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STUDIES ON SOUTH AFRICAN CARDIAC GLYCOSIDES

I. ISOLATION OF TOXIC PRINCIPLES OF *HOMERIA GLAUCA* (W. & E.) N.E. BR. AND OBSERVATIONS ON THEIR CHEMICAL AND PHARMACOLOGICAL PROPERTIES*

T. W. NAUDÉ[1] and D. J. J. POTGIETER[2]

ABSTRACT

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The main toxic principle (1α, 2α-epoxyscillirosidin, a new bufadienolide cardiac aglycone) was isolated by very mild isolation techniques constantly correlated with semi-quantitative toxicity determinations in guinea-pigs.

Extraction from plant material was effected at room temperature by suspending it in acetic acid solution and then extracting with chloroform. The residue was extracted with ethanolic citrate buffer and from this phase the toxic components were re-extracted with chloroform. The final separation was done by column chromatography on silica gel. The main toxic component (MTC) constituted 0,044% by mass of the dried plant material. The presence of several other related toxic components was indicated.

Certain physical and chemical characteristics of the MTC were determined.

The MTC had a subcutaneous LD_{50} of 0,194 (0,183 to 0,203) mg/kg for guinea-pigs and 3,6 (2,9 to 4,46) mg/kg for mice. The clinical signs were nervous in nature: in guinea-pigs a generalized curare-like paralysis resulted in death from respiratory failure while in mice a convulsive syndrome was encountered. The MTC had potent local anaesthetic properties.

INTRODUCTION

Tulp poisoning is caused by the genera *Homeria* and *Moraea* of the family Iridaceae (Steyn, 1934; Steyn, 1949; Watt & Breyer-Brandwijk, 1962). The genus *Homeria* is endemic to South Africa only and contains 37 species. Ninety species of the genus *Moraea* have been described of which 61 occur in South Africa. This genus is distributed throughout Africa, Madagascar and Australia (Phillips, 1951). The term "tulp" is generally used both in Afrikaans and English; although the English translation would be tulip, it is not used. The true tulip belongs to the family Liliaceae.

Almost all the 98 species of tulp found in South Africa are regarded as poisonous, the following being the most important economically: *Homeria breyniana* (L.) Lewis, *H. miniata* (Andr.) Sw., *H. pallida* Bak., *H. glauca* (W. & E.) N.E. Br., *Moraea polystachya* Ker., *M. polyanthos* Thunb., and *M. spathulata* (Lf.) Klatt.

Several of the *Homeria* spp., especially *H. breyniana* and *H. miniata*, have been introduced into Australia and New Zealand where they have become so widely established that they now constitute a serious toxic hazard (Watt & Breyer-Brandwijk, 1962; Anon., 1964).

Tulp poisoning occurs frequently in South Africa and is known to most farmers. The earliest recorded case is that noted by Ecklon in 1830, according to Watt & Breyer-Brandwijk (1962).

All classes of stock are susceptible when fed or dosed with tulp but cattle, sheep, goats and donkeys (in this order) are more liable to suffer poisoning under natural conditions. Man is also susceptible and cases of poisoning and deaths have been recorded when the bulbs were mistaken for edible "uintjies".

Normally only newly introduced stock that are not familiar with tulp or animals that are being moved ("trekvee") will eat it. Under advérse grazing conditions, however, even stock that normally avoid the plant will eat it, as it is often green during winter when all other grazing is dead. Since it often grows on cultivated lands it may be cut with forage and then cause poisoning as desiccation has little or no effect on its toxicity.

In general the clinical signs of poisoning by the different species of the two genera are the same. The syndrome is characterized by:

(a) A severe gastro-enteritis with tympany, colic and pronounced diarrhoea.
(b) Nervous involvement characterized by hypersensitivity, nervousness and muscular fasciculations, followed by a progressively increasing ataxia and posterior paresis, general muscular weakness and marked dullness.
(c) Heart involvement in which bradycardia, tachycardia, arrhythmia, etc., are encountered to a varying extent.
(d) The animal usually dies in a state of severe depression, paresis, dehydration and collapse.

At necropsy the outstanding feature is the gastro-enteritis but no pathognomonic or typical lesions are observed. Subepi- and subendocardial haemorrhages, congestion and oedema of the lungs and congestion of other organs may be present. The long, thin, fibrous tulp leaves are frequently still recognizable among the ruminal contents.

A notable exception to this syndrome occurs in *H. glauca* poisoning, where constipation instead of diarrhoea is observed.

Investigators working on the chemistry of tulp poisoning have only reported preliminary results, some of which are contradictory. MacKenzie (1910) did a pre-

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(1)Section of Toxicology, Veterinary Research Institute, Onderstepoort

(2)Department of Biochemistry, Faculty of Agriculture, University of Pretoria

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liminary pharmacological investigation on *H. collina* (Vent.) var. *miniata*. This was probably *H. breyniana* (L.) Lewis according to A. Amelia Mauve (Botanical Research Institute, Pretoria, personal communication, 1963). He found an ether extract of the corms pharmacologically inert. An alcoholic extract, however, produced the following effects:

(a) Nervous system: The reflex response of the spinal cord was initially increased and later abolished by direct effect. Motor nerve function was also impaired and abolished. A direct central action on respiration was suspected.
(b) Skeletal muscle: Excitability was decreased and finally abolished.
(c) Heart: A typical digitalis action was encountered.

Using classical chemical isolation procedure Rindl (1924) worked on *H. pallida* that had been tested for toxicity. Apparently he assumed that the toxic principle was an alkaloid and fractions not giving positive tests for alkaloids were presumably discarded, nor were any toxicity tests reported during isolation. He isolated a resinous, amorphous residue which gave positive tests for alkaloids. A portion of this was soluble in water and was reported to have a typical digitalis action. No further purification could be obtained and the "new" crude alkaloid was named homeridine.

Gunn & Brown (1932) investigated alcoholic tinctures of *H. breyniana* and *H. bulbilifera* and observed typical digitalis actions on the heart. [According to A. Amelia Mauve (Botanical Research Institute, Pretoria, personal communication, 1963) the plants referred to as *H. lucassi* and *H. aurantianica* are subspecies of *H. breyniana*]. The tinctures were chemically negative for alkaloids and they concluded that these plants contained cardioactive glycosides.

The attempts by Dry (1950) to isolate the toxic principle from *Moraea polystachya* Ker. met with moderate success. During his investigation he found that a toxic fraction (tested on a sheep) could be extracted with chloroform from an aqueous alkaline medium. He therefore concluded that the toxic principle had to be of an alkaloidal nature. In making this deduction he was apparently also influenced by the work of Rindl (1924).

This investigator employed classical chemical isolation procedures based on the assumption that he was probably dealing with an alkaloid and combined this with limited toxicity trials on sheep. He managed to isolate a basic residue which he regarded as an alkaloid: it was lethal to a sheep at *c.* 5 mg/kg. This basic substance constituted 0,04% of the dried plant. It contained nitrogen, was only partially soluble in weak alkaline solutions, had strong reducing properties and was still toxic (no quantities mentioned) after boiling for 3 to 4 minutes in 1N HCl. Strong alkali, however, destroyed its toxicity.

To summarize, the investigations by MacKenzie (1910) and Gunn & Brown (1932) were basically pharmacological and pointed to a toxic principle (or principles) with, *inter alia,* a digitalis action. The work of Rindl (1924) must be criticized on two counts: he assumed from the beginning that the toxic principle was alkaloidal and no toxicity tests were done during the process of isolation. Dry (1950) only carried out limited toxicity tests on sheep and isolated an impure, though very toxic, amorphous alkaloidal mass. Limited chromatographic purification was attempted, without success.

The classical approach was unsuccessful. Attempts to isolate the toxic principle had only been partially achieved: it appeared to be alkaloidal in nature with, among other things a digitalis-like action. Twenty-six years elapsed between the work of Rindl (1926) and Dry (1950) and, apart from a preliminary report on this paper by Naudé & Potgieter (1966) no other investigations on this toxicologically important group have been reported. This indicates that it is extremely difficult to isolate the toxic principles and that they are probably very labile.

Tulp poisoning constitutes a serious stock problem in South Africa and is also encountered in Australia and New Zealand. To date treatment has been symptomatic only and frequently completely empirical. It was therefore necessary to isolate and identify the toxic principles of these plants in order to investigate the pharmacological and, particularly, the biochemical changes which occur during poisoning. Only after this had been achieved could rational treatment be applied based on sound scientific principles. Even the development of a specific antidote is not impossible. Finally there was always the possibility that the pharmacological properties of these substances might prove useful in other spheres of medicine.

At the outset of this investigation, 2 kg of toxic, botanically identified *H. glauca* was available and it was therefore decided to work on this plant. As far as could be ascertained no chemical data had been published on this particular species.

It was decided to deviate completely from the classical chemical isolation procedure in view of the unsuccessful attempts already discussed. In its place very mild, basically biochemical techniques were used. Selective testing of material from each isolation step for toxicity on a semi-quantitative basis was regarded as an essential prerequisite for success.

Part I: Isolation of Some of the Toxic Principles and Some of their Chemical Properties

Materials and Methods

1. *Plant material*

The plant investigated was *Homeria glauca* (W. & E.) N.E. Br., commonly known as the Natal Yellow Tulp, of the family Iridaceae (Fig. 1).

The long thin leaves have a light grey appearance due to a waxy substance which can be rubbed off, revealing a glabrous, shiny, green surface. It has a flattish, star-shaped, yellow flower with small brown spots on the inner aspects of the six petals. This species is distinguished from other *Homeria* spp., especially *H. pallida* which it resembles very closely, by the fact that it is bulbiliferous, i.e. it bears small bulbils in the axils of one or more leaves (see arrow on Fig. 1).

Except for a few preliminary experiments done on three small batches of material available at Onderstepoort at the beginning of 1963, all the investigations were done on one batch of 10 bags of material from Estcourt. These plants were all in the pre-flowering stage and were collected during the latter part of July 1963 from three localities in this district - all in harvested lands along rivers. Flowering specimens collected about a month later from the three localities were all identified as *H. glauca* by A. Amelia Mauve, (Botanical Research Institute, Pretoria, personal communication, 1963).

To prevent rotting, the fresh plant material was sent to Pretoria by passenger train on the evening of the day it was collected and, on receipt the next morning, was kept at 4°C for a maximum of 2 days or until it could be put through a green feed cutter. The short pieces were then spread out in a thin layer on a cement floor in a

FIG. 1 *Homeria glauca* (W. & E.) N.E. Br., the Natal Yellow Tulp (Inch scale) (1 in. = 2,54 cm)

shed and turned intermittently for 2 to 3 weeks until dry. It was then collected in bags and stored at 4°C.

Immediately before extraction each batch was finely ground by putting it twice through a hammer mill, fitted with an 18 mesh sieve.

2. *Experimental animals*

For toxicity trials a mixed strain of rabbits was used initially, but they were found to be rather too big (average mass 4,5 kg) and were often very erratic in their response to tulp poisoning. Albino guinea-pigs (Wistar strain) were then used for all tests. For a while rabbits were still used for testing of plant material and plant residues because these fibrous materials were difficult to dose to guinea-pigs, but eventually, as dosing techniques improved, only guinea-pigs were used for all tests. With very few exceptions, only males between 0,5 and 1 kg, were used.

Compared with the rabbit, the guinea-pig was found to be a far more suitable experimental animal for assaying tulp toxicity. Apart from being much smaller and thus requiring smaller amounts of toxic material, it was found to be approximately twice as susceptible. Furthermore, the toxic dose was a quarter to a third of the lethal dose and very definite and characteristic signs could be produced at low dosage levels. The severity of signs and even time of onset was remarkably closely correlated with the dose. The rabbit, on the other hand, usually died from a specific dose and showed almost no signs from a slightly smaller dose.

3. *Reagents and apparatus*

All reagents and solvents were of ordinary reagent grade type except where otherwise specified. The chloroform was either commercial or B.P. grade. Where specially purified or dried reagents or solvents were required, these were prepared according to standard procedures (Vogel, 1957).

All evaporations, unless otherwise stated, were done on a Buchler Rotatory Flash Evaporator at 40°C at water aspirator pressure. Melting points were determined in a well stirred, electrically heated silicone bath apparatus using standardized thermometers.

4. *Toxicity trial procedure*

(a) *General dosing procedure*

Except where specifically stated, the toxicity of all fractions was tested *per os*. Wherever possible the material was dissolved, emulsified or suspended in water only. Usually, however, it first had to be taken up or suspended in a minimum quantity of ethanol before a suitable suspension was obtained but the dose of ethanol was kept below 1 ml/kg. Controlled experiments revealed signs of lethargy and flaccid abdominal muscles at doses higher than 1 ml ethanol/kg. A semiparalytic, totally phlegmatic state, however, only ensued after a dose of 2,5 ml/kg and even at this dosage rate total recovery took place after 24 hours.

With plant or other materials where difficulties were encountered in obtaining a suspension, the problem was solved with the aid of a 1% (w/v) of Cellofas (soluble carboxy methyl cellulose - medium viscocity, Imperial Chemical Industries) in water. Clinical observations were done whenever practicable and examinations *post mortem* carried out when it was deemed necessary.

The following abbreviations are used:-

$LD_{gp}(LD_r)$ = Lethal dose for guinea-pig (rabbit).
MLD = Minimum lethal dose.
LD_{50} = Median lethal dose.

Similarly, TD (toxic dose), MTD (minimum toxic dose) and TD_{50} (median toxic dose) refer to doses that cause visible toxic signs eg. typical neck paresis as observed in guinea-pigs.

The total toxicity of a specific fraction is expressed as, for example, 3 000 kg LD_{gp}. This means that it contains enough poison to kill 3 000 kg in mass of guinea-pigs. This expression is independent of the individual masses of the experimental animals and is therefore more convenient and exact than expression of this amount in terms of experimental animal LD, based on the average mass of the species.

It must be emphasized that all tests (except where otherwise specified) were semiquantitative and that both criteria (TD and LD) were subject to individual variations among experimental animals: It was impractical to determine a statistically significant LD_{50} or TD_{50} at each isolation step. Fortunately, however, relatively small individual variations were encountered in guinea-pigs. Their reactions were remarkably consistent. Toxicity assays, in the course of the isolation, could thus be carried out with relatively few experimental animals.

(b) *Clinical signs and gross pathology*

In the guinea-pig, the first sign after administration of toxic material was hypersensitivity. This was followed by a progressively worsening curare-like paresis, affecting the neck particularly in a very typical way during the early stages of intoxication. This paresis developed into almost total paralysis, dyspnoea developed and consciousness was lost after apnoea had set in. The heart kept on beating for several minutes after consciousness was lost.

In the rabbit the signs were very similar, with the exception that the typical neck paresis, observed in the guinea-pig, was only occasionally encountered.

Apart from a marked cyanosis, general congestion and moderate oedema of the lungs with occasional subpleural pulmonary haemorrhages, the necropsy was negative.

In a limited number of experiments on guinea-pigs with fresh, green plant material cultivated at Onderstepoort, exactly the same clinical signs, etc. were produced as was the case with the dried plant.

Results

I. *Extraction and separation of the toxic principles*

1. *Preliminary experiments*

Correlation of regular semiquantitative toxicity determinations with specific extraction steps soon revealed that the toxic principle(s) could be extracted from finely ground dried plant material with water at room temperature under neutral, acidic (pH 3,3) or alkaline (pH 8 to 9) conditions. The latter, however, led to an overall loss in toxicity.

From this aqueous phase it could be readily extracted with chloroform, but direct chloroform extraction of plant material at room temperature was completely unsuccessful. Very effective extraction could, however, be effected by repeatedly extracting a watery suspension of plant material with chloroform by shaking it at room temperature.

After evaporation to dryness of this chloroform phase, the toxic principle(s) could be extracted from the residue by citrate buffer (pH 3,25, 0,2M): ethanol (1 : 1, v/v) resulting in a very toxic, clear supernatant and a largely non-toxic tarry residue. Extraction of the former with chloroform resulted, after evaporation to dryness, in a residue with an LD of *c.* 5 mg/kg for the guinea-pig as compared with an LD of 1 g/kg for the plant material used.

Attempts to separate this fraction on cation exchange resin (Zeocarb 225 - X4, Permutit Co. Ltd., London) proved unsuccessful as a severe overall loss in toxicity yield was experienced. However, it resulted in a fraction having an LD of 3 mg/kg for the guinea-pig. With an anion exchange resin (Dowex 1 - X4, Dow Chemical Co., U.S.A.) complete loss of toxicity resulted and it was later proved that this specific resin destroys the main toxic principle.

Further attempts at fractionation by means of liquid extraction and precipitation by virtue of solubility differences in methanol, petroleum ether and ether, were unsuccessful.

2. *Successful separation on silica gel*

(a) *Thin layer chromatography (TLC) on silica gel*

An aliquot of the toxic fraction mentioned above with an LD of 3 mg/kg was dissolved in chloroform and spotted on thin layer plates of silica gel G (Merck, according to Stähl) of *c.* 200 μ thickness prepared in the standard manner and activated at 105°C for 30 to 60 min. The chromatograms were developed by ascending chromatography to a height of 10 to 15 cm in glass cabinets sealed with a paste of bentonite in glycerine and saturated with the vapour of the developing medium.

After various experiments it was found that in chloroform the spot was strongly adsorbed and hardly moved from the origin, whereas in 96% ethanol it moved as a more or less undifferentiated mass near the

front. Combinations of ethanol and chloroform resulted in successful separation of the extract's components. The organic phase of chloroform shaken up with ethanol and water in the proportion 80 : 10 : 10 (v/v/v) was used initially but approximately the same results were obtained with a solution of 8% absolute ethanol in chloroform. This was subsequently used because it was less tedious to prepare.

Because of the curare-like paralysis observed in experimental animals, the spray reagent employed by Schmid, Kebrle & Karrer (1952) for the detection of curare alkaloids, i.e. 1% $Ce(SO_4)_2$ (ceric sulphate) in 2N H_2SO_4, was used and found to be satisfactory. It was discovered by chance that if the solution was made up in 4N, instead of 2N H_2SO_4, better results were obtained and the stronger acid was therefore preferred. After development, the chromatogram was dried with a hair dryer and sprayed. It was then left in an oven at 105°C for 3 to 5 min. to allow the spots to develop. On exposure to ultra-violet light, (wavelength maximum 365 mμ) several fluorescent spots become visible. In addition, certain other non-fluorescent spots showed up more clearly than they did under natural light. Examination was, therefore, always undertaken in both natural and ultra-violet light.

A sketch of a TLC of this fraction appears in Fig. 2.

It was, therefore, possible to separate a fraction that had an LD_{gp} of 3 mg/kg into a series of detectable components. The main one had a tan-brown colour with a green fluorescent cap. The R_f of this spot was 0,6 to 0,7.

Unless otherwise specified, all TLC referred to in the rest of the present investigation, was done on silica gel G layers, developed in 8% ethanol in chloroform and sprayed with 1% $Ce(SO_4)_2$ in 4N H_2SO_4.

(b) *Separation by column chromatography on silica gel*

The successful separation obtained on TLC with silica gel was adapted to column chromatography with silica gel (Merck - less than 0,08 mm). Eight attempts, using different variations of mobile phase composition, load, fraction size, temperature, etc., were made before satisfactory separation of the individual components, visible on TLC, could be obtained. Working on a semi-quantitative basis on the pooled fractions obtained during these initial attempts, it was soon discovered that the main toxic fraction was represented by the compound giving the tan-brown spot with a green fluorescent cap (Fig. 2). It was, however, clear that certain other fractions were also toxic, but to a lesser extent.

Initially the separation of the fluorescent spot from the tan spot presented problems but a satisfactory resolution and yield was eventually obtained with a mobile phase of chloroform: ethanol: water in a ratio of 950: 25: 25 (v/v/v). The tan spot itself proved to be the main toxic component (MTC). Soon after the initial isolations, the MTC was found to be rather labile to exposure to sunlight, and to a lesser extent to heat, and the necessary precautionary measures were then taken.

With a load of 1 to 2 mg of crude extract/ml of packed silica gel, a satisfactory yield of the main component could be obtained. It was, however, invariably contaminated with a yellow background colour. The latter did not show up as a specific component on a chromatogram but moved as a yellow patch at the front and gave no definite colour with ceric sulphate. It interfered with crystallization in that too much washing fluid was needed to obtain colourless crystals. By applying a bigger load of up to 5 mg/ml, the toxic fraction collected contained contaminants running anteriorly and posteriorly, as well as the yellow background colour. Repetition of the chromatography on a second column (same dimensions) at a load of 1 to 2 mg/ml gave good resolution, only a slight yellow background colour being retained.

Because of the chloroform in the mobile phase, only glass and teflon tubing could be used in the system. Ground glass joints were lubricated with a paste of bentonite in glycerine.

(c) *A typical successful isolation correlated with toxicity determinations in guinea-pigs*

(1) Isolation

The whole isolation procedure is summarized in Fig. 3.

A batch of 1 kg of finely ground, dry plant material (I) was suspended in 4 l of 0,2 M acetic acid in a 10 l glass-stoppered bottle and left to soak overnight at 4°C. Four litres of chloroform was added and the suspension shaken for 2 hours at room temperature. After removal of the chloroform phase the process was repeated twice

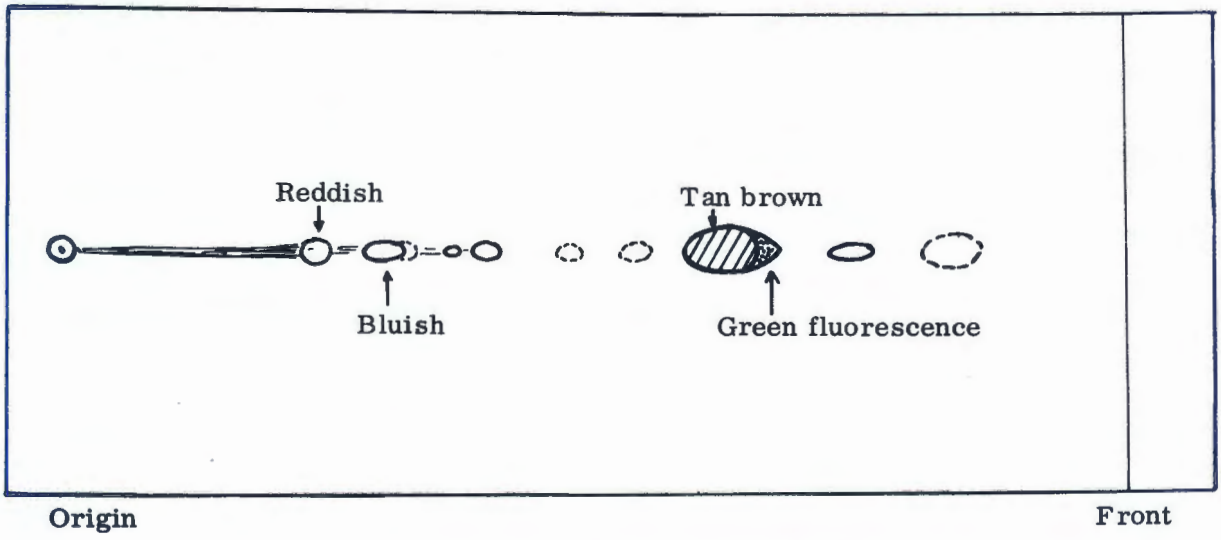


FIG. 2 Thin layer chromatogram of the toxic fraction on silica gel G developed in the organic phase of chloroform: ethanol : water (8 : 1 : 1, v/v/v) and sprayed with 1% $Ce(SO_4)_2$ in 4N H_2SO_4

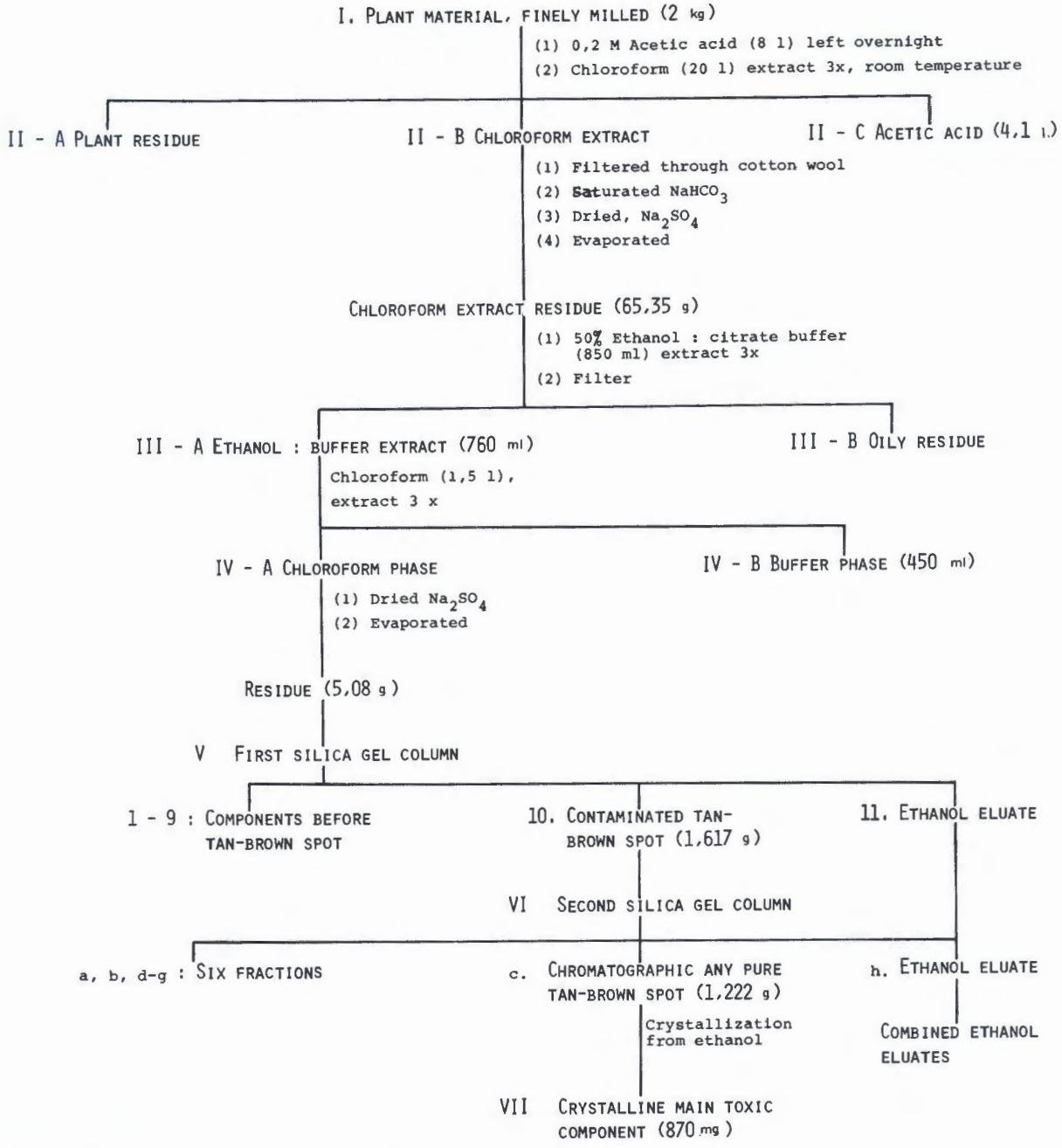


FIG. 3 Flow diagram of a typical successful extraction and chromatographic separation of the individual toxic components

with 3 l of chloroform each time. Another batch of 1 kg plant material was extracted in a similar manner.

The chloroform extract (II B) was siphoned off and the acetic acid (II C) expressed from the plant residue (II A). A sample of the latter was dried under negative pressure for toxicity determination. An aliquot of the acetic acid phase (II C, 4,1 l) was evaporated to an equivalent of 4 l total extract in order to remove all traces of chloroform before dosing. The chloroform extract (II B) was filtered through cotton wool and any acetic acid present in it was neutralized and extracted with saturated $NaHCO_3$ solution.

The chloroform was dried over anhydrous Na_2SO_4 and evaporated. A yield of 65,35 g of a green-black residue was obtained as determined on an aliquot which was thoroughly dried under negative pressure. This was suspended in ethanol and an equivalent volume of citrate buffer (pH 3,25; 0,2 M) added before shaking for one hour. The supernatant liquid was removed from the oily residue (III B) by decantation. The residue was extracted three times with a total of 850 ml of 50% ethanol: citrate buffer. The combined supernatant (III A, 760 ml) was clarified by filtration. TLC revealed that practically all the tan-brown component had been removed after two extractions. Fraction III A was then extracted with three portions of 0,5 l of chloroform. After drying the chloroform phase (IV A) over anhydrous Na_2SO_4 and evaporation to dryness, a residue of 5,08 g was obtained.

The buffer phase (IV B, 450 ml) was evaporated under negative pressure to 192 ml before dosing in order to remove most of the ethanol.

A column with a radius of 2,15 cm was packed to a height of 115 cm with silica gel (Merck - less than 0,08 mm diameter) as a slurry in the organic phase of a mixture of chloroform: ethanol: water in the proportion 95 : 2,5 : 2,5 (v/v/v), to yield a total packed volume of 1 675 ml. The column was set up in a dark room and the residue (5,08 g) obtained after evaporation of chloroform phase IV - A, adsorbed onto the column in 15 ml of the above organic phase. The load amounted to 3,15 mg/ml.

Elution took place with the same organic phase and the flow rate was adjusted ot 30 drops per min. Fractions of *c.* 10 ml were collected every 30 min by interval timing on a Beckman fraction collector. Collection was started 30 h after loading the column and a total of 355 fractions was collected over 7,5 days.

Several distinct coloured bands were visible on the column. A diffuse pale green band (that showed up as two distinct bands on a previous long, thin column) moved fastest and gave a pale green eluate in tubes 6 to 64. This was followed by a very sharp dark brown band which gave a dark brown eluate with a penetrating odour (V - 4, Table 1). This merged into a slowly fading green-yellow eluate up to tube 250, after which the eluate was pale yellow up to the last fraction collected. The main toxic fraction (V - 10) moved down the column as an opaque, oval area. It was visible up to three quarters of the way down the column. Three broad, dark green bands formed near the top of the column and moved very slowly. When collection was stopped, they had only moved a few cm.

Aliquots of 20 μl from every fifth tube, and where necessary in between, were now spotted on thin layers of silica gel G and developed in 8% ethanol in chloroform and sprayed as described earlier. The different fractions were grouped according to the distribution of spots on the chromatograms. The results are given in Table 1.

TABLE 1 Fractions collected from the first silica gel column

Fraction	Description	Tube No	Mass (mg)
V– 1	Blue spot at front*	1– 20	74
– 2	Red fluorescence (before spraying)*	21– 55	91
– 3	Yellow-brown spot	56– 64	46
– 4	Brown spot (penetrating odour)	65– 73	174
– 5	Pale green-brown spot* . . .	74– 95	159
– 6	Pink spot	96–115	77
– 7	Brown spot*	116–140	131
– 8	Beige spot	141–187	143
– 9	Green fluorescent spot . . .	188–230	164
–10 (i)	Transition from 9 to 10 (ii)* .	231–250	63
(ii)	Pure tan-brown spot. . . .	251–265	340
(iii)	Tan spot with blue spot* . .	266–290	903
(iv)	Tailing off of 10 (iii)* . . .	291–335	311
–11	Rest of tubes and alcohol washings of silica gel columns (First & Second)		2 019

*Not pure, but a mixture of components

At least eight components, some more and some less distinct, were discernible in varying concentrations - and several more can be expected if larger concentrations of extract is chromatographed and smaller fractions are collected - before the green fluorescent spot (V - 9) appeared. Some of the first eight grouped fractions were relatively pure chromatographically, whereas others contained a mixture of components (indicated by an * in Table 1).

When the green fluorescent spot's concentration began to diminish, it merged slightly with the tan spot [V - 10 (i)]. A portion of the latter [V - 10 (ii)] came through as a single component, but the main portion [V - 10 (iii)] was contaminated with a grey-blue spot having a much lower R_f value.

The silica gel was now poured out of the column, stirred in ethanol and filtered. As some colour remained, it was stirred for a second time with ethanol and left overnight before filtration. Very little colour was extracted.

Even fraction V - 10 (ii) was found to contain much yellow contaminant on concentration and the whole of fraction V - 10, amounting to 1,617 g, was therefore again subjected to chromatography on a second silica gel column. Successful resolution had previously been obtained at 4°C and the second column was, therefore, run in a dark cold room. The same silica gel and organic phase were used, except that the latter was made up and allowed to equilibrate at 4°C.

A column with a radius of 2,5 cm was packed to a height of 60 cm to give a packed volume of 1 180 ml. The whole of fraction V - 10 (1,617 g) was adsorbed onto the column and this amounted to a load of 1,37 mg/ml of packed silica gel. It was eluted as before. The flow rate was again 30 drops per minute and 5 ml fractions were collected on an L.K.B. Radi Rac fraction collector by siphoning. A total of 340 fractions was collected over 4 days.

The fractions were again spotted on thin layers as described before. The grouping of the fractions is given in Table 2.

TABLE 2 Fractions collected from the second silica gel column

Fraction	Description	Tube No	Mass (mg)
VI–a	All fractions preceeding tan spot	1–120	111
–b	Tan spot contaminated with fluorescent spot	121–139	33
–c	Pure tan-brown spot. . . .	140–212	1 222
–d	Tan spot contaminated with blue-brown spot	213–220	16
–e	Blue-brown spot	221–296	143
–f	Overlap of e and g	297–324	121
–g	Grey-blue spot	325–340	362
–h	Alcohol eluate of column . .		(Table 1)

A sharp brown band was once again observed on the column and was found to represent the blue spot moving at the front on TLC. This band was followed by 50 colourless fractions before the green fluorescent spot came through. The latter was contaminated with a small concentration of the desired component.

The main toxic component came through as a pure component (single spot) in relatively high concentration up to tube 221, when it decreased suddenly and merged into a component giving a slightly bluish-brown spot in low concentration over the next 100 fractions. Since this component had practically the same R_f as the main toxic one, it was at first thought that it was identical with the tan spot and that the difference in colour was due to the concentration. Thin layer chromatography of the concentrated fraction, however, showed it up as an intense green-brown spot on spraying with $Ce(SO_4)_2$, even before heating. Furthermore, its ultra-violet ab-

sorption spectrum and toxicity proved to be quite different from that of the main toxic component.

The column was now eluted with 1 l of ethanol for 2,5 days, which cleared it completely of all visible colour. This fraction (VI - h) was combined with fraction V - 11, the alcohol washings of the first column.

The fractions from both columns were evaporated to dryness under negative pressure at 40°C. The residues were dried for at least 24 hours under 25 mm Hg before the masses were determined. Several of these fractions had an oily consistency after evaporation and would not solidify even under high vacuum. Some of the masses are, therefore, only approximate. On the other hand, definite crystallization was observed after evaporation in fractions V - 3, - 6, - 7 and - 9 and in VI - e and - g.

A sketch of thin layer chromatograms obtained at three points during isolation, viz. the chloroform extract (II - B), the ethanol: buffer extract (III - A) and the pure main toxic component VI - c, is given in Fig. 4.

Crystallization of the pure main toxic component was effected as follows: Gentle heat was applied to dissolve 1 222 mg of dried residue of the pure main toxic component (VI - c) in 25 ml of ethanol. A small aliquot of the ethanol solution was removed for toxicity tests. As the remainder cooled thick needles formed slowly. Crystallization was performed as described later to yield a total of 870 mg of almost colourless, fine, needle-like crystals. This was equivalent to 435 mg of crystals/kg of dried plant material, i.e. a yield of 0,044 %.

(2) *Toxicity of individual fractions for guinea-pigs*

The complete toxicity trail is summarized in Table 3.

Plant material (I) and plant residue (II - A) were suspended in 1% (m/v) Cellofas solution. The acetic acid phase (II - C) and buffer phase (IV - B) were dosed, as such, after partial evaporation as described above. All the other fractions were either dissolved or suspended as well as possible in small quantities of ethanol and made up to volume in water or Cellofas solution.

The toxic dose (TD), instead of lethal dose (LD), was used in this isolation for humane reasons. Large doses of the toxic principle(s) killed the animal quickly with relatively little suffering whereas a MLD, which was the important dose in these isolations, caused long drawn-out suffering – the animal dying only after several hours of severe respiratory distress. With a MTD, however, only clinical signs developed and the animal suffered very little discomfort. The typical neck paresis, previously described, was used as the criterion. A MTD was defined as the amount of toxic principle(s) that caused a degree of neck paresis such that the guinea-pig could not keep its head off the floor for more than 10 seconds after it was lifted with a finger, within 6 hours of being dosed.

It must be re-emphasized at this stage that, since the TD_{50} of individual fractions had not been determined, the calculations appearing in the last four columns of Table 4 are only approximate, and that several apparent discrepancies are attributable to this fact. Nevertheless several important conclusions can be drawn from them.

After extraction, the plant residue (II - A) was ten times less toxic than the plant material (I), most of the toxic principle(s) being concentrated in the chloroform phase (II - B) from which it was almost completely extracted (after evaporation of the chloroform) by the 50% ethanol : citrate buffer.

Toxicity of the acetic acid phase (II - C) was constantly encountered. It was, however, always atypical in that clinical signs only developed after a latent period of several hours and lasted several days, whereas with the unextracted plant material, total recovery from a TD was usually observed after 24 hours.

Although the thick, oily residue (III - B) was not tested for toxicity (it was an extremely difficult fraction to dose quantitatively) the high toxicity of the following chloroform extract residue (IV - A), as well as the complete lack of toxicity of the buffer phase (IV - B), indicated that both extractions III and IV had been almost complete.

Of the 17 individual fractions obtained by chromatography the 12 consisting of more or less individual components, as well as the combined ethanol eluates of both columns were tested for toxicity. Seven, viz. V - 4, - 5, - 6, and - 9 and VI - c and - e, as well as the combined ethanol eluates of both columns (V - 11 and VI - h) were definitely toxic. Fractions V - 1 and - 3 might have been toxic had higher doses been administered - both guinea-pigs were debilitated after they were dosed with 40 and

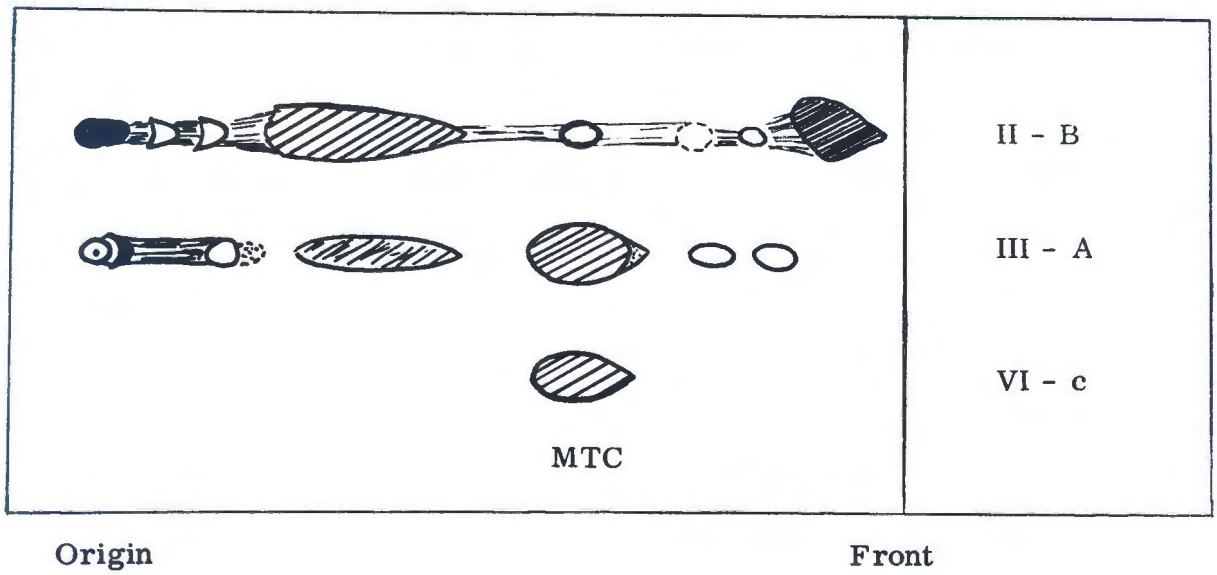


FIG. 4 A thin layer chromatogram of three specific fractions during a typical successful isolation procedure

TABLE 3 A typical successful isolation: the toxicity for guinea-pigs of individual fractions

Fraction No.	Description	Mass or Volume	Dose per kg to guinea-pigs (per os) Non-toxic	Toxic	Lethal	Estimated MTD	Total kg TD_{gp}	Toxicity increase	% Yield of toxic fraction
I	Plant material	2 kg	100, 200 mg	300 mg	1,25 g	300 mg	6 666	0	100
II–A	Plant residue	—	1, 2 g	3 g	—	3 g	666	—	10
–B	Chloroform extract	65,35 g	10 mg	15, 20 mg	30 mg	12,5 mg	5 225	24	79
–C	Acetic acid	4 l	10 ml	15,20 ml+	—	15 ml	267	—	4
III–A	50% Ethanol : citrate buffer extract	—	—	—	—	—	—	—	—
–B	Thick oily residue	—	—	—	—	—	—	—	—
IV–A	Chloroform extract residue	5,08 g	—	1, 2 mg	—	1 mg	5 080	300	76
–B	Buffer phase	192 ml	20, 30 ml	—	—	—	—	—	—
V	*First silica gel column*								
–1	Blue spot at front*	74 mg	25, 40 mg	—	—	?	—	—	—
–2	Red fluorescence*	91	25, 50 mg	—	—	—	—	—	—
–3	Yellow-brown spot	46	25, 35 mg	—	—	?	—	—	—
–4	Dark brown spot (penetrating odour)	174	25 mg	50 mg	—	40 mg	4	—	—
–5	Pale green-brown spot*	159	25 mg	37,5 mg+	50 mg	37,5 mg	4	—	—
–6	Pink spot	77	25 mg	40 mg	—	40 mg	2	—	—
–7	Brown spot*	131	25, 50, 75 mg	—	—	—	—	—	—
–8	Beige spot	143	25, 50, 75 mg	—	—	—	—	—	—
–9	Green fluorescent spot	164	5 mg	10, 12,5 mg	25 mg	10 mg	16	—	—
–10	Contaminated tan-brown spot	1 617	—	—	—	—	—	—	—
VI	*Second silica gel column*								
–a	All fractions preceding tan spot	111 mg	—	—	—	—	—	—	—
–b	Tan spot, contaminated with green fluorescent spot	33	—	—	—	—	—	—	—
–c	Pure tan-brown spot	1 222	0,5, 0,6 mg	0,4, 0,5, 0,6, 0,7 mg	—	0,4 mg 0,5 mg	3 060 2 444	750 600	46x 37x
–d	Tan spot contaminated with blue-brown spot	16	—	—	—	—	—	—	—
–e	Blue-brown spot	143	—	25 mg	—	25 mg	6	—	—
–f	Overlap of –e and –g	121	—	—	—	—	—	—	—
–g	Grey-blue spot	362	25, 50, 100 mg	—	—	—	—	—	—
V–11 VI–h	Ethanol eluates of both columns	2 019 g	—	—	12,5, 50, 100 mg+	10 mg	202	—	—
VII	Crystals from VI – c (ethanol)	870 mg	0,1, 0,4, 0,5 mg	0,2, 0,3 mg	—	0,2 mg 0,25 mg	4 350 3 480	1 500 1 200	65x 52x

Legend: ? Possibly toxic
* Not pure but a mixture of components
\+ Atypical signs - see text
x Estimated on two possible MTD

35 mg/kg respectively. With fraction V - 5, 50 mg/kg was lethal. At 37,5 mg/kg the guinea-pig was very limp and phlegmatic, but showed no neck paresis. Fraction V - 9, which exhibited green fluorescence, was the individual component judged to be the second most toxic. It was lethal at 25 mg/kg and toxic at 10 mg/kg and, in addition to the typical paralysis, severe convulsive signs were observed.

There was no doubt that Fraction VI - c, comprising the tan-brown spot, was the main toxic component. As little as 0,4 mg/kg, was toxic, and the crystals (VII) obtained from the ethanol solution were toxic even at 0,2 mg/kg. It was, however, obvious from these results that marked individual variations in susceptibility were encountered at these low dose rates (Table 4). This accounts for the variations of as much as 870 kg TD_{gp} in the calculation of total kg TD_{gp} summarized in Table 4.

Of the total of 5,08 g of chromatographed extract, *c.* 0,4 g was not regained with ethanol from the silica gel. The ethanol eluates (V - 11 plus VI - h) gave rise to 2,019 g of a residue which consisted of a whole series of components. This residue was quite toxic, being lethal at 12,5 mg/kg. In this instance a marked deviation in onset and duration of clinical signs was again observed. At 100 mg/kg typical neck paresis was in evidence within 1¼ hours and the guinea-pig died after 2 hours. In two animals, dosed at 12,5 mg/kg, neck paresis set in after 2 to 3 hours and was pronounced at 6 to 12 hours. These animals did not recover and the severe paresis persisted until death occurred on the 4th and 8th day, respectively. At this stage it was difficult to distinguish between paresis and weakness due to inanition and thirst.

Therefore, apart from the acetic acid phase and ethanol eluates of the columns, six (or possibly eight) more or less single components were found to be toxic. There was no doubt as to which one was the main toxic component; it comprised 0,044% by mass of the original (dried) plant material.

Subsequent experiments revealed that lowering of the pH of the 50% aqueous ethanol, used in extraction of the original chloroform residue (step III, Fig. 3), resulted in the removal of more contaminants at this stage. With 50% ethanol in distilled water an intensely turbid grey-green extract was obtained and this could not be clarified even by high speed centrifugation. When the pH of the aqueous ethanol was lowered stepwise (HCl), it was found that the clarity of the extract increased and eventually with 50% ethanol in 0,1 N HCl a clear yellow extract was obtained. Experimentation also revealed that the solubility of the MTC in water was such that, in later isolation 33% ethanol in 0,1 N HCl was used for extraction instead of the 50% ethanol: citrate buffer (0,2 M, pH 3,25). This required more extractions of the residue with larger volumes of the 33% ethanol in 0,1 N HCl but resulted in a clear yellow extract containing far less chlorophyl and other plant pigments.

As experience was gained, column chromatography was also improved so much that the toxic fraction from a 2 kg batch of plant material could be separated most satisfactorily on a single column. The latter had a volume of *c.* 2 l and a length of *c.* 70 cm. A slight increase in the amount of water adsorbed onto the column was found to retard the movement of the components to a remarkable extent. At the same flow rate of 30 drops per minute the time of separation was very much longer but resulted in much better resolution (3 weeks instead of 1 week). Although a certain degree of contamination of the MTC with the yellow background contaminant was still encountered, the MTC could be satisfactorily purified by crystallization, with the result that a second column was unnecessary.

II. *Physical and chemical characteristics of the main toxic component (MTC)*

1. *Crystallization from different solvents*

The solubility of the pale yellow residue (tan-brown spot) was tested in different solvents. It seemed highly soluble in chloroform, p-dioxane, acetone, ethyl acetate, isopropanol and methanol. It was slightly less soluble in ethanol, only partially soluble in benzene and only very slightly soluble in water. It was apparently insoluble in toluene, ether, petroleum ether and carbon tetrachloride.

Once crystallization was obtained it was found that the melting points (m.p.) were difficult to determine. They varied according to the solvent from which crystallization had taken place and subsequent studies (Enslin, Naudé, Potgieter & Van Wyk, 1966) revealed that practically all crystals contained solvent of crystallization, including benzene. This resulted in very gradual sintering long before the crystals actually started to melt. It was, therefore, extremely difficult to decide when the actual melting point was reached. Slow decomposition near the melting point was an added complication.

(a) *Petroleum ether precipitate*

A fine, white, powdery material was obtained by dissolving dried syrup (360 mg) in ethyl acetate and, while stirring continuously at 0°C, adding cold petroleum ether (b.p. 40 to 60°C) drop by drop until precipitation was complete. The precipitate was filtered at the pump, washed with cold petroleum ether (b.p. 40 to 60°C) and dried at 0,05 mm Hg. Two more crops of precipitate were obtained by repetition of the precipitation procedure on the mother liquor. The combined yield was 231 mg of powder (64%). This material did not have a sharp m.p., but sintered at 150°C and decomposed from 180 to 190°C.

The final mother liquor was evaporated to dryness. After four days crystallization occurred. These crystals were used for seeding and subsequently no trouble was experienced with crystallization of the main toxic component.

(b) *Crystallization from methanol*

A syrupy ethyl acetate solution was seeded with a few of the abovementioned crystals and scratched with a glass rod. On the addition of a few drops of methanol, crystallization occurred.

All the available pure material was now dissolved in methanol and filtered on a steam Buchner funnel. The filtrate was seeded and left to crystallize at room temperature for several hours before it was cooled to 4°C in a refrigerator. The crystals were collected at the pump and washed thoroughly with petroleum ether (b.p. 40 to 60°C). After drying under negative pressure a yield of 296 mg of fine, white, needle-like crystals was obtained. This material sintered from 146 to 147°C and melted with decomposition at 160 to 170°C.

$[\alpha_D]^{25,67°C} = -16° \pm 1°$ (C4, chloroform)

Three recrystallizations of 262 mg, using cold methanol as washing fluid, yielded only 6,5 mg (2,5% yield). These crystals exhibited the same melting point characteristics as the starting material.

(c) *Crystallization from ethanol*

Before seed crystals were available, an attempt was made to crystallize 50 mg of pure component from 0,5

ml of ethanol. Water was added to cloud point and the solution left at 4°C for 7 weeks. No crystallization occurred and it was found that degradation had occurred as evidenced by the reappearance, amongst others, of the green fluorescent spot on TLC.

In a second attempt 1,22 g of the pure component whose isolation is specifically described above was dissolved in 25 ml of ethanol by gentle warming to obtain an aliquot for toxicity determination. After standing overnight, long, thick crystals were formed. These were redissolved by gentle warming and the volume reduced to *c.* 15 ml. On cooling, small needle-like crystals grew slowly. These crystals were collected at the pump and washed with ethanol previously cooled to *c.* —20°C. Repeated evaporation and crystallization of the mother liquor yielded the following amounts in six successive crops:

	Mass	*Yield*
	mg	%
	585,9	48,0
	172,8	13,6
	52,1	5,2
	28,8	2,4
	19,1	1,55
	11,5	1,0
Total	870,2	71,75

Although more crystals could have been obtained from the final mother liquor, the colour of the latter was so intense that even washing them with cold ethanol failed to remove all the colour. These crystals began sintering at 147 to 148°C and melted slowly over a wide temperature range until complete liquefaction occurred at 160°C.

(d) *Crystallization from benzene*

A syrupy solution of the single component could be dissolved in benzene by heating. On cooling, hard, brittle, orthorhombic crystals were deposited. These had a high specific gravity and were only partially soluble, even in boiling benzene. The crystals so obtained were still contaminated with a yellow component.

To obtain pure crystals from benzene, crystals obtained from ethanol were recrystallized from chloroform and benzene. The best results were obtained with a ratio of 100 mg of crystals dissolved in 1 ml of chloroform to 10 ml of boiling benzene added under reflux. On cooling, the solution was seeded and allowed to crystallize. The crystals had to be scraped off the bottom of the flask before collection at the pump and were washed with cold benzene.

Crystals from ethanol (787 mg) were recrystallized in this manner. Two more crops were obtained from the mother liquor:

Mass	Yield	Melting points	
		Sintering	Completely melted
mg	%		
679,7	86,5	155–157°C	164–165,5°C
93,4	11,9	153–154°C	165°C
36,7	4,7	151–152°C	165–166°C
Total 809,8	103		

The yield of more than 100% can be attributed to the large amount of benzene of crystallization. It has been established that the crystals contain 0,5 mole of benzene per mole of the main toxic component (Enslin *et al.*, 1966). Recrystallization of 630 mg of the first batch, obtained above, from the same solvent mixture, resulted in a yield of 465,0 mg (74%). These crystals began sintering at 148 to 149°C and were completely melted at 155 to 158°C. A portion from this batch was used for elementary analysis, NMR studies and mass spectral analysis.

2. *Infra-red spectrum*

An infra-red spectrum of the crystalline MTC obtained from ethanol, is shown in Fig. 5. It was obtained with a cell path length of 0,2 mm at a concentration of 100 mg/ml in chloroform on a Beckman IR 8 Spectrophotometer.

Aliphatic character was indicated by strong absorption just below 3 000 cm^{-1}, with υ max at 2 950 cm^{-1}. A certain degree of absorption of this same band just above 3 000 cm^{-1}, as well as a sharp narrow band with υ max at 1 630 cm^{-1} was, however, indicative of a certain degree of unsaturation.

Three striking absorption bands indicative of the following groups were present:

1. 3 500 cm^{-1}: Hydroxyl group(s) (from the elementary analysis this could not have been due to nitrogen).
2. 1 740 to 1 700 cm^{-1}: Carbonyl group(s).
3. 1 240 to 1 190 cm^{-1}: Ester group(s).

Furthermore, the fact than the band at υ max 1 450 cm^{-1} had a weaker intensity that the one at υ max 1 370 cm^{-1} was indicative of an acetoxy group. A sharp band just below 1 380 cm^{-1} (in this case 1 370 cm^{-1}) was a further indication of a methylketone grouping. These interpretations are according to Nakanishi (1964).

3. *Ultra-violet spectrum*

The spectrum obtained for the MTC is illustrated in Fig. 6. This was obtained on a Beckman DK2A ratio-recording spectrophotometer at a concentration of 0,067 mg/ml of distilled water. (The base line was obtained with distilled water).

$E^{1\,cm}_{1\,per\,cent}$	126,5 (H_2O)
max (H_2O)	298 mμ
min (H_2O)	242 mμ
Strong absorpiton below	240 mμ

No shift in λ max, was observed on alkalinization (Na_2CO_3, pH 10 to 12) or acidification (HCl, pH 1 to 2) after 180 and 90 min, respectively, in solution in 10% aqueous ethanol. On alkalinization, however, the initial absorption at 360 mμ gradually increased while the absorption at 298 mμ gradually decreased. TLC afterwards revealed that degradation had taken place. No change whatsoever took place in acid solution. P.R. Enslin (National Chemical Research Laboratory, C.S.I.R. Pretoria, personal communication, 1965) pointed out that this absorption at *c.* 300 mμ was possibly due to an α-pyrone chromophore system.

4. *Elementary analysis*

Qualitative elementary analysis according to the standard sodium melt method (Mann & Saunders, 1960) was done on chromatographically pure syrup. A negative test for sulphur and nitrogen and a positive chlorine test was obtained. However, subsequent Beilstein tests on crystalline material were consistently negative and it was concluded that the positive chlorine tests on the syrup were due to minute quantities of chloroform still present in it.

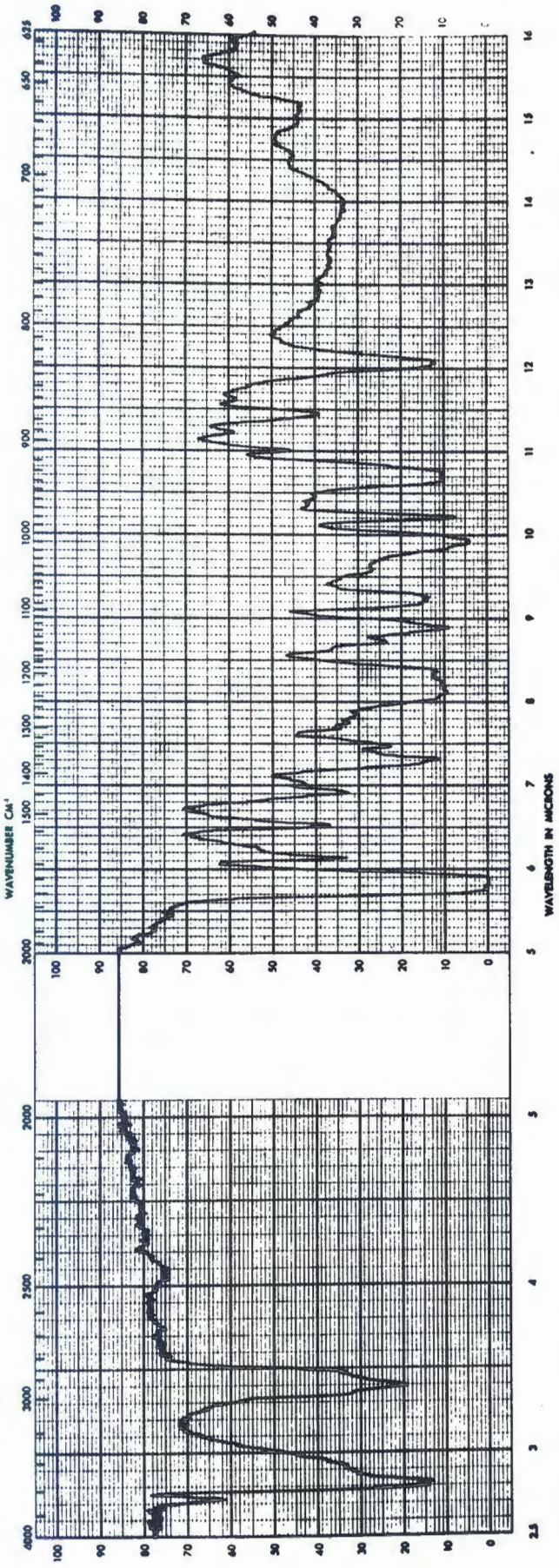


FIG. 5 Infra-red spectrum of the main toxic component

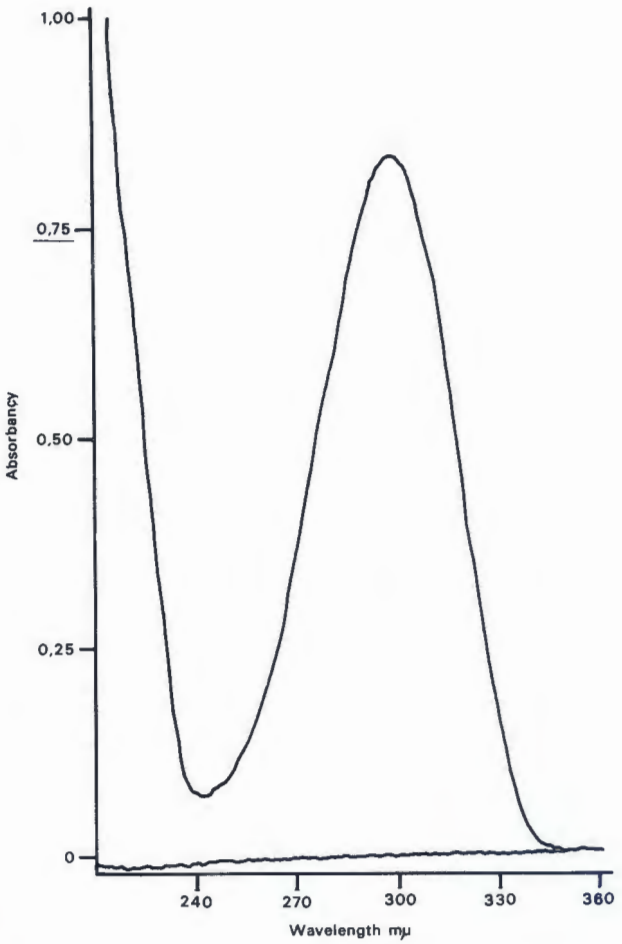


FIG. 6 Ultra-violet spectrum of the main toxic component

Quantitative elementary analyses, as well as the Rast molecular mass determination, were done by the Microanalytical Section, National Chemical Research Institute, C.S.I.R., Pretoria. Analyses were done on thrice recrystallized material (once from ethanol and twice from benzene). The crystals were dried in a drying pistol at a pressure of 0,05 mm Hg over boiling petroleum ether (b.p. 40 to 60°C) for 24 hours:

C:	67,84%; 67,78% - average	67,81%
H:	6,75%; 6,85% - average	6,80%
O:	25,36%; (determined)	25,36%
	Total	99,97%

Calculation of empirical formula:

C	H	O
67,81/12	6,8/1	25,36/16
5,65	6,8	1,585
24,99	30,03	7 i.e. $C_{25}H_{30}O_7$ (M.M. 442)

The Rast molecular mass determination gave a value of 319. However, decomposition began at 130°C with the result that the molecular mass was rather inaccurate (P. R. Enslin, N.C.R.L., C.S.I.R., Pretoria, personal communication, 1965).

Subsequent NMR and X-ray diffraction studies (Enslin *et al.*, 1966) revealed the presence of benzene of crystallization (0,5 mole/mole of MTC) as well as chloroform in these crystals. The above empirical formula was, therefore, regarded as being somewhat inaccurate. However, it was subsequently found to be in good agreement

with the final formula of $C_{26}H_{32}O_8.\frac{1}{2}C_6H_6$ which requires C, 68,1: H, 6,9: O, 25,0 as determined by Enslin *et al.* (1966).

5. *Chemical reactions and derivatives*

(a) *TLC spray reagents*

One per cent ceric sulphate in 4N H_2SO_4 proved to be the reagent of choice because it coloured many components found in the plant extract, as well as those found on degradation. The smallest quantity of MTC discernible on TLC was 0,5 μg and this showed up as a pale yellow spot under ultra-violet light.

Phosphomolibdic acid (10%) in ethanol was extremely sensitive to the MTC, showing up as little as 0,1 μg. The disadvantage, however, was that all components showed up as blue spots and no colour differentiation was obtained. Dragendorf's reagent (Randerath, 1963) coloured the MTC but no reactions were obtained with ammoniacal silver nitrate or aniline hydrogen phthalate.

(b) *Ester group test*

The standard test (Mann & Saunders, 1960) with alkaline hydroxylamine and ferric ions, gave a definite positive test with the MTC and is in agreement with the infra-red absorption band at 1 240 to 1 190 cm^{-1}.

(c) *Acetylation*

From the infra-red spectrum the presence of hydroxyl groups was indicated and acetylation under mild conditions was, therefore, attempted. Crystalline MTC (100 mg) was dried at a pressure of 0,05 mm Hg for 48 hours and then dissolved in 1 ml of anhydrous pyridine in a 25 ml glass-stoppered, round-bottom flask. Acetic anhydride (0,5 ml) was added and the solution shaken for 45 min at room temperature. At this stage solidification commenced and fine, white, needle-like crystals were formed.

TLC revealed that complete acetylation had occurred. Starting material was completely absent while a single new component with an R_f value of 0,825 had appeared (see Fig. 7). Crystallization was done from p-dioxane. Further chemical work on this derivative is reported elsewhere (Enslin *et al.*, 1966). As for the present investigation, the formation of an acetate confirmed the presence of a free hydroxyl group.

(d) *Heat lability*

By spotting *c.* 50 μg quantities of MTC on thin layers of silica gel G and then exposing this to heat at 105°C for varying periods, it was found that after 15 min definite degradation could be detected in the form of additional spots which appeared on development of the chromatogram. After 35 min exposure a whole series of components was detectable. It was, therefore, evident that the MTC was heat labile and that treatments involving heat were inadvisable.

(e) *Degradation in light*

During column chromatography it was noted that the pure component dissolved in chloroform and ethanol eluting fluid degraded at room temperature when direct rays of the sun (through the window) fell on it for 2 to 3 hours per day. By spotting *c.* 50 μg quantities on thin layers of silica gel, exposing this for different periods to light of different wavelengths and then developing the TLC, this phenomenon was confirmed. Direct sunlight, filtered through a window pane, resulted in a whole series of new components after as little as 15 min exposure. Under ultra-violet light (wavelength maximum of 365 mμ) the same degradation pattern was observed but was not as pronounced. With infra-red, white and neon lights and indirect sunlight only minor breakdown was visible after several hours.

(f) *Stability at extreme pH values*

Acid pH: Only very slight degradation took place at pH 1 to 2 but with strong acids, especially on warming, severe degradation was observed.

Alkaline pH: The detrimental effect of alkali on toxicity was already encountered during preliminary attempts at isolation. It was observed even under very mild alkaline conditions: only 2 drops of pyridine in 5 ml of water containing 5 mg MTC caused chromatographically detectable degradation within 24 hours.

The concentration of MTC (R_f value of 0,6, TLC on silica gel G) decreased under mild alkaline conditions and a new component (A1, first alkali degradation product, R_f value of 0,275) appeared. Depending on the concentration of alkali and time of exposure several other spots, particularly one at the origin (A2), appeared at the expense of A1.

An attempt to hydrolise the MTC with an anion exchange resin (Dowex 1–X4) and to isolate the products was unsuccessful: the greatest portion of the hydrolysis products was too firmly adsorbed on to the resin.

(g) *Isolation of the mild alkaline hydrolysis product (component A1)*

After several attempts it was found that with 0,1N $Ba(OH)_2$ in 50% aqueous ethanol, mild enough conditions could be created for rapid degradation of the main toxic component to component A1, without concomitant degradation to other products. The MTC was dissolved in 50% aqueous ethanol at 5 mg/ml and 0,1N $Ba(OH)_2$ added carefully to a pH of 8 to 9 as determined with Universal pH indicator paper (Merck). Aliquots of 20 μl were taken at regular intervals and spotted on TL silica gel plates and developed immediately to determine the degree of hydrolysis. At the same time the pH was determined and readjusted to 8 to 9 by the addition of alkali.

The starting material was rapidly degraded to component A1 (R_f value 0,275): more than 50% conversion had taken place after 2,5 min. Even before complete conversion of the starting material had occurred, secondary degradation of A1 to the component at the origin was visible. The reaction was stopped by bubbling CO_2 through the reaction mixture until a pH of 6 was reached. This was done as soon as most of the starting material had disappeared but before too much A2 had appeared. Normally this stage was reached after 60 to 90 min.

The aqueous ethanol reaction mixture was then extracted with chloroform and an additional three minor components were visible as determined by TLC. These six components were then separated by column chromatography at a load of less than 1 mg/ml of silica gel G (Merck) packed in 5% ethanol in chloroform, to a height of 10 cm. Elution was performed with the same phase. The separation of component A1 from the rest was most satisfactory.

Since the A1 component proved to be labile while in syrupy form and degraded to several components on drying under negative pressure, the solvent was evaporated and the residue dried under high vacuum for a short period of time only. Crystallization was done from ethanol containing 5 drops of water in 2 ml. Fine, white, needle-like crystals were obtained which were collected at the pump and washed with cold 95% ethanol (m.p. 243 to 245°C with slight decomposition after sintering from 234 to 235°C).

Component A1 had the following properties:

1. It was labile to alkali at a pH of 11 to 12 - on TLC being converted to a series of components that did not move from the origin. After exposure to strong alkali, it became non-toxic to guinea-pigs at 50 times the subcutaneous LD_{50} of the MTC. Exposure in an aqueous medium for 24 hours to a pH of 1, however, had no apparent effect.
2. Product A1, contrary to expectation, was still very toxic, the subcutaneous LD_{50} being 0,996 mg/kg compared to 0,194 mg/kg for the MTC (see Part II below). Like the starting material it also still had a local anaesthetic effect when brought into contact with the buccal mucous membrane.
3. When chromatograms of component A1 were sprayed with alkaline hydroxylamine and ferric nitrate (Randerath, 1963), it gave the same typical ester colour reaction as the main toxic component and, therefore, still had ester characteristics. However, products from further, more drastic alkaline degradation did not exhibit this characteristic.

(h) *Acetylation and chromatographic comparison of component A1 with the acetylated MTC*

At this stage work on the structure of the main toxic component by Enslin *et al.* (1966), had established the presence of only one free secondary hydroxyl group plus a second esterified with acetic acid. It was, therefore, suspected that mild alkaline hydrolysis might have led to the splitting of the acetate ester bond.

Crystalline component A1 (*c.* 2 mg) was dissolved in 1 drop of anhydrous pyridine and 1 drop of acetic anhydride added in an attempt to resynthesize the original main toxic component. Cooling in ice after 1 hour's reaction time did not lead to solidification but TLC revealed that A1 had disappeared almost completely (Fig. 7). Two new products had appeared, the minor one with an R_f value of the acetylated MTC, and the major component just behind it with an R_f value of 0,725. The MTC was not present.

These apparently confusing results were resolved when acetylated main toxic component (*c.* 1 mg) was dissolved in 50% aqueous ethanol and hydrolized at pH 8 to 9 with $Ba(OH)_2$ solution. The TLC obtained is presented in Fig. 7. All these components gave the same tan-brown colour on spraying.

A probable reaction scheme to explain the reactions that had taken place is given in Fig. 8.

The R_f values of these products on silica gel could also be explained by this reaction scheme. The desacetyl MTC (A1), having two hydroxyl groups, would be strongly adsorbed. The isomer of the main toxic component (MTCI), having the more reactive hydroxyl group blocked by acetate, would move faster than the MTC. The acetylated MTC (MTCAc), having both hydroxyl groups blocked, would obviously be only slightly adsorbed.

Part II: Toxicological and Pharmacological Investigations

For the sake of convenience and clarity the methods, results and a brief discussion of each aspect are dealt with separately. These are only preliminary investigations, resulting from phenomena encountered during the isolation and LD_{50} determinations.

I. *Clinical signs and gross pathology due to intoxication with* H. glauca

In guinea-pigs (Wistar strain) hypersensitivity was the first sign. A sharp, sudden noise close to the animal, e.g. giving the cage a rap with a hard object, caused the animal to jerk its head back sharply. Hypersensitivity was followed by a progressively worsening, general, curare-like paresis which gradually over-shadowed the hypersensitivity. Neck paresis was conspicuous. At first the animal tended to rest its head, slightly turned to one side, on the cage floor. The latter was a very typical and consistent early sign of poisoning in guinea-pigs.

Ataxia followed the paresis, the animal still being severely hypersensitive at this stage. On being stimulated it often rushed about in a pronounced ataxic manner and fell. Generalized paresis was followed by an almost total paralysis. The guinea-pig lay flat on its abdomen with the head turned to one side and the legs spread out. Reaction to pain stimuli persisted up to death and it was, therefore, concluded that this was a motor paralysis only.

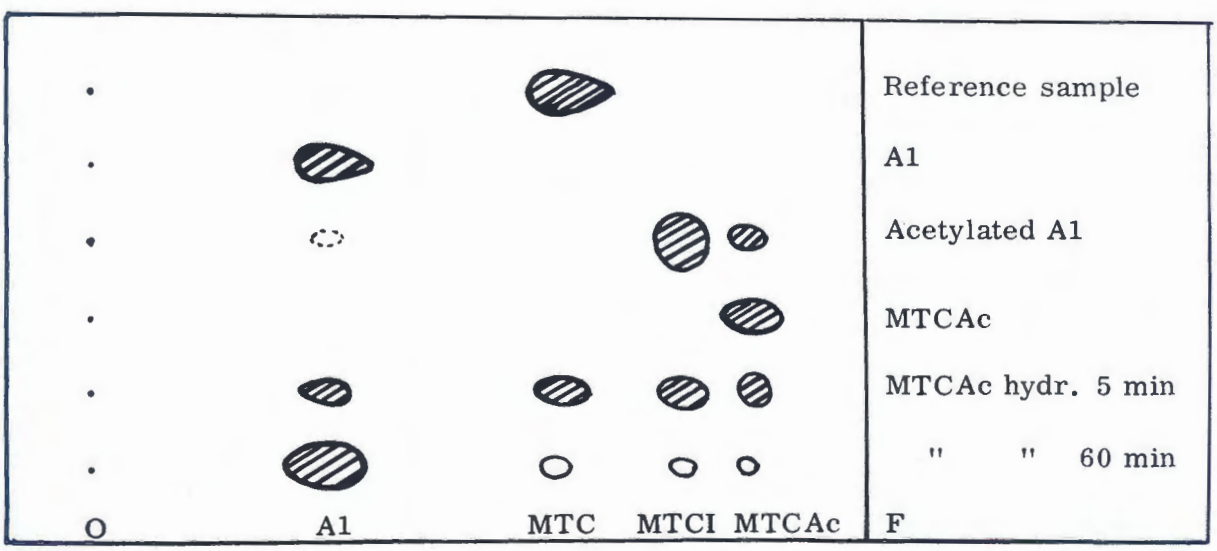


FIG. 7 A sketch of chromatograms obtained after acetylation of component A1 and hydrolysis of the acetylated main toxic component
Legend: O = origin F = front MTC = Main toxic component MTCI = Main toxic component isomer MTCAc = Main toxic component acetate

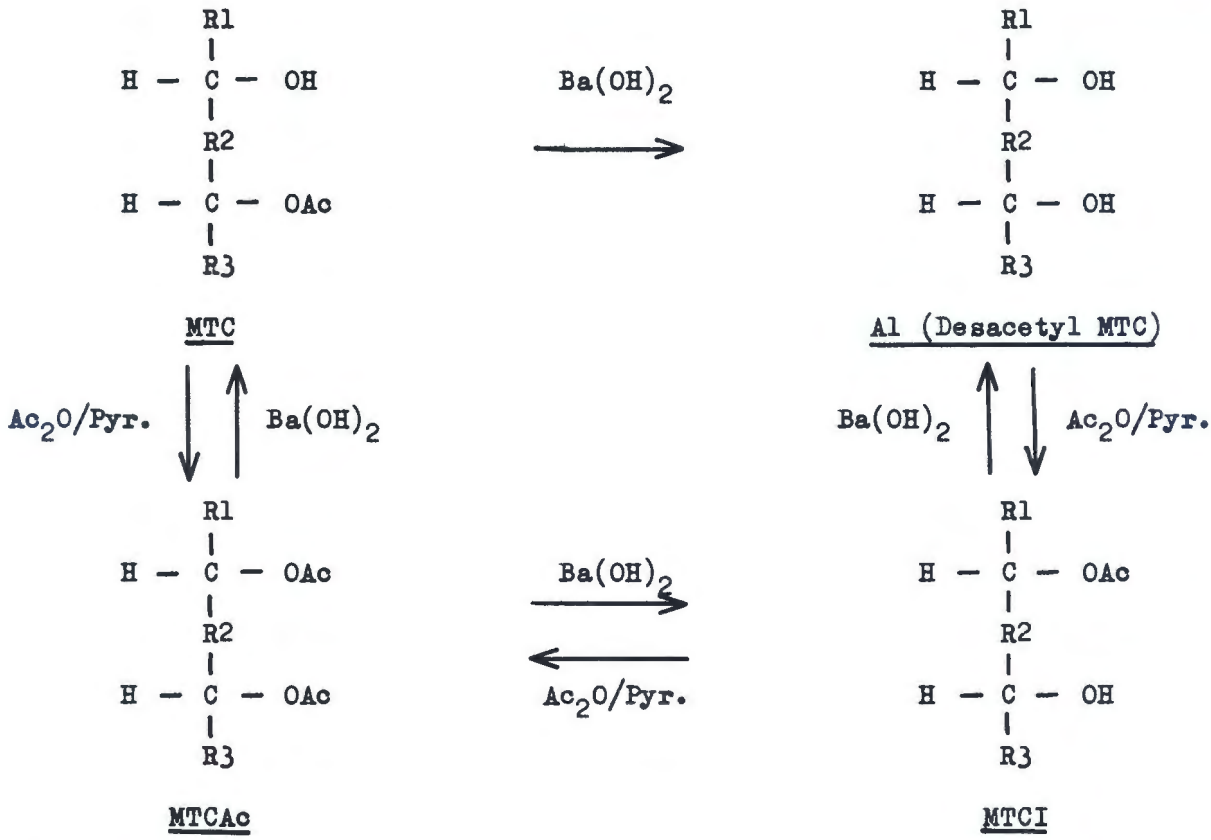


FIG. 8 A probable reaction scheme of alkaline degradation and acetylation of the main toxic component and its products

Dyspnoea set in at the same time as the paralysis. Breathing gradually became abdominal, shallow, irregular and laboured and was followed by apnoea. As cyanosis increased the colour of the eyes changed from pink to a dark, dull red. The animal finally gave a few frantic gasps, and if paralysis was not too far advanced, thrashed about violently before finally losing consciousness.

The cardiac rate and rhythm was slow and regular and a certain degree of arrhythmia was observed only when dyspnoea became advanced. The heart kept on beating in an irregular fashion long after apnoea had set in.

A flow of saliva, usually slight but occasionally copious was often encountered in the terminal stages.

The minimum time in which an animal was observed to die after oral administration of the plant was 20 min. All those that died from intoxication usually did so within 6 hours. On recovery, paresis often persisted for 24 to 48 hours and even longer in exceptional cases.

On *post mortem* examination general cyanosis and congestion were the most outstanding changes observed. On exposure to air the blood and organs, especially the lungs, changed in colour from a bluish-grey to normal bright red. The heart was invariably in diastole, there was hyperaemia and oedema of the lungs and frequently petechiae or ecchymoses under the pleurae.

Rabbits were found to be about half as susceptible to intoxication as guinea-pigs. In general the symptomatology in the rabbit was similar to that in the guinea-pig except that clinical signs were not as readily produced at sublethal doses - animals often showing no symptoms at a certain dose while death occurred when the dose was increased slightly. The typical neck paralysis in guinea-pigs was rarely observed in rabbits.

From these observations it was concluded that the toxic principle(s) in this plant caused a curare-like paralysis with respiratory paralysis as the final cause of death. The oxygen-carrying capacity of the blood did not seem to be affected, as demonstrated by the marked change in colour when the cyanotic blood and organs were exposed to air.

II. LD_{50} *determinations*

1. *Materials and Methods*

All the determinations were done by subcutaneous injection (s/c) from a 1 ml tuberculin type of syringe, graduated in divisions of 0,01 ml.

Young male albino guinea-pigs, with a mass of between 400 and 700 g, were deprived of food and water for approximately 24 hours prior to the determination. In the case of mice, young male albinos (Wistar strain) of between 17 and 27 g, were used. They were not starved beforehand. Immediately prior to the experiments the animals were randomized and then distributed in groups with the aid of a table of random numbers (Table 23, Arkin & Colton, 1950). Their masses were determined and they were marked distinctly.

Stock solutions of the compound to be tested were made by dissolving an accurately measured quantity in isotonic saline. Solution was facilitated by the addition of ethanol where necessary. Dilutions were then made in volumes suitable for injections with a 1 ml syringe. Ethanol was not required for solution of the MTC when guinea-pig determinations were done because 1 mg dissolved satisfactorily in 1 ml of saline. For mice much higher doses were required, with the

result that a 1% ethanol solution had to be used. However, not more than 0,2 ml of pure ethanol/kg was ever administered. Control experiments revealed that a dose of 2 ml of ethanol/kg elicited no clinical signs in mice. At 5 ml/kg signs were observed but even at 10 ml/kg only one of two test animals died. In the case of the desacetyl compound, A1, a 25% solution of ethanol in saline was required to dissolve 1 mg in 1 ml and this solution had to be kept at *c.* 40°C to prevent crystallization. Of this solution the maximum dose administered was 1,32 ml/kg and in control experiments up to 4 ml of 25% ethanol/kg produced no signs other than slight lethargy in guinea-pigs.

After injection, the clinical signs and the time of death were noted. *Post mortem* examinations were performed in order to exclude any mortality from conditions other than those caused by the injected material.

In all cases a pilot test was done with one or two animals per dose in order to determine the approximate range that had to be covered during these tests.

2. *Results*

(a) *Main toxic component*

(i) *Guinea-pig subcutaneous* LD_{50}*:* According to the method of Litchfield & Wilcoxon (1949) this was determined as 0,194 mg/kg with the 95% confidence limits as 0,183 to 0,203 mg/kg. These results are presented graphically in Fig. 9.

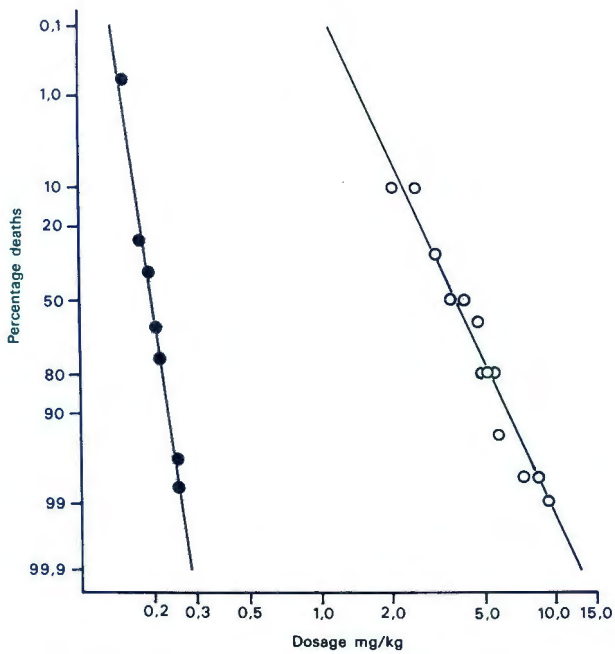


FIG. 9 A graphic determination of the subcutaneous LD_{50} of the main toxic component in guinea-pigs (left) and mice (right) on logarithmic probability paper (No. 3128, Codex Book, Co. Inc., Massachusetts)

Eleven groups of eight guinea-pigs each were used. All those that died from the toxic principle did so within 5 hours.

The clinical signs and gross pathology were identical to those encountered with plant material. In addition, however, a varying degree of uneasiness or pain at the point of injection, was observed in virtually all cases. A few minutes after the injection, retching movements without any actual vomition were often observed. In animals that recovered, paresis often persisted after 24 hours but, except for slight malaise in rare instances, no signs were present after 48 hours.

(ii) *Mouse subcutaneous* LD_{50}*:* This was determined as 3,6 mg/kg with 95% confidence limits of 2,9 to 4,46 mg/kg. For the determination of the confidence limits the special formula for heterogeneous data had to be employed. The results are also reflected in the graph (Fig. 9).

Thirteen groups of 10 animals each, were used and two distinct groups of clinical signs were observed: A slight hypersensitivity followed by paresis and a progressively worsening dyspnoea was observed within minutes after injection. The first group of mice died within 45 minutes of respiratory paralysis which followed the initial severe paresis. Death within 35 min occurred in 90% of this group.

To a large extent the second group seemed to recover from the severe dyspnoea though the paresis persisted. They then developed severe nervous signs and convulsions and died more than 60 min after injection. Actually 90% of this group died from 2 to 8 hours after injection. The nervous signs consisted of ataxia, loss of equilibrium and convulsive attacks (spontaneous or elicited by external stimuli) characterized by opisthotonus, continuous running movements, tail flailing, rolling on the long axis of the body and moving in circles to the side on which the injection had been given which was more paralyzed than the other side. Between these fits the animal lay prostrate showing marked abdominal breathing. In a few cases mice were observed at the moment of death, which took place during a fit with the head bent down onto the chest and both front and hind legs kicked out stiffly backwards. When recovery took place, nervous signs were often still in evidence after 24 hours but after 48 hours only slight disturbances in equilibrium and unthriftiness were observed.

Post mortem examinations were performed on several carcases: nothing unusual was observed.

(b) *Desacetyl main toxic component (Mild alkaline hydrolysis product)*

Due to the limited quantity of material available the method of Weil (1952) was followed and the determination was done by subcutaneous injection of guinea-pigs; only six groups of five each were used. The LD_{50} was found to be 0,9956 (0,9111 to 1,087) mg/kg.

The clinical signs and gross pathology were very similar to those described above for the MTC. However, retching movements were not observed. All showed neck paralysis within 15 to 30 min of being injected and those that died did so between 25 and 50 min. The recovery of the remainder was remarkably rapid when compared with the MTC. Severely affected guinea-pigs appeared to be completely recovered within 5 hours of being injected.

3. *Conclusions*

A very pronounced species difference in susceptibility to the MTC was discovered between guinea-pigs and mice, the latter being 18,5 times less susceptible. (Approximately the same total quantity of the MTC had to be given to a mouse to cause its death as was dosed to a guinea-pig.) Nervous signs, although occurring in guinea-pigs, were far more pronounced in mice.

In guinea-pigs very homogeneous results were obtained, the LD_1 and LD_{99} differing by only 0,1 mg/kg. In mice large individual differences were encountered, the LD_1, and LD_{99} differing by as much as 7,5 mg/kg.

Moreover, special formulae for heterogeneous results had to be used to calculate the confidence limits of the LD_{50}.

Mild alkaline hydrolysis of the MTC, known to take place between pH 7 to 8, resulted in de-acetylation and caused a five-fold drop in toxicity.

III. *Test for the cumulative effect of the MTC*

Five guinea-pigs received a daily dose by subcutaneous injection, at the rate of a quarter of the guinea-pig LD_{50} for 20 days. The dose was therefore 0,05 mg/kg and a total of five LD_{50} was administered to each guinea-pig. Except for a transient painful irritation at the point of injection and a slight hypersensitivity shortly thereafter, no effect could be observed. On challenging these guinea-pigs with an $LD_{99,9}$ (i.e. 0,3 mg/kg) from 3 to 9 days later, all died within 30 min after typical clinical signs.

Under these experimental conditions, therefore, there was no indication of a possible cumulative effect nor of any tolerance to this compound.

IV. *Histopathology subsequent to intoxication with the MTC*

Specimens from the brains, hearts, lungs, livers, kidneys and adrenals of the guinea-pigs in the latter experiment, were collected in 10% formalin and submitted for histopathological examination. J. D. Smit (Veterinary Research Institute, Onderstepoort, personal communication, 1965) reported that no significant lesions, other than severe congestion of all organs, were to be seen. This agrees with the histopathology of natural cases of acute tulp poisoning.

V. *Local anaesthetic action*

Throughout the attempts to isolate the toxic principle(s) (Part I), it was noted that the toxic fractions had a numbing, anaesthetic-like action when they were accidentally brought into contact with the mucous membrane of the lips and mouth. This effect was not noticed with plant material as such. This was first noted during concentration of the chloroform extract of the plant from an aqueous medium (e.g. Fraction II - B, Part I, Fig. 3).

MTC crystallized from benzene and chloroform was used to investigate this action.

1. *Intradermal injection in man*

The inside of the forearm of a volunteer was injected with 0,1 ml of the diluted drug to form an intracutaneous weal of *c.* 0,5 cm in diameter. Sensitivity to pain was tested by means of the prick or penetration of a hypodermic needle.

Initially the drug was dissolved in distilled water but control experiments revealed that distilled water alone also produced very definite local anaesthesia. A severe burning sensation was experienced for 30 seconds immediately after injection. This was followed by progressively deeper anaesthesia which was complete after 5 min and which lasted for an hour or more.

Four concentrations of the MTC in isotonic saline were then made for injection, viz. 0,01; 0,005; 0,0025 and 0,001%. Isotonic saline was used as a control. Anaesthesia was observed with all four concentrations but not with the saline - no burning sensation was produced as was the case with distilled water. With the 0,0025% solution slight insensitivity occurred after 15 min and complete local anaesthesia set in from 40 to 50 min after injection and lasted for as long as 100 min in one case and 180 minutes in another. Even the 0,001% solution produced a certain degree of anaesthesia between 15 to 35 min after injection.

Moderately severe side reactions were experienced. This consisted of a red, blotchy appearance and painful hyperaesthesia of the skin for 1 to 2 cm around and 4 to 5 cm distal to the point of injection. In view of the toxicity of the MTC and unpleasant side reactions, more comprehensive experiments on these lines were not conducted and the results presented here represent only a limited number of injections in one volunteer.

2. *Cornea of the rabbit and guinea-pig*

Solutions were made up in isotonic saline after first dissolving the crystals in a minimum quantity of ethanol. The maximum concentration of ethanol in the final solution was 0,6 %.

The rabbits used were of a mixed strain and consisted of males and females of different ages. After the eyelashes were cut away with a pair of scissors, a quantity of 0,1 ml was instilled into the conjunctival sac by means of a pipette and carefully distributed over the entire eyball. Five min later the eye was irrigated with 2 ml of isotonic saline to remove any remaining MTC. The sensitivity of the corneal reflex was then tested by means of touching the eyeball with the smoothly rounded end of a 2,5 mm diameter glass rod.

Three grades of anaesthesia were registered, viz. complete anaesthesia where even hard rubbing or pressing of the eyeball produced no reflex; anaesthesia where the eyeball could be touched or gently rubbed without eliciting the reflex and partial anaesthesia where the treated eyeball was definitely less sensitive than the other which was used as a control.

To exclude the effect of ethanol, six rabbits were treated in the same way with 5% ethanol in isotonic saline. No anaesthesia whatsoever was observed over a 2 hour period in which tests were done at 15 min intervals.

The following concentrations were tested: 0,01; 0,05; 0,1; 0,5 and 1,0%. The lowest concentration at which complete anaesthesia was observed, was 0,05%. In five out of eight rabbits anaesthesia was complete. It lasted between 15 and 45 min. In two other rabbits partial anaesthesia was present after 15 min. At higher concentrations anaesthesia was produced in every case, viz. in all five rabbits at 0,1 and all eight rabbits at 0,5%.

With the 1% solution, complete anaesthesia was obtained after 5 min in all ten rabbits tested and it lasted for the following periods:

Number of rabbits	2	1	1	2	1	1	1	1
Duration of local anaesthesia in h	0,5	1,0	1,5	1,75	2,5	4,0	4,5	5,5

The mean duration of complete local anaesthesia, therefore, was 127,5 min with a range of 30 min to 5,5 hours. Anaesthesia lasted from 2,5 to 27 h and partial anaesthesia was observed up to 57 h in one rabbit. This extreme variation can probably be attributed to the fact that a heterogeneous group of rabbits was used.

A marked miosis was observed from the 0,1% concentration and higher, being progressively more marked as the concentration increased. With the 1% solution it set in 15 to 30 min after instillation in all ten cases and lasted from 1,5 to 4 hours.

In ten guinea-pigs 0,025 ml of a 0,5% solution, instilled into the conjunctival sac and similarly washed out

TABLE 4 A comparison of certain properties of common local anaesthetics with those of the main toxic principle of *Homeria glauca*

	Threshold anaesthetic concentration (%)		Anaesthesia duration, rabbit cornea (min)		Subcutaneous LD_{50} (mg/kg)	
	Rabbit cornea	Human i.c. weal	1% soln.	0,1% soln.	Mouse	Guinea-pig
1. Cocaine	0,32	0,02	22	—	189	50
2. Procaine	4,0	0,04	0–2	—	800	400
3. Dibucaine (Nupercaine)	0,003	0,002 5	—	51	—	11,2
4. Tulp: main toxic principle	0,05	0,002 5	128	30–60	3,6	0,194

after 5 min with isotonic saline, also produced complete anaesthesia within 5 min. This lasted from 2 to 6,5 hours with a mean of 3,5 hours. Instead of miosis, however, a marked mydriasis set in in all cases between 15 and 30 min and lasted from 1 to 4,5 hours. In addition, all ten guinea-pigs developed hypersensitivity and neck paresis. Absorption from the mucous membrane of the eye was therefore good, since for an estimated mean weight of 600 g a dose of 0,21 mg/kg had been instilled.

Except for a very slight transient irritation seen in some animals immediately after instillation, no untoward effects on the eyes were observed. No ulceration or inflammation whatsoever occurred in 36 rabbits and ten guinea-pigs on which the compound was tested. Vision did not seem to be affected in any way during anaesthesia.

Certain properties of the MTC are compared with those of known local anaesthetics and these are listed in Table 4, which has been adapted from Goodman & Gilman (1955) and Hirschfelder & Bieter (1932). Strictly speaking, these results cannot be compared directly because the exact techniques by which they had obtained their data were not available. However, the approximate order of the local anaesthetic property of the MTC of tulp can be assessed in this manner.

DISCUSSION

At the outset it was realized that successful isolation would depend on a different approach to that followed by previous workers in respect of conditions of isolation, e.g. temperature, pH and type of solvent. Furthermore, the possibility that the toxic principle might not be an alkaloid, as previous workers had suspected, was also borne in mind. Attempts, therefore, were aimed at isolation of a toxic principle - whatever it was - rather than isolation of a specific type of compound such as an alkaloid, glycoside, etc. The general chemical and physical properties of these compounds were, however, always borne in mind and constantly compared with those of the toxic fraction. The isolation process was followed by toxicity trials performed at every step of fractionation. The specific toxicity of a particular fraction was determined as well as the total number of LD in it. These results undoubtedly contributed considerably to the ultimate success of isolating the toxic principles.

Initial extraction of plant material in chloroform revealed the insolubility of the toxic principles in this solvent. Extraction of the plant material however, with aqueous alkali or acid and subsequent chloroform extraction of the aqueous extracts, resulted in complete removal of the toxic principles from the aqueous extracts. This finding clearly demonstrated that the known solubility of a particular component in a particular solvent does not necessarily mean that such a component will be extractable from plant material in that solvent.

It was discovered that loss of toxicity occurred in an alkaline medium. When working with biologically active material of unknown nature, the necessity of regular semi-quantitative toxicity determinations at each isolation step and the avoidance of any pH extremes, is evident.

A vital 80% loss in toxicity might have been experienced right at the start and would possibly never have been discovered if initial extraction had taken place under relatively mild alkaline conditions,e.g. at pH 10. and had regular toxicity determinations not been done at each step. This can be confirmed by comparing the toxicity of the MTC with that of desacetyl MTC.

A knowledge of other solubility characteristics of the toxic fraction, contributed much to the development of a successful isolation procedure, e.g. its insolubility in ether, petroleum ether, carbon tetrachloride and toluene, slight solubility in water and benzene and good solubility in chloroform, acetone, ethyl acetate, ethanol and methanol. Insolubility in petroleum ether as against solubility in alcohols and water clearly demonstrated the polar nature of the toxic principle(s). On the other hand its ready solubility in chloroform also indicated a certain degree of non-polarity. The probability, therefore, of a relatively large molecule with a few strongly polar groups was indicated.

The fact that separation of the components of the toxic fraction could not be affected on a cation exchange resin, was the first indication that it was probably not of an alkaloidal nature. On the other hand, the complete loss in toxicity experienced on the anion exchange resin was a further confirmation on the lability of the toxic principle(s) under alkaline conditions.

The successful application of TLC on silica gel and consequent resolution of the components of the toxic fraction, as well as the discovery of the excellent qualities of ceric sulphate as a spraying reagent, constituted important advances. TLC soon revealed that the toxic fraction was completely adsorbed onto silica gel in chloroform but that the degree of adsorption was inversely proportional to the percentage of ethanol present in the chloroform. TLC also revealed that water in the developing medium resulted in a lowering of the Rf value of the main component of the toxic fraction.

These empirical characteristics enabled adaptation of TLC to successful column chromatography on silica gel. Column chromatography soon revealed the component in which the main toxicity was vested. This technique was investigated under different sets of conditions such as load applied, ethanol concentration and especially water content of the chloroform phase. Eventually the conditions were established under which the main

toxic component could be satisfactorily separated from all other components.

In the successful isolation described in Part I, overloading of the first column had taken place. This resulted in contamination of the MTC with a small quantity of the green fluorescent spot (Part I, Table 1), the yellow "background" contaminant, and the blue-brown and grey-blue spots (Part I, Table 2). The resolution of this mixture (1,62 g obtained from 2 kg of plant material) necessitated the use of a second silica gel column. This finally resulted in a dried, resinous film (1,22 g) containing the MTC as a single component. Crystals (870 mg) were obtained from ethanol and this constituted a yield of 0,044% by mass of the original (dried) plant material. This quantity of MTC represented a recovery of 50 to 60% of the total toxicity present in the (dried) plant material.

The experiments described above clearly demonstrated the extreme difficulty of resolving a complex plant extract into pure components on a single column. Partial purification on one column, followed by complete resolution on a second column is the usual procedure and has definite advantages in terms of load and, therefore, yield. On the other hand, if success can be achieved on one column only, at a very low load, the time and labour saved is considerable. This was subsequently done at a load of less than 2 mg extract/ml of packed silica gel. The requirement of very low load is especially true for column packing materials with a low capacity such as silica gel, alumina, etc. Initially 50% ethanol: citrate buffer (0,2 M, pH 3,25) was used to extract the first chloroform residue obtained by extraction of aqueous extracts of plant material (Part I, Fig. 2). Subsequently 33% ethanol in 0,1 N HCl was used. The high purity of the toxic fraction obtained by this method was probably due to the fact that the plant pigments were less polar at a pH of 1 than at a pH of 3,25 and were, therefore, not extracted by the rather polar 33% aqueous ethanol.

During chromatographic separation another five toxic components were demonstrated. These were classified as being different from the MTC in terms of the clinical signs they produced, their R_f values and yield of pure components. In addition, the combination of the ethanol eluates of the two silica gel columns was toxic and contained a whole series of components, amongst others the desacetyl product of the main toxic component. Experiments on degradation of the MTC produced components which, on TLC, closely resembled those encountered during column chromatography of plant material. It is suspected that some of the latter might be either degradation products or precursors of the MTC.

The overall loss in total toxicity experienced during isolation (Part I, Table 3), may possibly be attributed to the following factors:

(1) Degradation (e.g. deacetylation) of these principles during isolation.
(2) Strong adsorption of the more polar toxic components on the silica gel (ethanol elution did not result in complete recovery of the material applied to the columns).
(3) A synergistic action between the different toxic components encountered in the plant which might result in enhancement of toxicity.

Crystallization could be effected easily from different solvents but it was clear that solvent of crystallization was present in most cases. This resulted in difficulties during melting point determinations. Sharp melting points were rarely obtained, despite repeated recrystallizations. The fact that the MTC (and its derivatives) were definitely proved to be heat labile at 105°C, is in all probability a further important reason for these indefinite melting points, i.e. decomposition started to occur before melting took place.

Spectroscopic investigations yielded some clues as to the identity of the main toxic component. In the ultraviolet absorption spectrum the strong absorption at 298 mμ, which was not affected by short exposure to mild alkaline or acidic changes, was indicative of an α-pyrone group. Its aliphatic character and the presence of hydroxyl, carbonyl, ester, acetate and methylketone groups were indicated by typical absorption bands in the infra-red spectrum.

Elementary analysis revealed that the MTC contained only C, H and O and no N. Solvent of crystallization prevented calculation of an accurate empirical formula or the determination of an accurate molecular mass. The approximate empirical formula was found to be $C_{25}H_{30}O_7$ and the molecular mass 442.

The lability of the MTC to extremes of pH, heat and light was unequivocally demonstrated. Eventual successful isolation was to a large extent attributable to the meticulous avoidance of such conditions, e.g. even under extremely mild aqueous alkaline conditions deacetylation took place and resulted in a five-fold decrease in toxicity. Under strictly controlled conditions a good yield of the desacetyl derivative of the MTC could be isolated, but further alkaline degradation led to a non-toxic series of components that had lost their ester characteristics.

A limited number of toxicological and pharmacological investigations was done (Part II). The MTC was found to be highly toxic for guinea-pigs, but mice were considerably more resistant to its effects. This type of interspecific variation is commonly experienced.

It was concluded that the MTC was mainly a nervous poison resulting in respiratory paralysis as the final cause of death in guinea-pigs. In mice either respiratory paralysis in the early stages of poisoning or, subsequent to a most unusual and very typical nervous syndrome, strychnine-like convulsions in the later stages resulted in death. Any cardiac effects observed were secondary to the nervous and respiratory actions. Other toxic components isolated from the plant produced similar clinical signs in guinea-pigs.

Although the main toxic component was found to have potent local anaesthetic properties, its high toxicity and unpleasant side effects make its possible use for this purpose rather doubtful. It is strongly suspected that the acetate and desacetyl products of the MTC also act as local anaesthetics. The local anaesthesia caused by the intradermal injection of distilled water is probably explained by the fact that the intracutaneous localization of a volume of distilled water, in a position from which it could not easily escape, caused a temporary localized electrolyte imbalance with a consequent disturbance in nervous conduction. At this stage no explanation can be offered as to why the MTC should cause miosis in rabbits, but mydriasis in guinea-pigs.

After the present investigation was concluded, the structure of the main toxic component was determined in collaboration with P. R. Enslin of the National Chemical Research Laboratory, Council for Scientific and Industrial Research, Pretoria, and A. J. van Wyk of the Research Institute for Soils, Department of Agricultural Technical Services, Pretoria. This work is reported by Enslin *et al.* (1966). These workers found that the main toxic principle was 1α, 2α - epoxyscillirosidin,

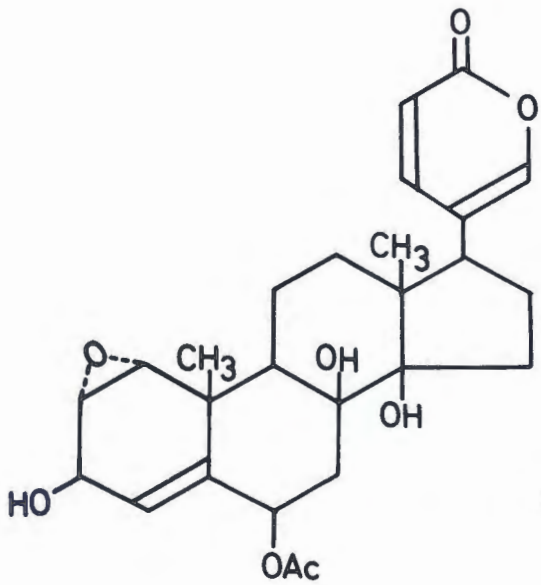
($C_{26}H_{32}O_8$, M.M. 472), the aglycone of a new bufadienolide cardiac glycoside (Fig. 10A). This is closely related to the known scillirosidin (Fig. 10B), the main toxic principle of Red Squill, *Urginea* (*Scilla*) *maritima* (L.) Bak. var. *rubra*.

The insolubility of the poisonous principles when plant material was extracted with chloroform in comparison to the high solubility in chloroform from aqueous extracts of the plant, can probably be explained as follows:-

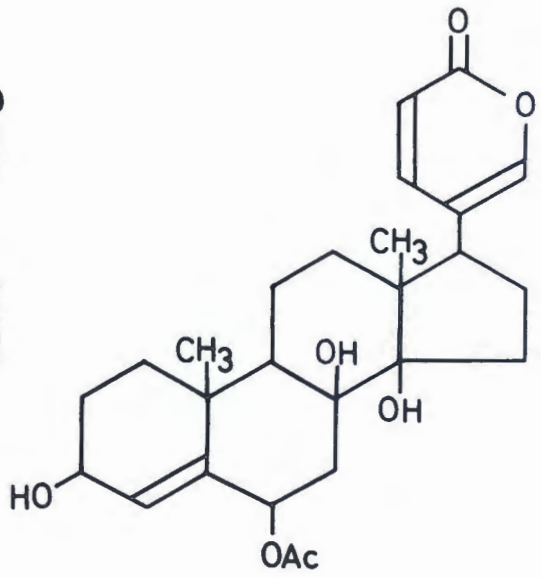
In the plant these compounds usually exist as glycosides, the glycosidic bond being at C_3 of the aglycone. The compound would therefore be too polar to dissolve in chloroform. Under mild alkaline or acidic aqueous conditions hydrolysis of this bond may easily be effected, especially if this sugar residue should be a 2-deoxy derivative (Fieser & Fieser, 1959). This would result in the liberation of the aglycone which would be readily soluble in chloroform.

It is very probable that the more polar, toxic fraction eluted from the silica gel columns with ethanol, contained, *inter alia*, the unhydrolyzed glycoside. The toxicity of the acetic acid phase, after the main toxic fraction had been extracted with chloroform, is very probably due to unhydrolyzed glycosides.

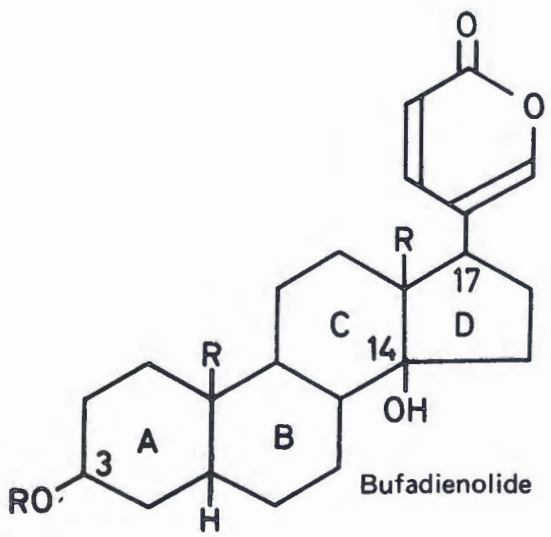
Furthermore, scillirosidin itself may be one of the plant's components. Chromatographically it resembles the green fluorescent spot, obtained in these plant extracts, very closely (A. J. van Wyk, Research Institute for Soil, Pretoria, personal communication, 1966).



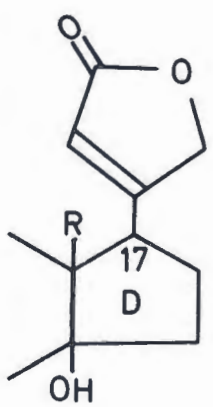
A. 1α, 2α - epoxyscillirosidin



B. Scillirosidin



Bufadienolide



Cardenolide

C. Stereospecificity for digitalis action

FIG. 10 Structural formulae of relevant cardiac aglycones

During investigations into the instability of the MTC in alkali and subsequent acetylation of the product obtained, a reaction scheme was postulated to explain the results (Part I, Fig. 8). Since the structure became known a more definite scheme can now be postulated as illustrated in Fig. 11.

Acetylation of epoxyscillirosidin (I, Fig. 11A) would take place at the very reactive secondary hydroxyl group at C_3 giving epoxyscillirosidin-acetate (IV) with acetate groups at both C_3 and C_6. Mild alkaline hydrolysis of epoxyscillirosidin resulted in the loss of an acetate group at C_6 giving desacetyl-epoxyscillirosidin (II). On reacetylation of the latter, the reaction took place first at the more reactive C_3 hydroxyl group, resulting in the 3-acetyl, 6-desacetyl derivative of epoxyscillirosidin (III), and only thereafter at C_6 to give epoxyscillirosidin acetate (IV). The complete loss of toxicity and ester character after exposure to strong alkali can, in all probability, be attributed to hydrolysis of the α-pyrone side-chain at C 17 (Fig. 10A).

Some of the toxicological and pharmacological properties of scillirosidin, especially the nervous syndrome in rodents, as described by Rothlin & Schalch (1952) and Gold, Modell, Cattell, Benton & Cotlove (1947), closely resemble those of epoxyscillirosidin.

The following stereo-specificity (Fig. 10C) is essential for a digitalis cardiac action (Chen & Henderson, 1954):-

1. Ring attachment: A - B preferably cis; B - C definitely trans; C - D definitely cis.
2. The following constituents must be present and the configuration must be β: A hydroxyl group at C_3, free or in a glycosidic bond; a hydroxyl group at C_{14}; a five- or six-membered lactone ring at C_{17}, preferably unsaturated.

All these requirements are met with in epoxyscillirosidin. The very typical terminal clinical signs described in digitalis intoxication, however, were never observed with this specific tulp or its active principles during isolation procedures in either guinea-pigs or rabbits. These typical signs are complete heart block resulting in severe arrhythmia with the heart stopping in systole, breathing being only affected secondarily. During the early part of the structure determination, a very strong suspicion was expressed by our co-workers Enslin & Van Wyk that the MTC was a cardiac aglycone.

The MTC was administered to a rat under pentobarbital anaesthesia. An electrocardiographic examination was carried out by K. C. Holemans (Department of Physiology, Medical Faculty, University of Pretoria, personal communication, 1965). This animal also died of respiratory distress and the heart kept on beating long after apnoea had set in. However, J. M. Combrink (517 Sanlam Building, Pretoria, personal communication, 1965), who interpreted the electrocardiogram, pointed out several typical digitalis changes prior to the onset of anoxia. These were:

(1) Progressive bradycardia
(2) Prolongation of the P–R interval.
(3) Change in direction of the T-wave in one lead.

Thus the pharmacological observations on the digitalislike action of crude or partially purified extracts from different tulp species by MacKenzie (1910), Rindl (1924) and Gunn & Brown (1932) were confirmed.

As far as can be ascertained, a local anaesthetic action has never been described before for a member of the cardiac glycoside group. It is well known that cardiac glycosides inhibit the sodium and potassium dependent adenosine triphosphatases which are essential for the action of the intracellular sodium pump and the consequent trans-membrane potential (Hoffmann, 1962). As the latter is absolutely essential for nervous conduction, it may be postulated that this is the basic explanation for the local anaesthetic effect as well as for the paralytic syndrome encountered. In the work of Hoffman (1962), the active trans-membrane transport of sodium and potassium was studied *in vitro* in erythrocyte ghosts. Cardiac glycosides were specifically used to inhibit and study the sodium pump's action. This method seems directly applicable to *in vitro* studies on antidotes for cardiac glycosides on the cellular level. Future work with tulp toxic principles on these lines might prove useful.

Subsequent to this work a further aglycone from *H. glauca* has been isolated and identified (Van Wyk & Enslin, 1969). This proved to be 14-hydroxy, 1α, 2α-epoxyscillirosidin, one of the more polar toxic principles of this species.

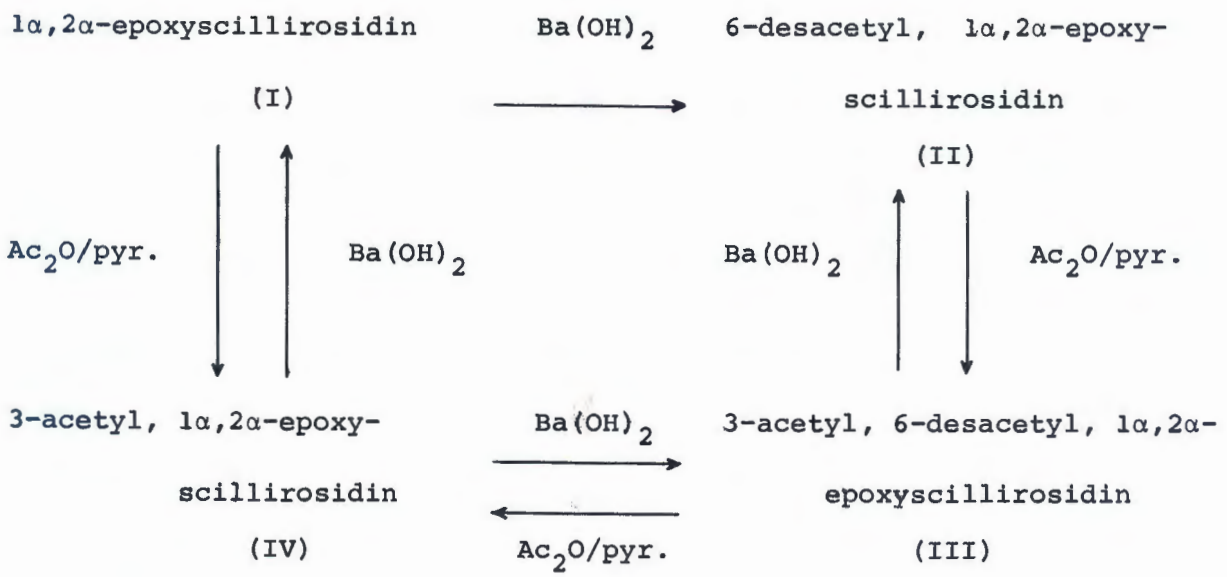


FIG. 11 A revised reaction scheme of alkaline degradation and acetylation of the MTC and its products

The fact that cardiac bufadienolide glycosides have been isolated from the genus *Moraea* of the same family, e.g. *M. polystachya* Ker. and *M. graminicola* Oberm. (Van Wyk & Enslin 1968) strongly indicates that the tulp group as such contains bufadienolides as toxic principles.

Important recent discoveries are those by Van Rooyen & Pieterse (1968) that *Cotyledon wallichii* Harv. (Crassulaceae) also contains a bufadienolide glycoside and by Anderson & Koekemoer (1968, 1969) who isolated several bufadienolides from *Melianthus comosus* Vahl.

In conjunction with the discovery by Louw (1952) that *Urginea sanguinea* Shinz. (= *U. burkei* Bak.) contains bufadienolides, this now indicates that poisoning is due to bufadienolide cardiac glycosides or aglycones in three economically significant groups of toxic plants in South Africa, i.e. Slangkop (Liliaceae), Tulp (Iridaceae) and Krimpsiektebossie (Crassulaceae).

Rational treatment of animals suffering from cardiac glycoside poisoning, specifically bufadienolides, therefore, now becomes a subject which should receive urgent attention. Cosmides, Miya & Carr (1956), reported interesting preliminary results on the use of relatively simple furane derivatives for the treatment of cardenolide poisoning. The use of pyrane derivatives for bufadienolide poisoning must be investigated.

It is unnecessary to stress the importance of cardiac glycosides in the treatment of cardiac insufficiency in man. There is a constant search for safer, more effective cardiac glycosides. It is not impossible that the tulp group may yet yield this ideal drug. If the attempts at treatment of tulp poisoning in animals prove successful, it may be directly applicable to cardiac glycoside overdosage in man, for which to date no specific and really effective antidote exists.

It is, therefore, just possible that this scourge of the farmer may, after all, be applied to benefit mankind.

Summary

The main toxic principle of *Homeria glauca* (W. & E.) N.E. Br. was obtained by employing mild isolation procedures correlated with regular semi-quantitative toxicity determinations in guinea-pigs. Some of its chemical and pharmacological properties were studied.

The toxic fraction could be extracted from plant material by water under mildly acidic or alkaline conditions. An almost quantitative removal of the toxicity from this aqueous phase could be effected with chloroform. Direct chloroform extraction of plant material under mild conditions was, however, ineffective.

The successful isolation procedure eventually employed consisted of suspending dried plant material in acetic acid (0,2 M) and extracting the toxic fraction from this aqueous suspension with chloroform. The latter was then evaporated and the residue extracted with ethanol: citrate buffer (0,2 M, pH 3,25), 1 : 1 (v/v). Extraction of this aqueous ethanol phase with chloroform produced a highly toxic residue containing 76% of the original total toxicity.

The presence of one major and several minor components was revealed by thin layer chromatography (TLC) on silica gel. Successful TLC separation resulted in the development of a column chromatographic method (silica gel) through which the individual components could be isolated.

The main toxic component (MTC) was toxic at 0,4 mg/kg while five other components were toxic at between 10 and 50 mg/kg. In addition, the ethanol eluates of the silica gel columns contained a mixture that was lethal at 12,5 mg/kg. The MTC was crystallized from ethanol and constituted 0,044% by mass of the original (dried) plant material. This was equivalent to a yield of between 50 and 65% of the original total toxicity of the plant material extracted.

Crystallization of the MTC could be effected from different solvents but sharp melting points could not be obtained because solvent of crystallization was present and because decomposition took place at high temperatures. Trouble was also experienced in the determination of the molecular mass and empirical formula.

Infra-red and ultra-violet spectroscopy and elementary analysis revealed that the MTC contained only C, H and O, that it was aliphatic in nature and that it had strong hydroxyl, carbonyl and ester characteristics as well as the possibility of an α-pyrone chromophore system. The estimated molecular mass was 442 and it had an empirical formula of $C_{25}H_{30}O_7$. (The structure of the MTC was subsequently elucidated. It is a new bufadienolide cardiac aglycone, 1α, 2α-epoxyscillirosidine, $C_{26}H_{32}O_8$, M.M. 472).

As determined by TLC this compound was rather labile to heat, light (especially of low wavelength) and pH extremes, particularly on the alkaline side. Very mild alkaline hydrolysis resulted in the successful isolation of the desacetyl product of the MTC. An acetyl derivative was obtained without difficulty.

The subcutaneous LD_{50} of the MTC was found to be 0,194 (95% confidence limits 0,183 to 0,203) mg/kg for male Wistar guinea-pigs and 3,6 (95% confidence limits 2,9 to 4,46) mg/kg for male Wistar mice. The desacetyl product proved to be five times less toxic for guinea-pigs. The clinical signs observed with the pure MTC were very similar to those caused by the plant. In guinea-pigs nervous signs and a generalized curare-like paralysis were observed and death resulted from respiratory paralysis. In mice a convulsive syndrome was encountered. The MTC proved to be a potent local anaesthetic.

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