ISOLATION AND IDENTIFICATION OF THE TOXIC PRINCIPLE OF GASTROLOBIUM GRANDIFLORUM

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SUMMARY

Gastrolobium grandiflorum **is one of the most toxic of Queensland plants and has caused serious stock losses in Central Queensland.**

The toxic principle was isolated from the leaf and identified as monofluoroacetic acid. Identity was established by gas chromatography, infra-red absorption spectroscopy, and micro-fluorine analysis.

An extract of *G. grandiflorum* **administered orally to both sheep and rats resulted in clinical symptoms, biochemical changes, and post-mortem findings similar to those obtained following the administration of an equivalent amount of authentic monofluoroacetic acid.**

I. INTRODUCTION

Gastrolobium grandiflorum F. Muell. is a shrub extending up to 6 ft in height, with several slender stems from a woody tuber just below ground level. It is known locally as heart-leaf poison bush but is not the true "heart-leaf", which grows only in Western Australia (Everist 1947). It is known also as desert poison bush and wallflower poison bush. The species grows on yellow sandy soil in poor forest country in northern and central Queensland. It occurs also in the Northern Territory and the north of Western Australia (Everist 1947). It is the only toxic species of *Gastrolobium* which extends beyond the boundaries of Western Australia and is the only toxic species found in the tropics (Gardner and Bennetts 1956, p. 70).

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The plant was first collected in the Whithrington Range in northern Australia by J. McD. Stuart on an exploration expedition and was characterized by F. von Mueller (1862). Its toxic nature was discovered early in its history, and Bailey (1900, p. 353) referred to it as "The most toxic of any in the Australian flora". Many instances of the toxicity have been recorded (Unpublished Reports, Official Records of the Queensland Department of Primary Industries). In these records many instances of sheep being killed with as little as 200 g or less are listed, while estimates of annual losses have been placed at 150–300 bullocks on one property and at 500 bullocks on another; the loss of 2,000 sheep, at one time, on another property in the affected area is also recorded.

In both sheep and cattle, poisoning is characterized by suddenness of death. Although several hours may elapse between exposure and the first apparent symptoms in the animal, it is frequently only minutes between onset of symptoms and death. Excitement, exercise or fright may precipitate symptoms.

The isolation and identification of the toxic principle in the leaf of *G. grandiflorum* is now reported.

II. MATERIALS AND METHODS

(i) *Source of Material.*—The leaf of *G. grandiflorum* was collected from widely separated areas in central Queensland. The samples were identified the Queensland Herbarium and the specimens were filed there under the numbers BRI 023743, BRI 025176, BRI 027202 and BRI 036495. Some specimens were air-freighted from the point of collection and were deep-frozen pending chemical examination. The bulk of the material was shade-dried, rail-freighted, hammer-milled and stored in sealed plastic or aluminium containers.

(ii) *Experimental Animals.*—Female albino rats of weight range 120–160 g and female white mice of weight range 25–30 g were used in experimental toxicity testing. Merino wethers of weight range 25–35 kg were used in the large-animal testing. Both rats and sheep were dosed by stomach tube with aqueous solutions which had been adjusted to pH 7. Mice were dosed by intraperitoneal injection. Sheep received volumes of up to 1 l, rats up to 10 ml, and mice up to 1·0 ml. All dosings were related to known quantities of air-dried leaf. Experimental toxicity testing with mice and/or rats was carried out after each chemical treatment of the extract.

Jugular blood samples from sheep were taken for biochemical testing directly into tared bottles containing 8 per cent. trichloroacetic acid solution. Samples of tissue were collected by routine necropsy methods immediately after death, weighed and macerated with 8 per cent. trichloroacetic acid solution.

(iii) *Citric acid Estimations.*—The estimation of citric acid was by a method developed in this laboratory and incorporating the better features of techniques reported by Weil-Malherbe and Bone (1949), Buffa and Peters (1949), Ettinger,

Goldbaum, and Smith (1952) and Taylor (1953). Samples of blood and tissues of sheep and organs of rats were examined. Delay in processing was avoided, as this caused a considerable reduction in the level of citric acid.

(iv) *Silicic Acid Chromatography.*—Two procedures were used. Method 1 was essentially that defined by the Association of Official Agricultural Chemists (1960, p. 359). Method 2, a modification of this method (Oelrichs and McEwan 1962) using the two mobile solvents fractionated chloroform and fractionated chloroform (950 ml) containing fractionated n-butanol (50 ml), was used to obtain a more complete separation of monofluoroacetic acid.

(v) *Esterification.*—Butyl esters were formed using butanol, free acid and concentrated sulphuric acid at 100°C for 1 hr in a sealed ampoule. After neutralization with aqueous sodium bicarbonate solution, the esters were extracted with a small volume of sulphuric ether, washed with water and dried.

2-chloroethanol esters were formed by the method of Oette and Ahrens (1961) by treating the acid with 2-chloroethanol containing 5–7 per cent. hydrogen chloride in a sealed tube for 1–2 hr. The mixture was then diluted with 5 volumes of water, extracted with hexane, the hexane washed with water and dried.

(vi) *Quantitative Estimation of Monofluoroacetic Acid.*—The method used was that of Oelrichs and McEwan (1962).

(vii) *Specialized Equipment.*—Micro-fluorine analyses were done by the method described by Harvey (1952). A Perkin-Elmer Model 154 D Vapor Fractometer with Flame Ionisation Detector and a 1 mV Honeywell Brown Strip Chart Recorder were used for vapour fractionation. In chromatography of free acids a metre stainless steel column was packed with acid-washed "Embacel" kieselguhr (7·5 g) coated with Tween 80 (2·5 g), a slight variation of the method of Emery and Koerner (1961). Esters were separated on a 2-m column packed with finely ground diatomaceous earth coated with polypropylene glycol—Perkin-Elmer Column R. For infra-red spectometry a Perkin-Elmer Model 21 instrument was used.

III. EXPERIMENTAL AND RESULTS

(a) Toxicity Testing

During the extraction and purification of the toxin from *G. grandiflorum,* solutions were toxicity tested with mice and rats. Mice were injected intraperitoneally with neutral aqueous solutions in a range of dosages, three mice being used at each dose rate. Initially, correlation of these results with toxicity to rats by oral dosage was good. However, as separation proceeded, particularly in relation to partition into butanol from aqueous solutions of varying pH, poor correlation indicated that the mouse test was not satisfactory.

In toxicity testing with rats using purified extracts, the symptoms of muscle tremors and convulsive spasms were very similar to those produced by monofluoroacetic acid. This directed attention towards the measurement of biochemical changes in blood and tissue.

Sheep were used in the terminal stages of the investigations to confirm that the extracted toxin produced clinical symptoms, biochemical changes and post-mortem findings similar to those obtained both with whole plant and with authentic monofluoroacetic acid. The residual plant material after extraction produced no clinical symptoms and no significant biochemical changes in sheep.

(b) Chemical Extraction

Preliminary work showed that the toxin was water-soluble. Investigation to determine a better extracting solvent, particularly from the point of view of obtaining complete extraction with minimum amounts of contamination, showed that methanol extracted the toxin readily, and that continuous extraction with ethanol in an all-glass Soxhlet apparatus yielded complete extraction. Ethanol extraction followed by concentration, addition of water, filtration, and further concentration of the neutralized solution provided a solid residue of maximum toxicity.

Acidification to approximately 1 per cent. with sulphuric acid, filtration, continuous extraction of the filtrate with alcohol-free sulphuric ether, and partition into N sodium bicarbonate solution yielded an extract which was extremely toxic to rats. Citric acid levels obtained from one rat dosed with this extract are recorded in Table 1 (Rat No. 2). A sheep dosed with a similarly prepared extract from 600 g of air-dried leaf died showing typical convulsions in 3½ hr; levels of citric acid in blood and tissue are included in Tables 2 and 3.

TABLE 1

CITRIC ACID LEVELS IN ORGANS OF RATS

Rat No.	Treatment	Organ	Citric Acid (p.p.m.)
1	Aqueous extract equivalent to 25 g leaf/kg	Kidney	1040
		Liver	102
2	Ether extract of aqueous solution equivalent to 28 g leaf/kg	Kidney	628
		Liver	254
3	Ether solution from silicic acid chromatography equivalent to 25 g leaf/kg	Kidney	1190
		Liver	141
4	Monofluoroacetic acid 3·5 mg/kg	Kidney	959
		Liver	112
5	Monofluoroacetic acid 3·0 mg/kg	Kidney	1096
		Liver	123
6	Undosed control—slaughtered	Kidney	37
		Liver	17
7	Undosed control—slaughtered	Kidney	42
		Liver	19

TABLE 2

CITRIC ACID LEVELS (P.P.M.) IN BLOOD OF SHEEP

Time of Sampling	Sheep No. 1. Dosed with Extract of 600 g of *G. grandiflorum* Leaf	Sheep No. 2. Dosed with 50 mg of Monofluoro-acetic Acid	Sheep No. 3. Dosed with 1,200 g of Extracted Leaf	Sheep No. 4. Undosed Control
Predosing ..	14·3	24·6	13·4	17·8
After 30 min ..	8·1	24·9	10·4	19·7
After 90 min ..	26·0	28·8	18·4	18·6
After 120 min ..	44·0	29·7	20·0	19·2
After 150 min ..	59·6	34·5	—	—
After 180 min ..	76·9	54·3	25·6	20·5
After 220 min ..	—	40·9	20·6	—
After 300 min ..	—	46·2	18·5	18·2
After 390 min ..	—	45·3	18·3	19·8

TABLE 3

CITRIC ACID LEVELS (P.P.M.) IN TISSUES OF SHEEP

Tissue	Sheep No. 1. Dosed with Extract of 600 g *G. grandiflorum* Leaf	Sheep No. 2. Dosed with 50 mg Monofluoroacetic Acid	Sheep No. 4. Undosed Control
Heart	304	400	39
Kidney	191	265	20
Diaphragm	76	100	39
Spleen	57	80	22
Liver	17	35	9
Cerebellum	15	58	25
Medulla	15	68	16
Cerebrum	23	95	12

Repetitive partition of the toxin obtained from 4 kg of leaf between ether and aqueous media by changing pH resulted in an ether solution containing 480 mg monofluoroacetic acid, estimated by gas chromatography. This solution (100 ml) was dried over anhydrous sodium sulphate (10 g) and activated carbon (5 g), filtered, and distilled through a semi-micro fractionating column, using an oil-bath temperature up to 180°C. Maximum distillation temperature obtained in this procedure was 130°C. Removal of volatile acids in this way reduced the total acid concentration by approximately 90 per cent. as estimated by titration with N sodium hydroxide. No appreciable toxicity was detected in the distillate.

The neutral solution obtained by titration of the residue was filtered acidified to 1 per cent. with sulphuric acid, extracted with ethanol-free sulphuric ether, extracted with sodium bicarbonate solution and carefully exaporated to dryness.

(c) Silicic Acid Chromatography

Half of the solid obtained by chemical extraction was acidified with 50 per cent. sulphuric acid, dried with a minimum quantity of anhydrous sodium sulphate and transferred to a silicic acid column (Method 1). The column was prepared by grinding 10 g silicic acid with 5·0 ml of 0·5N sulphuric acid and forming a slurry with the mobile solvent consisting of fractionated chloroform containing 10 per cent. by volume of fractionated n-butanol. Air bubbles were removed and the column packed under pressure. The eluate from the column was collected in 3-ml fractions. Examination of the fractions by gas chromatography revealed the presence of peaks corresponding to monofluoroacetic acid in fractions 10-12. These fractions were combined and the silicic acid chromatographic Method 2 used to obtain a more complete separation. In this procedure 15 g silicic acid was ground with 7·5 ml 0·5N sulphuric acid, mixed with fractionated chloroform and poured into a column. After preparation of the column and addition of the sample the acids were developed using fractionated chloroform (100 ml) and then eluted, using fractionated chloroform (95 ml) containing fractionated n-butanol (5 ml). Fractions (3 ml) were collected from the eluate and peaks corresponding to monofluoroacetic acid were found by gas chromatographic examination of fractions 28–30. These were combined, and quantitative gas chromatographic estimation indicated a recovery of 190 mg monofluoroacetic acid.

After evaporation to dryness from a sodium bicarbonate solution, the solid was dissolved in 1 per cent. sulphuric acid and extracted with a minimum quantity of distilled ethanol-free sulphuric ether. The ether solution was dried over anhydrous sodium sulphate and evaporated at −50°C, with precautions to prevent introduction of moisture, to a final volume of 0·5 ml.

(d) Gas-liquid Chromatography

In the preliminary studies gas chromatography was used to detect the presence of an acid in plant extracts and in fractions purified by silicic acid chromatography having the same retention time as monofluoracetic acid. Subsequently the gas chromatograph was used to prepare a pure, moisture-free sample of the toxin for infra-red absorption spectroscopy.

In this step the nitrogen carrier gas was thoroughly dried by passage through a mixture of phosphorus pentoxide and powdered asbestos. Comparison of the ether solution (0·5 ml) obtained from the silicic acid chromatography with monofluoroactic acid (Figure 1) was carried out to determine accurately the times of collection. To collect the pure sample a U-tube of pyrex glass of 7-mm internal diameter, 11 cm in height, packed with a dry purified sand and immersed in a freezing bath (−80°C) was connected to the column outlet at the predetermined times following three injections (each of 50 μl) into the gas flow. The sample was dissolved by heating with carbon tetrachloride (1ml) and was removed by means of a syringe. This solution was examined by infra-red spectroscopy.

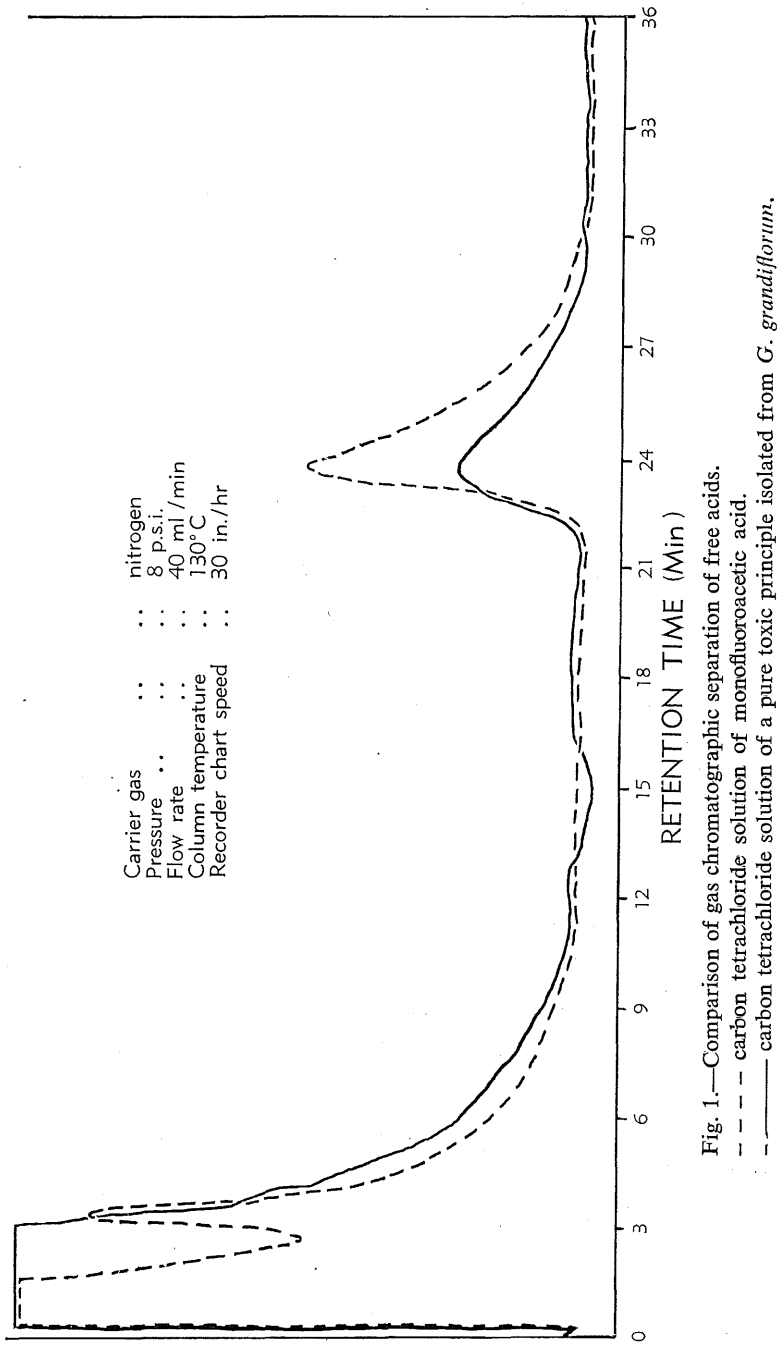

Fig. 1.—Comparison of gas chromatographic separation of free acids.
– – – – carbon tetrachloride solution of monofluoroacetic acid.
———— carbon tetrachloride solution of a pure toxic principle isolated from *G. grandiflorum*.

The remainder of the material adhering to the sand was removed with dry sulphuric ether and evaporated to dryness at —50°C. This material, together with the remainder of the ether solution obtained from silicic acid chromatography,

was used to form butyl and 2-chloroethanol esters for comparison with these esters of monofluoroacetic acid. Comparison by gas chromatography of the butyl esters is shown in Figure 2, and comparison of the 2-chloroethanol esters in Figure 3.

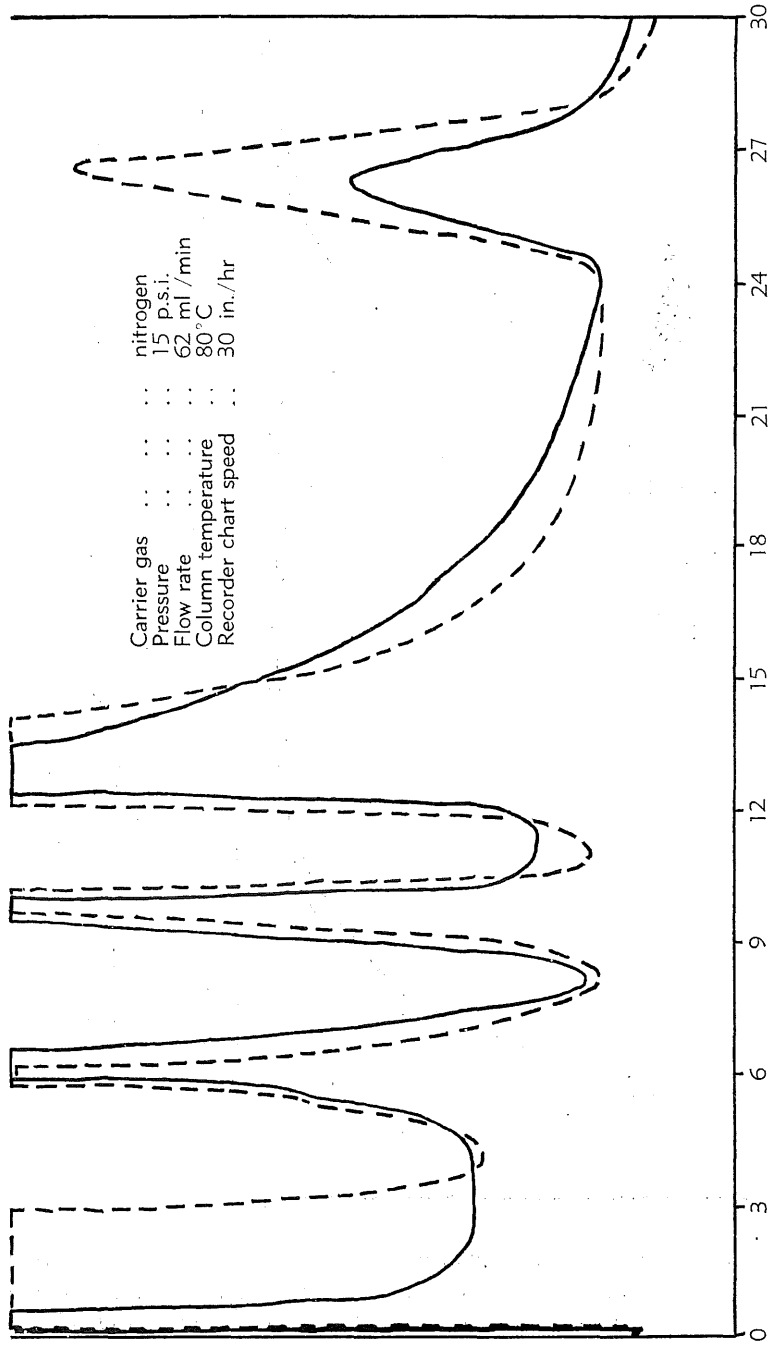

Fig. 2.—Comparison of gas chromatographic separation of butyl esters.
- - - - ether solution of ester prepared from monofluoroacetic acid.
——— ether solution of ester prepared from pure toxic principle isolated from *G. grandiflorum*.

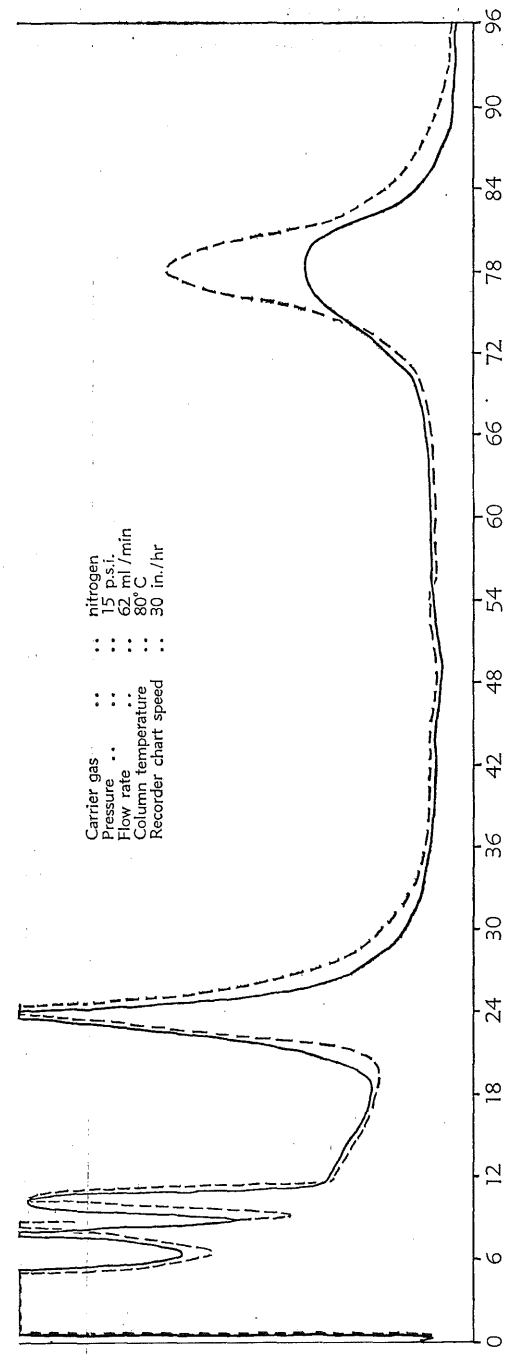

Fig. 3.—Comparison of gas chromatographic separation of 2-chloroethanol esters.
– – – – ether solution of ester prepared from monofluoroacetic acid.
——— ether solution of ester prepared from pure toxic principle isolated from *G. grandiflorum*.

(e) Infra-red Absorption Spectroscopy

The infra-red spectra of carbon tetrachloride solutions of the sample collected after gas chromatography and of monofluoroacetic acid are shown in Figure 4.

Both spectra show the absorption bands characteristic of a short-chain aliphatic carboxylic acid together with a band at 1095 cm^{-1} characteristic of a carbon-fluorine stretching mode.

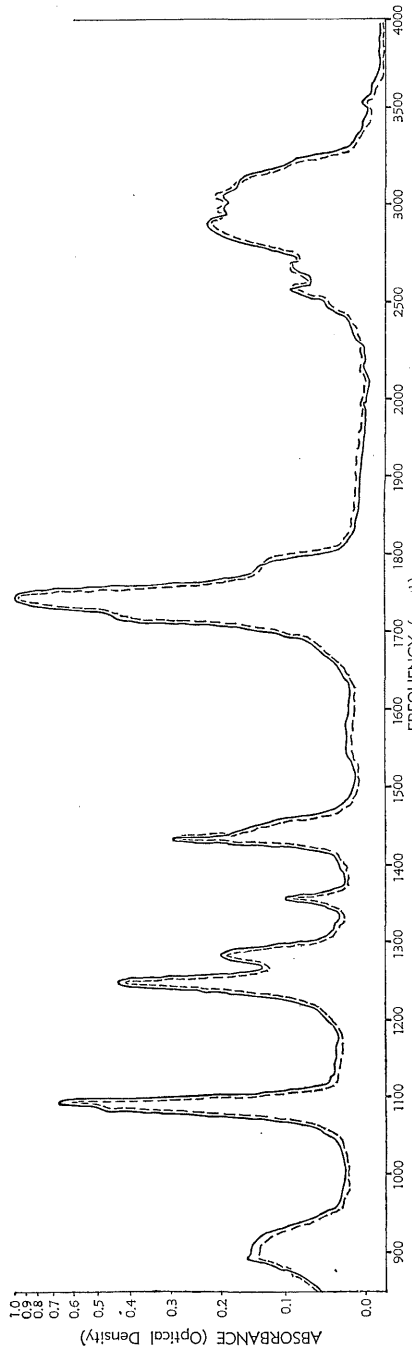

Fig. 4.—Infra-red absorption spectra of carbon tetrachloride solutions of monofluoroacetic acid – – – – and the acid isolated from *G. grandiflorum* ————

This evidence confirms the identification of monofluoroacetic acid in *G. grandiflorum*.

(f) Citric Acid Estimation

Estimations of citric acid concentration were made on the livers and kidneys of rats and on the blood and tissues of sheep. Samples were removed into 8 per cent. trichloroacetic acid solution immediately after death, weighed, macerated and filtered. Citric acid estimations were carried out on the filtrate; results are given in Tables 1–3.

(g) Fluorine Estimations

Quantitative fluorine estimations on monofluoroacetic acid and on the pure acid isolated from *G. grandiflorum* gave similar recoveries of fluorine.

(h) Quantitative Estimation of Monofluoroacetic Acid

Samples of *G. grandiflorum* from three different areas in Queensland were examined quantitatively. Results are given in Table 4.

TABLE 4

CONCENTRATION OF MONOFLUOROACETIC ACID IN *G. grandiflorum*

(mg/kg on air-dried basis)

Material	Area 1. Charters Towers District	Area 2. Clermont District	Area 3. Aramac District
Leaf air-freighted immediately after collection	150	185	—
Leaf air-dried in the field	120	160	105

IV. DISCUSSION

The large-animal toxicity testing of *G. grandiflorum* (Official Records of the Queensland Department of Primary Industries) showed that the mature plant was toxic whether fed fresh or air-dried. To facilitate the identification of the toxic principle, it was necessary to find a suitable laboratory test animal which could be used at least in the initial isolation procedures.

Considerable variation was found in the reaction of mice to extracts of *G. grandiflorum* and oral administration to rats proved much more satisfactory. Toxicity testing with rats was also a guide to the nature of the toxic principle. Symptoms of muscle tremors and tetanic spasms, following the administration of partially purified extracts, closely resembled those found when rats were dosed with purified extracts of *Acacia georginae* (Oelrichs and McEwan 1962). Biochemical analysis or organs showed the presence of high levels of citric acid and suggested that the toxin of *G. grandiflorum* might also be the monofluoracetic acid radical.

Using the procedure already developed for *A. georginae,* an extract was obtained from 600 g of leaf which produced characteristic monofluoroacetic acid poisoning and elevated levels of citric acid in the blood and organs of a sheep. The lower levels of citric acid in the brain of the sheep dosed with plant extract could be due to the rapidity of death ($3\frac{1}{2}$ hr as compared with 6 hr in the sheep which received monofluoroacetic acid), or to the slightly greater time lapse between death and the sampling of brain tissue in the sheep poisoned with *G. grandiflorum* extract. Dosing of a sheep with massive doses of residual plant material showed that extraction had removed virtually all the toxic principle.

Examination of samples of the toxic extract by gas chromatography revealed the presence of an acid identical in retention time with monofluoroacetic acid. Further extraction, silicic acid partition chromatography and passage through a gas chromatographic column provided a solution in carbon tetrachloride which gave an infra-red absorption spectrum identical with that of pure monofluoroacetic acid in saturated solution in carbon tetrachloride. Micro fluorine analysis confirmed the presence of fluorine in concentration equivalent to monofluoroacetic acid.

All evidence is in agreement with the conclusion that monofluoroacetic acid is the toxic principle of *G. grandiflorum.*

The first report of monofluoroacetic acid as the active principle of a toxic plant was that of Marais (1944) in the leaf of *Dichapetalum cymosum* in South Africa. Serious stock losses from this plant had been reported by Rimington (1935), and Marais (1943) had succeeded in isolating the toxin. A level of 15 mg monofluoroacetic acid per gram of the dry plant has been quoted by Watt and Breyer-Brandwijk (1962, p. 380).

Naturally occurring monofluoroacetic acid toxicity was next reported by Oelrichs and McEwan (1961), who identified the fluoroacetate ion as the toxic principle in the leaf and pod of *Acacia georginae* in Queensland. Subsequently, Murray, McConnell, and Whittem (1961) recorded the suspected presence of the acid in *A. georginae* in the Northern Territory. Oelrichs and McEwan (1962) reported levels usually ranging from 10 to 40 mg/kg in leaf, pod and seed, with one sample of young, immature seed showing 390 mg/kg on an air-dried basis.

The levels found in *G. grandiflorum* ranged from 105 to 185 mg/kg in the air-dried leaf, but some field reports suggest that under some circumstances these levels must be greatly exceeded.

The symptoms and post-mortem changes reported by Bennetts (1935) for sheep poisoned with four species of *Oxylobium* and 16 species of *Gastrolobium* in Western Australia are strikingly similar to those associated with *G. grandiflorum* toxicity and with varying doses of monofluoroacetic acid. It seems most probable that the same toxin—monofluoroacetic acid—or a toxin containing this radical is responsible for the toxicity of these species.

V. ACKNOWLEDGEMENTS

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ADDENDUM

Since completion of this manuscript information has been received from Dr. J. R. Cannon, Chemistry Department, University of Western Australia, that he and his co-workers have recently obtained evidence independently that monofluoroacetic acid is present in the toxic Western Australian plants *Gastrolobium callistachys* and *Oxylobium parviflorum.*

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