidised to the corresponding acid.

PHYSICAL METHODS OF IDENTIFICATION & STRUCTURE DETERMINATION

ULTRA-VIOLET ABSORPTION SPECTRA

Ultra-violet absorption spectra, as an aid to the elucidation of the structure of the cardiac glycosides were first used by Elderfield and his co-workers (Paist, Blout, Uhle, and Elderfield, 1941), to support the chemical evidence that the side chain attached at C_{17} in the digitaloid glycosides and aglycones was a $\Delta^{\alpha\beta}$-butenolide and not the $\Delta^{\beta\gamma}$-butenolide proposed by Jacobs (Jacobs, Hoffmann, and Gustus, 1926). These digitaloid compounds have a strong absorption band at 216-218 mμ, $\log \varepsilon \doteqdot 4.2$, which is due to the $\Delta^{\alpha\beta}$-butenolide ring. The doubly unsaturated cyclopentenolide ring of the squill glycosides absorbs strongly at 300 mμ, $\log \varepsilon \doteqdot 3.7\text{-}3.8$. These chromophore systems are common to all the naturally occurring digitaloid and squill glycosides, respectively.

The strophanthus glycosides, and those derived from corotoxigenin (Fig. 18) have an aldehyde group at position C_{10} in the steroid nucleus. This group shows a typical carbonyl absorption band in the region 295-310 mμ, $\log \varepsilon \doteqdot 1.4\text{-}1.6$. By reduction of the aldehyde group to the corresponding primary alcohol, this peak is replaced by a

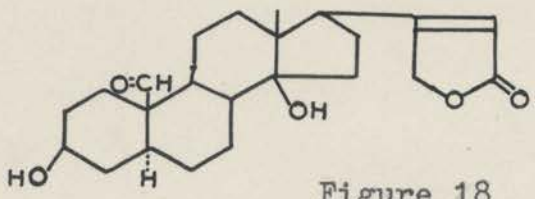


Figure 18

point of inflexion near 300 mμ. Corotoxigenin and its derivatives undergo autoxidation to the C_{19} acid (uzarigenic acid) (Stoll, Pereira, and Renz, 1949; Hunger and Reichstein, 1952), which also shows no absorption maximum in the 300 mμ region.

Compounds in which the chromophoric $\Delta^{\alpha\beta}\gamma$-lactone is further conjugated by the introduction of nuclear double bonds have the absorption maximum shifted to longer wave lengths. Removal of the C_{16} hydroxyl group in gitoxigenin derivatives produces 16-anhydro compounds of the type (Fig. 19). These compounds have the maximum at 270-280 mμ, log $\mathcal{E} \doteqdot 4.1 - 4.3$.

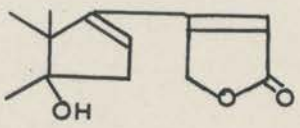


Figure 19

Further conjugation of the $\Delta^{\alpha\beta}$-γ-lactone, as in compounds of type (Fig. 20) (14,16-dianhydrogitoxigenin derivatives)

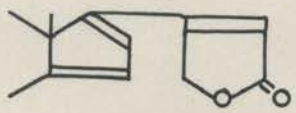


Figure 20

shifts the main absorption band to the 330 mμ region ($\log \varepsilon \doteqdot 4.2 - 4.3$).

The presence of a carbonyl group (as a keto group) in the nucleus or in the side chain can be detected by ultraviolet absorption in the 300 mμ region. Keto groups usually absorb at slightly shorter wave-lengths (275-290 mμ) than an aldehyde group (295-310 mμ) and the intensity of the keto band is similar to that of the aldehyde so that there is no definite division between these values. $\Delta^{\alpha\beta}$-unsaturated ketones have a characteristic absorption band at 230-260 mμ ($\log \varepsilon \doteqdot 4.0-4.20$), attributed to the conjugated system, and a second band of low intensity with a maximum at 315-320 mμ which has been attributed to carbonyl absorption.

Schindler and Reichstein (1954,a)in the investigation of the structure of caudogenin (Fig. 21) have shown that the C_{12} hydroxyl group has a marked effect on the position

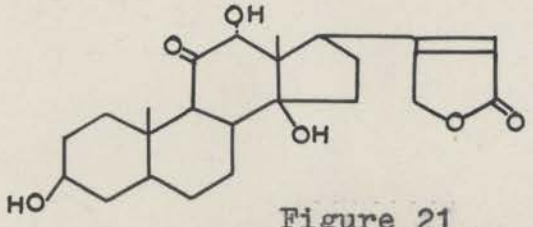


Figure 21

of the keto band. These workers as well as Baumgartner and Tamm (1955) have shown that the orientation of the hydroxyl group has an even greater effect on the position of the absorption maximum, than the relative positions of the

hydroxyl and keto groups. Using 3α-acetoxy-11-ketocholanic acid methyl ester (Fig. 22) and 3α-hydroxy-12-ketocholanic acid methyl ester (Fig. 23) as the reference compounds, they found that the introduction of the equatorial 11α or

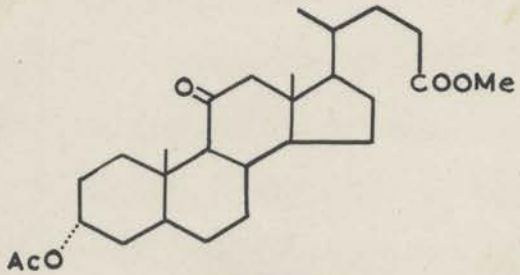


Figure 22

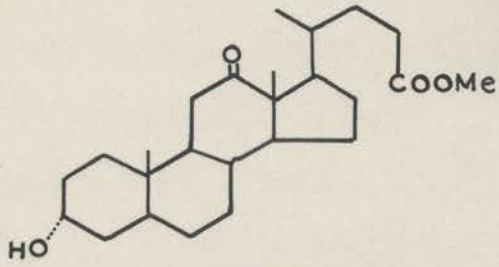


Figure 23

12β hydroxyl groups shifted the maximum by 11.5-12.5 mμ towards shorter wave length. The 11β- and 12α-axial hydroxyl groups shifted the maximum by 12-17mμ towards longer wave length. Acetylation of those compounds containing the axial hydroxyl (at 11β or 12α) shifts the maximum to shorter wave length (5-8 mμ), whereas acetylation of those compounds containing equatorial hydroxyl groups (at 11α or 12β) causes a shift of the maximum by 6-7 mμ towards longer wave length. However these compounds which have been compared do not contain the 14β-hydroxyl group which is present in all the normal cardiac glycosides and aglycones and hence must be taken into consideration when analogies are drawn between these synthetic compounds and the naturally occurring heart poisons.

Ultra-violet absorption data for steroids has been collected by Gillam and co-workers (1940, 1941, 1943, 1945) and Dorfman (1953), whilst some empirical rules for the prediction of absorption maxima have been deduced by Woodward (1941, 1942). The application of these rules has been illustrated by Fieser and Fieser (1949) in the correlation of the available data with structure.

It may be emphasised here that the ultra-violet absorption maxima of a compound alone is insufficient evidence on which to base conclusions regarding structure. However, ultra-violet spectra are most helpful in that they indicate possible structures which can then be confirmed by chemical reactions. This is particularly the case with compounds which have a carbonyl group in the molecule. Usually it is not possible to say whether the absorption in the 300 mμ region is due to an aldehyde or ketone group. However, these can be readily distinguished by oxidation reactions. On the other hand, comparison of spectra with those of known compounds, as in the case just quoted (Schindler and Reichstein, loc.cit.) of caudogenin and sarmentogenin, can be of great value.

INFRARED ABSORPTION SPECTRA

Absorption of ultra-violet light by unsaturated groups is attributed to the nature and ease of excitation of the orbital electrons involved in the unsaturated bond. The

absorption of light in the infrared region is due to the vibration of the atoms of the molecule in both longitudinal (stretching) and transverse (bending) modes.

In a complicated structure such as a steroid molecule, the longitudinal and transverse vibrations are associated with many hydrogen atoms, with methyl groups, and with any hydroxyl, carbonyl, or other groups present, as well as the vibrations due to the interaction of these groups. Consequently the infrared spectrum of a steroid is usually a highly complicated combination of absorption bands.

The infrared spectra of steroids, and cardiac glycosides in particular, consist of three main regions:

(a) <u>The region from 2.5 - 4 (4,000-2,500cm^{-1})</u>

This region contains absorption bands due to the O-H stretching frequency of the free hydroxyl group between 2.74 and 2.79 μ (3650-3584 cm^{-1}). The hydrogen bonded hydroxyl groups also absorb in this region, single bridge compounds absorbing between 2.82 and 2.90 μ (3546-3448 cm^{-1}). For those compounds in which polymeric association of the hydroxyl groups occurs, the absorption band is found between 2.90 and 3.125 μ (3448-3200 cm^{-1}). The position of the absorption band of the free hydroxyl group will vary with solvent and concentration. [The quoted wave lengths are for carbon disulphide solutions.] In more concentrated solutions, the position of the hydroxyl absorption band will shift towards longer wave length as the tendency increases for the

hydroxyl group to hydrogen bond with the hydroxyl of other molecules. In the extreme case for example, in nujol or crystal films, all the hydroxyl groups are hydrogen bonded and there is no band at 2.8 μ due to the free hydroxyl group. This fact has been used by Reichstein and co-workers (Simpson, Tait, et al., 1954) as an aid to the elucidation of the structure of aldosterone. These workers used as a model the spectrum of γ-hydroxy-valeraldehyde, which exists in the cyclic hemiacetal form, to demonstrate the increased intermolecular hydrogen bonding between the hydroxyl groups with increasing concentration. As the spectrum of aldosterone behaved in a similar manner, they were able to deduce that there was a similar structure in aldosterone, namely strong intra-molecular hydrogen bonding apart from the normal inter-molecular association.

It is possible, by using quantitative measurements, to determine the number of free hydroxyl groups in the compound, but no positional effects have been discerned.

In saturated steroids, a strong absorption band occurs in this region at 3.37 μ (2967 cm^{-1}) due to the C-H stretching vibration. In β-anhydro cardiac aglycones ($\Delta^{14.15}$), the C_{15}, C-H vibration gives rise to a well defined band near 3.27 μ (3055 cm^{-1}) (Bladon et al., 1951). [Fieser and Fieser (1949) state that this band is seen at 3.312 μ (3020 cm^{-1}) as a shoulder (or inflexion) at the base of the main C-H stretching band. However, as this band is frequently seen

in the spectra of normal cardiac glycosides and aglycones, as well as in the corresponding anhydrous compounds it is unreliable as an indication of the presence of the Δ^{14-15} double bond.]

This absorption band (or its absence) may be used as an aid in distinguishing between α- and β- anhydroaglycones. The α-anhydroaglycones ($\Delta^{8.14}$) contain a tetra-substituted double bond which shows no absorption bands in the 3.3 μ (3030 cm^{-1}), 6.0 μ (1667 cm^{-1}), and 12 μ (833 cm^{-1}) regions of the infrared spectrum (Cardwell and Smith, 1954; Bladon et al., 1951; Cardwell, 1953). Transparency of the anhydrogenin in the 12 μ (833 cm^{-1}) region is confirmation of the presence of a tetra-substituted double bond.

(b) <u>The region 5-7 μ (2,000-1,429 cm^{-1})</u>

This region is important as most of the bands of diagnostic value occur between these wave lengths. Correlation of absorption maxima occurring in this region with structure, has been extensively investigated by Jones and co-workers (Jones and Dobriner, 1949; Jones and Herling, 1954). The $\Delta^{\alpha\beta}$-γ-lactone side chain of the cardiac glycosides gives rise to two bands at 5.69-5.78 μ (1757-1730 cm^{-1}) and 6.12-6.15 μ (1635-1625 cm^{-1}) due to the stretching frequency of the C=O and the C=C respectively. Saturation of the butenolide shifts the absorption maxima of the carbonyl C=O stretching frequency to 5.62-5.60 μ (1780-1786 cm^{-1}). A saturated

6 membered lactone ring, e.g. uzarigenic acid -(19)- 19→3- lactone has an absorption maximum at 5.8 μ (1725 cm^{-1}) (Fig. 24 A) which cannot be differentiated from the band due to the butenolide. However, saturation of the butenolide shifts its absorption maximum to shorter wave length 5.65 μ (1770 cm^{-1}) and allows resolution of these two bands (cf. Hunger and Reichstein, 1952) (Fig. 24 B).

Carbonyl groups in the nucleus give rise to bands between 5.7 and 5.9 μ (1754-1695 cm^{-1}) which show some positional specificity, e.g. 3-C=O $\lambda^{CS_2}_{max.}$ 5.82 μ (1718 cm^{-1}), but a keto group at C_{11}, $\lambda^{CS_2}_{max.}$ 5.84 μ (1712 cm^{-1}), cannot be distinguished in the presence of other keto carbonyls. Diketones in which the two keto groups are sufficiently separated, e.g. 3 : 11, 3 : 12, $(CO)_2$ show the maxima of the two individual carbonyl groups, but in compounds which contain α- or β- diketones, vibrational interaction occurs with overlapping and displacement of the absorption bands.

The carbonyl group of the acetoxy group (at C_3) absorbs at 5.74μ (1742 cm^{-1}) which in the case of the cardiac glycosides, usually overlaps with the C=O stretching band of the butenolide. The acetyl carbonyl group however, gives rise to a strong band at 8.08 μ (1238 cm^{-1}) due to the C-O stretching vibration and can therefore be detected in the cardiac genin acetates.

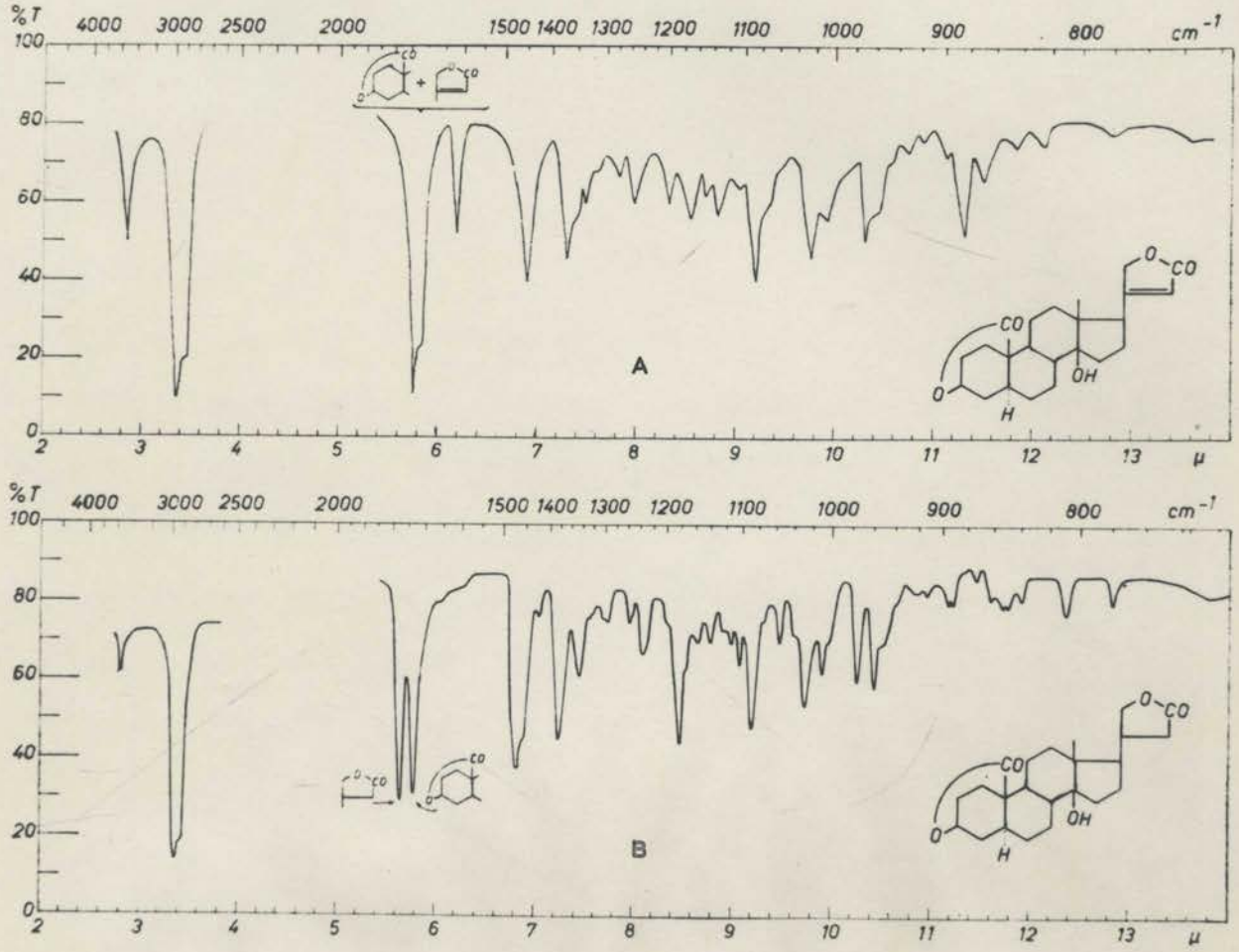


Figure 24

Infrared absorption spectra in nujol. NaCl prism.

A = Uzarigenic acid -(19)- 19→3 lactone.

B = Dihydrouzarigenic acid (19), -19→3 lactone.

(Reproduced from Helv.Chim.Acta, 1952, 35, 1080)

The carbonyl group at C_{19} absorbs at a wave length so close to that of the butenolide carbonyl group that differentiation depends on the solvent and the resolving power of the instrument.

(c) The region 8-12 μ (1250-800 cm^{-1})

This region contains a large number of complex absorption bands of varying intensity and is usually referred to as the 'finger-print' region. This series of bands is unique for every individual compound and so provides a sure means of identification.

However, there are a number of strong absorption bands in this region which have been studied by various workers (Jones, Humphries, Herling, and Dobriner, 1951; Cole, Jones, and Dobriner, 1952; Furst, Kuhn, Scotoni, and Gunthard, 1952; Cole, 1952). They have established that the band at 10 μ (1,000 cm^{-1}) (due to C-O- stretching vibrations) is dependent on whether the hydroxyl group at C_3 is equatorial or axial. Also, for C_3 acetates the complexity of the acetoxy band at 8.64 μ (1240 cm^{-1}) is dependent on the equatorial or axial nature of the acetoxy group. For example, in the trans A/B (allo) series of steroids, if the hydroxyl group at C_3 is β-equatorial), the absorption maximum is at 9.62 μ (1040 cm^{-1}); the α (axial) hydroxyl group has the absorption maximum at 10 μ (1000 cm^{-1}). In the cis A/B (normal) series of steroids, the C_3β-OH (axial)

absorbs at 9.65 μ (1036 cm^{-1}) and the $C_3\alpha$-OH (equatorial) has the absorption maximum at 9.58 μ (1044 cm^{-1}). The positions of the absorption maximum of the $C_3\beta$-OH groups of the cis A/B and trans A/B series differ by only 4 cm^{-1} (1036 cm^{-1} and 1040 cm^{-1} respectively). This small difference in frequency could be resolved, and the results would be significant, provided the spectra were determined on the same instrument and in the same solvent.

In the trans and cis A/B series, the C_3 acetoxy compounds are distinguished by the complexity of the absorption band at 8.07 μ (1240 cm^{-1}). Cholestan-3 β-ol-acetate (equatorial) has a simple absorption band, whereas cholestan-3 α-ol-acetate (axial) has a complex band at this wave length. Coprostan-3 β-ol-acetate (axial) has a complex band and coprostan-3 α-ol-acetate (equatorial) has a simple absorption band at 8.07 μ (1240 cm^{-1}). The complexity of the acetoxyl group absorption band is interpreted as enhanced steric hindrance to freedom of rotation in the axial type acetates. The nature of the acetate absorption band is of greater value in determining the conformation of the substituent at C_3, than the position of the hydroxyl absorption. These results are summarised in Table 2.

TABLE 2

		OH absorption band $\lambda_{max.}^{CS_2}$ cm^{-1}	Nature of -OAc band at 1240 cm^{-1}
Trans A/B	3β (e)	1040	simple
	3α (a)	1000	complex
Cis A/B	3β (a)	1036	complex
	3α (e)	1044	simple

These effects have been observed in carbon disulphide solution, but in paraffin mulls the effects may be reversed, e.g. a 3β-acetoxycholane derivative in paraffin may show a simple band in the 8 μ region, but a complex band due to a 3α-acetoxycholane derivative in paraffin has not yet been observed (Schindler and Reichstein, 1952). Consequently, simple bands observed in paraffin mulls in this region may not be reliable, but complex bands would probably be significant.

In the case of the cardiac aglycones, in which the C_3 hydroxyl group is invariably β-orientated, it is therefore possible to make significant deductions regarding the stereochemistry of the A and B rings from the position of, and nature of the absorption bands due to the C_3 hydroxyl and the C_3 acetoxyl groups. To avoid ambiguity these spectra should be determined in carbon disulphide solution.

The cardiac aglycones are usually insoluble in the solvents used for the determination of infrared spectra in this region (CS_2, CCl_4) so that most of the available data is for spectra determined in paraffin mulls. Consequently it is extremely difficult to obtain sufficient data for comparison with spectra of new cardiac aglycones.

The angular methyl groups at C_{18} and C_{19} in the steroid nucleus have absorption bands at 7.25 μ (1378 cm^{-1}) and 7.22 μ (1384 cm^{-1}) (Jones and Cole, 1952) respectively. For the detection of these bands it is necessary to use carbon tetrachloride or carbon disulphide as the solvent, as chloroform will absorb in the 7.22 μ (1384 cm^{-1}) region. These bands are helpful in determining the structure of those compounds which contain groups other than methyl (e.g. -CHO, $-CH_2OH$, -COOH and 19-lactones or lactols) at C_{19} or C_{18}. Thus it may be seen that the infrared spectrum of a compound may yield valuable information regarding its structure.

MOLECULAR ROTATION

As the steroid nucleus contains many asymetric centres, the stereochemistry is most complicated, and this becomes even more abstruse when the nucleus is substituted. Thus when reactions are performed on steroids, or when new compounds are prepared, a large number of isomers are formed, and although they could be predicted on a theoretical basis, the actual identification of any single isomer is difficult.

Formerly chemical methods were the only means of identifying these isomers, but in many cases the ambiguous nature of the results made specific determination of the structure very difficult. In recent years a great deal of physical data has been obtained from measurements on compounds of proven chemical structure. Consequently, greater use is being made of physical measurements to detect differences between the various isomers. Many physical methods have been applied in this field, but of these, two appear to be the most reliable, namely infrared spectroscopy, which has been dealt with in the previous section (p. 46), and molecular rotational differences which will be dealt with here.

Correlation of steroid structure and optical rotation was initiated by Callow and Young (1936 A) and extended by Lettre (1937), and Wallis et al., (1941). Later Barton perfected a method for the analysis of molecular rotational differences (Barton et al., 1945, 1946, 1948, 1949; Barton and Klyne, 1948. 1949).

The contributions of certain functional groups to steroid molecular rotation ($[M]_D = [\alpha]_D$ x mol. wt. x 10^{-2}) are characteristic of the positions occupied. These rotational contributions of the groups are independent of one another only if highly unsaturated groups (e.g. αβ-unsaturated ketones,lactones etc.) are absent, and if other functional groups in the molecule are separated from the group in question by a certain number of carbon atoms. If highly unsaturated groups are present, or if

the functional groups are close together they may exert 'vicinal' action upon one-another, thus influencing their rotational contributions. Vicinal action between hydroxyl and keto groups does not occur if they are separated by at least three saturated carbon atoms and this is usually true also for olefinic double bonds and hydroxyl groups. For larger substituent groups such as acetoxyl or benzoxyl, vicinal action between these groups and hydroxyl, keto, or olefinic double bonds is negligible only if they are separated by at least five saturated carbon atoms. Keto and olefinic groups also require five carbon atoms separating them to remove the effects of mutual interference. Thus the possibility of vicinal action occurring in any given compound must be taken into consideration when using this method of analysis.

Molecular rotational contributions of particular functional groups (Δ values) are defined as:

$[M]_D$ of the compound containing the substituent minus the $[M]_D$ of the corresponding compound containing a saturated carbon atom in the position considered. e.g. $[M]_D$ stenol - $[M]_D$ stanol = the molecular rotational contribution of the olefinic bond.

The molecular rotational contributions are dependent on the position and nature of the substituent in the ring system. For substituents in or near rings A and B, the

Δ values are determined by the configuration at C_5, but the Δ values of substituents in rings C and D are independent of the C_5 configuration. However the Δ values of the substituents in rings C and D are dependent on the configuration at C_{14} and C_{17}.

In order to indicate the value of this method as an aid to the determination of molecular structure its use may be divided into the following sections:-

(a) To determine the nature of the A/B ring junction. Evidence obtained by molecular rotational differences combined with infrared spectral data should establish the configuration at C_5 (see p. 52).

(b) To determine the presence and position of olefinic bonds in the nucleus. This measurement may be used to distinguish between α- and β-anhydro cardiac genins.

(c) To determine the orientation of nuclear substituents (cf. Baumgartner and Tamm, 1955).

(d) To show the position and orientation of lactone rings attached to, and within the nucleus.

(e) To give information regarding the nature of the carbohydrate unit in glycosides.

Molecular rotational contributions form important evidence of homogeneity and molecular type, and are most useful in making predictions regarding molecular structure. However these predictions must be supported by chemical evidence.

The $[M]_D$ of a compound varies with the solvent to the extent of approximately 15° (dioxane), 30°(acetone or ethanol) greater than the value in chloroform. Differences in temperature from 15° - 35°C and in concentration from 0.5 - 5% make no significant differences to $[M]_D$ values. The usual permissable error in $[\alpha]_D$ is ±2°-3° giving an error in $[M]_D$ of ±10°. The sodium D line is usually employed as the light source. Observations with light of different wave length, e.g. the mercury green line at 5461A, cannot be compared with those made with sodium vapour light.

Although the optical rotations of numerous steroids and their derivatives are known, it is not always possible to apply this method of structural analysis. This is due to lack of sufficient reference data, particularly in the case of the cardiac glycosides and aglycones. The compounds isolated in this investigation could not be analysed by this method as they appear to have structures which are not analagous to known compounds.

CARBOHYDRATE CONSTITUENTS

Hydrolysis of the glycosides yields one or more carbohydrate molecules and an aglycone (or genin) or a secondary product of dehydration. A number of glycosides may have the same aglycone; thus digoxin, acetyl digoxin, and digilanid C all have the same genin, digoxigenin and all three glycosides can be detected in the plant. The enzymes which occur in the plants are able to hydrolyse glucose from a desoxysugar, but quantitatively they show a certain specificity towards the substrate with which they exist in the plant. For example, digilanidase - the enzyme obtained from Digitalis lanata - splits off glucose more readily from the digilanids than does digipurpidase - the enzyme obtained from Digitalis purpurea.

Probably the best example of the step-wise degradation of a genuine glycoside is provided by k-strophanthoside, the primary glycoside obtained from Strophanthus kombe seeds. k-Strophanthoside is a trioside.

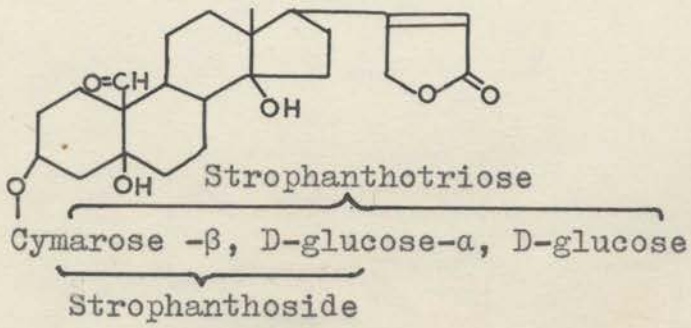


Figure 25

Stoll and co-workers (Stoll, Renz, and Kreis, 1937) were able to show that the three glycosides k-strophanthoside, k-strophanthin-β, and cymarin, were related to one another and have the common aglycone, strophanthidin. Thus, α-glucosidase, from yeast - which cleaves only α-glucosidic linkages - hydrolyses k-strophanthoside to k-strophanthin-β and one molecule of glucose. Strophanthobiase, obtained from the seeds of Strophanthus courmonti (Jacobs and Hoffman, 1926,a) cleaves the β-glycosidic link in k-strophanthin-β, producing cymarin (strophanthidin + cymarose) and glucose. The linkage between the aglycone and the sugar chain is broken by acid. Hydrolysis of k-strophanthoside yields strophanthotriose, k-strophanthin-β yields strophanthobiose, and cymarin yields cymarose. These reactions are summarised in Figure 25.

It was found in these investigations by Stoll, that the well known α-glucosidase of yeast was capable of splitting the linkage between the two glucose residues in k-strophanthoside, so that the genuine glycoside could be converted to k-strophanthin-β, by means of an enzyme from a foreign source. This showed that the glucosidic linkage between the terminal sugar residues was α. Whilst the sugar residue remains attached to the aglycone the linkage between cymarose and glucose can be broken only by the use of the specific enzyme strophanthobiase. However, strophanthobiose (cymarose + β-glucose can be hydrolysed to cymarose and glucose by the well

known β-gluscosidase, emulsin. Consequently this linkage between cymarose and glucose must be of the β-glucosidase type. Also, strophanthobiase must belong to the β-glucosidase class of enzymes. Stoll and Renz (1939), have shown that the other well known specific enzymes, digilanidase, digipurpidase, and scillarenase belong to the β-glucosidases. There are many enzymes known which will split off 2-glucose molecules from di- and tri- glycosides. The full summary is given by Stoll and Renz (1939). Hunger and Reichstein (1950) have used the enzyme from the seeds of Adenium multiflorum, and Frerejaque (1947, 1948) has used the hepatopancreatic juice of the snail. Further literature on the use of enzymes is given by Huber et al (1951).

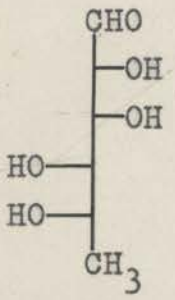
The hydrolysis of cardiac glycosides by treatment with acid does not always lead to the production of the aglycone; frequently the product is the corresponding α- or β-anhydrogenin. This effect was first explained by Jacobs and Bigelow (1932) after careful examination of the behaviour of a number of glycosides towards acids. They postulated that only those glycosides in which the genin (aglycone) is linked directly to a 2-desoxy sugar are easily hydrolysed. These conditions are fulfilled, for example in the case of digoxin, where the carbohydrate linked to the aglycone is the 2,6-desoxyhexose, digitoxose (configuration established by Micheel, 1930; synthesised by Gut and Prins, 1947), but not in the case of

digitalinum verum, in which the sugar linked to the genin is digitalose, the 3-methyl-ether of a methyl pentose (Schmidt, Mayer, and Distelmaier, 1943; Synthesis - Schmidt and Wernicke, 1947). Thus when a 2-hydroxysugar (e.g. rhamnose, glucose, digitalose) is linked directly to the aglycone, fission of the glycosidic link can be achieved only under such drastic conditions that the aglycone is simultaneously dehydrated. Voss (1936), found that with model glycosides, alcoholysis sometimes proceeds as much as one hundred times as fast as hydrolysis, and used this technique for splitting the glycosides under less drastic conditions. Thus, by allowing convallamarin (a glucoside-dirhamnoside) to stand at 35°C for several days in 2% methanolic hydrogen chloride, convallamaretin which has the tertiary C_{14}-OH group intact was obtained. Another method which has been used frequently by Reichstein is that of Mannich and Siewert (1942), whereby the glycoside is cleaved by means of hydrogen chloride in acetone. Glycosides which are slightly soluble in acetone go into solution as the monoacetonide. After standing one or two weeks, the aglycone is obtained. In this way Mannich (loc. cit.) was able to obtain the true genin of ouabain. Antiarigenin has also been obtained by this method. Glycosides which are completely insoluble in acetone are not hydrolysed by this method, e.g. Hellebrin.

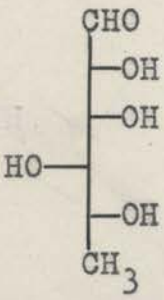
Reichstein has treated hydrolysis resistant glycosides with alkali to form the iso-glycosides which are then hydrolysed to the iso-aglycones without dehydration at C_{14} (Reichstein et al., 1949). With the exception of D-glucose and L-rhamnose, the sugars obtained by the hydrolysis of the cardiac glycosides have not been found elsewhere in nature. The structures of the sugars are shown in Table 3.

TABLE 3

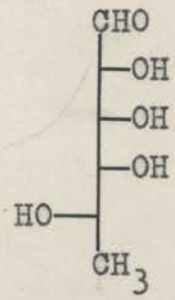
Normal Sugars (-OH in C_2)



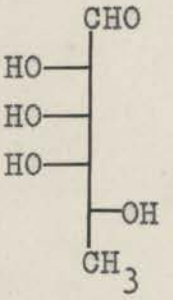
L-Rhamnose



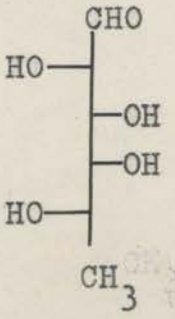
Antiarose = D-Gulo-methylose (i)



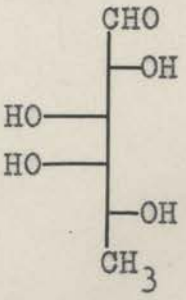
L-Talomethylose (ii)



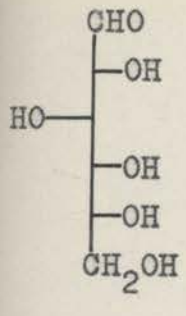
D-Talomethylose



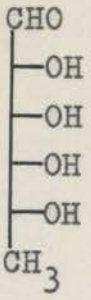
L-Fucose



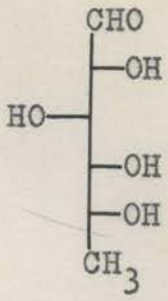
D-Fucose



D-Glucose

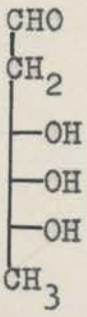


D-allomethylose (iii)

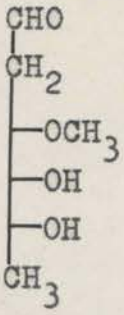


D-Quinovose

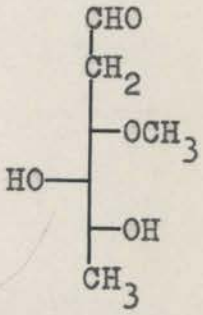
2,6-Desoxy Sugars and Methyl Ethers



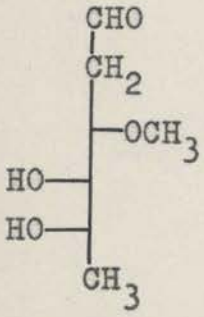
D-Digitoxose (iv)



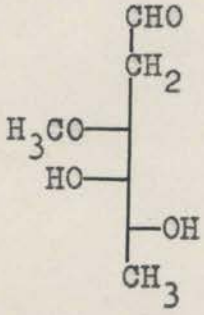
D-Cymarose (v)



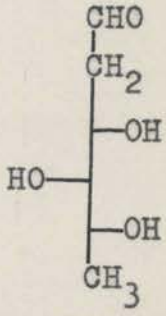
D-Sarmentose (vi)



L-Oleandrose (vii)

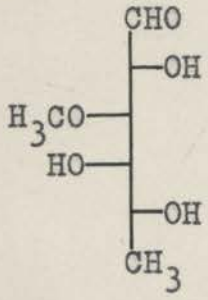


D-Diginose (viii)

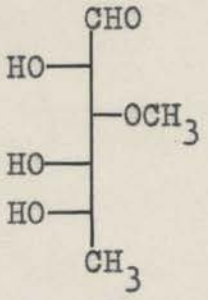


D-Boivinose (ix)

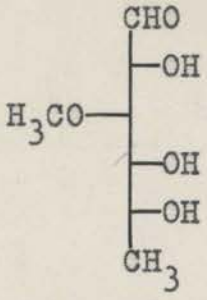
Methyl Ethers of 6-desoxy sugars



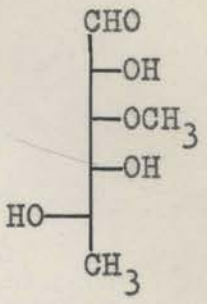
D-Digitalose (x)



L-Thevetose (xi)



D-Thevetose (xi)



Acovenose (xii)

(i) Antiarose: Reichstein et al., Helv.Chim.Acta, 31, 1948, 688

(ii) L-Talomethylose: Schmutz, Ibid, 1948, 31, 1719.

(iii) D-Allomethylose: Reichstein et al., Helv. Chim. Acta., 1952, 35, 1074.

(iv) D-Digitoxose: Micheel, Ber., 1930, 63, 347.

(v) D-Cymarose: Prins, Helv. Chim. Acta., 1946, 29, 378

(vi) D-Sarmentose: Reichstein et al., Ibid, 1949, 32, 446.

(vii) D.Boivinose: Reichstein et al., Ibid, 1952, 35, 730
1953, 36, 302

(viii) L-Oleandrose: Blindenbacher and Reichstein, Ibid, 1948, 31, 2061.

(ix) D-Diginose: Shoppee and Reichstein, Ibid, 1942, 25, 1611.

(Synthesis) Tamm and Reichstein, Ibid, 1948, 31, 1630.

(x) D-Digitalose: Reher and Reichstein, Ibid, 1946, 29, 343.

Reichstein and Tamm, Ibid, 1949, 32, 163.

(xi) L-Thevetose = cerberose: Frerejaque and Hasenfratz, Compt. rend., 1946, 222, 815.

= L-Glucomethylose: Blindenbacher and Reichstein, Helv.Chim.Acta, 1948, 31, 1669.

(xii) Acovenose: Tamm and Reichstein, Ibid, 1951, 34, 1224.

It is noteworthy that all the methoxy sugars have the methoxy group in the C_3 position. The 2-desoxy sugars have characteristic high reactivity and give the Keller-Kiliani reaction [formation of a blue ring at the interface of a solution in acetic acid containing Fe^{+++} ions, with sulphuric acid (H. Kiliani, 1896, 1913)].

PART III

THE CARDIAC GLYCOSIDES OF GOMPHOCARPUS FRUTICOSUS (R.Br.)

A mixture of impure glycosides was obtained from the dried whole plant by continuous extraction, firstly with light petroleum and then with a mixture of chloroform - methanol (1:1). The chloroform - methanol extract, after evaporation and dilution with water, was shaken with carbon tetrachloride to remove pigments. The aqueous alcoholic extract was then evaporated to dryness, and redissolved in methanol. The hot methanol solution was diluted with water until slight precipitation was obvious, and then slowly evaporated. The mixture of glycosides crystallised from this solution (yield 0.084%).

In order to compare this extraction method with the usual process, which involves the precipitation of tannins and pigments by lead hydroxide or basic lead acetate, a second extraction was carried out on another quantity of plant material from the same sample. In this experiment the plant was defatted with petroleum ether and then extracted with 80% ethanol - water. The aqueous alcoholic extract after evaporation and dilution with water, was treated with basic lead acetate, filtered, and extracted with chloroform. The yield of mixed glycosides from this extraction was 0.074%. It was found that the lead precipitate contained a small amount of the cardiac glycosides.

As the first method of extraction did not involve this loss of material (which can be recovered by dissolving the

lead precipitate in dilute sulphuric acid and re-extracting with chloroform), it was used for the three subsequent extractions.

Paper chromatography of the mixed glycosides by the methods of Svensen and Jensen (1950), and Silberman and Thorp (1953), would not separate the component glycosides, but by using formamide impregnated paper and 10% benzene in chloroform (Schindler and Reichstein, 1951, c) as the mobile phase, it was possible to separate the mixture into three components (Fig. 26 A,p.70). A similar result was obtained by using a mixture of chloroform - benzene - n.butanol (78:12:5) on formamide impregnated paper. In this case, the slow running spots had been moved further along the paper, but the positions of the two fast running constituents had not altered greatly (Fig. 26, B, p.70).

The component glycosides of this mixture were separated by chromatography on neutral alumina. The glycosides were applied to the column in a mixture of chloroform - benzene (1:1) and eluted by varying the polarity of the eluting solvent. (Fig. 27, p.71; see also p.142). The material obtained from fractions 5 - 10 has been named Gomphoside and that obtained from fractions 16-23, Afroside. Paper chromatography, using the formamide systems already referred to showed gomphoside to be a pure substance, but afroside consists of two components (Fig. 28, p.72).

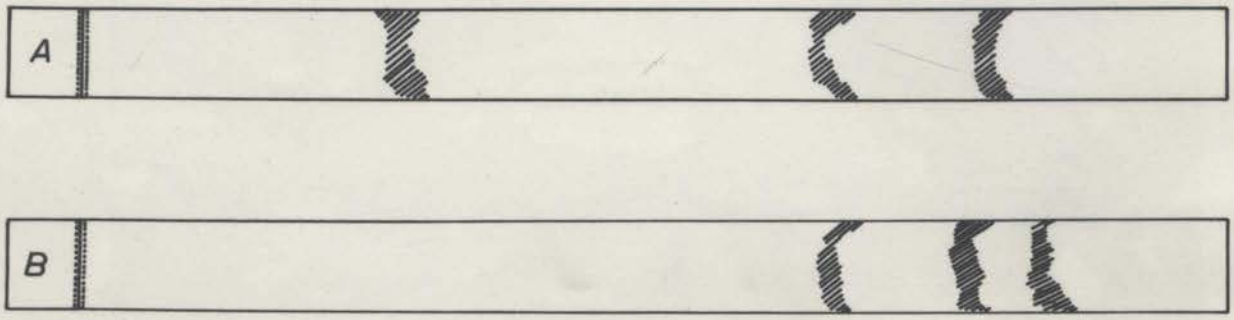


Figure 26

Chromatograms on formamide impregnated paper of the glycosides of Gomphocarpus fruticosus.

A = Developing solvent $CHCl_3$: C_6H_6, 9:1

B = Developing solvent $CHCl_3$: C_6H_6 : BuOH, 78:12:5

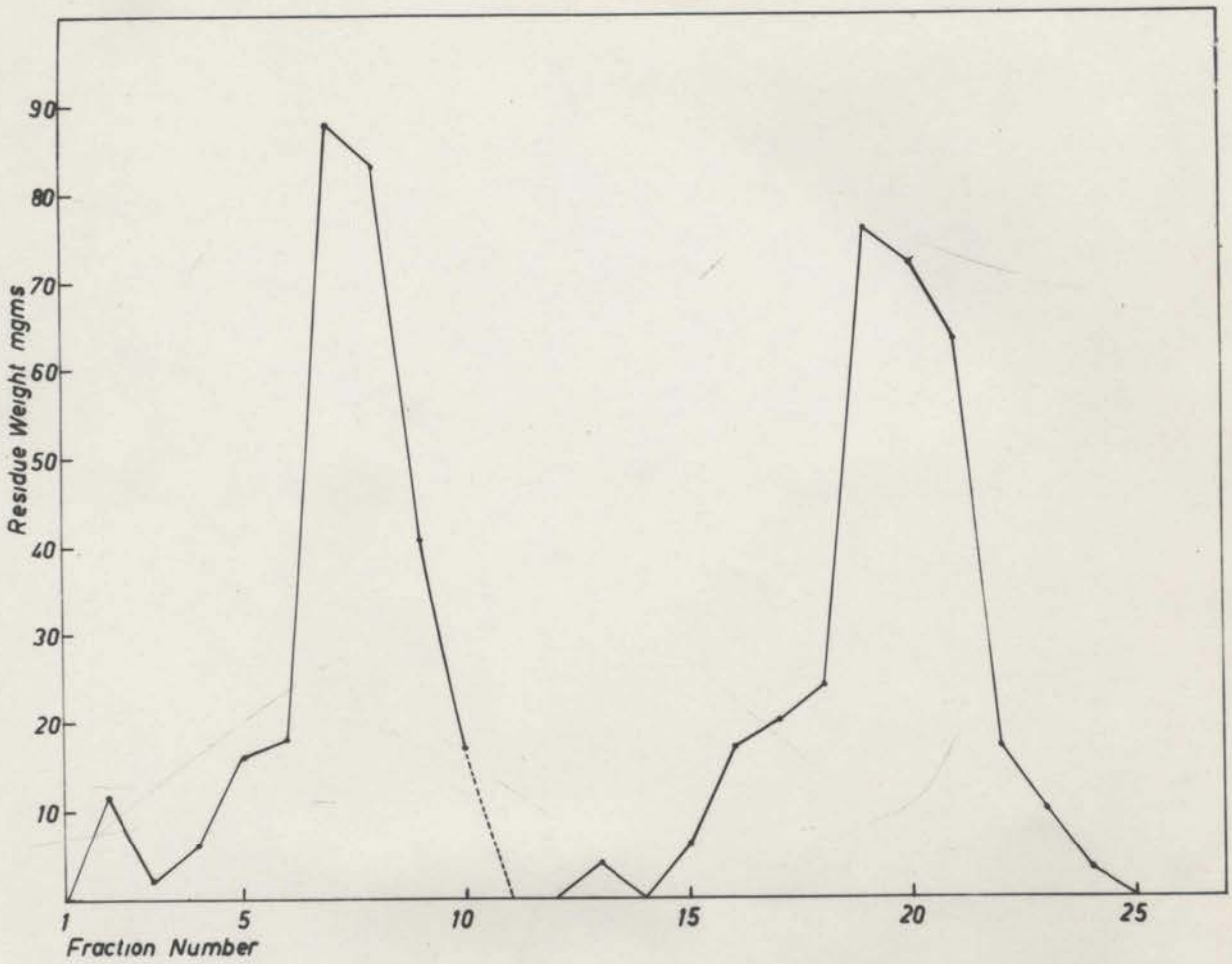


Figure 27

Separation of Gomphoside and Afroside on Alumina.

(cf. p.142)

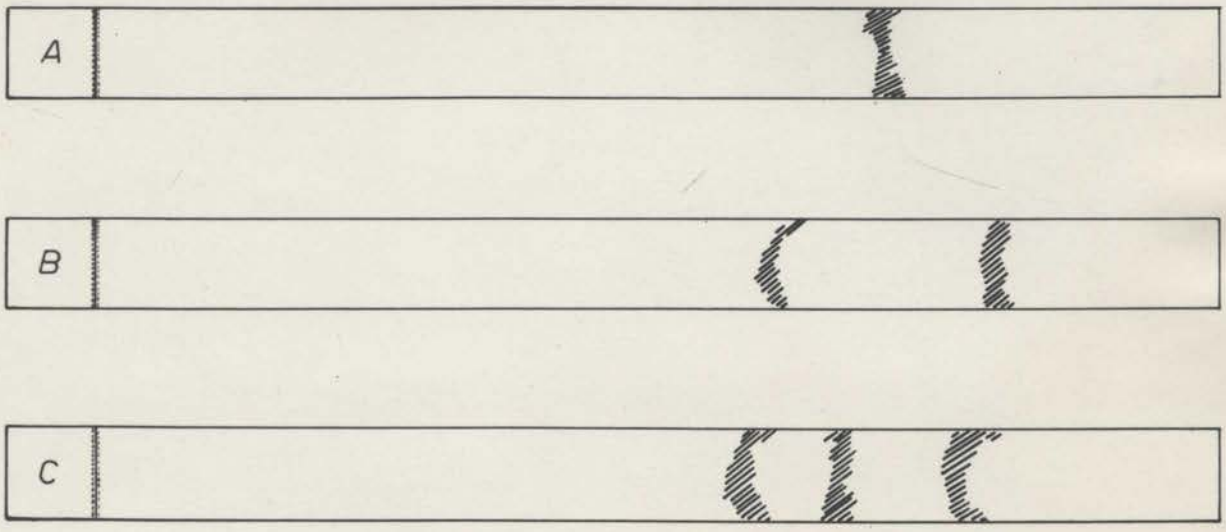


Figure 28

Chromatograms on formamide impregnated paper.

Developing solvent - $CHCl_3$: C_6H_6 : BuOH, 78:12:5

A = Gomphoside

B = Afroside

C = A + B

GOMPHOSIDE

The yield of gomphoside, separated on the alumina column, was 7.3% of the total quantity of mixed glycosides applied to the column. This yield of gomphoside varied from approximately 2 - 10% of the total glycosides obtained from the various plant samples which were extracted. The total quantity of gomphoside obtained in this investigation was 1.243 g.

Gomphoside, m.p. 234-242°C., analyses for a formula $C_{29}H_{44}O_8$.[This compound was reported as having the formula $C_{35}H_{52}O_{10}$ but further analyses of gomphoside and acetyl gomphoside have favoured the formula $C_{29}H_{44}O_8$ (Watson and Wright, 1953)] It gives positive Legal and Raymond reactions (p.134) and carbohydrate test (p.143), but a negative Keller-Kiliani reaction (p.135). It has the typical ultra-violet absorption maximum of the butenolide ring at 218 mμ (log ε = 4.22) (Fig. 29, p.74).

Gomphoside contains neither methoxyl nor acetyl groups. Acetylation with acetic anhydride in pyridine gives a diacetate, m.p. 252-255°C., which analyses for a formula $C_{33}H_{48}O_{10}$. When this acetate was treated with chromium trioxide in acetic acid, oxidation occurred, but the only substance which was obtained from the reaction was 12% of unchanged acetyl-gomphoside. No oxidation products could be isolated.

The infrared spectra of gomphoside and acetyl gomphoside, are shown in Fig. 30 (curves A, & B). In these spectra, the C = O and C = C stretching absorption bands of the butenolide ring occur at 5.73,6.12 μ (1745,1634 cm^{-1}) and 5.75,6.15 μ (1740, 1626 cm^{-1}) respectively.

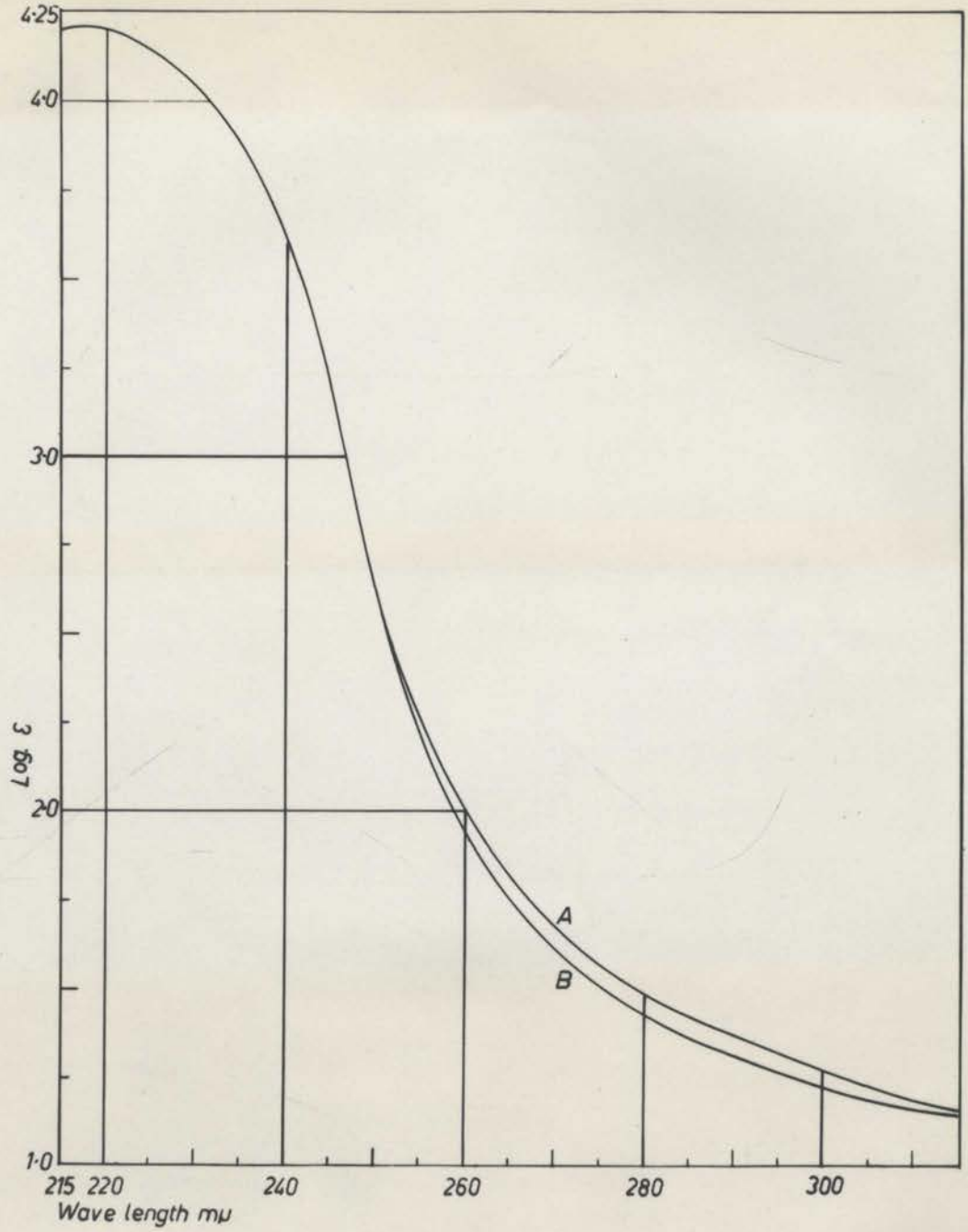


Figure 29

Ultra-violet absorption spectra

A = Gomphoside ($C_{29}H_{44}O_8$) λ max. = 218 mμ, log ε = 4.22

B = Gomphogenin ($C_{23}H_{34}O_5$) λ max. = 218 mμ, log ε = 4.22

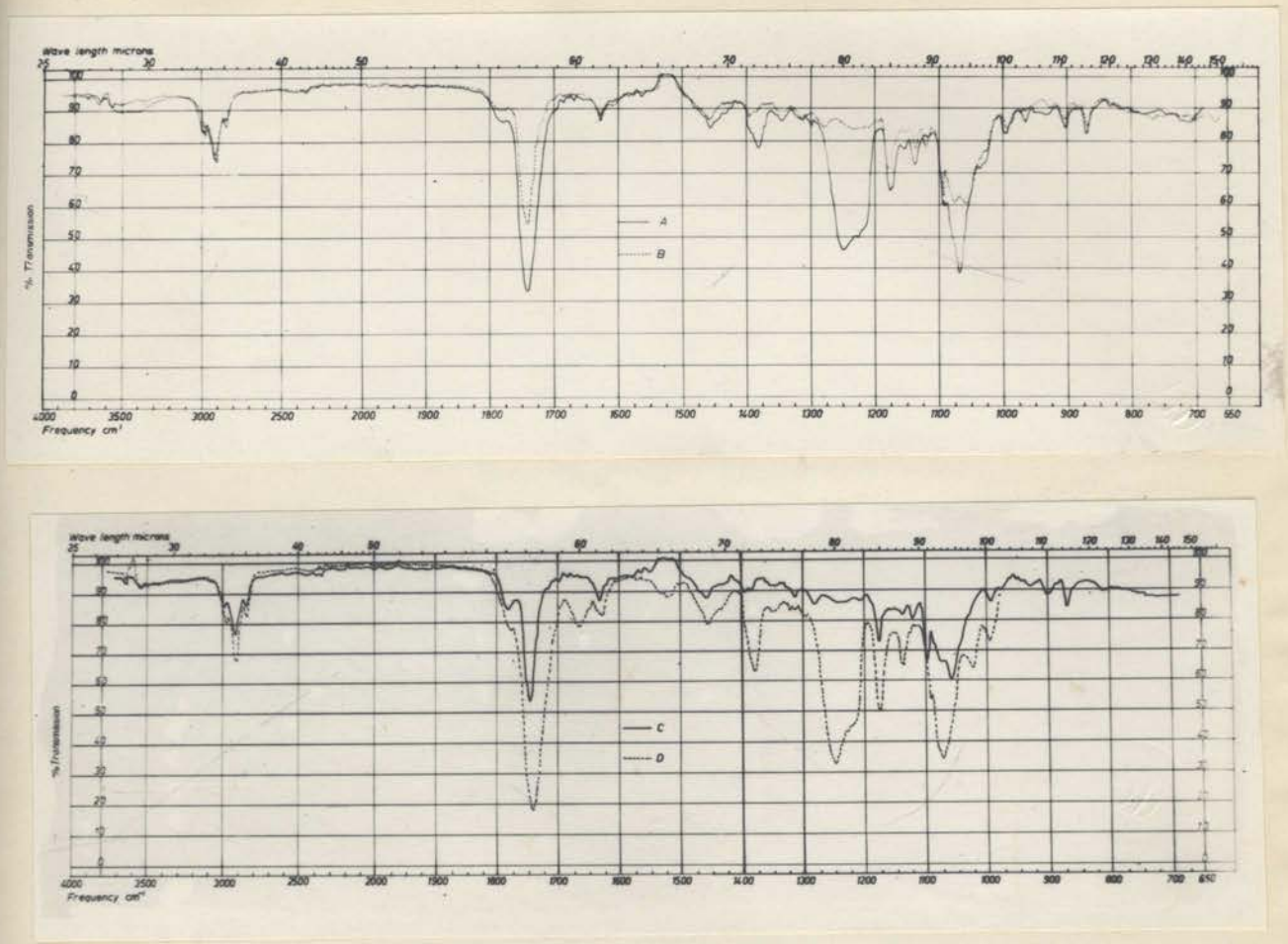


Figure 30

Infrared absorption spectra

A = Acetyl-gomphoside

B = Gomphoside

C = Gomphogenin

D = Acetyl-gomphogenin

Saturated solutions in chloroform

NaCl prism.

An attempt to hydrolyse gomphoside by refluxing with Kiliani's mixture (HOAc : H_2O : HCl, 3.5 : 5.5 : 1.0) for two hours resulted in the recovery of unchanged gomphoside. Gomphogenin was obtained from gomphoside by hydrolysis with 5% sulphuric acid in 75% aqueous methanol. This compound analysed for a formula $C_{23}H_{34}O_5$ and gave positive Raymond and Legal reactions, but a negative tetranitromethane test. The ultra violet spectrum of gomphogenin shows the typical absorption maximum of the butenolide ring at 218 mμ, log ε = 4.22 (Fig. 29. p.74).

The infrared absorption spectrum of gomphogenin is shown in Figure 30 (cruve C). The absorption bands due to the C = O and C = C stretching frequencies of the butenolide ring occur at 5.73 μ (1745 cm^{-1}) and 6.12 μ (1633 cm^{-1}) respectively.

Acetylation of gomphogenin yields a monoacetate which analyses for a formula $C_{25}H_{36}O_6$. This reaction indicates that there is only one hydroxyl group in the structure of gomphogenin which may be esterified under the conditions of acetylation (acetic anhydride in pyridine at 20°C). Therefore it is reasonable to assume that this hydroxyl group is in position 3 in the nucleus. The infrared absorption spectrum of acetyl gomphogenin (Fig. 30, curve D), shows the C = O and C = C stretching bands of the butenolide ring at 5.74 μ (1743 cm^{-1}) and 6.13 μ (1630 cm^{-1}) respectively. Also the

absorption band of the C - O stretching frequency of the acetyl group at 8.00 μ (1250 cm^{-1}) shows the complex structure associated with axially orientated substituents.[This spectrum was determined in chloroform solution.] As pointed out in the section on infrared spectroscopy (p. 52), the cardiac genins of known structure have a β orientated hydroxyl group at C_3. For this group to be β and axial, the A/B ring junction is likely to be cis. The infrared absorption spectrum of acetyl gomphogenin also shows an absorption band at 2.75 μ (3640 cm^{-1}) which is assigned to absorption of unassociated hydroxyl groups. [This band is not well resolved, as the only prism available was of sodium chloride.] The nature of this hydroxyl absorption does not indicate the number of hydroxyl groups which are present in the molecule.

Acetyl gomphogenin was oxidised by chromium trioxide in acetic acid, but an insufficient quantity of this material was available for physical or elemental analyses. This indicates that although the hydroxyl group cannot be acetylated, it can be oxidised.

From these reactions it is possible to draw some conclusions relating to the structure of gomphoside and gomphogenin. The ultra-violet and infrared absorption spectra indicate that these compounds contain the normal butenolide side chain, common to all cardiac genins of the digitalis type. Also, the infrared spectrum of gomphogenin acetate indicates that the A/B ring junction may be cis. The acetylation of gomphoside shows that there are only two

hydroxyl groups in the molecule which can be esterified, and as gomphogenin forms only a monoacetate, both of these hydroxyl groups must be located in the carbohydrate moiety. Therefore, besides the two hydroxyl groups at C_3 and C_{14} in the aglycone, there is a third hydroxyl group which cannot be esterified. As the ultra-violet absorption spectrum of gomphogenin does not show the absorption plateau between 270 - 300 mμ (log. $\varepsilon \doteqdot 1.25$-$1.4$) which is present in the spectrum of coroglaucigenin (Hunger and Reichstein, 1952), it is reasonable to assume that this hydroxyl group is not a primary hydroxyl situated at C_{19}. However, the reactions which have been carried out do not indicate the position of this group in the nucleus.

To summarise these reactions and conclusions, they are interpreted in partial structural form in Figure 31 (p.79).

The partial formula assigned to the carbohydrate part of the glycoside requires it to be a di-desoxy hexose. However, as the Keller-Kiliani test was negative, the possibility of it being a 2-6 desoxy hexose may be eliminated. A quantitative oxidation of gomphoside by periodic acid indicated that one mole of the oxidising agent was required for the reaction. Therefore the hydroxyl groups present in the carbohydrate must be on adjacent carbon atoms. However, the constitution of this sugar is unknown as only small quantities of gomphoside were available for hydrolysis reactions.

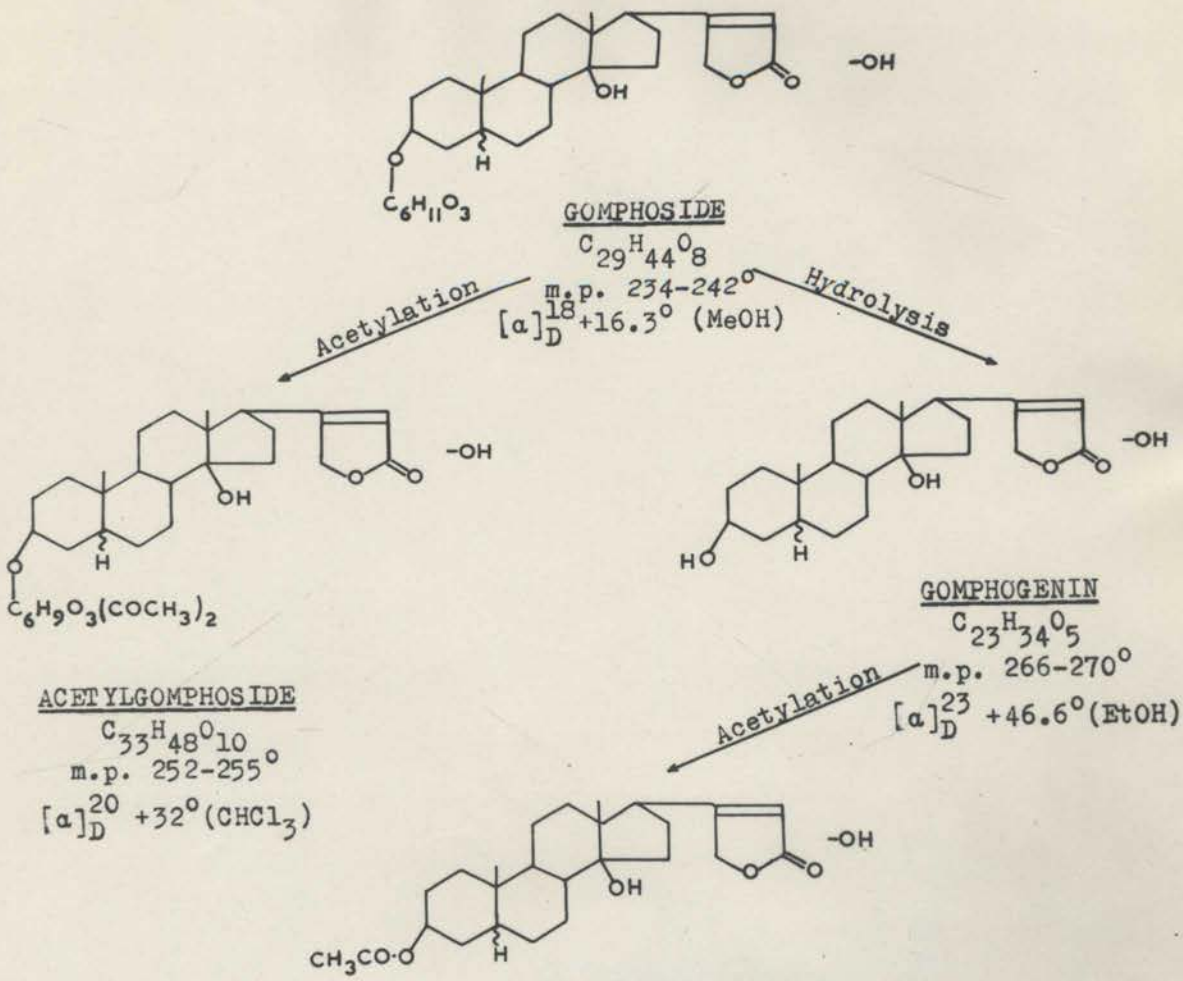
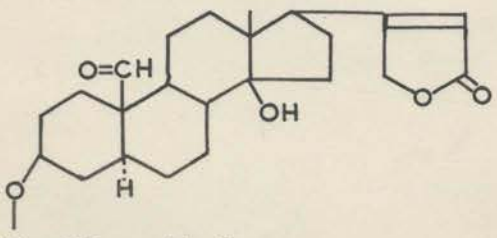

-OH
OH
H
$C_6H_{11}O_3$
GOMPHOSIDE
$C_{29}H_{44}O_8$
m.p. 234-242°
$[\alpha]_D^{18}$ +16.3° (MeOH)
Acetylation
Hydrolysis
-OH
OH
H
$C_6H_9O_3(COCH_3)_2$
ACETYLGOMPHOSIDE
$C_{33}H_{48}O_{10}$
m.p. 252-255°
$[\alpha]_D^{20}$ +32°($CHCl_3$)
-OH
OH
HO
H
GOMPHOGENIN
$C_{23}H_{34}O_5$
m.p. 266-270°
$[\alpha]_D^{23}$ +46.6°(EtOH)
Acetylation
-OH
OH
CH_3CO·O
H
ACETYLGOMPHOGENIN
$C_{25}H_{36}O_6$
m.p. 150-156°
$[\alpha]_D$ +38°($CHCl_3$)

Figure 31

AFROSIDE

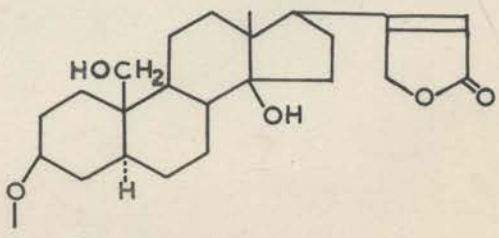
This constituent consists of a mixture of two components which cannot be separated on alumina. Paper chromatography, using formamide as the stationary phase, and benzene - chloroform, or benzene - chloroform - n.butanol mixtures as the mobile phase, were the systems used to separate this mixture into its two components. The result of the paper chromatography indicates that the two components of this mixture are present in approximately the same proportion.

Recently, Keller, Hunger, and Reichstein (Keller and Reichstein, 1949; Hunger and Reichstein, 1952) have described the extraction and chemical constitution of two cardiac glycosides, gofruside and frugoside from the seeds of Gomphocarpus fruticosus (R.Br.) obtained from South Africa. These glycosides have the structures shown in Figures 32 and 33. The components of afroside (and also the compound gomphoside) have been shown to differ, by comparative chromatography on formamide impregnated paper, from gofruside and frugoside, samples of which were kindly supplied by Professor T. Reichstein (see Fig. 34, p.83).



D-allomethylose
Gofruside

Figure 32



D-allomethylose
Frugoside

Figure 33

Afroside (m.p. 258-262°C) analyses for the formula $C_{29}H_{42}O_9$ and gives positive Legal and Raymond reactions, but a negative Keller-Kiliani reaction. It gives a positive test for the presence of a carbohydrate moiety. Afroside has the ultra-violet absorption maximum typical of the butenolide ring at 217 mμ (log ε = 4.22). The ultra-violet absorption curve (Fig. 35, p.84) also shows a second maximum at 292-293 mμ (log ε = 1.57) which may indicate the presence of a C_{10} aldehyde group. However, when the solution of afroside has been stored for some time (in this case, 4 weeks) this second maximum at 292-293 mμ is replaced by an inflexion in the curve at 292 mμ (Fig. 35, p.84). This would indicate that the aldehyde group at C_{10} is being oxidised to the corresponding acid or that some other transformation occurs on standing, which alters the nature of the chromophoric group at C_{10}.

Hunger and Reichstein (loc. cit) have shown that gofruside, kept in solution, undergoes auto-oxidation of the aldehyde group to form the corresponding acid. Also, acid hydrolysis of gofruside [and also christyside (Schindler and Reichstein, 1953)] leads to the production of uzarigenic acid -(19), 19 ⟶ 3 lactone (Fig. 36, p.82). The auto-oxidation of an aldehyde group at C_{19} in the trans A/B series of cardiac glycosides had also been shown to occur in bovoside A (Katz, 1953), which has the formula shown in Figure 37 (p.82).

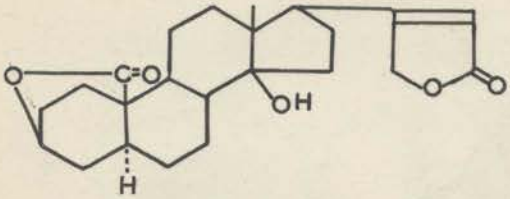
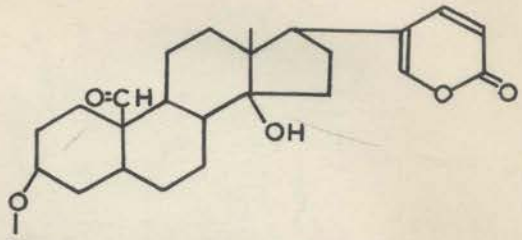


Figure 36



L-Thevetose

Figure 37

Reduction of afroside by sodium borohydride gave a chromatographically homogeneous product ($C_{29}H_{44}O_9$), which showed an ultra-violet absorption curve similar to that of the stored afroside solution, except that the intensity of absorption of the reduced compound in the region 265-325 mμ was somewhat greater than that of the stored compound (see Fig.38, p.85).

Chromatography of the reduced compound on formamide impregnated paper showed it to be different from both of the constituents of afroside (Fig. 39, p.86). This compound was named Afrosidol.

Treatment of afroside with hydrochloric acid (Mannich hydrolysis procedure) resulted in the recovery of a single substance which was shown by paper chromatography (Fig. 39, p.86) to be identical with one of the constituents of afroside (yield 78%). The ultra-violet absorption curve of this compound is shown in Figure 38 (p.85). This constituent analysed for a formula $C_{29}H_{42}O_9$ and was named Afroside B. [Apparently no hydrolysis of the glycoside was effected by this reaction.]

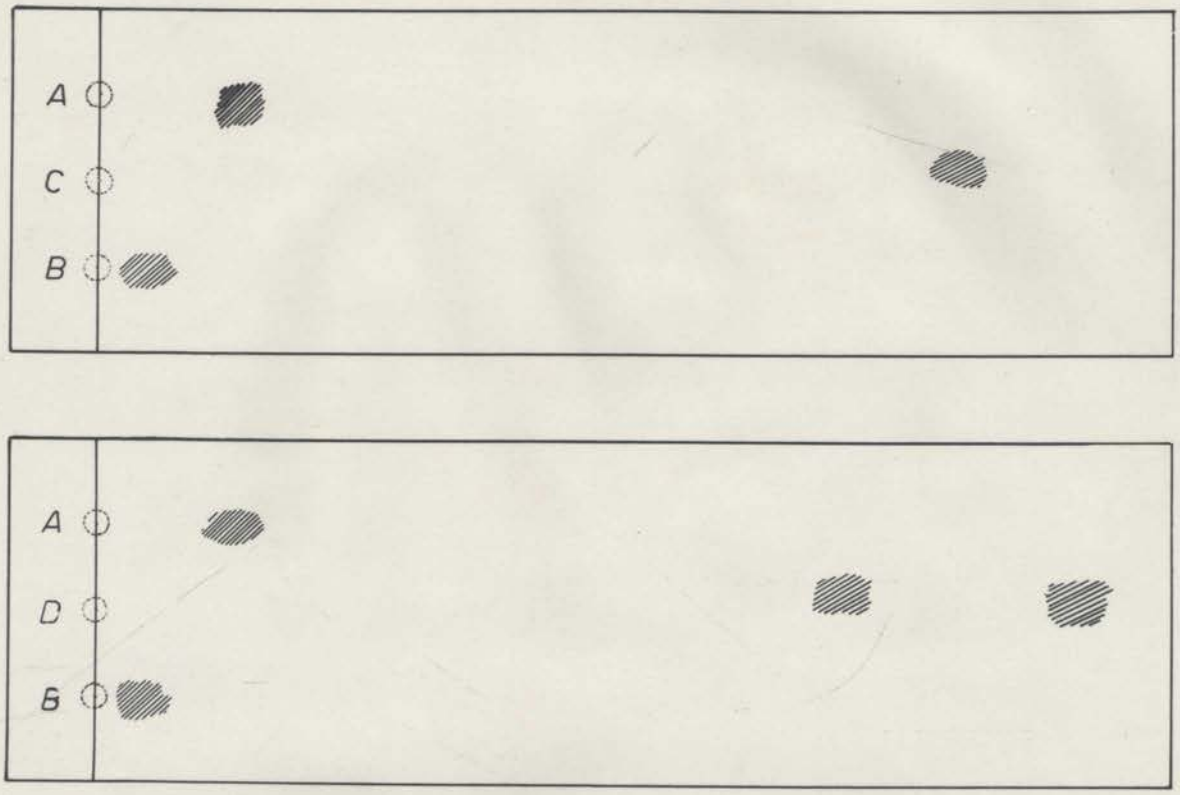


Figure 34

Chromatograms on formamide impregnated paper.

Developing solvent, $CHCl_3$: C_6H_6 : BuOH, 78:12:5.

A = Gofruside

B = Frugoside

C = Gomphoside

D = Afroside

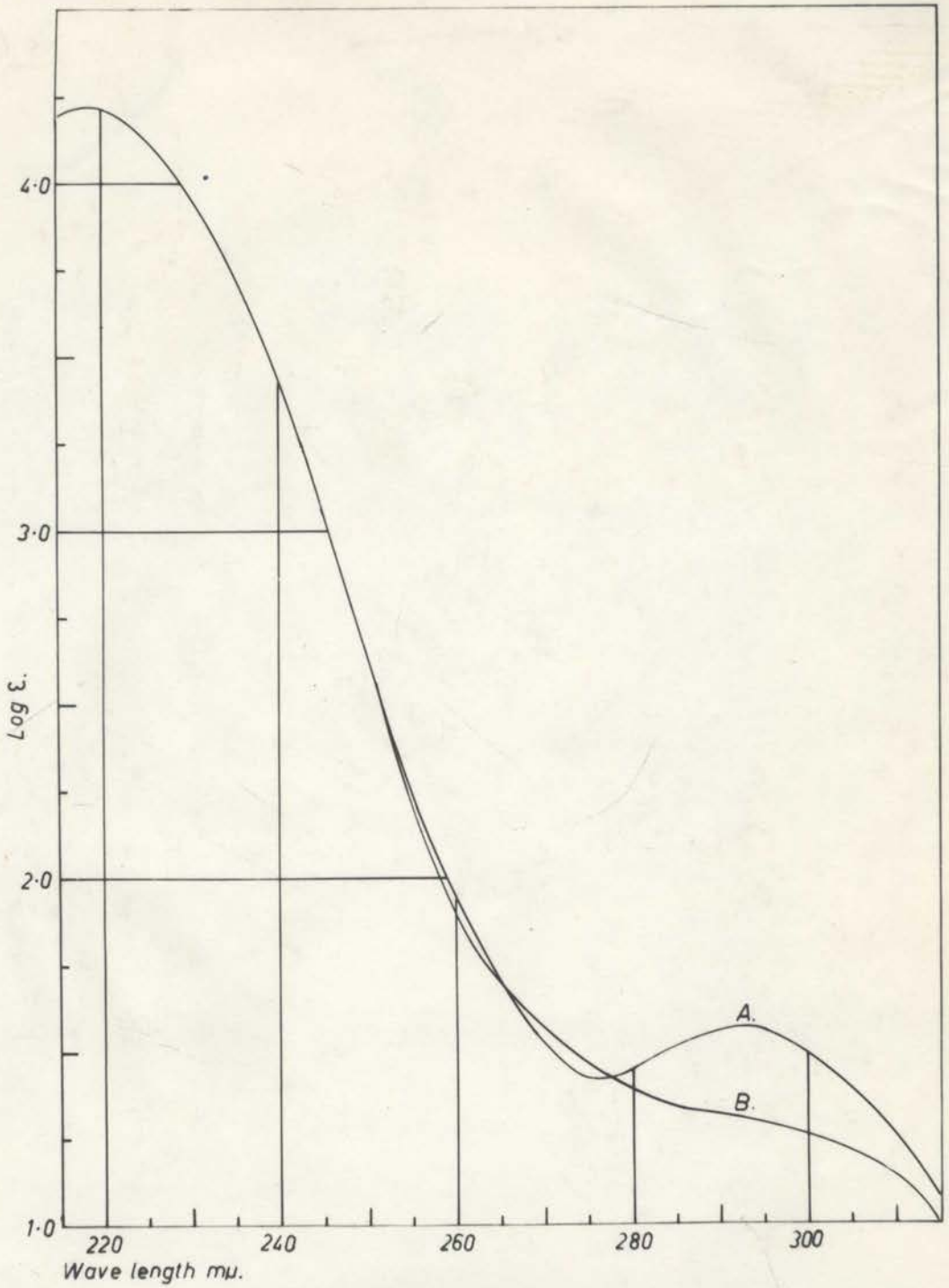


Figure 35

Ultra-violet absorption spectra.

A = Afroside ($C_{29}H_{42}O_9$), fresh solution, λ max. = 217 mμ, log ε = 4.22
λ max. = 292-293, log ε = 1.57.

B = Afroside, solution stored for 4 weeks.
λ max. = 217 mμ, log ε = 4.22; Inflexion 292 mμ, log ε = 1.26.

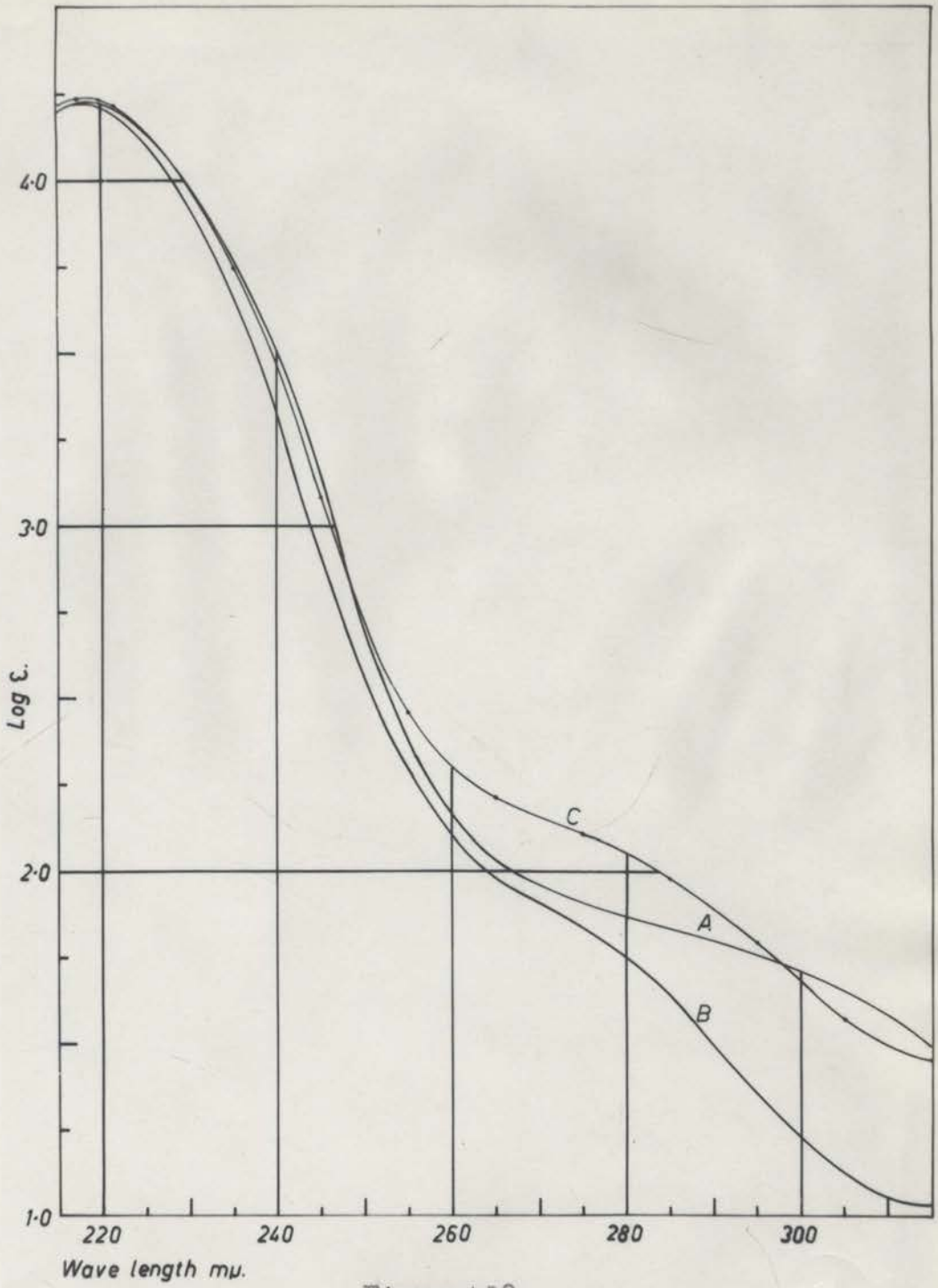


Figure 38

Ultra-violet absorption spectra.

A = Afrosidol ($C_{29}H_{44}O_9$), λ max.= 218 mμ, log ε = 4.22

B = Afroside B ($C_{35}H_{46}O_9$), λ max.= 217 mμ, log ε = 4.21

C = Acetyl-afroside B ($C_{35}H_{46}O_{12}.H_2O$), λ max.= 216 mμ, log ε = 4.24.

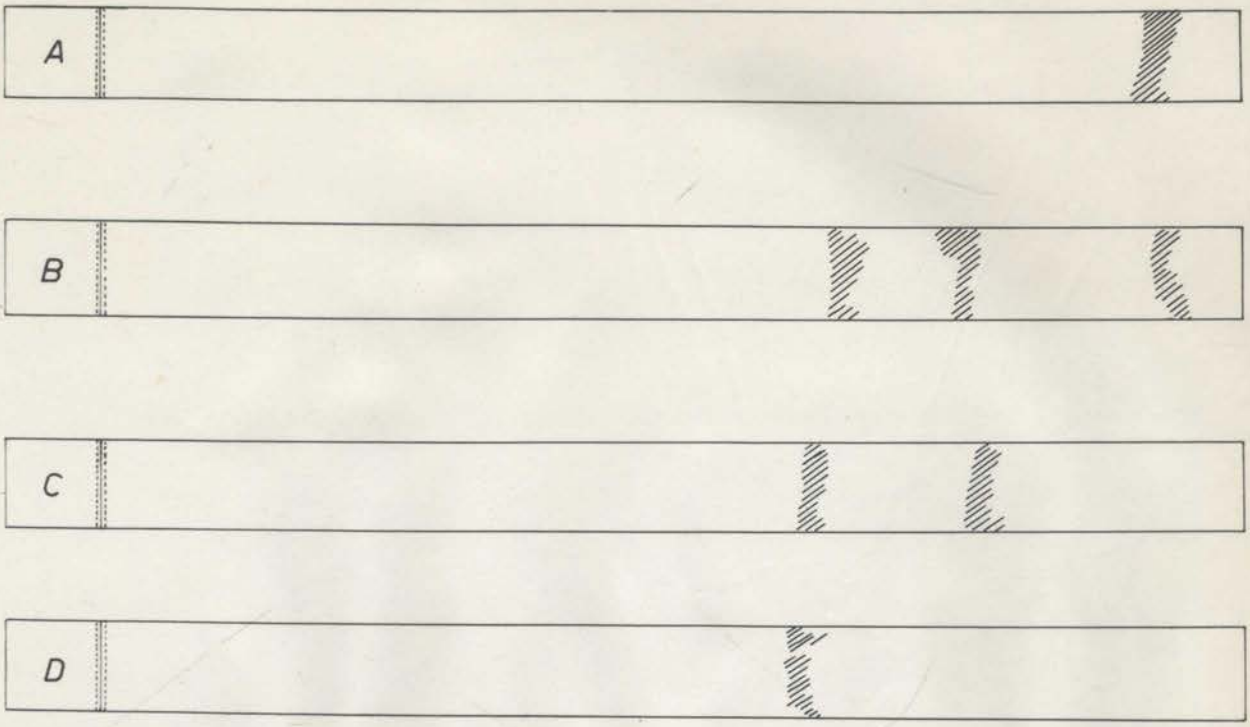


Figure 39

Chromatograms on formamide impregnated paper.

Developing solvent, $CHCl_3$: C_6H_6 : BuOH, 78:12:5

A = Afrosidol

B = Afrosidol + Afroside

C = Afroside

D = Afroside B

An attempt to separate the two components of afroside by chromatography of the acetates resulted in a yield of approximately 85% of an homogeneous acetylated product, 14% of unacetylated starting material, and a trace of a third apparently acetylated compound (see p.152). The main fraction (the acetylated compound) from this experiment, after recrystallisation, analysed for a formula $C_{35}H_{46}O_{12}\ H_2O$. An acetyl determination indicated the presence of three acetyl groups.

Acetylation of afroside B gave a compound which was shown by analyses, specific rotation, melting point, and paper chromatography to be identical with the acetate obtained from afroside. This compound is acetyl-afroside B. Saponification of this acetate by potassium bicarbonate in aqueous methanol gave afroside B. [Benzoates of either afroside or afroside B could not be obtained in crystalline form even after chromatography on alumina.]

When afroside was refluxed with hydroxylamine in the presence of sodium acetate, a crystalline substance was obtained which, when chromatographed on formamide impregnated paper was shown to consist of two constituents, one of which was unchanged afroside B (Fig. 40). Chromatography on alumina did not separate these two compounds. The infrared spectrum of the mixture showed a weak indefinite absorption band between 6.01 and 6.18 μ (1664-1618 cm^{-1}), which could be due to the overlapping of the C = C stretching band of the butenolide

ring, with C = N stretching band of an oxime (Fig. 41, p.89). Elemental analysis of the mixture showed the presence of 0.74% nitrogen. Although these results are not conclusive evidence for the presence of an oxime in the mixture, it is evident that Afroside A has undergone some structural modification.

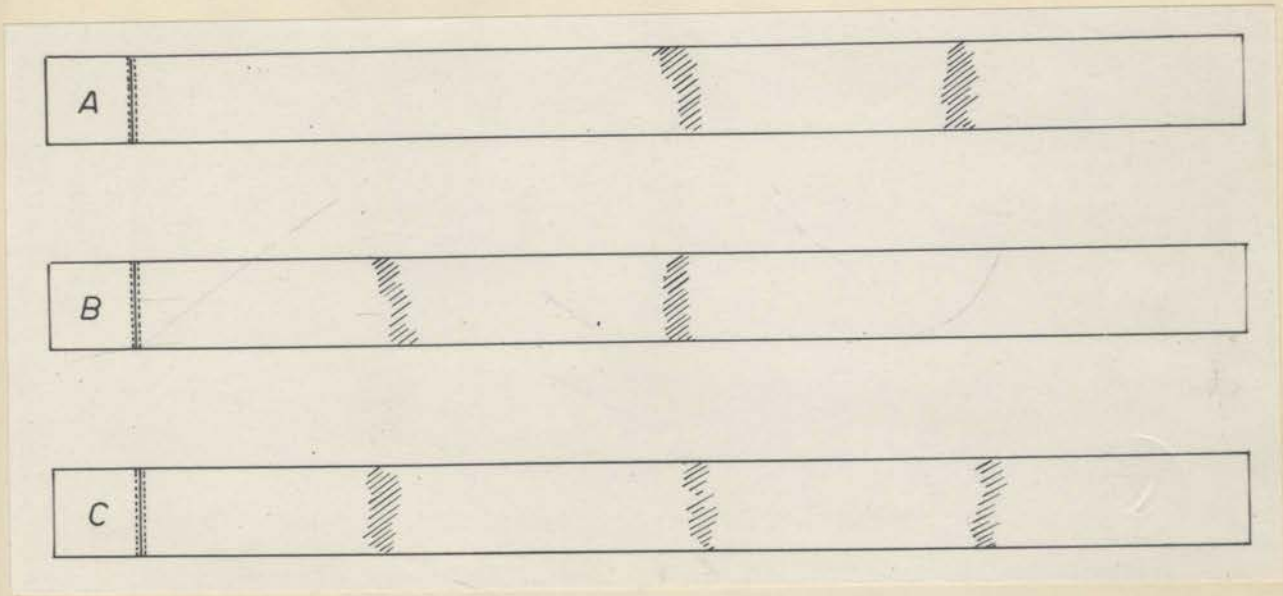


Figure 40

Chromatograms on formamide impregnated paper.

Developing solvent, $CHCl_3$: C_6H_6, 9:1.

A = Afroside

B = Product from attempted formation of an oxime.

C = A + B

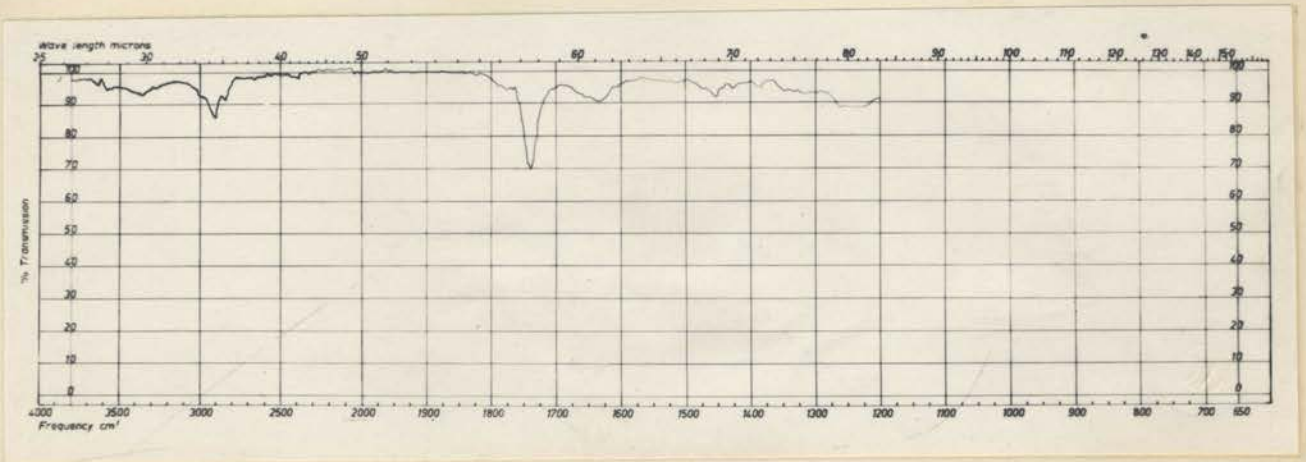


Figure 41

Infrared absorption spectrum of the mixture from the attempted formation of an oxime of Afroside.

Saturated solution in chloroform - NaCl prism.

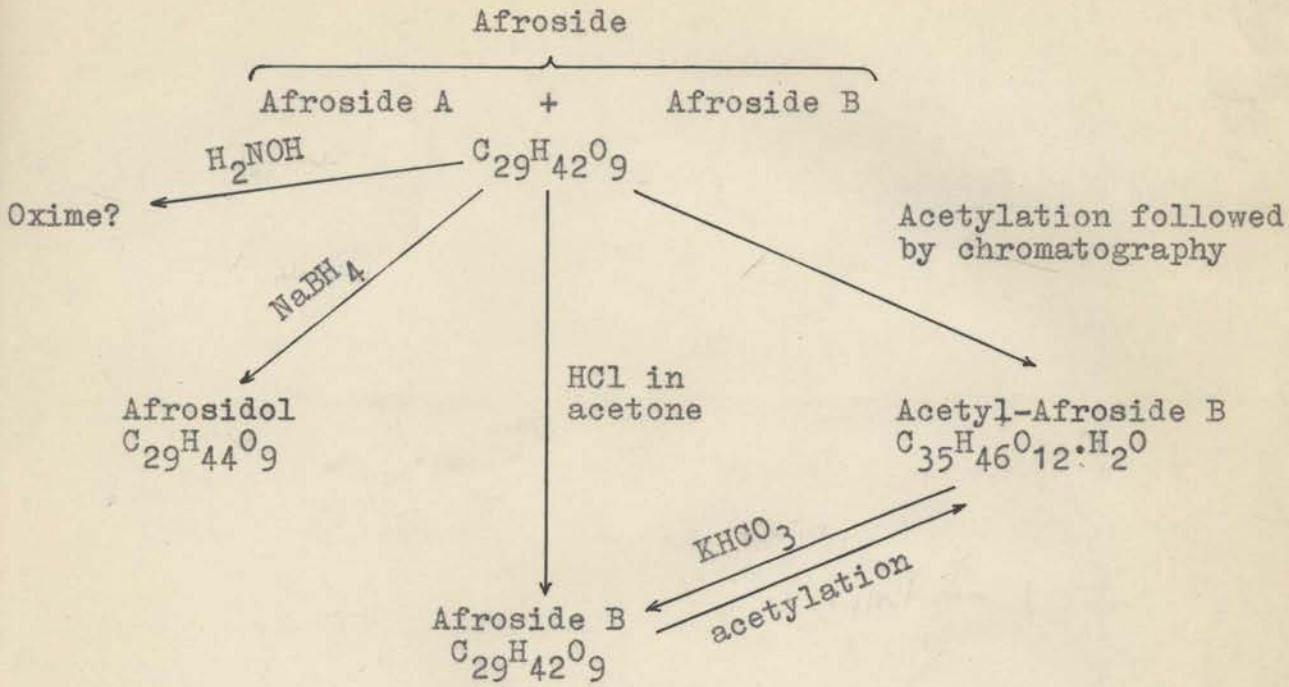


Figure 42

The infrared absorption spectra of afroside B, afrosidol, and acetyl-afroside B (in nujol) are shown in Figure 43 (p.92). Afroside B has the absorption bands due to the butenolide ring at 5.77 μ (1733 cm^{-1}) (C = O stretching frequency) and 6.10 μ (1639 cm^{-1}) (C = C stretching frequency), whereas afrosidol has these bands at 5.82 μ (1718 cm^{-1}), and 6.17 μ (1621 cm^{-1}). In the spectrum of acetyl-afroside B, the carbonyl absorption band occurs at 5.73 μ (1745 cm^{-1}) and the double bond at 6.16 μ (1623 cm^{-1}). These are the only main absorption bands which are of particular significance in these spectra.

As stated previously, afroside B was recovered unchanged when submitted to the Mannich hydrolysis procedure. Treatment of afroside with 0.1N H_2SO_4 in a mixture of methanol - water (1:1) under reflux for 30 minutes also had no effect on the glycosides and afroside was recovered unchanged. Hydrolysis under more drastic conditions (5% H_2SO_4 in methanol - water, 75:25) gave a crystalline product, in nearly theoretical yield, which was shown to be an anhydrogenin. The anhydrogenin analysed for a formula $C_{23}H_{30-32}O_6$, and gave positive Legal, Raymond, and tetranitromethane reactions.

Cardiac glycosides which have a 2-desoxy sugar linked directly to the steroid nucleus are easily hydrolysed by acids (e.g, cymarin, i.e. strophanthidin + cymarose) but of those which have a normal 2-hydroxy sugar in this position, (e.g. convallotoxin, i.e.strophanthidin + rhamnose) only those which are soluble in acetone or will form a soluble acetonide can be hydrolysed by the Mannich hydrolysis method (1% HCl in acetone) to give the normal genin. Certain glycosides, e.g. neriifolin, can be hydrolysed only under such conditions that the aglycone is produced in an anhydrous form, from which almost always two isomers and other transition-products arise. These anhydrogenins are named according to the position of the double bond formed by the elimination of the C_{14} hydroxyl group. The α-anhydrogenins have the $\Delta^{8.14}$ bond and the β-anhydrogenins have the $\Delta^{14.15}$ bond as in Figures 44 and 45,(p.93).

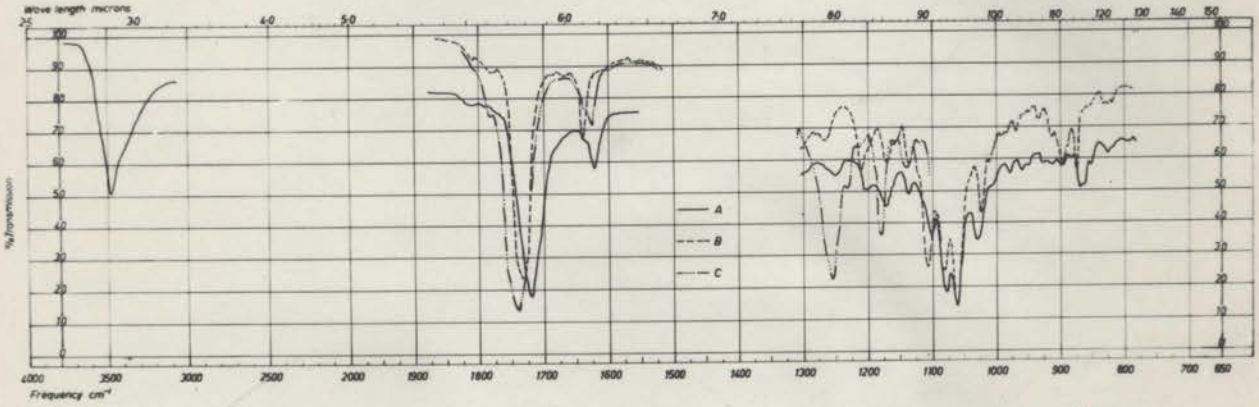


Figure 43

Infrared absorption spectra in nujol. NaCl prism.

A = Afroside B

B = Afrosidol

C = Acetyl-Afroside B

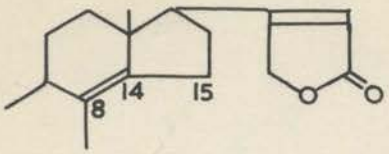


Figure 44

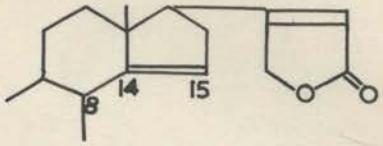


Figure 45

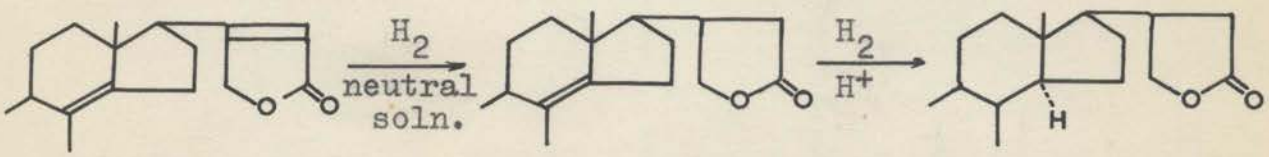
Recently Cardwell and Smith (1954) in their hydrogenation experiments and spectral studies on the α and β anhydro-digitoxigenins and digoxigenins have shown that the dextro-rotary α-anhydrogenins are unsaturated in the 8:14 position, and the laevorotary β-anhydrogenins are the 14:15 unsaturated isomerides. A double bond in the 14:15 position is readily hydrogenated, whilst an 8:14 double bond is inert to hydrogenation under neutral conditions; in the presence of mineral acid, partial migration of double bonds from 8:14 to 14:15 takes place with consequent hydrogenation.

The β-anhydrogenins show characteristic absorption bands in the 3.3, 6.0, and 12.0 μ regions of the infrared spectrum (Bladon et al, 1951), due to the C - H stretching of the trisubstituted double bond, whereas the α-anhydro-genins are transparent in these regions, the $\Delta^{8:14}$ double bond being tetra-substituted (see p. 49).

These experiments have been carried out on the anhydrogenin obtained by the hydrolysis of afroside. The rotation of the

anhydroafrogenin is $[\alpha]_D^{17}$ + 60.8 ±2° (c = 0.855 in MeOH), indicating that it is possibly the α-isomer.

Hydrogenation of anhydroafrogenin in neutral solution, using platinum oxide as catalyst, gave the corresponding butanolide; the double bond in the nucleus being unchanged as indicated by the positive tetranitromethane test. This recovered material was then hydrogenated in acetic acid with 1% hydrochloric acid using platinum oxide as catalyst. The product from this reaction was the fully hydrogenated genin, as was shown by the negative results of the Legal, Raymond, and tetranitromethane colour reactions. These hydrogenation experiments are summarised in Figure 46.



+ve Raymond	-ve Raymond	-ve Raymond
+ve Legal	-ve Legal	-ve Legal
+ve Tetra-nitromethane	+ve Tetra-nitromethane	+ve Tetra-nitromethane

(Colour Reactions)

Figure 46

The infrared spectrum of the anhydrogenin showed a very weak absorption band in the 12 μ region (Fig. 47, p.97), but

no band in the 6 μ region, which would indicate that there was a small quantity of the $\Delta^{14:15}$ compound present as an impurity in the sample. However, it is apparent from these reactions and measurements that the bulk of the product from the acid hydrolysis of afroside is the α-anhydroafrogenin. Acetylation of α-anhydroafrogenin gave a crystalline acetate, which analysed for a formula $C_{25}H_{34}O_8$. The acetyl determination showed the presence of one acetyl group, but analysis figures did not fit the theoretical values exactly (calculated for 1 -OAc 9.1%, found 12.5%).

Oxidation of acetyl-anhydroafrogenin with chromium trioxide in acetic acid, gave a product which was different from the starting material when chromatographed on formamide impregnated paper, but there was not sufficient for analyses or constant determinations. However, the reduction of the chromium trioxide is not direct evidence for the presence of an unacetylated hydroxyl group as the methylene groups adjacent to the $\Delta^{8:14}$ bond would undergo allylic oxidation with the formation of the 15-, or 7-oxo-8(14) enyl oxide (Wintersteiner and Moore, 1943).

Recently Poos et al.,(1953) have described the use of chromium trioxide in pyridine for the oxidation of hydroxyl groups to ketones or aldehydes. This reagent has the advantage over chromium trioxide in acetic acid, in that it does

not oxidise double bonds. When acetyl α-anhydroafrogenin was treated with chromium trioxide in pyridine, no oxidation occurred and the starting material was recovered unchanged. This reaction indicates that there are no free secondary hydroxyl groups present in the structure of acetyl α-anhydroafrogenin.

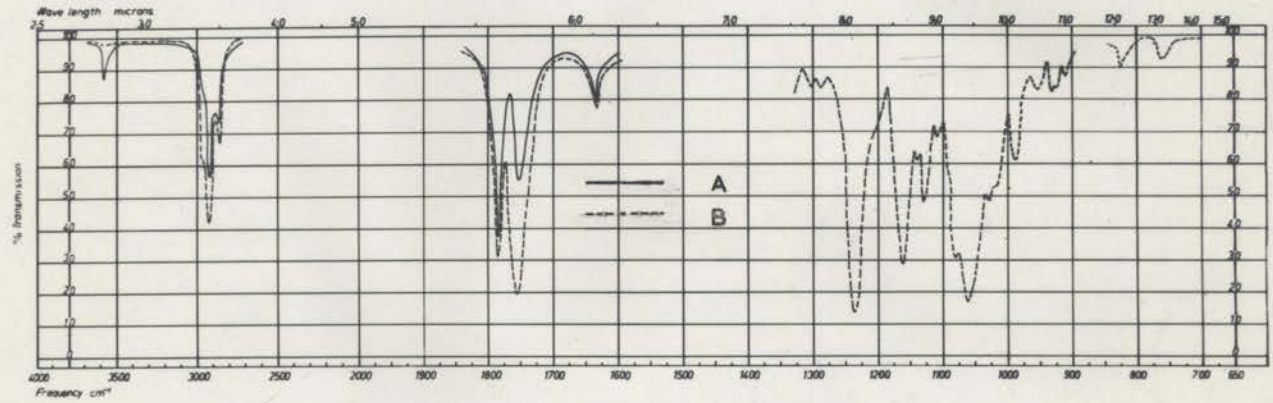


Figure 47

Infrared absorption spectra

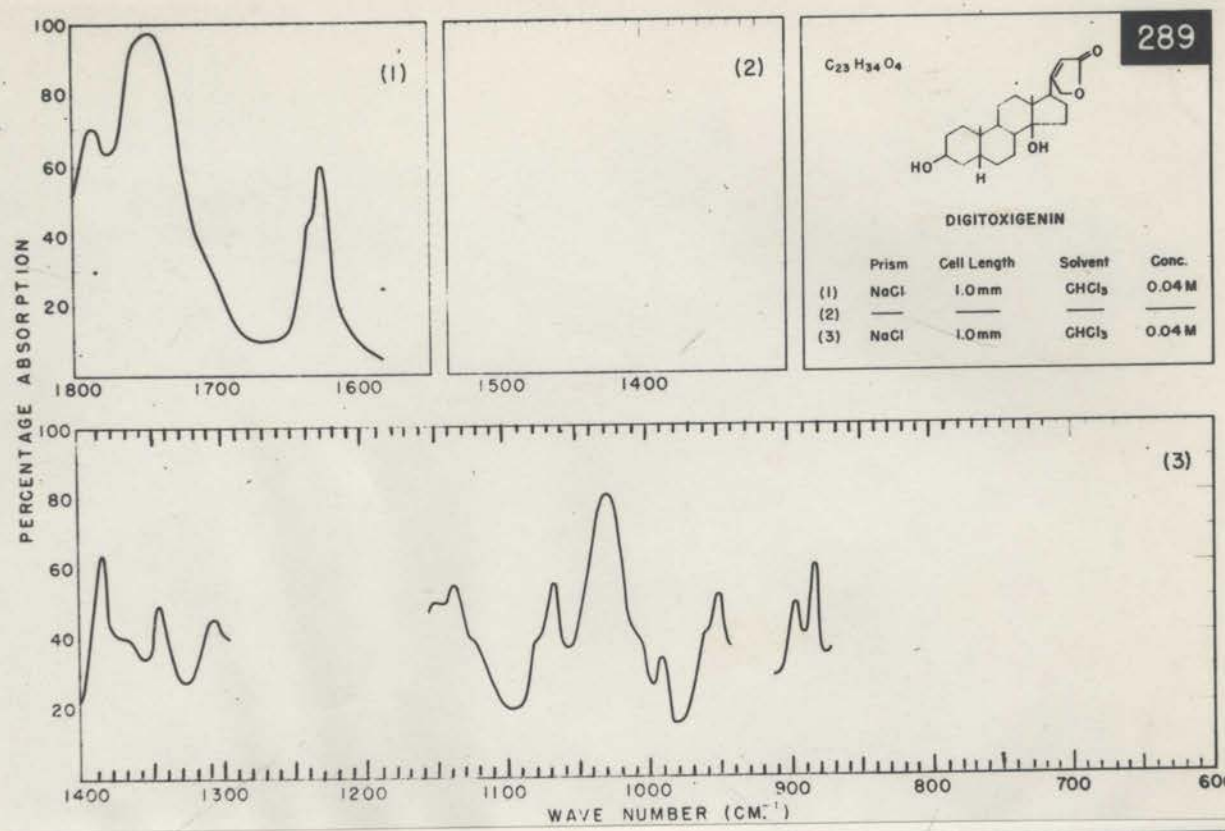
2.5 - 6.25 μ, saturated solution in CS_2 - CaF_2 prism.

7 - 14 μ, saturated solution in CCl_4 - NaCl prism.

A = α-anhydroafrogenin

B = acetyl-α-anhydroafrogenin

(These spectra were recorded by Dr A.R.H. Cole, University of Western Australia.)



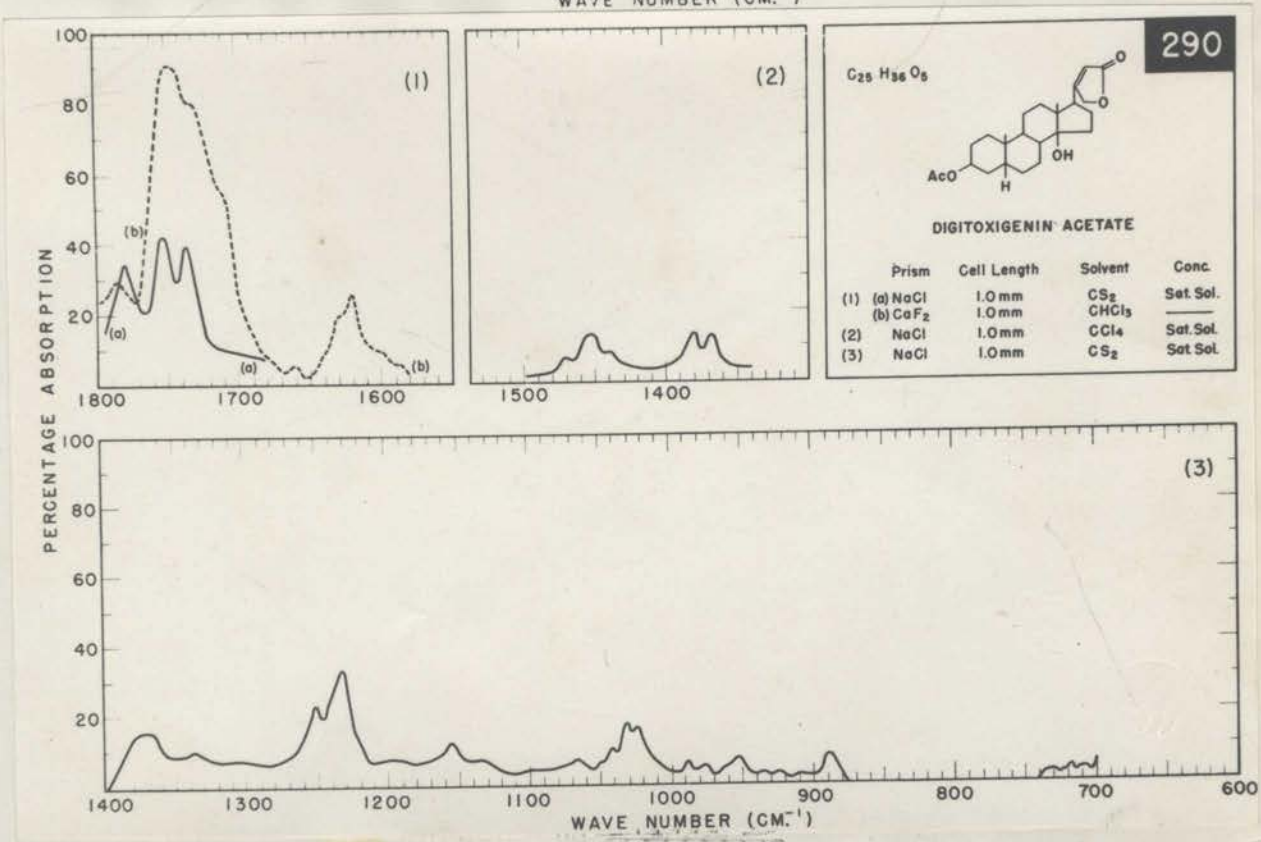


Figure 48

Reproduced from "Infrared Absorption Spectra of Steroids", Dobriner, Katzenellenbogen & Jones; Interscience, 1953, New York.

INFRARED SPECTRAL ANALYSIS

The infrared spectra of α-anhydroafrogenin and acetyl α-anhydroafrogenin are shown in Figure 47 (p.97). In both spectra, apart from the normal butenolide absorption bands at 6.12 μ (1633 cm^{-1}), C = C stretching frequency, and 5.70 μ (1755 cm^{-1}), C = O stretching frequency, there is an intense absorption band at 5.60 μ (1786 cm^{-1}). The infrared spectra of all the naturally occurring cardiac glycosides and aglycones show an absorption band of very weak intensity at this wave length. Jones (1954) ascribes this absorption maximum to the C = O stretching of the butenolide ring. [This band is apparent in the spectrum of digitoxigenin shown in Figure 48 (p.98).] However the carbonyl C = O stretching frequency band of the saturated γ-lactones, as in the dihydro-cardiac aglycones, occurs between 5.60 and 5.65 μ (1786-1770 cm^{-1}) (see p. 50, and Fig. 24 B, p.51), and has a high intensity. Consequently, the intense absorption band at this wave length in the spectra of α-anhydroafrogenin and its acetate must be due to a saturated γ-lactone ring present in the structure of these compounds. In the spectrum of the anhydrogenin, the intensity of the 1786 cm^{-1} band is greater than that of the 1755 cm^{-1} band, whereas in the spectrum of the acetate, these intensities are reversed. This is accounted for by the overlapping of the acetyl and butenolide carbonyl absorption bands in the spectrum of the α-anhydro-

afrogenin shows the true relationship of the intensities of these two bands.

The possibility of the 1786 cm^{-1} band being due to the presence of the small quantities of the corresponding butanolide has been excluded by chromatography on the formamide impregnated system, the systems of Svensen and Jensen (1950), and of Silberman and Thorp (1954), as well as developing with trichloracetic acid which will detect butanolides. No trace of anything other than the α-anhydroafrogenin, or its acetate, could be detected. Also, the intensity of the 1786 cm^{-1} band is such that a large amount of the butanolide would have to be present as an impurity.

In the spectrum of α-anhydroafrogenin acetate, an intense absorption band, due to the C - O stretching vibration of the acetate, occurs at 8.08 μ (1238 cm^{-1}). This band has the simple structure associated with equatorially orientated substituents at C_3 (see p. 52). As all the naturally occurring aglycones of known structure have a β-orientated hydroxyl group at C_3, for the substituent in this position to be equatorial, the A/B ring junction is probably trans. The spectrum of acetyl-digitoxigenin (Fig. 48, p.98), in which case the A/B ring junction is cis and the β-orientated substituent at C_3 is axial, shows a complex absorption band at 8.08 μ.

The ultra-violet absorption curve of α-anhydroafrogenin is shown in Figure 49 (p.102). Although there is no discrete absorption maximum in the 290-310 mμ region, there is a point of inflexion at 290 mμ ($\log \varepsilon = 1.95$), which is probably due to carbonyl absorption. The significance of this point of inflexion is emphasised by calculating the difference curve (curve Δ, Fig.49, p.102), which is derived by subtracting the ε values of gomphogenin from those of α-anhydroafrogenin and replotting the log Δ values [i.e. $\log \Delta = \log (\varepsilon_{\text{acetyl α-anhydroafrogenin}} - \varepsilon_{\text{gomphogenin}})$]. The "difference curve" shows a discrete maximum at 292 mμ ($\log \varepsilon = 1.73$), which confirms the presence of carbonyl absorption in the spectrum of α-anhydroafrogenin. This absorption may be due to the carbonyl group of the saturated five membered lactone ring.

The carbonyl group of a saturated lactone does not normally give rise to an absorption band in the ultra-violet region. However, certain six membered saturated lactones, as in uzarigenic acid -(19), 19→ 3 lactone show only very weak carbonyl absorption at 290 mμ, but 3β-allomethylosido-uzarigenic acid has an absorption plateau between 280-300 mμ. ($\log \varepsilon \doteqdot 1.25$). A similar absorption band is shown by uzarigenic acid between 270-290 mμ ($\log \varepsilon \doteqdot 1.2$). (Hunger and Reichstein, 1952). Although the intensity of the absorption plateau of α-anhydroafrogenin at 290 mμ ($\log \varepsilon = 1.95$) is greater than that of either of the above compounds, it is reasonable to assume that this absorption is due to a carbonyl group.

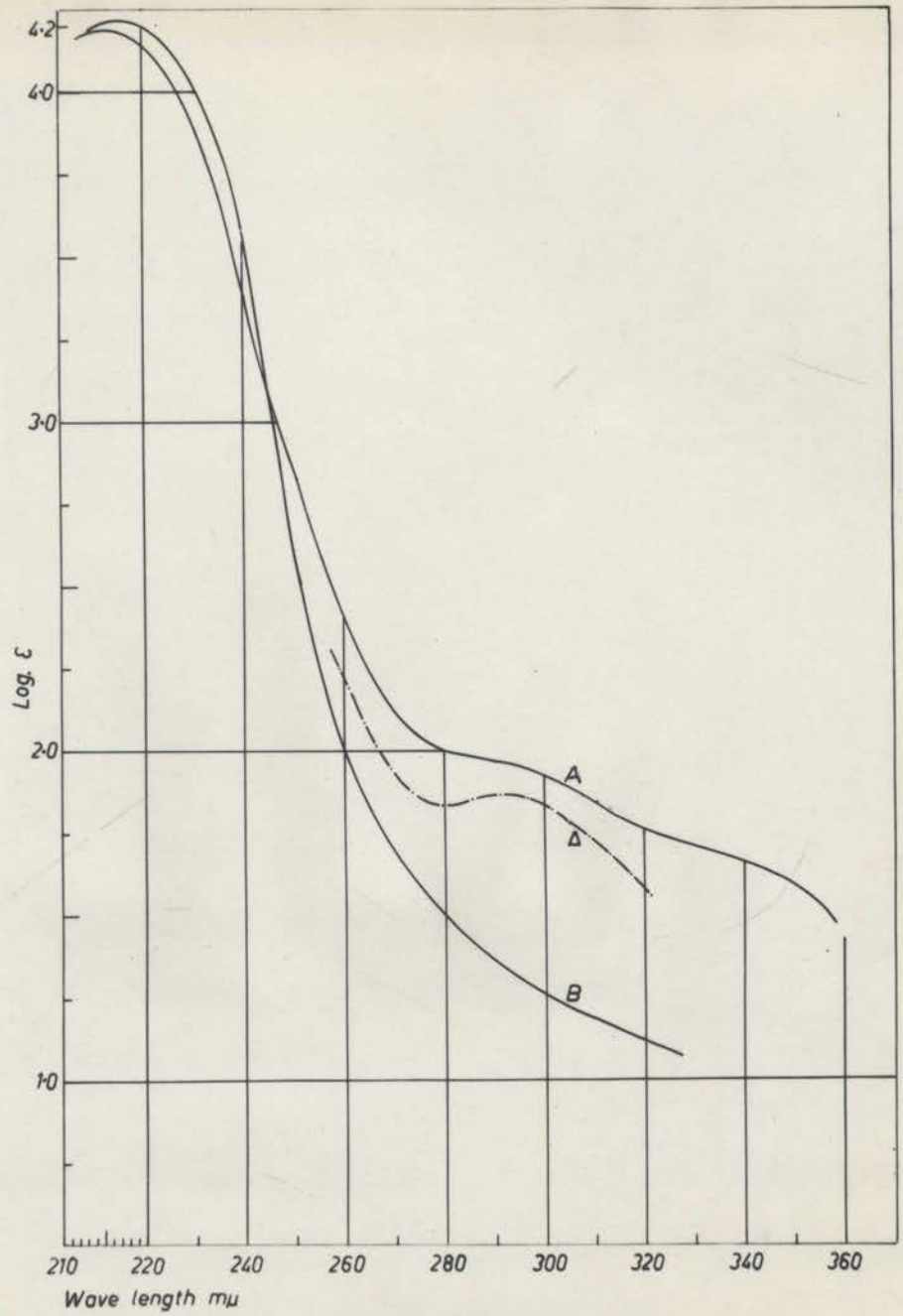


Figure 49

Ultra-violet absorption spectra

A = α-Anhydroafrogenin ($C_{23}H_{30-32}O_6$), λ max.= 214 mμ,
log ε = 4.20; inflexion λ = 290 mμ. log ε = 1.95.

B = Gomphogenin ($C_{23}H_{34}O_5$), λ max.= 217 mμ, log ε = 4.21

Δ = "Difference curve", λ max.= 292 mμ, log ε = 1.73,
log Δ_ε = log ($\varepsilon_A - \varepsilon_B$).

From the above reactions and physical measurements it is possible to deduce a partial structure of α-anhydroafrogenin (Fig. 50), the position of the γ-carbon atom of the saturated five membered lactone ring being undecided.

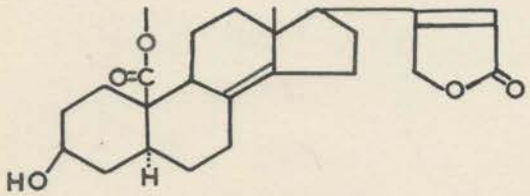


Figure 50

As the acid hydrolysis of the glycoside would tend to increase the rate of oxidation of the aldehyde group (at C_{10}) to the corresponding acid, it would also facilitate the formation of a lactone of the carboxylic acid with a γ-hydroxy group. Consequently, in order to obtain a genin which does not contain the γ-lactone ring, it is necessary to reduce the aldehyde group of the glycoside to the corresponding primary alcohol, before acid hydrolysis.

Reduction of afroside with sodium borohydride gave afrosidol ($C_{29}H_{44}O_9$, see p. 82), which on acid hydrolysis gave an anhydrogenin, anhydroafrogenol ($C_{23}H_{32}O_5.H_2O$). Anhydroafrogenol gave positive Legal, Keller-Kiliani, and tetranitromethane reactions and was shown to be different from α-anhydroafrogenin by paper chromatography (see Fig. 51, p. 104).

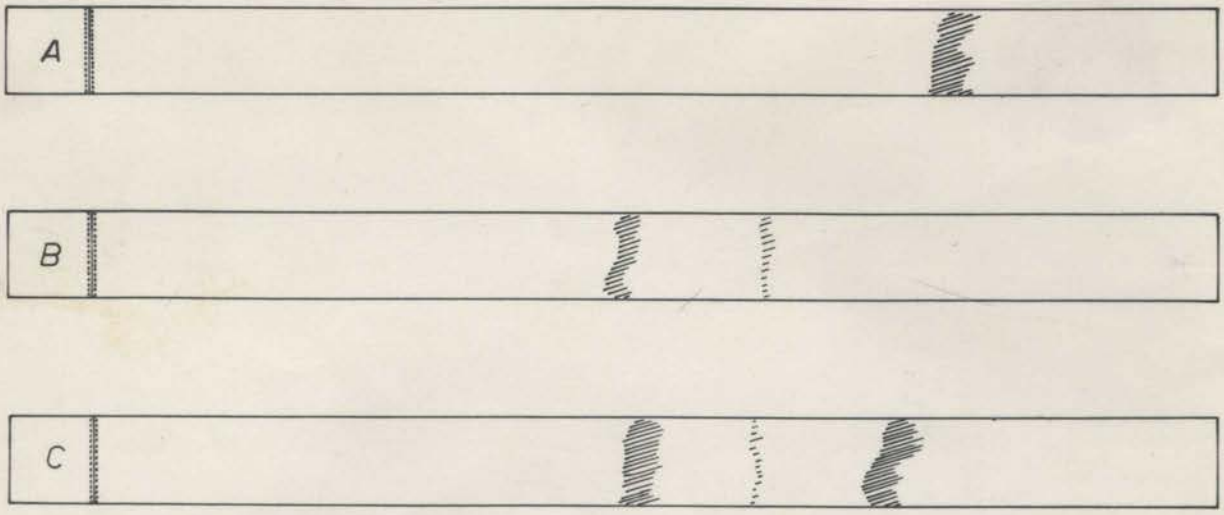


Figure 51

Chromatograms on formamide impregnated paper.
Developing solvent, $CHCl_3$: C_6H_6, 9:1.

A = α-anhydroafrogenin

B = anhydroafrogenol

C = A + B

The infrared absorption spectrum of anhydroafrogenol (Fig. 52, p.105) shows only a very weak absorption band at 5.60 mμ (1786 cm^{-1}), as well as the two characteristic bands of the butenolide side chain at 5.70 mμ (1755 cm^{-1}), and 6.12 μ (1633 cm^{-1}). The absence of the strong absorption band at 5.60 μ indicates that the saturated β-lactone ring is not present in the structure of anhydroafrogenol.

Also, the ultra-violet absorption spectrum of anhydroafrogenol shows no obvious carbonyl absorption in the 290-300 mμ region (Fig. 53, p.106). The spectrum of this compound is comparable with that of a compound containing a primary alcoholic group at C_{19} (c.f. coroglaucigenin; Hunger and Reichstein, 1952).

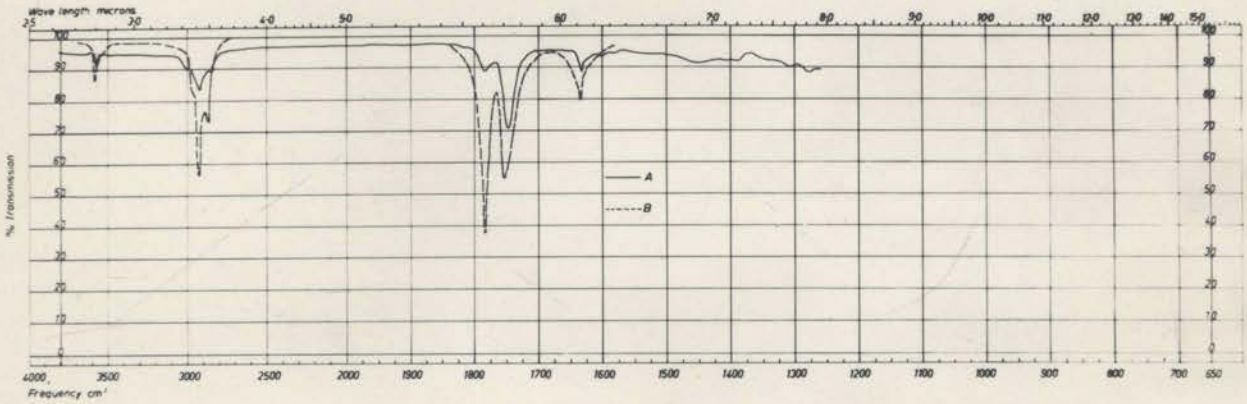


Figure 52

Infrared absorption spectra

A = Anhydroafrogenol

B = α-Anhyroafrogenin

A, saturated solution in $CHCl_3$ - NaCl prism.

B, 2.5 - 4 μ, solution in Cs_2 - CaF_2 prism,

5.5 - 6.5 μ, solution in CCl_4 - NaCl prism.

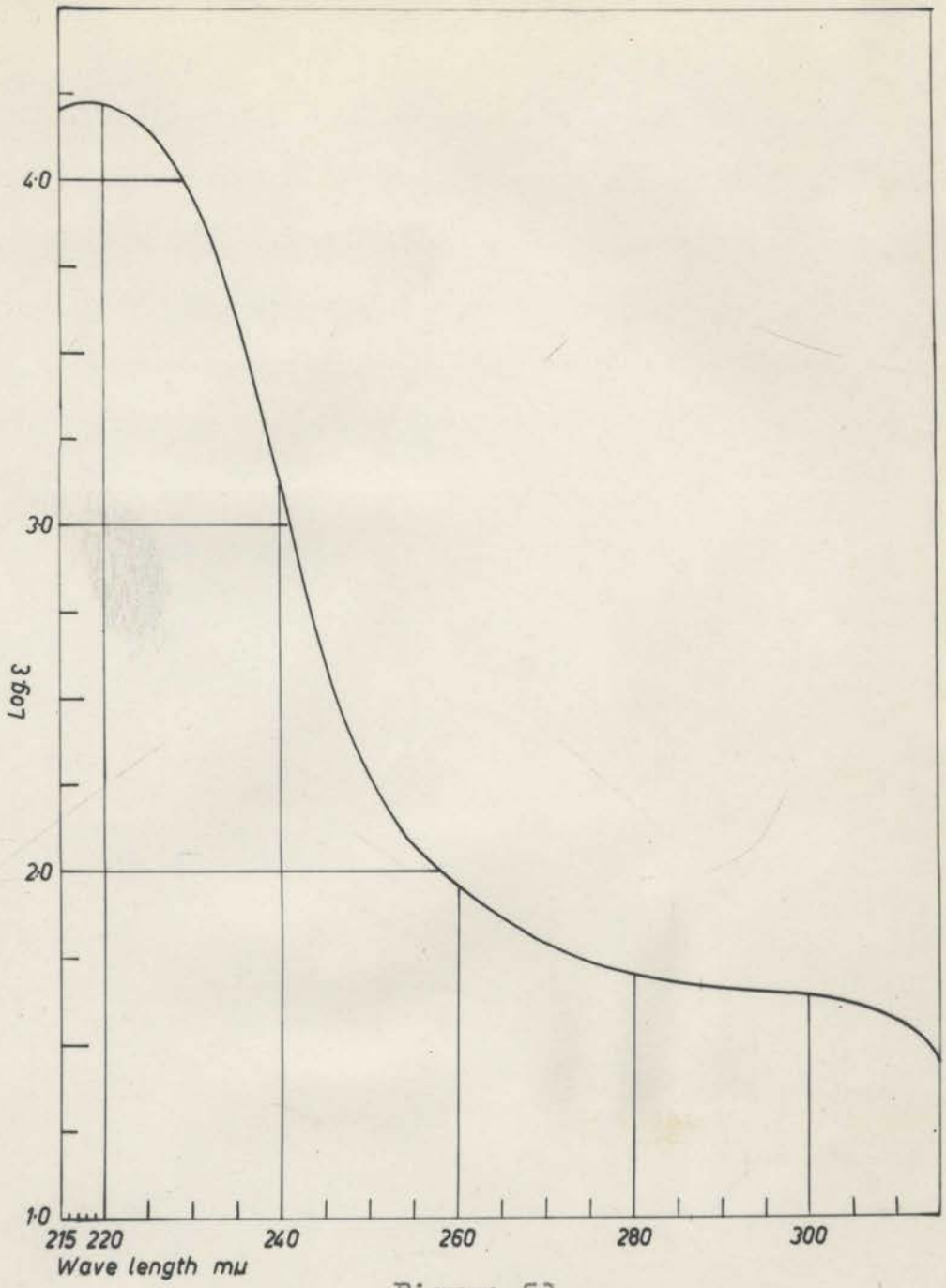


Figure 53

Ultra-violet absorption spectrum

Anhydroafrogenol ($C_{23}H_{34}O_6$)

λ max. = 218 mμ, log ε = 4.23;

inflexion λ = 293 mμ, log ε = 1.65

The results of these analyses indicate that the aldehyde group of afroside has been reduced by sodium borohydride to the primary alcohol which does not oxidise and form a lactone with the γ-hydroxy group during acid hydrolysis.

The presence of a saturated γ-lactone in the structure of an α-anhydro cardiac genin may be accommodated in only a limited number of positions which are indicated in Figure 54.

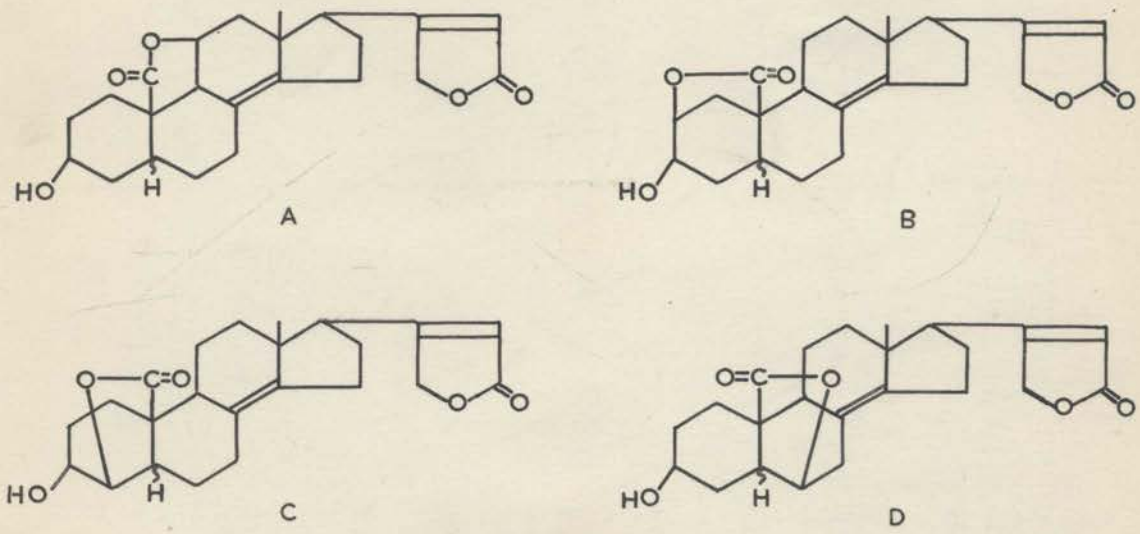


Figure 54

Of these, structures B and C necessitate the presence of an α-glycol grouping in the structure of anhydroafrogenol. These two structures can be eliminated therefore, as anhydroafrogenol is not oxidised by potassium periodate. Structure A is favoured, as there are no cardiac genins of known structure with a hydroxyl group at C_6, which is necessary for the structure of substance D (Fig. 54).

Furthermore the C_6-β hydroxyl group is not easily esterified, (Reich and Lardon, 1946) and its axial conformation would favour the formation of the lactone with the carboxylic acid at C_{10}. Conversely the C_6-α (equatorial) hydroxyl group would be easily esterified, but it would not form a lactone with the C_{10} carboxylic acid group as readily as the C_6β hydroxyl group. Therefore the proposed structures for α-anhydroafrogenin and anhydroafrogenol could be those of Figures 55 and 56, but the structures in Figures 57 and 58 are favoured by analogy with cardiac genins of known constitution.

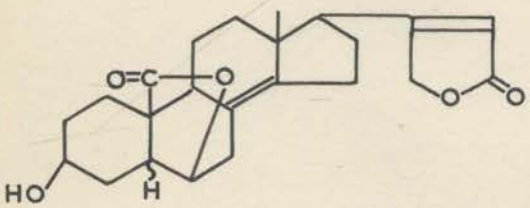


Figure 55

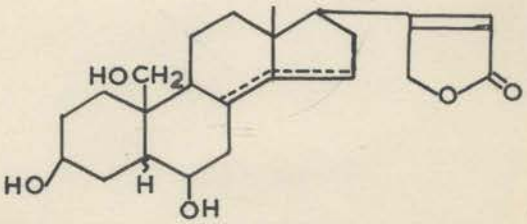


Figure 56

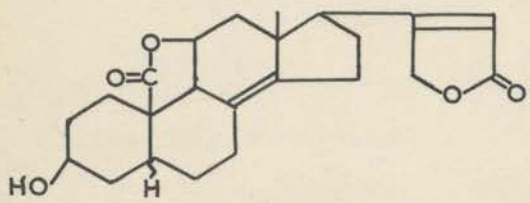


Figure 57

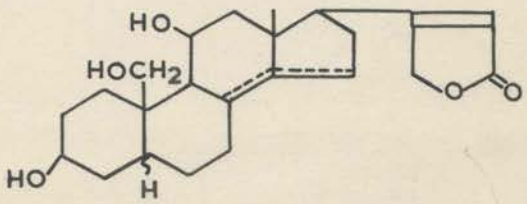


Figure 58

Anhydroafrogenol, on acetylation gave a product which could not be crystallised, but analysed for a formula $C_{27}H_{38}O_8$, which indicates the presence of two acetyl groups (acetyl determination:

found 14.4%, calc. for 2-OAc 15.4%). If the hydroxyl group which is in the γ-position with respect to the aldehyde group at C_{10} is at C_{11}, and is β-orientated it would not esterify under the conditions used for the acetylation (acetic anhydride in pyridine). Furthermore, the β-orientation of the hydroxyl group in this position would be the more favoured for the formation of the γ-lactone ring, as the orientation of the aldehyde group at C_{10} is also β. The model of this compound is such that the C_{11} α-hydroxyl group could also form a γ-lactone with a carboxylic acid group at C_{10}, but it would esterify under the conditions of acetylation.

The formation of a diacetyl compound from anhydroafrogenol may be accounted for by the esterification of the hydroxyl groups at C_3 and C_{19}.

Ehrenstein and Neumann (1951) in their investigation of the dehydration products of 3β, 5, 14, 19-tetrahydroxyl-14 iso etianic acid (obtained from the degradation of strophanthidol) isolated a compound which would form only a monoacetate, and which did not give a positive tetranitromethane reaction. The ethyl ester of this compound was designated as ethyl 3β,5-dihydroxy-8,19-oxido etianate (Fig. 59).

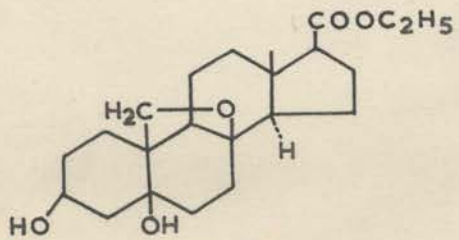


Figure 59

Hunger and Reichstein have proposed a similar structure for α-anhydrocoroglaucigenin (Fig. 60).

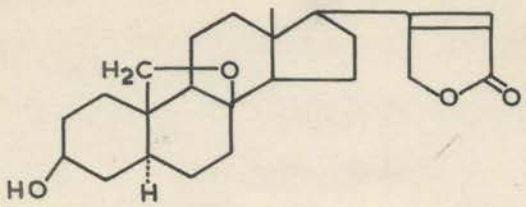


Figure 60

As anhydroafrogenol forms a diacetate and gives a positive tetranitromethane reaction, it is unlikely that it contains the tetrahydrofuran ring which is present in these compounds. Similarly, the analyses which have been obtained for anhydroafrogenol and acetyl-anhydroafrogenol could not be accommodated in formulae which would contain only four and five oxygen atoms.

The proposed C_{11}-β hydroxyl group of anhydroafrogenol could be easily eliminated by dehydration (11β-OH/9 α-H), and the double bond formed in this reaction would give a positive tetranitromethane test. However, the $\Delta^{9:11}$ bond would be expected to hydrogenate in acid solution in the presence of platinum oxide catalyst. Acetyl-anhydroafrogenol, on hydrogenation in alcohol containing 2% hydrochloric acid and 10% platinum oxide, gave a compound which showed a positive tetranitromethane reaction, but a negative Raymond test, indicating that the nuclear double bond is resistant to hydrogenation. A $\Delta^{14:15}$ bond would also be expected to be saturated under these conditions as β-anhydrocardiac genins can be fully hydrogenated in neutral solution with platinum oxide

as catalyst. Thus it appears that the anhydroafrogenol which has been obtained from the hydrolysis of afrosidol is the α-isomer (Fig. 61).

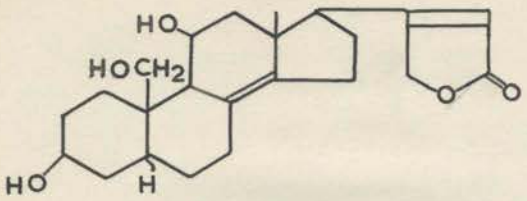


Figure 61

Acetyl anhydroafrogenol is not stable to oxidation by chromium trioxide in acetic acid. An attempted oxidation of this compound by the chromium trioxide - pyridine complex in pyridine, which as indicated previously, does not oxidise unsaturated linkages, gave only a few milligrams of a neutral fraction which has not been identified. Whilst not conclusive evidence, this reaction would tend to support the presence of an unesterified hydroxyl group.

On the basis of these deductions it is possible to draw some conclusions regarding the constitution of the glycosides of afroside. Afroside, $C_{29}H_{42}O_9$, consists of two isomeric glycosides which may exist as a mixture of the two forms shown in Figures 62 and 63.

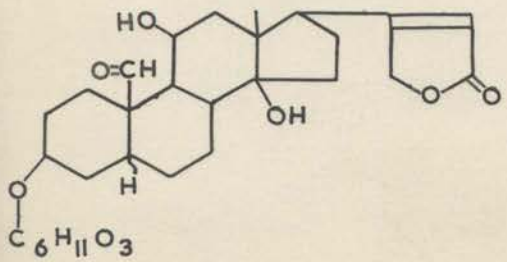


Figure 62

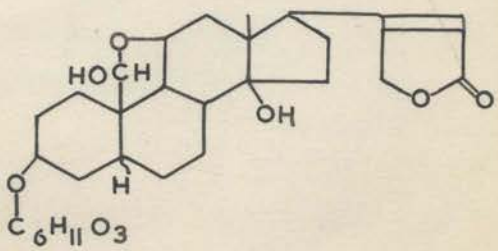


Figure 63

It has not been demonstrated conclusively that these two compounds exist in equilibrium, but some of the reactions which have been carried out on afroside, indicate that the two forms may be converted readily from one to the other. This type of equilibrium is considered by Reichstein (loc.cit) to exist in solution between corotoxigenin and the 19→ 3 cyclic hemiacetal forms (Fig. 64).

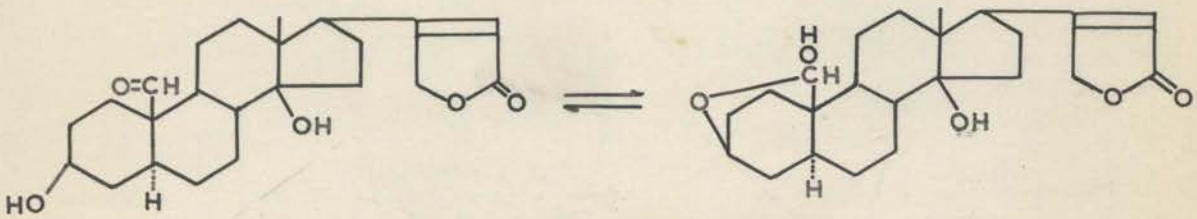


Figure 64

Also, Jacobs and Gustus (1927) demonstrated that strophanthidin under the influence of acid, isomerises to pseudostrophanthidin (Fig. 65).

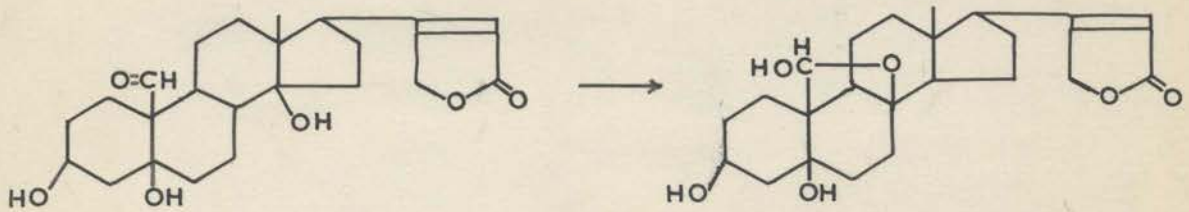


Figure 65

Similarly, aldosterone, the mineral corticoid hormone isolated from the adrenal cortex (Simpson et al, 1953; Mattox et al, 1953), has been shown by Schindler and Reichstein (1954b) to exist in equilibrium between the C_{18} aldehyde and the C_{18}→ C_{11} cyclic

hemiacetal forms (Fig. 66).

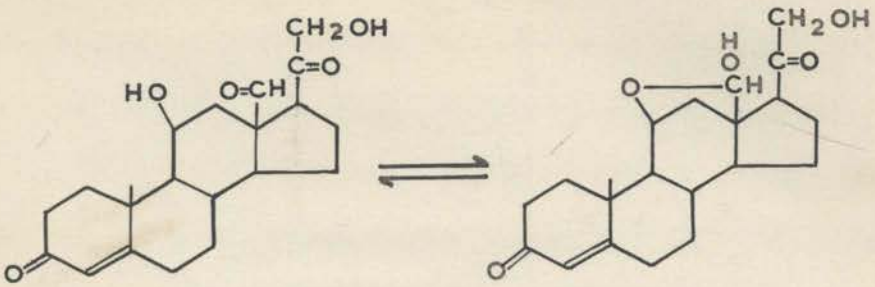


Figure 66

When afroside is treated with hydrochloric acid in acetone, the sole product is afroside B (see p. 87), which has an ultra-violet absorption spectrum consistent with that of an hydroxyl functional group at C_{19} rather than a carbonyl group. [Also, the spontaneous disappearance of the absorption maximum at 292 mμ in the ultra-violet absorption spectrum of afroside can be interpreted as the formation of the cyclic hemiacetal form, rather that oxidation to the acid, as stored solutions of afroside do not show acidic reactions.]

Acetylation of afroside results in the production of an homogeneous triacetate (acetyl-afroside B) which also shows no evidence of carbonyl absorption in the ultra-violet spectrum (see Fig. 38, p. 85). The hydroxyl group at C_{19} in the cyclic hemiacetal form of afroside (afroside B, Fig. 63, p.111) would acetylate under the conditions of acetylation. Reduction of afroside by sodium borohydride produces only afrosidol (see p.82).

The fact that in each of these reactions only one compound is obtained, indicates that there is either an equilibrium existing between the two compounds of afroside, or that one form is easily converted to the other.

The reactions which have been carried out, and the conclusions which have been drawn from them are interpreted in structural form in Figure 67 (p.115). Although these structures have not been proven beyond doubt it is suggested that they represent a reasonable interpretation of the experimental work.

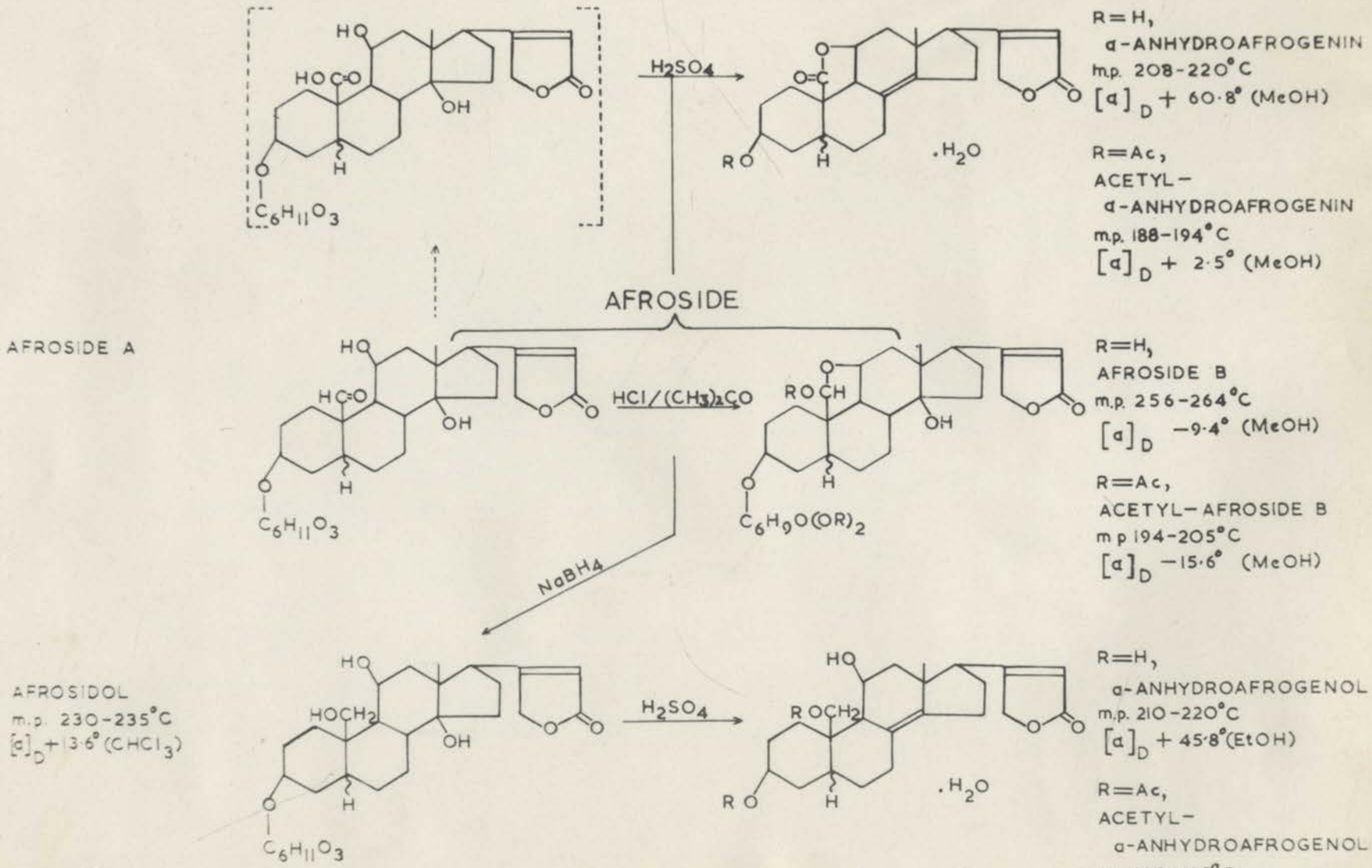

AFROSIDE
AFROSIDE A
H_2SO_4
$HCl/(CH_3)_2CO$
$NaBH_4$
H_2SO_4
$C_6H_{11}O_3$
$C_6H_9O(OR)_2$
$.H_2O$
R=H,
α-ANHYDROAFROGENIN
m.p. 208-220°C
$[\alpha]_D$ + 60·8° (MeOH)
R=Ac,
ACETYL-
α-ANHYDROAFROGENIN
m.p. 188-194°C
$[\alpha]_D$ + 2·5° (MeOH)
R=H,
AFROSIDE B
m.p. 256-264°C
$[\alpha]_D$ −9·4° (MeOH)
R=Ac,
ACETYL-AFROSIDE B
m p 194-205°C
$[\alpha]_D$ −15·6° (MeOH)
AFROSIDOL
m.p. 230-235°C
$[\alpha]_D$ +13·6° ($CHCl_3$)
R=H,
α-ANHYDROAFROGENOL
m.p. 210-220°C
$[\alpha]_D$ + 45·8° (EtOH)
R=Ac,
ACETYL-
α-ANHYDROAFROGENOL
m.p. 160-165°C

Figure 67

A PRELIMINARY INVESTIGATION OF THE CARDIAC GLYCOSIDES OF CERBERA FLORIBUNDA, CERBERA DILATATA, AND CERBERA MANGHAS

A number of plants of the Cerbera (Tanghinia) species has been investigated by other workers (Matsubara, 1937, Frerejacque, 1945, 1948; Helfenberger and Reichstein, 1948, 1952; Sigg, Tamm and Reichstein, 1955), but the three varieties which have been used in this investigation are not among those previously examined. Recently Reichstein and co-workers (Helfenberger and Reichstein, loc. cit.; Sigg, Tamm, and Reichstein, loc. cit.) have isolated from Tanghinia venenifera Poir. (Cerbera tanghinia Hook.),the glycosides tanghinin, desacetyl-tanghinin, acetyl-neriifolin (=cerberin), and tanghiferin. The structure of tanghiferin is unknown, but acetyl neriifolin has digitoxigenin as the aglycone and acetyl-thevetose as the carbohydrate moiety. Tanghinin and desacetyl tanghinin have the partially characterised genin, tanghinigenin (Fig. 68) as the steroid portion of the molecule and acetyl-L-thevetose and L-thevetose respectively, as the carbohydrate constituents. Helfenberger and Reichstein (1952) have suggested that tanghinigenin may contain the extra oxygen atom present in the nucleus in an oxide ring.

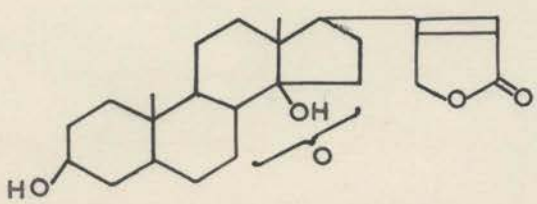


Figure 68

As the pericarp of the Cerbera fruit did not contain detectable amounts of cardiac glycosides it was necessary to use mature ripe fruit in which the kernels were of maximum size. In immature fruit the kernel is not properly formed and often in over-ripe specimens the kernels have begun to decompose. The fresh ripe fruits of these plants were obtained from Northern Queensland. Although the glycoside content of the kernels of these fruits is sometimes as high as 10% of the weight of the kernel, this represents only about 0.05% of the weight of the fruit. Only small quantities of the fruits were available for investigation.

The kernels were removed from the fruit, mixed with kieselguhr in a mortar and ground to a powder. This powder was packed into soxhlet thimbles and extracted, firstly with petroleum ether to remove the oil (20% of the kernels by weight) and then with aqueous ethanol. Evaporation of the ethanol gave an oil which was shown in each case to be a complex mixture of cardiac glycosides.

Cerbera floribunda

The petroleum ether extraction of the fresh kernels gave approximately 20% by weight of a yellow oil, which has not been investigated. Evaporation of the alcohol extract gave 8.8% of a mixture of cardiac glycosides. This mixture was shown by paper chromatography (on formamide impregnated paper) to consist of two compounds which have been designated as F1 and F2.

F1, by the distance which it has moved on the paper chromatograms (Fig. 69, p.118) appears to be a highly polar

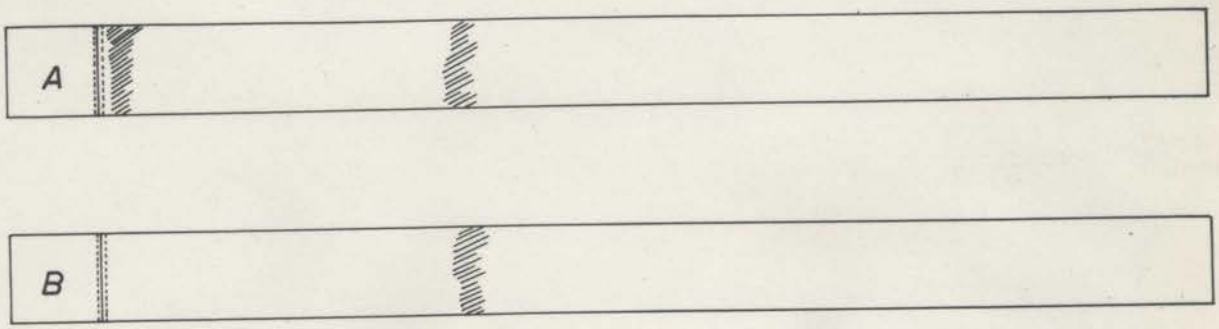


Figure 69

Chromatograms on formamide impregnated paper.

Developing solvent, $CHCl_3$: C_6H_6 : BuOH, 78:12:5

A = Cerbera floribunda glycosides (F1+F2)

B = F_2

compound. F1 is insoluble in chloroform, and may be separated from F2 by extraction of the mixture with chloroform. An acetone solution of F1 after a period of three months, deposited a few crystals which had a melting point of 188-190°C. However, when F1 was left in contact with the air it became a brown tarry mass.

F2 separated from the mixture by chloroform extraction and when recrystallised, analysed for a formula $C_{32}H_{46-48}O_{10}$. Acetyl and methoxyl determinations indicated the presence of one acetyl and one methoxyl group. The ultra-violet spectrum of F2 (Fig. 70. p.120) shows the typical absorption maximum of the butenolide ring at 217 mμ ($\log \varepsilon = 4.22$). F2 gives positive Raymond and Legal reactions, but a negative Keller-Kiliani reaction. The test for carbohydrate (p.143) indicates that this compound is a glycoside.

Acetylation of F2 gave a compound which analysed for a formula $C_{34}H_{48-50}O_{11}$. By analysis, this compound was shown to contain two acetyl groups and one methoxyl group. This substance is referred to as acetyl F2. A crystalline benzoate of F2 could not be obtained.

F2 could not be hydrolysed by the method of Mannich and Siewert (1942), but sulphuric acid hydrolysis gave a crystalline genin which analysed for a formula $C_{23}H_{34}O_5$. Acetylation of this genin gave a yellow oil which could not be crystallised even after chromatography on alumina.

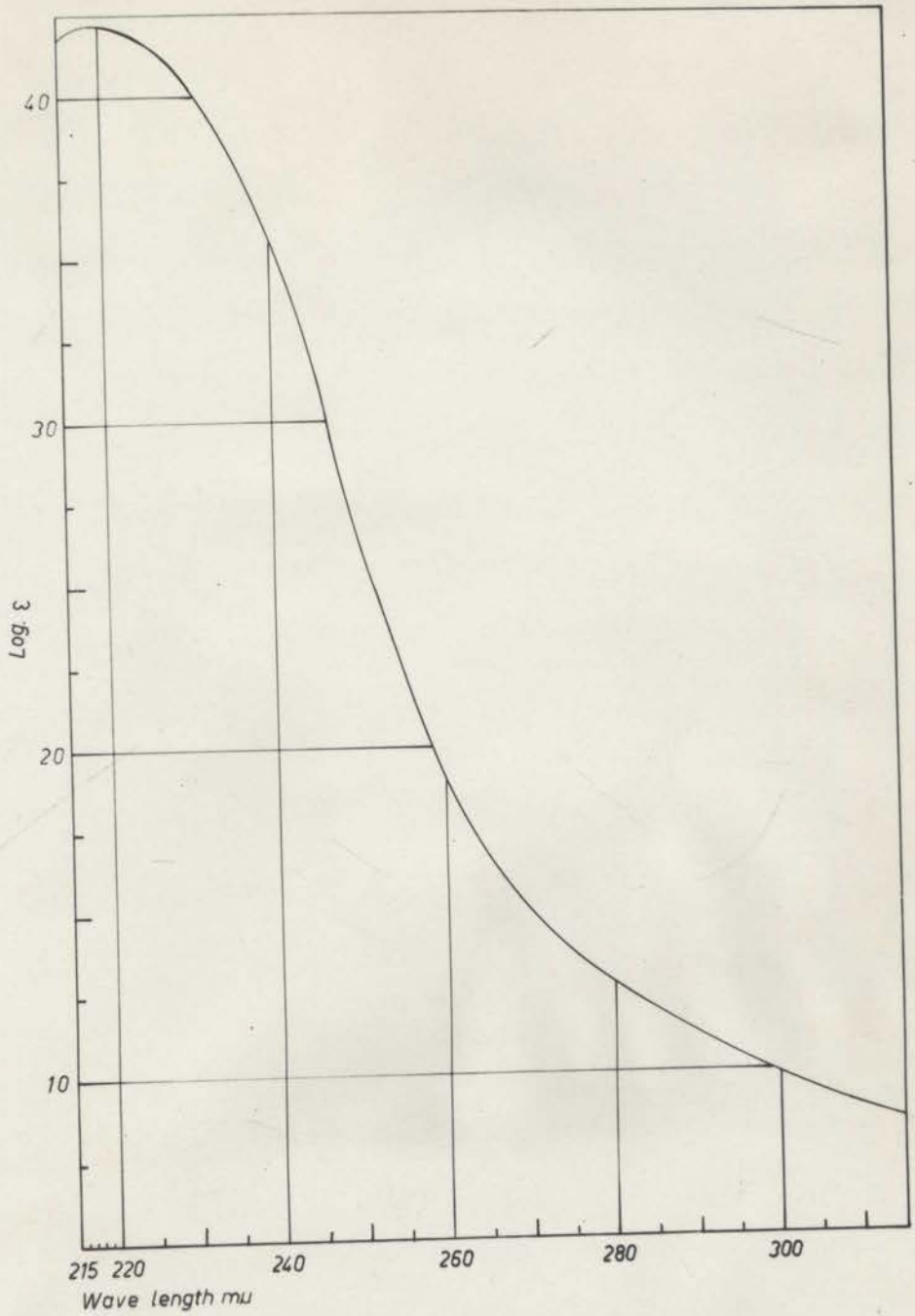


Figure 70

Ultra-violet absorption spectrum.

F2, λ max. 218 mμ, log ε = 4.22

F2, acetyl-F2, and F2-genin are isomeric with tanghinin, acetyl-tanghinin, and tanghinigenin (and 3-epitanghinigenin) respectively, which have been isolated from the seeds of Tanghinia venenifera Poir (=Cerbera tanghinia Hook.) by Sigg, Tamm, and Reichstein (1955). However, the physical constants of the compounds of Cerbera floribunda do not agree with those of tanghinin and its derivatives. These constants are shown in the following table:

Substance	Formula	m.p.°C.	$[\alpha]_D$
Tanghinin	$C_{32}H_{45-48}O_{10}$	128	-81 (methanol)
F2	$C_{32}H_{46-48}O_{10}$	215	-48 (ethanol)
Acetyltanghinin	$C_{34}H_{48-50}O_{11}$	189	-88 (ethanol)
Acetyl-F2	$C_{34}H_{48-50}O_{11}$	134	-51 (ethanol)
Tanghinigenin	$C_{23}H_{32-34}O_5$	187	+14.1 (chloroform)
3-epitanghinigenin	$C_{23}H_{32-34}O_5$	230-235	+28.2 (chloroform)
F2-genin	$C_{23}H_{32-34}O_5$	255	+20.6 (ethanol)

F2-genin has a similar specific rotation to both tanghinigenin and 3-epitanghinigenin (allowing for solvent differences), but the melting point is higher than that of either of these compounds. The melting point of 187°C for tanghinigenin was determined on a sample obtained as colourless prisms from an acetone petroleum ether mixture, and that of 3-epitanghinigenin was determined on material obtained as colourless prisms from acetone (m.p. 230-235°C) (Sigg, Tamm, and Reichstin,1955).

F2-genin was crystallised in the form of colourless needles from ethanol - water (8:1). However, in order to show that one of the isomers of tanghinigenin is identical with F2-genin it would be necessary to compare the infrared spectra of the two compounds. Unfortunately samples of the isomeric tanghinigenins were not available for comparison with F2-genin.

Acetyl-F2 could be separated from acetyl-tanghinin (a sample of which was kindly supplied by Professor T. Reichstein) by paper chromatography using the reversed phase system of Tschesche (with solvent system No. T1, p.138), but not on formamide impregnated paper (using the mixture chloroform: benzene:n.butanol, 78:12:5, as the developing solvent). The Rf values of acetyl-F2 and acetyl-tanghinin on the reversed phase paper chromatograms were $R_f^{24^\circ}$ 0.14 and $R_f^{24^\circ}$ 0.09 respectively (Fig. 73 ,p.128)

The infrared absorption spectra of acetyl-F2 and acetyl-tanghinin (in nujol) are shown in Figure 71 (p. 123). As these spectra are different in their absorption characteristics between 8 and 15 μ (1250-666 cm^{-1}) they cannot have the same chemical constitution. Thus, F2 is a compound which is isomeric with tanghinin but is not identical with that compound.

Summary: Two glycosides were obtained by extraction of the fresh kernels of Cerbera floribunda fruit with 70% ethanol. These glycosides were separated by extraction of the mixture with chloroform. F1, the chloroform insoluble glycoside, is

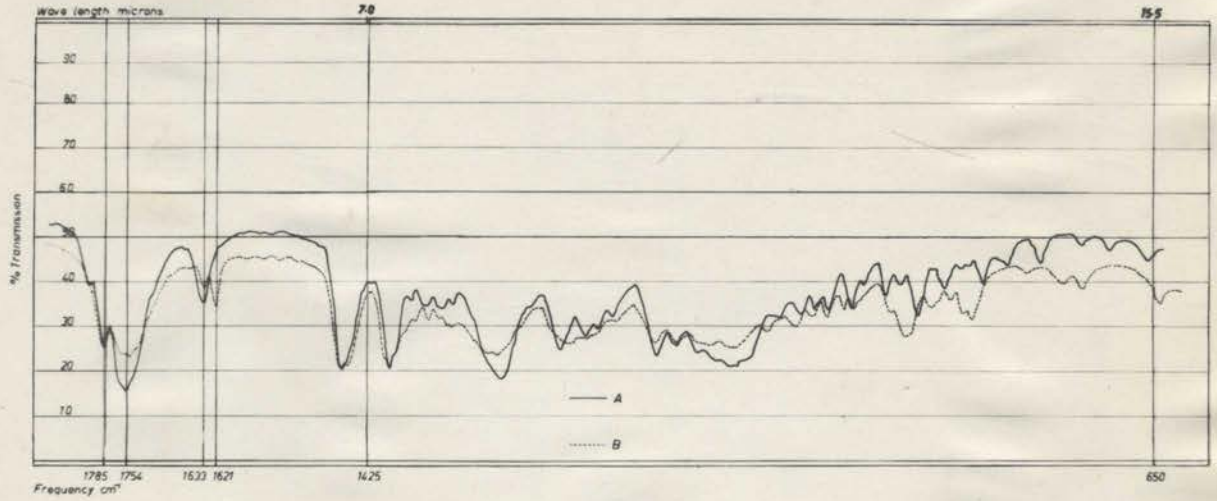


Figure 71

Infrared absorption spectra determined in nujol - NaCl prism

A = Acetyl-F2

B = Acetyltanghinin

a highly polar compound which is unstable when exposed to the atmosphere. This compound could not be obtained in a state sufficiently pure for analyses.

F2, the chloroform soluble glycoside is isomeric with tanghinin ($C_{32}H_{46-48}O_{10}$) and forms a diacetate. F2-genin obtained by sulphuric acid hydrolysis of F2, is isomeric with tanghinigenin and 3-epitanghinigenin ($C_{23}H_{34}O_5$), but the physical constants of F2-genin are not identical with those of tanghinigenin or 3-epitanghinigenin.

CERBERA DILATATA

The fresh kernels of the fruit were extracted with petroleum ether (b.p. 30-90°C.) and 70% ethanol - water, in the manner described previously (p. 117). From these kernels approximately 20% of oil (petroleum ether extract) and 5.3% of glycosides (ethanol extract) were obtained.

The crystalline mixture of glycosides was shown by chromatography on formamide impregnated paper to consist of four constituents (Fig. 72, p.127). This mixture was dissolved in chloroform - benzene (1:1) and chromatographed on alumina (see p. 183). Fractions 2 - 10 from the alumina column were shown to consist of one compound, which is referred to as D2, and fractions 13 - 24 were found to contain a mixture of three glycosides (Fig. 73, p.128).

D2 analysed for a formula $C_{32}H_{46-48}O_{10}$ and was shown by analyses to contain one acetyl and one methoxyl group. The physical constants were identical with those of F2, and these two compounds could not be separated by paper chromatography on either formamide impregnated paper, or on the reversed phase system. Acetylation of D2 gave a crystalline compound which analysed for a formula $C_{34}H_{48-50}O_{11}$ and was shown to contain one methoxyl and two acetyl groups. Paper chromatography [using the formamide and reversed phase systems with solvent systems No 2 (p.136) and No T1 (p. 138) respectively], mixed melting point, and optical rotation, showed that this compound was

identical with acetyl-F2. The mixture of glycosides contained in fractions 15-20 from the alumina column consisted of three compounds, one of which was identified as monoacetylneriifolin by reversed phase chromatography using solvent system No T1 (see Fig. 73, p.128).

The compounds D3 and D4 were shown by paper chromatography (see Fig. 71 p.128) to be different from D2, desacetyl-tanghinin, and acetyltanghinin, but have not been identified.

Thus, fresh kernels of Cerbera dilatata which were investigated, have been shown to contain acetylneriifolin and three other glycosides (D2, D3, and D4),of which D2 has been identified with the glycoside F2 obtained from Cerbera floribunda.

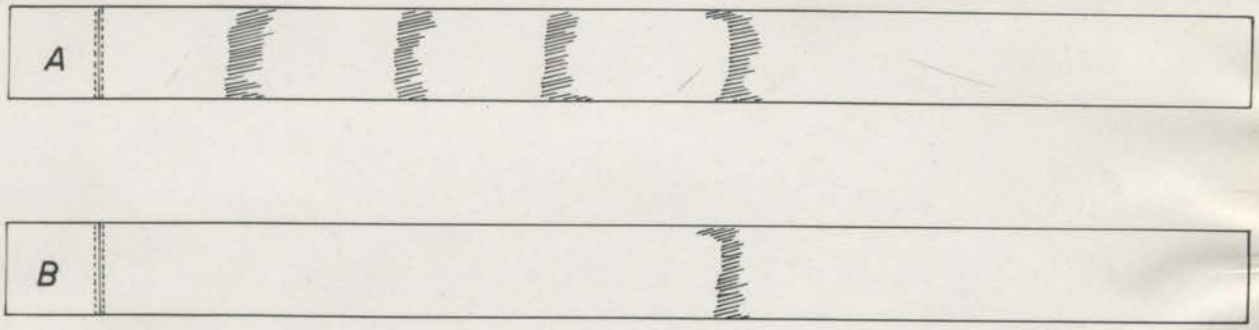


Figure 72

Chromatograms on formamide impregnated paper.

Developing solvent, $CHCl_3$: C_6H_6 : BuOH, 78:12:5.

A = Cerbera dilatata glycosides

B = D2

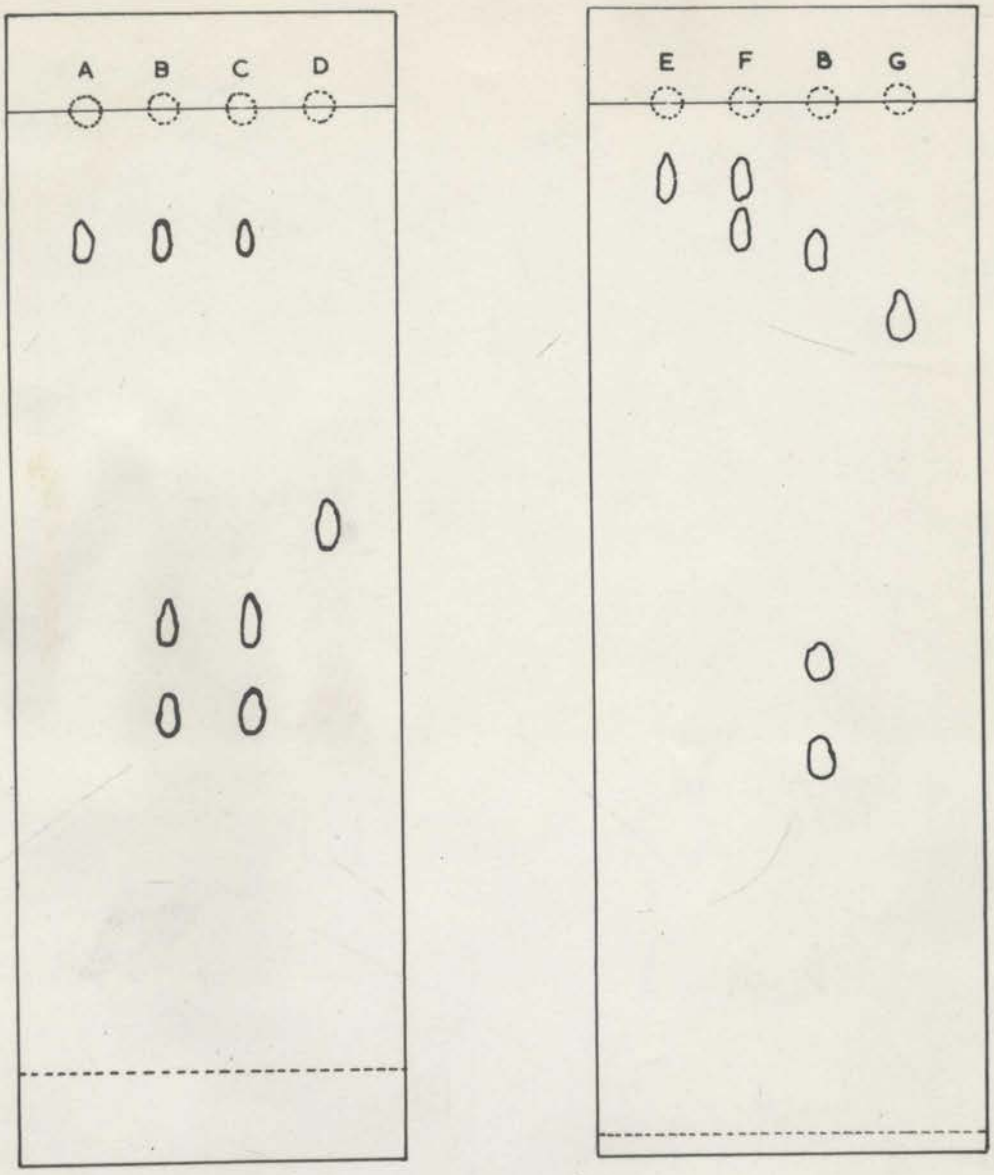


Figure 73

Paper chromatograms using the reversed phase system No.T1 (p.138)

A = Acetyl neriifolin,, Rf = 0.15

B = Cerbera dilatata glycosides (fractions 13-24),
Rf = 0.15, 0.55, 0.65

C = A + B

D = Desacetyl tanghinin, Rf = 0.45

E = Acetyl tanghinin, Rf = 0.09

F = Acetyl-F2, Rf = 0.14

G = F2, Rf = 0.25

CERBERA MANGHAS

Extraction of the fresh kernels of Cerbera manghas fruit by the method outlined previously (p.117) gave approximately 14% of oil, and 10% of a semi-crystalline mixture of glycosides.

The mixture of cardiac glycosides was shown to consist of two components by chromatography on formamide impregnated paper (Fig. 74, p.130). These glycosides were separated by extraction of the mixture with dry chloroform, the less polar compound being obtained from the chloroform solution. The chloroform insoluble compound is referred to as M1, and the chloroform soluble substance as M2.

M1 could not be crystallised even after chromatography on alumina, and the method of trituration of the compound with alumina used by Chen and Steldt (1942) for the purification of cerberoside was tried without success. This substance appears to be the same as F1, as it could not be separated from F1 on paper chromatograms (Fig. 75, p.131), and on exposure to the atmosphere it degenerates from a white powder to a brown sticky mass.

M2, the chloroform soluble constituent of the mixture, was obtained in insufficient quantity for analyses or constant determinations, but it appears to be identical with F2 and D2. Paper chromatography of M2 with F2 and D2 on the formamide and reversed phase systems would not separate these three compounds (Fig. 75, p.131). Thus the glycosides of the kernels of Cerbera manghas fruit which have been investigated, appear to be identical with those obtained from Cerbera floribunda.

The results of this preliminary investigation of the cardiac glycosides of Cerbera floribunda, Cerbera dilatata, and Cerbera manghas, are summarised in Figure 76 (p.132). Cerbera floribunda and Cerbera manghas appear to contain the same two constituents, whereas Cerbera dilatata contains four glycosides, one of which is identical with one of the compounds from Cerbera floribunda and Cerbera manghas. Of the other three glycosides of Cerbera dilatata, one has been identified as acetyl-neriifolin, and the remaining two compounds have not been identified.

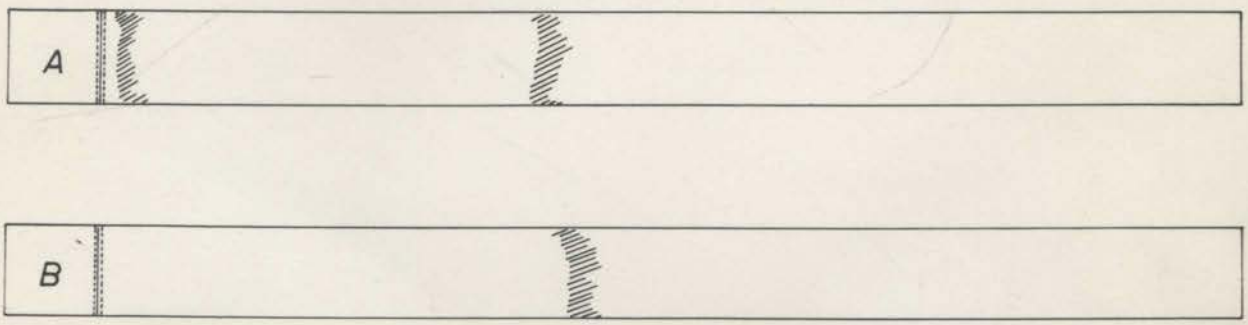


Figure 74

Chromatograms on formamide impregnated paper.

Developing solvent, $CHCl_3$: C_6H_6 ; 9:1

A = Glycosides of Cerbera manghas

B = M2

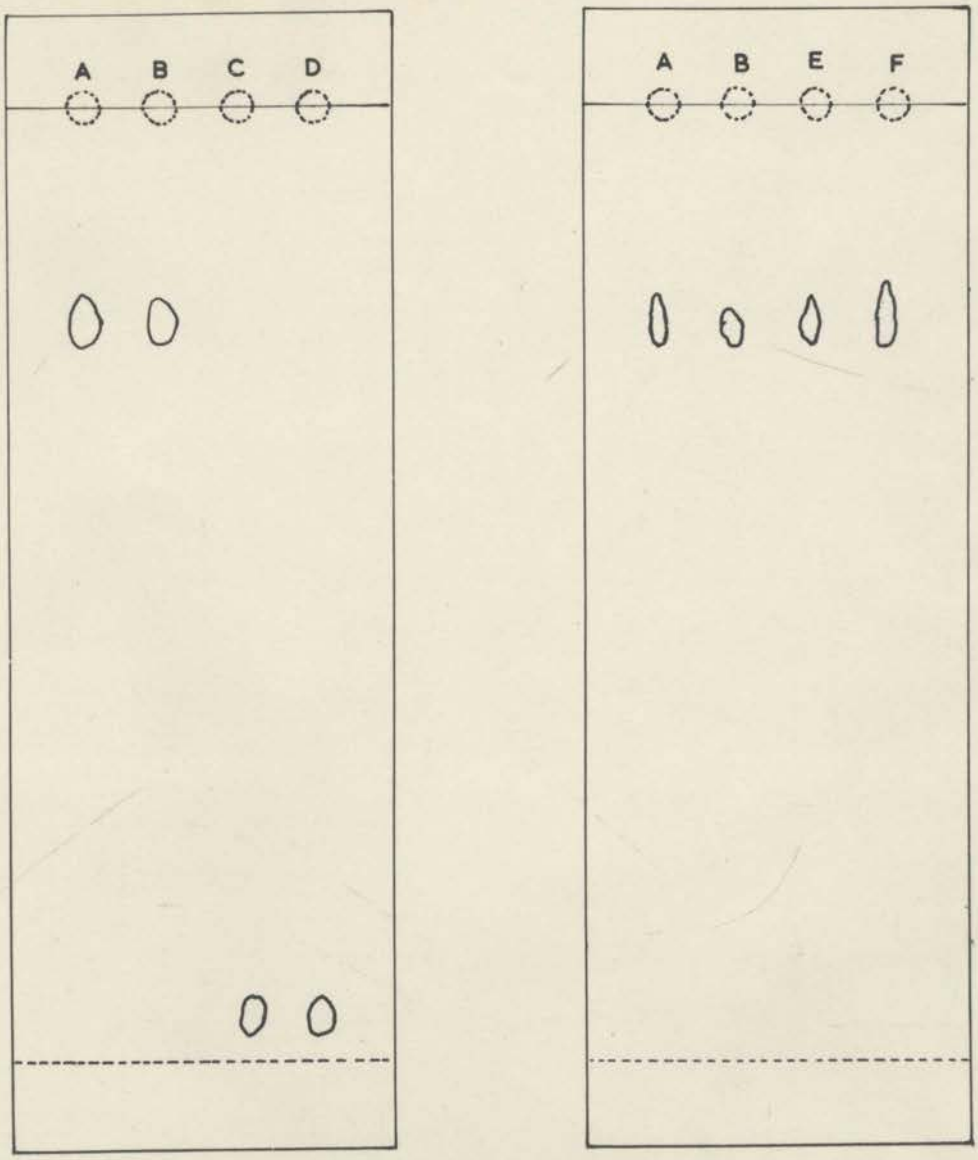


Figure 75

Paper chromatograms using the reversed phase system No.T1 (p.138)

A = F2, Rf = 0.25
B = D2, Rf = 0.25
C = M1, Rf = 0.975
D = F1, Rf, = 0.975
E = M2, Rf = 0.25
F = A + B + E

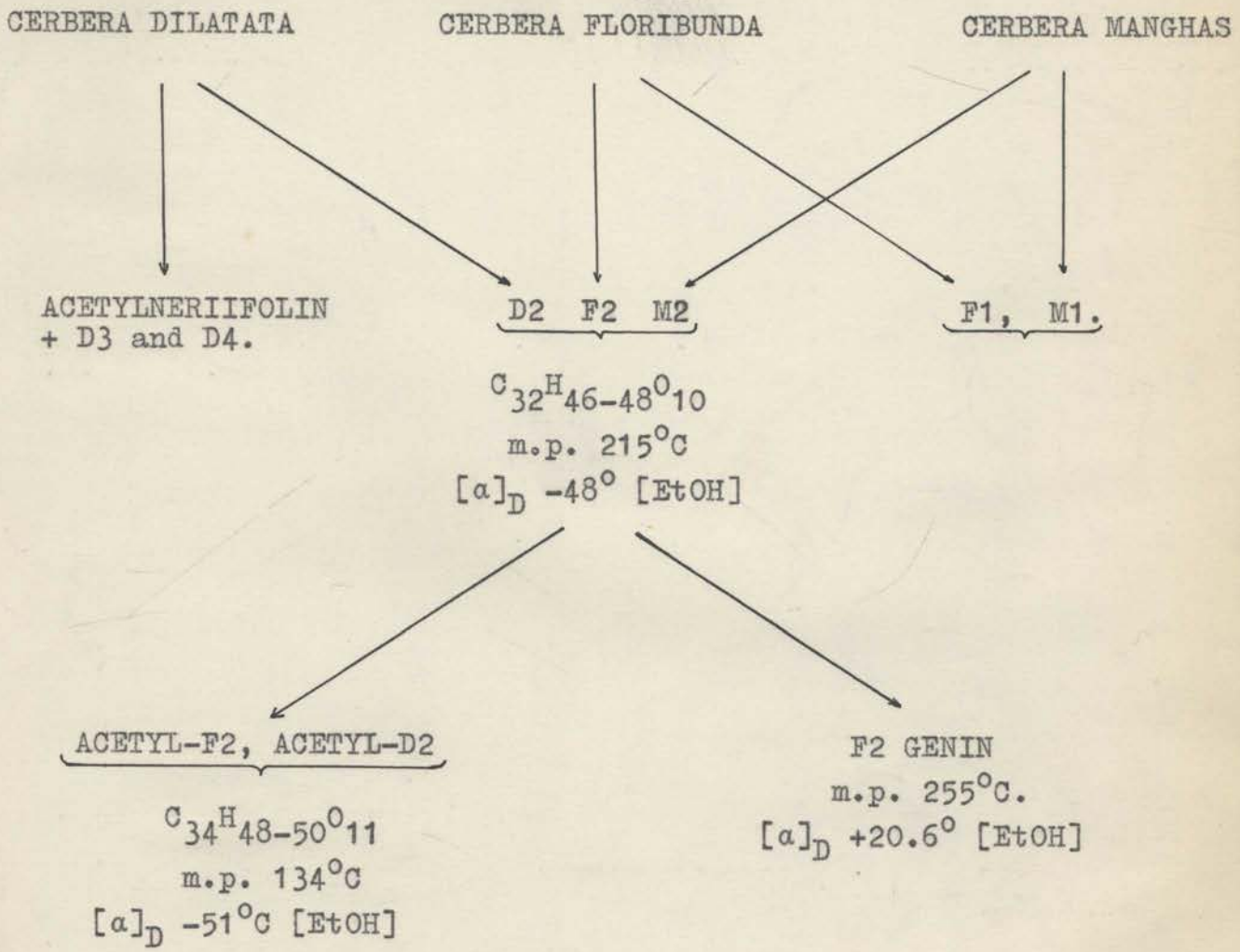

CERBERA DILATATA
CERBERA FLORIBUNDA
CERBERA MANGHAS
ACETYLNERIIFOLIN
+ D3 and D4.
D2 F2 M2
F1, M1.
$C_{32}H_{46-48}O_{10}$
m.p. 215°C
$[\alpha]_D$ -48° [EtOH]
ACETYL-F2, ACETYL-D2
$C_{34}H_{48-50}O_{11}$
m.p. 134°C
$[\alpha]_D$ -51°C [EtOH]
F2 GENIN
m.p. 255°C.
$[\alpha]_D$ +20.6° [EtOH]

Figure 76

PART IV

EXPERIMENTAL

GOMPHOSIDE, AFROSIDE

SOLVENTS

All solvents used for column and paper chromatography were dried and fractionated before use. Rectified spirit which had been refluxed over potassium hydroxide and then fractionated until its percentage transmission at the wave length of 220 mμ was 50-60% of that of water at the same wave length, was used for all measurements of ultra-violet absorption spectra.

CHROMATOGRAPHY MATERIALS

Alumina for column chromatography was neutralised and reactivated to activity II-III (Brockman and Schodder, 1941), by the method outlined by Reichstein and Shoppee (1949).

The paper used in the paper chromatography experiments was Whatman No. 4 or Schleicher-Schull 204 a - both papers being of approximately the same quality.

PHYSICAL MEASUREMENTS

Melting points were done on a hot stage mounted on a microscope, and are uncorrected.

Optical rotation measurements were determined in a 1 decimeter micropolarimeter tube.

Ultra-violet absorption spectra were measured on a Unicam Quartz Spectrophotometer model S.P. 500.

Infrared absorption spectra were recorded by Dr A.R.H. Cole of the University of Western Australia, and by Timbrol Pty. Ltd., of New South Wales. The instruments were Perkin-Elmer double beam models.

Elemental analyses were carried out by Dr K.W. Zimmerman, C.S.I.R.O. Micro Analytical Laboratory, Melbourne, Victoria.

COLOUR REACTIONS

The following colour reactions have been used throughout this work.

Raymond Reaction

For details see page 14. This reaction is specific for the $\Delta^{\alpha\beta}$-γ-lactone ring in the cardiac aglycones. The corresponding butanolides do not give a colour with this reagent. Consequently this reagent is most useful for following the course of the glycosides through extraction procedures, chromatography columns, and reactions such as isomerisation of the glycosides and genins to the corresponding iso compounds, and other reactions involving the butenolide side chain.

Legal Reaction

The red colour produced in this reaction is characteristic of all naturally occurring cardiac glycosides and aglycones. 1 - 2 mg. of the compound to be tested are dissolved in 1 - 2 ml. of pyridine and 1 - 2 mg. of sodium nitroprusside are dissolved in the same solution. The same volume of 10% aqueous solution of sodium hydroxide is added to the pyridine solution. A positive reaction is shown by the pyridine layer developing a bright red colour.

Keller-Kiliani Reaction

This reaction is specific for digitoxose or other 2-desoxy sugars. As those glycosides which contain a 2-desoxy sugar linked directly to the aglycone are easily hydrolysed (see page 62) this test can be used for determining the conditions under which the glycoside may be hydrolysed. 2 - 5 mg. of the glycoside are dissolved in 0.5 ml. of acetic acid and 1 - 2 drops of a 2% aqueous solution of ferric chloride added. This solution is underlayed with concentrated sulphuric acid. A positive reaction is shown by the formation of a blue or green ring at the interface of the two layers.

Tetranitromethane Reaction

This reagent is used to detect the presence of olefinic bonds in the nucleus of the cardiac genins. The double bond of the butenolide ring does not give a reaction with this reagent. 1 - 2 mg. of the substance to be tested are dissolved in 1 - 2 drops of chloroform and 2 drops of tetranitromethane added. A blank reaction, consisting of chloroform without the compound to be tested is carried out at the same time. A positive reaction is shown by the development of a bright yellow colour in the test solution, the blank remaining colourless.

Concentrated Sulphuric Acid

This test is used as a qualitative test to distinguish between two or more similar compounds. Individual compounds follow a definite series of colour changes when dissolved in concentrated or 80% sulphuric acid. Approximately 2 mg. of the substance to be tested are placed on a spotting plate and moistened with 1 - 2 drops of concentrated or 80% sulphuric acid. The colour changes are recorded at various time intervals.

PAPER CHROMATOGRAPHY

Systems using formamide impregnated paper

All the chromatography experiments on formamide impregnated paper were done by the horizontal method (Meredith and Sammons, 1952) at 33°C - this being the temperature of a biological incubator which was used as a convenient constant temperature chamber. A number of tanks (each 7½" x 12½" x 1½" containing 6 - 8, 3/4" wide paper strips) could be used in the incubator at one time. All chromatograms were done on paper strips 3/4" x 10" unless otherwise indicated.

Developing Solvents

Solvent System 1: Chloroform containing 10% benzene, saturated with formamide.

Solvent System 2: Chloroform : benzene : n.butanol, 78:12:5, saturated with formamide.

Preparation of Paper Strips

The paper strips were soaked in a solution of 50% formamide in methanol for 30 minutes. The strips were then taken out of this solution, spread out on filter paper and excess of the impregnating solvent removed by blotting with another sheet of paper. Solutions of the compounds to be chromatographed were then spotted on the starting line, a jet of warm air being used to dry off the solvent during spotting. The paper strips were then placed on a horizontal glass rack in the tank, and the end of the strips behind the starting line dipped into the developing solvent contained in the bottom of the tank. The tank was then covered with a plate glass lid, sealed with bentonite-glycerine paste, and placed in the incubator. The time of development was usually 16 hours, but occasionally shorter times were used to separate fast running compounds. It was found that by using 30% formamide in methanol for impregnation of the paper strips, the speed of movement of slow running compounds could be increased appreciably. Where these variations in time of development and amount of formamide used in the impregnation of the paper have been used, they will be indicated; otherwise the development time of 16 hours and impregnation of the paper with 50% formamide in methanol is implied.

Reversed Phase Systems (Tschesche, Grimmer, Seehofer, 1953).

The chromatography experiments using these solvents were done on sheets of Whatman No. 4 paper (4" x 12"), at room temperature by the downward method. The solvent mixtures were prepared by shaking the components together and allowing the mixture to equilibrate at room temperature for 24 hours. The organic phases of these solvent mixtures are used as the stationary phases, and the aqueous phases are the developing solvents.

Solvent System T1: Ethyl hexanol : pentanol : water : formamide, 6 : 2 : 8: 2.

Solvent System T2: Ethyl hexanol: pentanol : water : formamide, 6 : 2 : 1 : 4.

Method

The organic phase (less dense phase) is sprayed on to the paper and then excess solvent is removed by pressing the paper between sheets of filter paper. The substances to be chromatographed are applied as spots on the starting line and the chromatogram is developed with the aqueous phase. The development time is usually 3 - 4 hours. When these systems are used it is not necessary to equilibrate the chromatogram in the tank before development.

Location of the Developed Spots

The Raymond reaction (Raymond, 1938) was used to detect the position of the compounds after development on formamide

impregnated paper. The paper strips were removed from the chromatography tanks and allowed to dry at room temperature before the Raymond reaction was applied, (i.e. the developing solvent was allowed to evaporate, the formamide remaining). The strips were dipped in a 5% solution of metadinitrobenzene in benzene, allowed to dry, and then placed in 20% aqueous sodium hydroxide solution which had been spread on a white tile. Facsimiles of the chromatograms were made immediately as the blue colour produced by this reaction fades in 3 - 5 minutes.

For those compounds which were chromatographed on paper employing the reversed phase systems, after development the paper sheets were dried for 10 minutes at 100°C and then sprayed with a solution of 25% trichloracetic acid in chloroform. After being sprayed, the sheets were heated in an oven at 100-110°C for five minutes. The compounds were then located under ultra-violet light as blue fluorescent areas. The Raymond reaction was also used to locate the developed spots on reversed phase system chromatograms.

EXTRACTION OF GOMPHOCARPUS FRUTICOSUS

3.64 Kg. of dried powdered plant material were defatted with petroleum ether in a soxhlet apparatus, and then extracted with 50% chloroform-methanol solution for 20 hours. The chloroform-methanol extract (10 l.) was evaporated in a climbing film evaporator to a volume of 1.5 l., diluted to 2 l. with water, and then shaken out with 3 x 250 ml. portions

of carbon tetrachloride which removed appreciable quantities of chlorophyll and other pigment material. Extraction of the aqueous - methanolic extract with chloroform (4 x 250 ml.) brought the glycosides into the chloroform solution. Evaporation of the chloroform under reduced pressure left a tarry residue which was redissolved in methanol, diluted with water and allowed to stand. From this solution 3.03 g. of impure crystals of the mixed glycosides were obtained, which represents a yield of 0.084%.

During the chloroform extraction of the aqueous methanolic solution, 6.8 g. (equivalent to 1.87%) of Rutin separated at the interface. This pigment was identified by paper chromatography and colour reactions (cf. Casteel and Wender, 1953) of the glycoside and the derived génin (quercitin), and analyses and physical constants of these compounds.

A second extraction of the plant material was conducted using 2.5 Kg. of dried plant. This sample was defatted with petroleum ether and then extracted with 80% ethanol - water in a soxhlet apparatus for 26 hours. The alcoholic extract (10 l.) was evaporated under reduced pressure to 1.5 l. and then diluted to 3.0 l. with water. An aqueous solution of basic lead acetate was then added until no further precipitation occurred. The solution was filtered and excess lead acetate removed by precipitation with disodium phosphate solution (10%). After filtering, the extract was evaporated under reduced pressure to 2 l., and then extracted with chloroform in a

continuous liquid-liquid extractor. Evaporation of the chloroform left a tarry residue which was redissolved in methanol, diluted with water, and crystallised. The yield of mixed glycosides from this extraction was 1.86 g. which represents 0.074% of the plant material. It was found that the precipitation of the tannins etc., by the basic lead acetate removed a small amount of the cardiac glycosides. This could be recovered by treating the precipitate with dilute sulphuric acid, filtering, and then extracting the glycosides from the filtrate with chloroform.

As this difficulty was not encountered in the first method of extraction, it was used for the three subsequent extractions of the plant material. The total yield of crude glycosides obtained from the five extractions of plant material was 10.34 g.

SEPARATION OF THE CARDIAC GLYCOSIDES ON ALUMINA

1.2 g. of the mixed glycosides were dissolved in 200 ml. of dry chloroform and diluted to 400 ml. with benzene. This solution was applied to 36 gm. of alumina contained in a column 300 mm. long and 37 mm. internal diameter. The glycosides were eluted from the column with 400 ml. portions of solvent. The eluate (400 ml.) thus collected was evaporated under reduced pressure and the residue weighed. The composition of the eluting solvent and the weight of the residue is shown in the following table.

Fraction Number	Solvent	Residue wt. in mg.	Raymond Reaction.
1		-	-
2		12	-ve.
3	$CHCl_3$: C_6H_6, 1:1	2	-ve.
4	$CHCl_3$: C_6H_6, 2: 1	6	+ve.
5	$CHCl_3$: C_6H_6, 3:1	16	+ve.
6		18	+ve.
7		87	+ve.
8	$CHCl_3$	83	+ve.
9		41	+ve.
10		17	+ve.
11	$CHCl_3$: Et_2O, 4:1	-	-
12		-	-
13		4	-ve.
14		-	-
15		6	+ve.
16		17	+ve.
17		20	+ve.
18	$CHCl_3$: Et_2O , 1:1	24	+ve.
19		76	+ve.
20	$CHCl_3$: $(CH_3)_2$ CO, 9:1	72	+ve.
21	$CHCl_3$: $(CH_3)_2$ CO, 4:1	63	+ve.
22	$CHCl_3$: CH_3OH , 99:1	17	+ve.
23	$CHCl_3$: CH_3OH, 98:2	10	+ve.
24	$CHCl_3$: CH_3OH, 95:5	3	+ve.
25	$CHCl_3$: CH_3OH, 90:10	-	-
26	$CHCl_3$: CH_3OH, 4:1	-	-
27	$CHCl_3$: CH_3OH, 1:1	-	-

GOMPHOSIDE

Fractions 5 - 10 (262 mg.) from the alumina column were chromatographed on formamide impregnated paper using solvent systems No. 1 and No. 2, and were shown to consist of an homogeneous substance which was named Gomphoside (Fig. 28, p.72).

After three crystallisations from aqueous methanol, 223 mg. of gomphoside were obtained as hexagonal plates, m.p. 234-242°C, $[\alpha]_D$ + 16.3 ±2° (c = 1.09 in MeOH) which analysed for a formula,

$C_{29}H_{44}O_8$ (M.W. 520.6), found: C = 66.65, H = 8.24
C = 66.26, H = 7.98
C = 66.21, H = 8.36
C = 66.02, H = 8.32
calc.: C = 66.90, H = 8.52

Gomphoside gave positive Raymond and Legal reactions but a negative Keller-Kiliani reaction. With 80% sulphuric acid the following colour changes were noted:

(time in minutes) 0' yellow, 30' orange, 60' orange-red, 120' lilac, 240' red-brown.

The total quantity of pure gomphoside obtained from the five extractions of plant material was 1.243 g.

Test for the presence of carbohydrate

[cf. Bally, Mohr, and Reichstein, 1951]

5 mg. of gomphoside were placed in a test tube with 0.2 ml. Kiliani mixture (HOAc : H_2O : HCl, 3.5 : 5.5 : 1.0) and

heated at 100°C. for 1 hour. The solution was then evaporated to dryness in vacuo at 40°C. The residue was heated with 0.5 ml. water, cooled and extracted 4 times with 0.5 ml. chloroform, the chloroform layer being removed each time with a capillary pipette. The remaining aqueous solution was evaporated in vacuo until free from chloroform, made alkaline to phenolphthalein with 1 - 2 drops of 2 N sodium hydroxide and then two drops of freshly prepared Fehling's solution added. This solution was warmed gently for two minutes in a water-bath. Immediate reduction occurred, a brown deposit of cuprous oxide being formed, which indicated the presence of a reducing sugar.

Acetylation of Gomphoside

94 mg. of gomphoside were dissolved in 1 ml. of dry pyridine and 1 ml. of acetic anhydride added. The solution was allowed to stand at room temperature (20°C.) for 36 hours. It was then diluted to 4 ml. with water and the acetylated material extracted by shaking the solution with 4 x 10 ml. portions of chloroform. The chloroform solution was washed with dilute sulphuric acid (2 x 10 ml., 0.1 N.), then 3 times with 10 ml. portions of 10% aqueous sodium bicarbonate solution, and finally with 3 x 10 ml. of water. The chloroform extract was dried over anhydrous sodium sulphate, filtered, and evaporated to dryness under vacuum, leaving 83 mg. of colourless foam.

This foam was recrystallised three times from methanol - water, yielding 71 mg. of colourless needles, m.p. 252-255°C., $[a]_D^{20}$ + 32 ±2° (c = 0.741 in $CHCl_3$). This compound analysed for the formula,

$C_{33}H_{48}O_{10}$ (M.W. 604.33), found: C = 65.15, H = 7.68
C = 65.47, H = 7.47
calc.: C = 65.56, H = 7.94

Acetyl determination, found: 14.2%, 14.3%
calc.: 2-OAc, 14.25%

This compound had an Rf value of 0.15 when chromatographed on the reversed phase system No. T1 (p. 138).

Chromium Trioxide Oxidation of Acetyl-Gomphoside

95.8 mg. of acetyl-gomphoside were dissolved in 2.0 ml. of acetic acid and 1.0 ml. of a 2% solution of chromium trioxide in acetic acid was added. This solution was allowed to stand at room temperature for 16 hours, after which another 0.25 ml. of the chromium trioxide solution was added. [Reduction of the chromium trioxide had occurred as shown by the greenish colour of the solution.] The solution was allowed to stand for another three hours before being diluted with 5 ml. of methanol-water (1:1). This solution was left to stand until the reduction of the chromium trioxide was complete.

Sodium bicarbonate solution was added to the reaction mixture until the pH was 8 - 9, then extracted with chloroform (5 x 10 ml.). The chloroform solution was washed

with water (4 x 5 ml.), then dried over anhydrous sodium sulphate. Evaporation of the chloroform under vacuum gave 22 mg. of colourless foam. Recrystallisation from methanol gave 15 mg. colourless crystals (m.p. 250 - 255°C) which were shown by mixed melting point and paper chromatography to be unchanged acetyl-gomphoside.

The aqueous solution remaining after the chloroform extraction was acidified to congo-red and re-extracted with chloroform (5 x 10 ml). This chloroform extract was washed with water until the washings were neutral, dried over sodium sulphate, and the chloroform evaporated in vacuo. Approximately 5 mg. of oil remained which did not give a positive Raymond reaction. This residue was neutral to litmus paper but has not been identified.

Hydrolysis of Gomphoside

132 mg. of gomphoside were dissolved in 30 ml. of Kiliani's mixture (HOAc : H_2O : HCl, 3.5 : 5.5 : 1.0) and heated to 100°C. for 1½ hours. The solution assumed a pale yellow colour. On cooling, the solution was extracted 5 times with 10 ml. portions of chloroform. The chloroform extract was then washed with 10% aqueous sodium bicarbonate solution until neutral, and finally with water (3 x 10 ml.). This extract was dried over anhydrous sodium sulphate and evaporated to dryness in vacuo, leaving 84 mg. of yellow oil. Recrystallisation of this oil from methanol-water gave 62 mg. of white

crystals , m.p. 230-242°C, which were shown by analysis, mixed melting point, and carbohydrate test to be unchanged gomphoside. 107 mg. of gomphoside were dissolved in 12 ml. of methanol and diluted with 12 ml. of 10% sulphuric acid in 75% aqueous methanol. After refluxing this solution for 4 hours the methanol was removed by vacuum distillation, and the hydrolysis product allowed to crystallise. 64 mg. of colourless crystals were obtained, which on recrystallisation from aqueous methanol gave 53 mg. of colourless plates, m.p. 266-270°C, $[\alpha]_D^{23}$ + 46.6° ±2° (C = 0.60 in EtOH). This compound gave positive Raymond and Legal reactions, but a negative tetranitromethane reaction. With concentrated sulphuric acid the following colour changes were noted:-

(time in minutes) 0' yellow, 30' orange, 60' orange-brown, 120' brown, 240' reddish-violet.

It analysed for a formula,

$C_{23}H_{34}O_5$ (M.W. 390.5) found; C = 69.99, H = 8.15

calc.: C = 70.74, H = 8.78

Acetylation of Gomphogenin

30 mg. of gomphogenin were dissolved in 0.5 ml. of pyridine and 0.5 ml. of acetic anhydride was added. The solution was allowed to stand at room temperature for 48 hours after which it was extracted with chloroform and treated in the manner described for the acetylation of gomphoside (p.144). The chloroform extract was dried over anhydrous sodium sulphate and evaporated under vacuum. 26 mg. of colourless foam were

obtained and on recrystallisation from methanol gave 21 mg. of colourless crystals, m.p. 150-156°C, $[\alpha]_D$ +38 ±2° (c = 0.65 in $CHCl_3$), which analysed for a formula,

$C_{25}H_{34}O_6$ (M.W. 430.52), found: C = 69.20, H = 8.06

calc.: C = 69.74, H = 7.96

Acetyl determination, found: 8.8%

calc.: 1-OAc, 10.0%

Oxidation of Acetyl-Gomphogenin by Chromium Trioxide in Acetic Acid

Approximately 20 mg. of acetyl-gomphogenin were dissolved in 0.5 ml. of acetic acid (stable to CrO_3) and 0.5 ml. of a 2% solution of chromium trioxide in acetic acid was added. A blank reaction consisting of 0.5 ml. of acetic acid to which was added, 0.5 ml. of the 20% chromium trioxide in acetic acid, was carried out at the same time. After standing at room temperature for 16 hours the solution containing the acetyl-gomphogenin had assumed a greenish colour, whereas the blank solution remained reddish brown. 0.5 ml. of the 20% chromium trioxide in acetic acid solution was added to both blank and reaction mixtures, and these solutions were allowed to stand for a further 5 hours. After treatment of the reaction mixture in the manner described for the oxidation of acetyl-gomphoside (p. 145), only a trace of a neutral material was obtained. This could not be identified.

Oxidation of Gomphoside with Periodic Acid

Two portions of gomphoside were weighed into conical flasks, dissolved in 10 ml. of methanol and 10 ml.of 0.01523 M periodic acid solution were added. One solution was allowed to stand at room temperature for 48 hours and the other for 72 hours. The solutions were diluted with 10 ml. of water and neutralised with solid sodium bicarbonate. 20 ml. of 0.10 N sodium arsenite solution and 1 ml. of 10% potassium iodide solution were added to each solution, the excess sodium arsenite being estimated with 0.10 N iodine solution.

(i) 41.27 mg. = 0.0000793 g. mol (calculated for M.W. 520.64). Titrated after 48 hours. Volume of 0.10 N I_2 required = 16.95 ml. By calculation, the quantity of periodic acid used in the oxidation = 0.0000725 g. mol.

(ii) 26.69 mg. = 0.0000513 g. mol. (calculated for M.W. 520.64). Titrated after 72 hours. Volume of 0.10 N I_2 required = 18.05 ml. By calculation the quantity of periodic acid used in the oxidation = 0.0000550 g. mol.

AFROSIDE

The fractions 16-23 (total 308 mg) from the alumina column were chromatographed on formamide impregnated paper and showed the presence of two constituents in each of the fractions. By the intensity of the colour produced by the Raymond reaction it was estimated that both constituents of the mixture were present in approximately the same proportions. An attempt to separate these two constituents by chromatography on alumina resulted in the original material being recovered and no separation was effected. Recrystallisation of this material similarly produced no separation of the mixture but it was obtained as square plates from methanol-water (296 mg.). This mixture is referred to as Afroside and has m.p. 258-262°C, $[\alpha]_D^{21}$ +42° ± 2° (C = 1.02 in pyridine), and analyses for a formula,

$C_{29}H_{42}O_9$ (M.W., 534.6),	found:	C = 64.55, H = 7.81, O = 27.2
		C = 64.72, H = 7.86
		C = 64.91, H = 7.73
	calc.:	C = 65.15, H = 7.92, O = 26.93
Methoxyl determination,	found:	0.35%, 0.30%
	calc.:	1-OMe, 5.81%
Acetyl determination,	found:	1.67%, 1.62%
	calc.:	1-OAc, 8.05%

This compound gave positive Legal and Raymond reactions but negative Keller-Kiliani reaction. The test for the presence

of carbohydrate (see p. 143) was positive. Colour reaction with concentrated sulphuric acid was as follows: (time in minutes) 0' yellow, 15' orange-yellow, 30' brown-orange, 60' red-brown, 120' red-brown. The total quantity of afroside isolated by chromatography on alumina of the mixed glycosides obtained from the five extractions of the plant material was 3.480 g.

Acetylation of Afroside

98.4 mg. of afroside, m.p. 258-262°C, were dissolved in 2.0 ml. of dry pyridine and diluted with 2.0 ml. of acetic anhydride. The solution was allowed to stand at room temperature (18°C) for 48 hours. The acetylated product was extracted with chloroform in the same manner as for acetyl-gomphoside (see p. 144). 89 mg. of colourless syrup were obtained on evaporation of the chloroform. Recrystallisation of this material from methanol-water gave 73 mg. of colourless crystals, m.p. 196-198°C, $[\alpha]_D^{18}$ -18.3 ±2° (c = 0.92 in methanol). This compound was shown to be homogeneous by chromatography on formamide impregnated paper, using solvent system No.2 (50% $HCONH_2$:MeOH; developing time 5½ hours), and also by the reversed phase system No. T1 (Rf = 0.26). Acetyl-afroside analysed for a formula,

$C_{35}H_{48}O_{13}$ (M.W. 676.35), found: C = 62.15, H = 7.10

calc.: C = 62.09, H = 7.09

Acetyl determination, found: 18.5%

calc.: 3-OAc, 18.64%

This experiment was repeated on a larger scale, 0.592 g. being acetylated in 20 ml. of pyridine with 20 ml. of acetic anhydride. After treating in the manner described for acetyl-gomphoside (p.144), 0.852 g. of a yellow oil was obtained. Paper chromatography showed that the product contained some unacetylated afroside as well as the acetyl-afroside. The 0.852 g. of oil was dissolved in 5 ml. of chloroform and diluted to 15 ml. with benzene. This solution was chromatographed on 5 g. of neutral alumina (activity III-IV).

Fraction Number	Eluting Solvent ml.	Weight of Residue mg.	Identification by paper chromatography
2 - 5	5 $CHCl_3$ + 10 C_6H_6	651	Acetyl afroside
6	5 $CHCl_3$ + 10 C_6H_6	-	-
7 - 9	10 $CHCl_3$ + 5 C_6H_6	-	-
10 - 12	15 $CHCl_3$	Trace	-
13 - 14	5 MeOH + 10 $CHCl_3$		
15 - 16	10 MeOH + 5 $CHCl_3$	115	Afroside
17 - 18	15 MeOH	-	-

Recrystallisation of the acetyl-afroside gave 624 mg. of pure material, m.p. 198-204°C. A mixed melting point with a sample from the first experiment showed no depression.

Attempted Formation of an Oxime from Afroside.

32 mg. of afroside were dissolved in 5 ml. of ethanol, 54 mg. of hydroxylamine hydrochloride, and 102 mg. of sodium acetate ($CH_3\ CO_2\ Na.3H_2O$) in 2 ml. of water were added. This mixture was refluxed for 2 hours. The solution was diluted with 5 ml. of water, the ethanol removed in vacuum, and extracted with chloroform (4 x 5 ml.). The chloroform extract was washed with water (3 x 5 ml.), dried over anhydrous sodium sulphate, evaporated, and the residue redissolved in 1 ml. of methanol and transferred to a centrifuge tube. 5 drops of water were added and the solution slowly evaporated. The supernatant liquid was removed from the crystals which were then washed with aqueous methanol (9:1) and finally dried. 23 mg. of crystals were obtained, m.p. 225-227°C. Chromatography of this mixture on 1 g. of neutral alumina gave 16 mg. of crystalline material (m.p. 231 - 234°C.) which were shown to consist of the same two constituents by chromatography on formamide impregnated paper (solvent system No. 2). A nitrogen determination on this mixture showed the presence of 0.74% nitrogen.

Attempted Mannich Hydrolysis of Afroside

300 mg. of Afroside were dissolved in 15 ml. of methanol - chloroform (1:3) and evaporated under vacuum to dryness. 150 ml. of pure dry acetone were added and heated to boiling point, then cooled rapidly to room temperature (18°) and 1.5 ml. of concentrated hydrochloric acid added. The suspended afroside gradually went into solution. This solution was shaken at room temperature for 12 days.

The acetone was removed under vacuum, 50 ml. of water being added gradually as the acetone was removed. Then 50 ml. of methanol were added and the solution refluxed for 30 minutes. The methanol was removed by vacuum distillation and the aqueous solution allowed to crystallise. The crystals were collected and washed repeatedly until the filtrate was neutral. Recrystallisation from methanol - water gave 247 mg. of colourless crystals, m.p. 256-264°C, $[\alpha]_D^{17}$ -9.4 ±2°, (c=1.06 in MeOH) and analysed for a formula,

$C_{29}H_{42}O_9$ (M.W. 543.63): found, C = 65.26, H = 7.98
C = 64.93, H = 8.03
calc., C = 65.15, H = 7.92

It is apparent that treatment of Afroside with 1% hydrochloric acid in acetone does not effect hydrolysis of the glycoside.

Chromatography on formamide impregnated paper with solvent system No. 2, as developing solvent showed this compound to be identical with one of the components of the

original afroside mixture (see Fig. 39, p.86). This compound has been named Afroside B.

Acetylation of Afroside B

125 mg. of afroside B were dissolved in 2.5 ml. of dry pyridine and 2.5 ml. of acetic anhydride were added. The solution was allowed to stand at room temperature (20°C) for 48 hours. Treatment with the method used for the acetylation of gomphoside and recrystallisation from methanol - water gave 110 mg. of colourless crystals, m.p. 194-205°C, $[\alpha]_D^{20}$ -15.6 ±2° (c = 0.96 in MeOH), which analysed for a formula,

$C_{35}H_{48}O_{13}$ (M.W. 676.35), found: C = 62.09, H = 7.62

calc.: C = 62.09, H = 7.09

Acetyl determination, found: 17.1%

calc.: 3-OAc 18.65%

A mixed melting point determination with acetyl-afroside showed no depression. Also, acetyl-afroside and acetyl-afroside B could not be separated by chromatography on formamide impregnated paper [$HCONH_2$:MeOH, 1:1; developing time 5½ hours], using solvent system No. 2, or on the reversed phase system No. T1 (Rf = 0.24). Hence acetyl-afroside and acetyl- afroside B are the same compound.

Saponification of Acetyl-Afroside with Potassium Bicarbonate

62 mg. of acetyl-afroside were dissolved in 7.5 ml. of methanol and diluted with 1.5 ml. of an aqueous 5% potassium bicarbonate solution. The reaction mixture was allowed to

stand at room temperature for 12 days. Most of the methanol was evaporated under vacuum at 30°C and the remaining aqueous methanolic solution extracted with chloroform - methanol (9:1, 4 x 10 ml.). The chloroform solution after being washed with water and dried over anhydrous sodium sulphate was evaporated under vacuum to dryness. 43 mg. of colourless foam were obtained, which on recrystallisation from methanol - water gave 34 mg. of crystalline material, m.p. 252-258°C, which was identified as afroside B by mixed melting point (no depression), and chromatography on formamide impregnated paper using solvent system No. 2.

Reduction of Afroside with Sodium Borohydride

97 mg. of afroside were dissolved in 10 ml. of 75% dioxane-water. To this solution 24 mg. of sodium borohydride in 2.4 ml. of 75% dioxane - water were added over a period of 20 minutes. The solution was then allowed to stand at room temperature (18-19°C) for 4½ hours. The solution was just alkaline to phenolphthalein. Dilute sulphuric acid was added drop by drop to the solution (H_2 being evolved) until it was just acid to congo-red paper. 6.5 ml. of methanol - water (1:1) were added and the solution allowed to stand at room temperature (18°C) for 16 hours. [A slight precipitate appeared after the acidification, which redissolved on the addition of the aqueous methanol.] Evaporation of the methanol under vacuum caused the formation of a precipitate. This material was filtered off and the mother liquors extracted with 3 x 10 ml. portions of chloroform - methanol (9:1). A total of 93 mg. of white powder were obtained. This material was shown to be a boric acid complex by the following tests:

(i) A flax fibre was dyed with tumeric by boiling the fibre in an aqueous solution of tumeric for five minutes. Approximately 2 mg. of the compound were dissolved in 2 - 3 drops of concentrated hydrochloric acid on a spotting plate and the flax fibre dipped into this solution. The colour of the fibre was changed from yellow to red. The red portion of the fibre, when dipped into a solution of sodium hydroxide, turned purple. This is a positive test for the presence of boron.

(ii) Approximately 2 mg. of the compound were dissolved in 1 - 2 drops of a solution of 0.02% quinalizarin in concentrated sulphuric acid. The colour of the quinalizarin changed from purple to blue. This was a confirmatory test for the presence of boron in the compound.

Decomposition of the boric acid complex

The 93 mg. of material obtained from the sodium borohydride reduction of afroside were dissolved in the 5 ml. of methanol, and 0.5 gm. of D-mannitol were added. This solution was then diluted with 5 ml. of 0.1 N sulphuric acid and refluxed for 30 minutes. The methanol was removed in vacuo and the compound allowed to crystallise from the aqueous acid solution. 54 mg. of colourless crystals m.p. 230-235°C, $[\alpha]_D^{24}$ +13.6 ±2°(c = 0.65 in chloroform), and analysed for a formula,

$C_{29}H_{44}O_9$ (M.W. 536.64), found: C = 65.29, H = 8.25
calc.: C = 64.90, H = 8.26

This compound was named Afrosidol. The physical constants, mixed melting point and chromatography on formamide impregnated paper showed afrosidol to be different from afroside B and the other constituent of afroside. 500 mg. of afroside in 25 ml. of 75% dioxane - water were reduced by 120 mg. of sodium borohydride in 12 ml. of 75% dioxane - water by the above method. After decomposition of the boric acid complex, and recrystallisation from methanol - chloroform - water (9:1:1), 356 mg. of crystalline afrosidol were obtained, m.p. 235°C.,

Hydrolysis of Afrosidol with 5% Sulphuric Acid

172 mg. of afrosidol were dissolved in 25 ml. of methanol, and 25 ml. of 10% sulphuric acid in 75% aqueous methanol were added. The solution was refluxed for 5 hours, after which the methanol was removed by vacuum distillation and the hydrolysis product allowed to crystallise. 57 mg. of colourless crystals were obtained, m.p. 205-210°C, which gave positive Raymond, Legal, and tetranitromethane reactions. Recrystallisation of this material from a mixture of acetone, methanol, and water (2:4:1) raised the melting point to 210-220°C, $[\alpha]_D^{20}$ +45.8 ±2° (c=0.649 in EtOH). This compound, Anhydroafrogenol, analysed for a formula,

$C_{23}H_{34}O_6$ (M.W. 406.50), found: C = 66.43, H = 8.13

calc.: C = 67.95, H = 8.43

Extraction of the mother liquors of the hydrolysis reaction with chloroform-alcohol (9:1) gave 62 mg. of resin which after crystallisation from acetone-methanol-water (2:4:1) had m.p. 208-216°C (51mg.). A second hydrolysis of 84 mg. of afrosidol gave 58 mg. of anhydroafrogenol.

Treatment of Anhydroafrogenol with Periodic Acid

42.64 mg. (0.000105 gm. mol.) of anhydroafrogenol were dissolved in 10 ml. of a mixture of dioxane-water (1:1), and 10 ml. of 0.01950 M. periodic acid solution in water were added. This solution was allowed to stand at room temperature (22°C) for 24 hours. 10 ml. of water, 20 ml. of 0.1 N sodium arsenite solution, and 1 ml. of 10% potassium iodide solution were added and the solution allowed to stand for 20 minutes.

The excess sodium arsenite was estimated by titration with 0.1 N iodine solution. Titration after 24 hours:

Volume of 0.10 N I_2 required = 16.80 ml.

By calculation the quantity of periodic acid used in the reaction = 0.0000350 gm. mol.

Acetylation of Anhydroafrogenol

68 mg. of anhydroafrogenol were dissolved in 2 ml. of pyridine, and 2 ml. of acetic anhydride were added. This solution was allowed to stand at room temperature (18°C) for three days. After treatment in the manner described for the acetylation of gomphoside (p.144), 81 mg. of yellowish powder was obtained. This material could not be crystallised.

The 81 mg. of acetylated material were dissolved in 5 ml. of a mixture of benzene-chloroform (9:1) and chromatographed on 1.5 g. of neutral alumina

Fraction Number	Eluting Solvent 5 ml.	Weight of Residue mg.	Identification by Paper Chromatography.
1 - 5	C_6H_6 : $CHCl_3$, 9:1	11	Acetylated product
6 - 10	C_6H_6 : $CHCl_3$, 4:1	34	
11 - 15	C_6H_6 : $CHCl_3$, 2:3	19	
16 - 19	C_6H_6 : $CHCl_3$, 1:4	ca. 3	-
19 - 25	$CHCl_3$	ca.10	Anhydroafrogenol

The 64 mg. of acetylated material obtained from the alumina column could not be crystallised. This material had m.p. 160-165°C., and analysed for the formula,

$C_{27}H_{38}O_8$ (M.W. 488.56), found: C = 66.95, H = 7.55

calc.: C = 66.37, H = 7.43

Acetyl determination, found: 11.1%, 14.4%

calc.: 2-OAc 15.4%

Hydrogenation of Acetyl-Anhydroafrogenol

5 mg. of platinum oxide in 3 ml. of acetic acid were hydrogenated until the uptake of hydrogen ceased. Then a solution of 53 mg. of acetyl-anhydroafrogenol in 1.5 ml. of acetic acid containing 0.15 ml. of hydrochloric acid were added to the suspension of the catalyst, and the hydrogenation continued until the uptake of hydrogen ceased. The reaction mixture was filtered, diluted with water to 10 ml. and then extracted with chloroform (4 x 10 ml.). The chloroform solution was washed with sodium bicarbonate solution (2 x 10 ml.), water (2 x 10 ml.), and dried over anhydrous sodium sulphate. Evaporation of the chloroform solution gave approximately 50 mg. of yellowish resin. This compound gave positive Raymond and tetranitromethane reactions, indicating that reduction of the olefinic bonds had not taken place. [It is assumed that the catalyst was poisoned in some way.]

5 mg. of platinum oxide in 2 ml. of absolute alcohol were hydrogenated, and then the material recovered from the previous experiment (50 mg.), dissolved in 2 ml. of absolute alcohol containing 0.1 ml. of hydrochloric acid added. When the uptake of hydrogen had ceased, the reaction mixture was filtered, diluted to 10 ml. with water, and then extracted with chloroform as in the previous experiment. Evaporation of the chloroform gave 4.3 mg. of pale yellow resin which did not give a Raymond reaction, but gave a strong tetranitromethane reaction. This material was used in the following oxidation experiment.

Oxidation of the Hydrogenation Product of Acetyl-Anhydroafrogenol by Chromium Trioxide in Pyridine

The dihydro-acetyl-anhydroafrogenol (43 mg.) obtained from the previous experiment was dissolved in 2 ml. of dry pyridine, and 2 ml. of chromium trioxide - pyridine complex added. [Prepared by the method of Poos et al., 1953, from 43 mg. of chromium trioxide in 2 ml. of dry pyridine.] The reaction mixture was allowed to stand at room temperature for 20 hours, before pouring into 10 ml. of water. The aqueous solution was extracted with chloroform (4 x 5 ml.), the chloroform phase being filtered through super-cel to break the emulsion. The chloroform extract was washed with dilute sulphuric acid (1 x 10 ml.), sodium bicarbonate solution (2 x 10 ml.), water (2 x 10 ml.), and dried over anhydrous sodium sulphate. The chloroform extract was a reddish-orange colour. This extract was evaporated to dryness

under vacuum and redissolved in 5 ml. of chloroform - benzene (1:1), and chromatographed on 1 g. of neutral alumina.

Approximately 5 mg. of a colourless substance was obtained from fractions 3 - 6 (eluted by $CHCl_3$: C_6H_6, 2:1 - 4:1), which gave a positive tetranitromethane reaction. However, there was insufficient for crystallisation and constant determination.

Attempted Hydrolysis of Afroside with 0.05 N Sulphuric Acid

105 mg. of afroside were dissolved in 10 ml. of methanol and diluted to 20 ml. with aqueous 0.1 N sulphuric acid, and the solution refluxed for 30 minutes. [Shortly after the solution began to reflux the material precipitated.] Most of the methanol was removed by vacuum distillation and the solution allowed to crystallise. 98 mg. of colourless crystals were recovered. This material was shown by paper chromatography on formamide impregnated paper to be unchanged afroside. The same experiment was repeated using the 98 mg. of recovered afroside in 15 ml. of methanol to which was added, 5 ml. of 0.5 N sulphuric acid, and refluxing for one hour. After treating in the same manner as for the previous experiment, 93 mg. of unchanged afroside were recovered.

Hydrolysis of Afroside with 5% Sulphuric Acid in 73% Methanol - Water.

103 mg. of afroside were dissolved in 25 ml. of methanol and diluted with 25 ml. of a solution of 10% sulphuric acid in 75% methanol - water. After refluxing this solution for 3 hours, the methanol was evaporated under reduced pressure, 25 ml. of water being added gradually during the evaporation. On cooling, colourless crystals were deposited. They were collected and washed with water until the washings were neutral. 66.5 mg. of material were obtained in this manner. The mother liquors were extracted with chloroform (3 x 10 ml.), washed with 10% sodium bicarbonate solution (1 x 10 ml.), and then water (3 x 10 ml.).

The chloroform extract was dried over anhydrous sodium sulphate and evaporated - giving another 6 mg. of material.

Paper chromatography on formamide impregnated paper showed the presence of a small amount of the starting material as well as the hydrolysis product. This mixture was dissolved in 5 ml. of chloroform - benzene (1:1) and chromatographed on 3.5 g. of alumina. 56 mg. of the hydrolysis product were obtained by elution from the chromatography column.

Fraction Number	Eluting Solvent 5 ml.	Weight of Residue mg.	Identification by paper chromatography
1	$CHCl_3$: C_6H_6, 1:1	-	-
2 - 8	$CHCl_3$: C_6H_6, 1:1-3:1	56	Hydrolysis product.
9 - 10	$CHCl_3$	Trace	
12 - 16	$CHCl_3$: MeOH, 9:1-1:1	9	Afroside.

Recrystallisation of the hydrolysis product from aqueous methanol gave 51 mg. of colourless crystals, m.p. 208-220°C, $[\alpha]_D^{17}$ +60.8 ±2° (c = 0.855 in MeOH), which analysed for,

$C_{23}H_{30}O_6$ (M.W. 402.47), found: C = 68.31, H = 8.04

calc.: C = 68.63, H = 7.51

The alternative formula is,

$C_{23}H_{32}O_6$ (M.W. 404.49), calc.: C = 68.29, H = 7.98

280 mg. of afroside were hydrolysed under the same conditions, yielding 177 mg. of this material. This genin gave a positive reaction with tetranitromethane, indicating that it is an anhydrogenin. When treated with concentrated sulphuric acid, the following colour changes were observed:

(time in minutes) 0' yellow, 30' orange, 60' brown, 120' brown.

Hydrogenation of Anhydroafrogenin

18.8 mg. of anhydroafrogenin (in 2 ml. of ethanol) were hydrogenated at atmospheric pressure and room temperature (20°) in the presence of 2.2 mg. of platinum oxide suspended in 0.5 ml. of ethanol. [The platinum catalyst was reduced before the introduction of the anhydroafrogenin.] The hydrogenation was continued until the uptake of hydrogen ceased. The solution was filtered and evaporated to dryness in vacuo. The white crystalline residue gave a positive reaction to tetranitromethane, but negative reactions to the Legal and Raymond colour tests.

The recovered material was redissolved in 1.0 ml. of acetic acid containing 2 drops of concentrated hydrochloric acid, and hydrogenated in the presence of 2 mg of platinum oxide in 1.0 ml. of acetic acid, until the uptake of hydrogen ceased. The solution was filtered, diluted to 5 ml. with water, neutralised with sodium bicarbonate solution and extracted with chloroform (3 x 5 ml.). The chloroform solution on evaporation, gave a pale yellow oil which was redissolved in methanol, transferred

to a centrifuge tube and diluted with water until a precipitate began to form. The solution was evaporated slowly and then centrifuged. The supernatant liquid was removed with a capillary pipette and the residue washed three times with a mixture of water - methanol (5:1), and finally dried. This material gave negative results when tested with the Legal, Raymond, and tetranitromethane colour reactions.

Acetylation of α-Anhydroafrogenin

146 mg. of α-anhydroafrogenin were dissolved in 2 ml. of dry pyridine, diluted with 2 ml. of acetic anhydride and allowed to stand at room temperature (20°C) for 48 hours. This solution was treated in the same manner as for previous acetylation reactions. After 4 recrystallisations from aqueous methanol, 126 mg. of colourless crystals were obtained, which had m.p. 188-194°C, $[\alpha]_D^{18}$ +2.5 ±2° (c = 0.81 in MeOH) and which analysed for a formula,

$C_{25}H_{34}O_8$ (M.W. 462.52),	found:	C = 64.93, H = 7.70
	calc.:	C = 64.92, H = 7.41
Acetyl determination,	found:	12.5%
	calc.:	1-OAc, 9.3%

Treatment of Acetyl-α-anhydroafrogenin with Chromium Trioxide in Acetic Acid

Approximately 20 mg. of acetyl-α-anhydroafrogenin were dissolved in 0.5 ml. of acetic acid (stable to CrO_3) and 0.5 ml. of a 2% solution of chromium trioxide in acetic acid was added. After standing for 16 hours at room temperature, the reaction

mixture has assumed a green colour. A blank reaction carried out at the same time remained reddish-brown. The reaction mixture was treated in the manner described for the oxidation of acetyl-gomphoside. 5 mg. of a yellow oil was obtained as the neutral fraction, which would not be crystallised, but was shown to be different from the starting material by paper chromatography (formamide impregnated paper, using solvent system No. 1).

Treatment of Acetyl-α-anhydroafrogenin with Chromium Trioxide - Pyridine Complex in Pyridine

32 mg. of acetyl-α-anhydroafrogenin were dissolved in 0.5 ml. of dry pyridine and approximately 0.5 ml. of a chromium trioxide - pyridine complex was added. [This complex was prepared by the method of Poos et al., 1953. The chromium trioxide - pyridine complex contained 64 mg. of CrO_3 dissolved in 1 ml. of dry pyridine.] The reaction mixture was thoroughly mixed and allowed to stand at room temperature over night, before being treated in the manner described in the oxidation of acetyl-anhydroafrogenol (p. 162).

Evaporation of the chloroform extract gave approximately 8 mg. of a yellow oil, which could not be separated from a sample of the starting material when chromatographed on formamide impregnated paper (using 50% $HCONH_2$ - MeOH for the impregnation of the paper, and solvent system No. 1, with a developing time of 5½ hours).

CERBERA FLORIBUNDA

CERBERA DILATATA

CERBERA MANGHAS

CERBERA FLORIBUNDA

Extraction

97 g. of fresh kernels were mixed with 20 g. of kieselguhr in a mortar and crushed to a coarse powder. This material was placed in a soxhlet extraction apparatus and extracted with petroleum ether (b.p. 30-90°C) for 18 hours. The material was dried at room temperature before extraction with 70% ethanol. The extraction with ethanol was continued for 24 hours, fresh solvent being used after every 6 hours.

Evaporation of the petroleum ether extract gave approximately 9 g. of oil. Evaporation of the ethanol in vacuo gave 8.6 g. of a yellow syrup.

Separation of F2 from the mixture of glycosides

The 8.6 g. of yellow syrup obtained from the ethanol extraction was dried under high vacuum, and then shaken with dry, alcohol-free chloroform (4 x 25 ml.). The chloroform extract was evaporated to dryness by vacuum distillation, from which 3.2 g. of yellow resin were obtained. Recrystallisation of this material from ethanol - water (9:1) gave approximately 1.8 g. of colourless plates, m.p. 215-225°C, $[\alpha]_D^{18}$-48 ±2° (c = 1.125 in ethanol) which analysed for a formula, $C_{32}H_{46-48}O_{10}$ (M.W. 590.7-592.7), found: C = 65.41, H = 8.27

C = 65.24, H = 8.00

$C_{32}H_{46}O_{10}$ (M.W. 590.7),	Calc.: C = 65.04, H = 7.85
$C_{32}H_{48}O_{10}$ (M.W. 592.7)	Calc.: C = 64.93, H = 8.19
Acetyl determination,	Found: 5.9%, 7.6%
	Calc.: 1-OAc 7.3%
Methoxyl determination,	Found: 5.85%, 5.31%
	Calc.: 1-Ome 4.91%.

The Raymond and Legal tests were positive, but the Keller-Kiliani test was negative. It gave a positive reaction for the presence of carbohydrate (see p.143). The residue which remained after the extraction of the mixture of glycosides with chloroform weighed approximately 4.5 g. This material was extremely soluble in water, and dissolved readily in hot acetone. It could not be crystallised readily, but an acetone solution of the compound, after standing for three months, deposited a few colourless needle crystals which melted at 188-190°C. This substance was unstable when exposed to the atmosphere.

Acetylation of F2

340 mg. of F2 were dissolved in 3 ml. of pyridine and 3 ml. of acetic anhydride were added. This solution was allowed to stand at room temperature for 48 hours, after which it was poured into 50 ml. of ice water. The aqueous solution was extracted with ether (3 x 20 ml.). The ether fractions were combined and washed with dilute sulphuric acid (2 x 10 ml.), 10% sodium bicarbonate solution (2 x 10 ml.) and finally water (3 x 10 ml.). The ether solution was dried over anhydrous sodium sulphate, and evaporated to dryness. 270 mg. of white

crystalline materialwere obtained which on recrystallisation from ethanol - water (7:3) gave 240 mg. of colourless needle crystals, m.p. 134-135°C, $[\alpha]_D^{24}$-51 ±2° (c = 1.167 in absolute ethanol), which analysed for a formula,

$C_{34}H_{48-50}O_{11}$	(M.W. 632.7-634.7),	found: C = 63.36, H = 7.59
$C_{34}H_{48}O_{11}$	(M.W. 632.7)	calc.: C = 64.54, H = 7.65
$C_{34}H_{50}O_{11}$	(M.W. 634.7)	calc.: C = 64.40, H = 7.95
	Acetyl determination,	found.: 13.2%
		calc.: 2-OAc 13.7%
	Methoxyl determination,	found: 4.88%
		calc.: 1-OMe 4.90%

Attempted Hydrolysis of F2 by 1% Hydrochloric Acid in Acetone

100 mg. of F2 were dissolved in 100 ml. of purified acetone and 3.0 ml. of concentrated hydrochloric acid were added. The solution was allowed to stand at room temperature for 3 weeks, after which 50 ml. of water were added and the solution evaporated to approximately 50 ml. 50 ml. of methanol and one drop of conc. sulphuric acid were added and the solution refluxed for 30 minutes. Most of the methanol was removed by vacuum distillation and the remaining aqueous solution was extracted with chloroform (3 x 20 ml.).

The chloroform extract was washed with 10% sodium bicarbonate (2 x 10 ml.), water (3 x 10 ml.), and dried over anhydrous sodium sulphate. Evaporation of the chloroform gave 80 mg. of colourless crystals, which on recrystallisation from ethanol-water (7:1) had m.p. 200-205°C. This material gave a positive test

for the presence of carbohydrate, and was shown to be unchanged F2 by mixed melting point and paper chromatography (formamide impregnated paper, using solvent system No. 1).

Hydrolysis of F2 by 4% Sulphuric Acid in 75% Methanol-Water

230 mg. of F2 was hydrolysed by 4% sulphuric acid in 75% methanol-water, using the method given for the hydrolysis of gomphoside (p.146). 50 mg. of colourless needles were obtained, m.p. 255°C., $[\alpha]_D^{18}$ +20.6 (c = 0.97 in ethanol), which analysed for the formula,

$C_{23}H_{34}O_5$ (M.W. 390.5), found: C = 70.70, H = 8.42

calc.: C = 70.74, H = 8.78

This compound gave positive Raymond and Legal reactions, but it was not sufficiently soluble in chloroform to give a tetranitromethane reaction. A second hydrolysis of F2 was carried out, 227 mg. giving 106 mg. of F2-genin.

Acetylation of F2-genin

104 mg. of F2 were dissolved in 1 ml. of pyridine, and 1 ml. of acetic acid anhydride was added. After standing at room temperature for 24 hours the reaction mixture was treated in the manner described for the acetylation of F2. Evaporation of the ether extract gave approximately 100 mg. of brown syrup. This syrup was dissolved in 15 ml. of chloroform - benzene (1:1) and chromatographed on 3 g. of alumina contained in a column 9 mm. x 16 mm.

Fraction Number	Eluting Solvent 15 ml.	Weight of Residue mg.	Identification by Paper Chromatography
1 - 3	$CHCl_3$: C_6H_6, 1:1	-	-
5 - 9	$CHCl_3$: C_6H_6, 2:1	54	Apparently acetylated compound (yellow syrup)
10 - 15	$CHCl_3$	ca.10	F2-genin
15 - 18	$CHCl_3$: MeOH, 9:1	-	-

The fraction 5 - 9 from this chromatogram could not be crystallised.

CERBERA DILATATA

Extraction

315 g. of fresh white kernels from the fruit of this plant were crushed with 200 g. of kieselgühr in a mortar. This material was extracted with petroleum ether (b.p. 30-90°C) in the manner described for the extraction of Cerbera floribunda (p.169). Evaporation of the petroleum ether gave approximately 40 g. of yellow oil.

The defatted material was dried at room temperature and then extracted with 70% ethanol for 18 hours, fresh solvent being used after every six hours. The combined ethanolic extracts (600 ml.) were evaporated under vacuum leaving 16 g. of white semi-crystalline residue (yield approximately 5%). This mixture was shown by paper chromatography to consist of 4 constituents (on formamide impregnated paper, using solvent system No 2,p.136; see Fig. 72, p.127).

8 g. of this mixture were dissolved in 100 ml. of chloroform and then diluted to 200 ml. with benzene. This solution was applied to 240 g. of alumina (activity II-III) contained in a column 5 cm. x 80 cm. The composition of the eluting solvent and the nature of the residue obtained by evaporation of the fractions are given in the following table:

Fraction Number	Eluting Solvent 200 ml.	Weight of Residue mg.	Raymond Reaction
1	$CHCl_3$: C_6H_6, 1:1	10	-ve
2	$CHCl_3$: C_6H_6, 3:2	15	+ve
3 - 10	$CHCl_3$: C_6H_6, 2:1-3:1	2400	+ve
11 - 12	$CHCl_3$: C_6H_6, 4:1-5:1	20	+ve
13 - 20	$CHCl_3$	3670	+ve
21 - 24	$CHCl_3$: EtOH, 99:1-90:1	ca.80	+ve

The material obtained from fractions 2 - 10 (2415 mg.) was a homogeneous substance designated D2 (see Fig. 72, p. 127). The residue from fractions 13 - 14 (3750 mg.) was shown by paper chromatography, using the formamide and reversed phase systems [solvent systems No.2 and No.T1, respectively, (pp.136 and 138)] to consist of three constituents (see Fig.73,p.128).

Purification of D2

Recrystallisation of the 2.415 mg. of D2 obtained from the chromatography column, from ethanol - water (7:1), gave 2.36 g. of

colourless crystals, m.p. 225-230°C, $[\alpha]_D^{18}$-44.2 ±2° (c = 0.816 in ethanol), which analysed for a formula,

$C_{32}H_{46-48}O_{10}$ (M.W.590.7-592.7), found: C = 64.87, H = 7.92

$C_{32}H_{46}O_{10}$ (M.W. 590.7), calc.: C = 65.04, H = 7.85

$C_{32}H_{48}O_{10}$ (M.W. 592.7), calc.: C = 64.93, H = 8.19

Acetyl determination, found: 7.6%, 7.1%

calc.: 1-OAc 7.3%

Methoxyl determination, found: 5.31%, 5.31%

calc.: 1-OMe, 4.91%

D2 gave positive Raymond and Legal reactions, and a negative Keller-Kiliani reaction. The test for the presence of carbohydrate was positive. This compound was shown by mixed melting point and paper chromatography (using formamide and reversed phase systems; solvent system No.2 and No. T1 respectively) to be identical with F2 isolated from Cerbera floribunda.

Acetylation of D2

120 mg. of D2 were dissolved in 1 ml. of pyridine and 1 ml. of acetic anhydride was added. This solution was allowed to stand at room temperature for 24 hours, after which the acetylated material was extracted in the manner described for the acetylation of F2 (p.170). 82 mg. of colourless crystals were obtained, m.p. 130°C, $[\alpha]_D^{24}$-48 ±2° (c = 0.962 in ethanol), which analysed for a formula,

$C_{34}H_{48-50}O_{11}$ (M.W. 632.7-634.7), found: C = 63.52, H = 7.66

$C_{34}H_{48}O_{11}$ (M.W. 632.7), calc.: C = 64.54, H = 7.65

$C_{34}H_{50}O_{11}$ (M.W. 634.7), calc.: C = 64.40, H = 7.95

Acetyl determination, found: 12.7%

calc.: 2-OAc, 13.7%

Methoxyl determination, found: 5.7%

calc.: 1-OMe 4.9%

This compound was shown by a mixed melting point determination to be identical with acetyl-F2 (no depression).

CERBERA MANGHAS

Extraction

34 g. of the fresh kernels of the fruit of this plant were mixed with 50 g. of kieselguhr and ground to a powder. This material was extracted in a soxhlet apparatus in the manner described for the extraction of Cerbera floribunda (p.169). Evaporation of the petroleum ether gave approximately 6 g. of yellow oil.

The ethanolic extracts were combined and evaporated to dryness in vacuo. Approximately 3 g. of a yellowish mixture of glycosides were obtained. Extraction of this mixture with dry, ethanol-free chloroform (4 x 25 ml.), removed the less polar substituent of the mixture (substance M2). Evaporation of the chloroform left approximately 100 mg. of white semi-crystalline material. Recrystallisation of this substance from ethanol-water gave 80 mg. of white amorphous material, m.p. 190-203°C.

The chloroform insoluble residue (substance M1) weighed approximately 3 g. This material was ubstable in air and could not be crystallised. The substance M2 could not be separated from F2 or D2 on the reversed phase paper chromatograms, using solvent system No.T1 (p.138, and Fig. 74, p.131).

APPENDIX 1

The plant specimens, unless otherwise stated, were received in air-dried condition, the drying having been done at 54-60°C. for varying lengths of time.

The plants were ground to a coarse powder and 25 g. of the product were macerated with 70% alcohol for 48 hours. The alcoholic extract was then separated by filtration and concentrated in vacuo until the alcohol was almost completely removed. The concentrated extract was then diluted to 50 ml. with water and 0.45 g. of sodium chloride was added to render the solution more nearly isotonic with blood. The solution was filtered if there had been much precipitation and was then used for the biological test. Guinea pigs weighing 400-600 g. were used and anaesthetised with urethane, using a dose of 0.7 ml. per 100 g. of a 25% solution. The guinea pigs were arranged for artificial respiration and the thorax was opened in order to observe the heart. The injection was made by means of a motor-driven syringe giving a constant rate of injection of 0.5 ml. per minute and the needle was usually inserted into the jugular vein.

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APPENDIX 2

Perfusion of the Isolated Heart of a Rabbit

Langendorff's Method

A rabbit was killed by a blow on the head. The throat of the animal was then cut, and when the blood had drained out the chest was opened and the heart removed. It was dipped into a basin of Ringer's solution and gently squeezed to remove the blood from the aorta. The aorta was freed from its attachment to the pulmonary artery, and the canula tied in the aorta. When the canula was in place, a thread was attached from the tip of the ventricle to the recording arm of a kymograph.

After the heart had been beating normally for a few minutes, a solution containing 1 part in 20,000 of thiopentone was added through a side arm of the canula. The amplitude of the heart beat was reduced to approximately 60% of the normal amplitude. Then a solution containing 1 part in 200 of Gomphocarpus fruticosus (Asclepias fruticosa) extract was added which increased the amplitude of the heart beat to approximately 150% of the normal amplitude.

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A SURVEY OF THE OCCURRENCE OF CARDIO-ACTIVE CONSTITUENTS IN PLANTS GROWING WILD IN AUSTRALIA

I. FAMILIES *APOCYNACEAE* AND *ASCLEPIADACEAE*

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(Accepted for publication 18th May, 1953.)

The cardiac glycosides represent one of the few remaining groups of drugs derived solely from plant sources and for this reason are the subject of considerable research at the present time.

We have attempted to survey the occurrence of these drugs in Australian plants as part of the Australian Phytochemical Survey of the Commonwealth Scientific and Industrial Research Organization.

So far the families *Apocynaceae* and *Asclepiadaceae* have been almost completely examined from material collected in Queensland.

In order to obtain a quick evaluation of these plants the anaesthetized guinea pig has been used and extracts of the plants administered by continuous intravenous injection. This is essentially the official method of the British Pharmacopoeia (1948) for the assay of digitalis, and it provides a means of detection of almost any substance which has a direct action on the cardio-vascular system.

A cardiac glycoside injected in this way quickly slows the heart and makes the individual contractions more forceful. With more prolonged administration cardiac arrhythmias are observed with dropped beats and coupling of the auricular and ventricular contractions when the heart is very slow. This later gives place to ventricular fibrillation and finally cardiac arrest.

Some substances which are purely toxic to the heart cause an increase in rate together with increasingly feeble contractions. This is followed by progressive poisoning of the heart until it fails in diastole. With toxic effects of this kind no slowing or increase in ventricular contractile force is observed at any time during the experiment and it is reasonable to assume the absence of cardiac glycosides in such circumstances.

Method.

The plant specimens, unless otherwise stated, were received in air-dried condition, the drying having been done at 54–60° C. for varying lengths of time.

The plants were ground to a coarse powder and 25 gm. of the product were macerated with 70 p.c. alcohol for 48 hours. The alcoholic extract was then separated by filtration and concentrated *in vacuo* until the alcohol was almost completely removed. The concentrated extract was then diluted to 50 ml. with water and 0·45 gm. of sodium chloride was added to render the solution more nearly isotonic with blood. The solution was filtered if there had been much precipitation and was then used for the biological test. *Guinea pigs weighing* 400–600 gm. were used and anaesthetized with urethane, using a dose of 0·7 ml. per 100 gm. of a 25 p.c. solution. The guinea pigs were arranged for artificial respiration and the thorax was opened in order to observe the heart. The injection was made by means of a motor-driven syringe giving a constant rate of injection of 0·5 ml. per minute and the needle was usually inserted into the jugular vein.

TABLE 1.

Apocynaceae.

Plant	Part of plant tested	Equivalent wt. of dried plant per kilo of body wt. to produce toxic effect gm.	Remarks
Alstonia constricta (F. Muell)	Bark	1·7	Heart slowed and finally stopped
Alstonia scholaris R.Br.	Bark	15·0	Non toxic
Alyxia magnifolia	Leaves and stems	12·6	Non toxic
Alyxia spicata	Leaves and stems	10·6	Non toxic
Alyxia ruscifolia	Leaves	11·3	Non toxic
Carissa ovata R.Br.	Leaves and stems	3·0	Typical cardiac glycoside action
Cerbera dilatata	Kernels	0·35	Typical cardiac glycoside action
Cerbera floribunda	Kernels	0·31	Typical cardiac glycoside action
Cerbera manghas	Kernels (of fresh fruit)	0·20	Typical cardiac glycoside action
Ervatamia orientalis R.Br.	Leaves	13·2	Non toxic
Lochnera rosea (Reichb.)	Leaves and stems	16·8	Non toxic
Ochrosia elliptica (Labill.)	Leaves	14·8	Non toxic
Parsonsia eucalyptophylla (F. Muell.)	Leaves	14·5	Non toxic
Parsonsia straminea (R.Br.)	Leaves and stems	6·0	Heart normal then suddenly stopped
Parsonsia latifolia	Leaves and stems	18·0	Non toxic
Thevetia nereifolia	Leaves	3·2	Typical cardiac glycoside action
Wrightia millgar	Wood	14·5	Non toxic

RESULTS.

Tables 1 and 2 show the results obtained with plants of the Apocynaceae and Asclepiadaceae respectively. In each case the figures given are the weight of dried plant corresponding to the amount of the extract injected and represent the average of experiments on two to four guinea pigs. Where no toxic symptoms were observed the injection was continued for at least half an hour or until an equivalent of over 10 gm. of plant material had been injected.

TABLE 2.

Asclepiadaceae.

Plant	Part of plant tested	Equivalent wt. of dried plant per kilo of body wt. to produce toxic effect gm.	Remarks
Gomphocarpus fruticosus (L.) *Asclepia fruticosa* R.Br.	Leaves and stems	1·6	Typical cardiac glycoside action
Cryptostegia grandiflora R.Br.	Leaves and stems	0·4	Extremely toxic cardiac glycoside action
Hoya australis R.Br.	Leaves and stems	15·0	Non toxic
Hoya australis	Fresh leaves and stems	18·5	Non toxic
Hoya nicholsonia	Stems	9·0	Heart slowed, slight fibrillation before death
Marsdenia rostrata R.Br.	Bark	6·4	Heart slowed and stopped, no fibrillation, etc.
Secamone elliptica R.Br.	Leaves and Stems	16·3	Non toxic
Tylophora grandiflora	Leaves and Stems	0·96	Heart stopped suddenly (insufficient sample for repetition)

It will be seen that a typical cardiac glycoside action is seen with approximately 0·3 gm. of *Cerbera sp.* and with about the equivalent of 3·0 gm. of *Carissa ovata* or *Thevetia nereifolia,* the latter being in fact well known as a source of thevetin. Of the Asclepiadaceae both *Gomphocarpus fruticosus* and *Cryptostegia grandiflora* showed cardiac glycoside toxicity, and the latter has been shown to contain cryptograndosides (Aebi and Reichstein, 1950). Two other species of Asclepias (physocarpa and curassavica) are already known to contain cardiac glycosides (Cornforth, 1950) and hence have not been included in this survey. It has been reported that *Hoya australis* is toxic to the heart but this was not observed in our experiments. The inclusion of the fresh material was, in fact, an effort to make sure that the drying process had not caused a loss of the toxic material expected. Work is proceeding in these laboratories on the isolation and pharmacological examination of glycosides from *Gomphocarpus fruticosa* and the three *Cerbera* species listed above.

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SUMMARY.

Plants of the *Apocynaceae* and *Asclepiadaceae* families obtained from Queensland have been tested for cardiac activity by observing the effect on the exposed heart of a guinea pig when extracts of the plants are injected intravenously.

Of the *Apocynaceae* family, the *Cerbera spp.*, *Carissa ovata* and *Thevetia nereifolia* showed typical cardiac glycoside action, and of the *Asclepiadaceae*, *Gomphocarpus fruticosus* (R.Br.) and *Cryptostigia grandiflora* (R.Br.) were the only two to show a typical cardiac glycoside activity.

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THE CARDIAC GLYCOSIDES OF *GOMPHOCARPUS FRUTICOSUS* (R.Br.)

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In the course of a survey for the presence of cardioactive constituents in plants growing in Australia, samples of *Gomphocarpus fruticosus* (R.Br.) growing exotically in Queensland, were found to be highly toxic to guinea pigs.[1]

Recently, Hunger and Reichstein[2, 3, 4] have described the extraction and chemical constitution of two cardiac glycosides, gofruside and frugoside, from the seeds of *Gomphocarpus fruticosus* (R.Br.) obtained from South Africa. The plant we have investigated contains a mixture of glycosides which we have named gomphoside and afroside. We have shown them to differ from gofruside and frugoside by comparative chromatography on formamide-impregnated paper,[5] with samples of these glycosides kindly supplied by Prof. Reichstein.

The mixture of crude glycosides was obtained from the dried whole plant by continuous extraction, firstly with light petroleum and then with 50% chloroform-methanol. The chloroform-methanol extract, after evaporation and dilution with water, was shaken with carbon tetrachloride to remove pigments. The aqueous alcoholic extract was then evaporated to a small volume, from which the glycosides slowly crystallized. The gomphoside and afroside fractions were separated by chromatography on neutral alumina.

Gomphoside, after recrystallization from methanol-water, was obtained as hexagonal plates, m.p. 234—242°, $[\alpha]_D^{21} + 16{\cdot}3° \pm 2°$ (*c*, 1·09 in MeOH) (Found C, 66·65; H, 8·24. $C_{35}H_{52}O_{10}$ requires C, 66·5; H, 8·23%).

Afroside, as obtained from the alumina column, when chromatographed on formamide-impregnated paper system, invariably showed the presence of two constituents. Reduction of afroside with sodium borohydride gave a homogeneous product, indicating that the possible relationship between the two constituents of afroside is that one is a C_{10} alcohol and the other a C_{10} aldehyde. When afroside was treated with HCl in acetone,[6] one of these constituents was recovered unchanged. This constituent (called afroside B) was shown to be homogeneous when chromatographed on formamide paper. The other constituent of the mixture has not yet been separated.

Afroside crystallizes in square plates from methanol-water, m.p. 258—262°, $[\alpha]_D^{21} + 42° \pm 2°$ (*c*, 1·02 in pyridine) (Found: C, 64·55; H, 7·81; O, 27·2. $C_{29}H_{42}O_9$ requires C, 65·2; H, 8·0; O, 26·9%).

Afroside B has m.p. 256—264°, $[\alpha]_D^{17} - 9{\cdot}4° \pm 2°$ (*c*, 1·94 in pyridine). Found: C, 65·26; H, 7·98. $C_{29}H_{42}O_9$ requires C, 65·2; H, 8·0%). Acetylation of afroside B with acetic anhydride in pyridine yields a crystalline product, m.p. 196—205°, $[\alpha]_D^{17} = -15{\cdot}6° \pm 1°$ (*c*, 0·96 in MeOH) (Found: C, 62·15; H, 7·10; OAc, 18·5. $C_{35}H_{46}O_{12}$, H_2O requires C, 62·2; H, 7·10; 3OAc requires 18·65%). Hydrolysis of afroside with 5% sulphuric acid in MeOH yields an anhydrogenin (tetranitromethane test is positive), m.p. 210—220° $[\alpha]_D^{17} + 60{\cdot}8 \pm 2°$ (*c*, 0·855 in MeOH) (Found: C, 68·31; H, 8·24; $C_{23}H_{34}O_6$ requires C, 67·95; H, 8·34%). Acetylation of this anhydrogenin with acetic anhydride in pyridine yields a crystalline acetate, m.p. 188—194°, $[\alpha]_D^{17} = +2{\cdot}5° \pm 1°$ (*c*, 8·1 in MeOH) (Found: C, 64·93; H, 7·70; OAc, 12·5. $C_{25}H_{36}O_8$ requires C, 64·63; H, 7·81; 1OAc, 9·3%).

Melting points are uncorrected.

Work on these compounds is continuing and the results will be published elsewhere.

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