# "TOXINS IN CERTAIN INDIGENOUS KENYA PLANTS"

By

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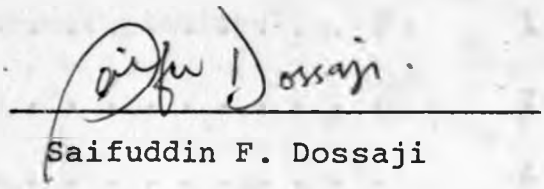
This thesis has not been submitted for a degree in any other university.



Saifuddin F. Dossaji

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## ABSTRACT

During the past years, several groups of natural products have been shown to be effective poisons, whether present in plants, micro-organisms or animal tissues. Such natural poisons may represent various classes of chemical structures, including alkaloids, saponins, cardiac glycosides, amines, polynuclear hydrocarbons, polypeptides, azoxyglycosides etc. On ingestion these toxins may have varied effects which may depend on the concentration level at which the toxin is ingested or on metabolic differences among species. Consequently the compound can be acutely toxic causing death within a few hours, most of the known alkaloids, cardiac glycosides and bacterial toxins fall into this group, or it may have varied effects leading in due course to chronic illness and even death. Certain hepato-toxins and carcinogens appertain to this type.

In Kenya there have been several reports on toxicity by natural products, especially among livestock, but very few toxins have been characterized. However, in special

circumstances, it has been possible to identify the plant producing either severe toxicity or delayed chronic diseases in a particular locality or in groups exposed to a common environmental factor.

The production of tumours of the liver, kidney and lungs following chronic feeding of crude meal prepared from the nuts of Encephalartos hildebrandtii was reported by Mugera and Nderito (1968). E. hildebrandtii provides a source of edible starch for the natives of Kenya of the Coast Province during famine and whenever there is shortage of food. It is also known to be eaten by livestock.

The water soluble hepato-toxin, macrozamin, (methylazoxymethanol-β-primeveroside, $CH_3\underset{\downarrow O}{N} = N.CH_2O.\ C_5H_9O_4.C_6H_{10}O_5$) has been isolated and characterized as the lethal chemical from the seeds of E. hildebrandtii. Macrozamin and its analogue cycasin, (methylazoxymethanol-β-glucoside ($CH_3\underset{\downarrow O}{N} = N.CH_2O.C_6H_{11}O_5$) which is found in the Cycas species is reported to be toxic and carcinogenic.

New impetus for further research on Cycads has been the recognition of a possible relationship between the ingestion of cycad material and the occurrence of a severe

paralytic condition. According to Mason and Whiting ingestion of leaves of species of four of the genera are reported to produce a neurological disorder in cattle, involving the irreversible paralysis of the hind quarters. A new etiological approach to this neurological disorder has been suggested by Vega and Bell who isolated a non-protein amino acid, α-amino-β-methylaminopropionic acid ($CH_3$-NH-$CH_2$-CH($NH_2$)-COOH), from seeds of Cycas circinalis (Cycadaceae) which they found neurotoxic in chickens during preliminary investigations.

The distribution of azoxyglycosides in the Cycads suggested a fair possibility of the presence of this unusual amino-acid in the Cycads indigenous to Kenya, but it could not be detected in either E. hildebrandtii or C. thuarsii.

The primary causes of most cancers are still unknown, however, the great activity and considerable diversity of carcinogenicity shown by N-nitroso-compounds have led to an increasing interest in them as possible environmental hazards.

A chemical investigation of certain species of plants from the valley of "Nasampolai", Kenya, one or more of which

are thought to induce oesophageal and rumenal cancer in cattle, was carried out for the presence of N-nitrosamines, and secondary amines, which are implicated as possible precursors of N-nitrosamines, in an attempt to account for some epidemiological aspects of the outbreak.

## ACKNOWLEDGEMENTS

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# CHAPTER I

## ABSTRACT

The water soluble hepato-toxin macrozamin, methyl-azoxymethanol-β-primeveroside ($CH_3\overset{+}{N}(O^-) = N-CH_2O - C_{11}H_{19}O_9$) has been isolated and characterized from the nuts of Encephalartos hildebrandtii. Macrozamin has been previously reported by other workers to be acutely toxic and carcinogenic (2) (13).

## INTRODUCTION

The members of the group of plants commonly referred to as Cycads (order Cycadales) belong to an ancient family of plants which predominated over other vegetation during the greater part of the Mesozoic. They are thought to represent an intermediate evolutionary step from fern to flowering plant. They are palm-like plants whose stems and seeds are rich in starch, and they belong to the order Cycadales which is composed of three families and nine genera. Stems and seeds of these plants provide a source of edible starch - and are used as food and fodder in many parts of tropical and subtropical areas. In some areas Cycads constitute an invaluable famine food as they frequently survive typhoon and drought when other food sources are destroyed. Several species of the family are poisonous to livestock as well as man. A review of the literature on the toxicity of Cycads has been compiled by Whiting (1).

Scientific interest in the role of Cycads in disease is not new. Search for the plant's toxic ingredient began in the 1870's. Little progress was made until 1941, when a glycoside was isolated from Marcrozamia spiralis by

Cooper (2). Several glycosides have since been isolated and found to be lethal to man and animals (1). New impetus for research on Cycads has been the recognition of a possible relationship between ingestion of cycad material and the occurrence of carcinogenic tumours and neurological disorders.

In Kenya the seeds of E. hildebrandtii are used as a source of edible starch by the Wasanya tribe during times of famine or whenever there is shortage of food. The Wasanya are aware of the presence of a toxic ingredient in the seeds which they remove by the following process. Seeds are cut into small pieces, dried in the sun and placed in a bag in running water or, alternatively, steeped in water for eight days; the water being changed daily. The water-soluble toxin in the seeds is thus removed by this treatment. Seeds so treated are dried, powdered, and the powder is used in the preparation of a local porridge (3).

Mugera and Nderito (4) have demonstrated that the crude meal prepared from E. hildebrandtii contains a potent liver and kidney toxin and the lesions induced in rats were

similar to those induced by cycasin, methylazoxymethanol-β-glucoside ($CH_3\overset{\downarrow}{N}\overset{}{O} = N-CH_2O-C_6H_{11}O_5$) and crude meal from Cycas circinalis. These observations indicated that non-detoxified starchy kernel flour prepared from E. hildebrandtii contains some potent toxic and carcinogenic factor or factors which produce tumours of the liver, kidney and lungs (4). The toxic and carcinogenic factor or factors of E. hildebrandtii have not been identified, but could be similar, if not identical, to those found in other Cycad plants.

The first biochemical isolation of a toxic glycoside, an azoxyglycoside, from Cycads was reported by Cooper (2) who obtained a crystalline substance from the seeds of Macrozamia spiralis, an Australian cycad, and named it macrozamin. It was reported to be present also in the seeds of other Cycads growing in Queensland, Australia (5) and in Encephalartos barkerii, an African Cycad, according to Lythgoe as cited by Riggs (5). Toxic properties of seeds of several species of Encephalartos have previously been described (1). The isolation of a glycoside from seeds of C. revoluta and the determination of its structure

were accomplished by Nishida et al. (6) who named the compound cycasin. Later, it was also found in C. circinalis (7) (26). It was chemically closely related to macrozamin except for the sugar moiety, which was D-glucose in the case of cycasin. Both cycasin and macrozamin had similar toxic effects, the toxicity residing in their identical aglycone moiety, methylazoxymethanol ($CH_3-\overset{\overset{O}{\downarrow}}{N} = N-CH_2OH$) (6) (7) (13).

## EXPERIMENTAL RESULTS

The crystalline material from the seeds of E. hildebrandtii was characterized as macrozamin by paper chromatography, melting point determination, ultraviolet and infrared absorption spectroscopy.

The pure white crystals melted at 199° - 200°C (decomp.). The melting point of macrozamin is reported to be 199° - 200°C (13). A mixed melting point with an authenticated sample of macrozamin did not show any change.

Paper chromatograms were developed as described on page 23. The crystallized material gave one spot when run alone and also when admixed with an authentic sample of macrozamin. Developed with pure cycasin two spots of different $R_f$ values were obtained.

The ultraviolet spectrum (fig. 1) of the crystals exhibited a strong absorption peak at 217 mμ, identical to that shown by cycasin (6) and macrozamin (11). This absorption peak is due to the azoxy structure of the aglycone (6, 11).

The infrared spectrum (fig. 2) is identical to that of macrozamin (11) in every respect. I.R. spectra were run

as KBr discs using a Beckman IR 4 spectrophotometer.

Green, D.F. and Hechet, S.S. (24) report that typical trans-azoxy compounds show strong I.R. absorption at both 1500 $cm^{-1}$ and 1300 $cm^{-1}$. Langley et al. (25) have investigated the I.R. spectra of a number of compounds containing the azoxy group and have shown that the symmetrical stretch of this group ranges between 1285 - 1345 $cm^{-1}$ and the asymmetric stretch ranges from 1495 to 1531 $cm^{-1}$. Absorptions due to the symmetric and asymmetric stretching modes are located approximately 200 $cm^{-1}$ apart for individual compounds.

Crystals from E. hildebrandtii and macrozamin show similar symmetric and asymmetric peaks at 1344 $cm^{-1}$ and 1540 $cm^{-1}$ respectively. The I.R. spectrum of cycasin (fig. 3) also shows typical symmetric and asymmetric peaks at 1338 $cm^{-1}$ and 1540 $cm^{-1}$ respectively. In addition the spectrum of macrozamin shows peaks at 1370 $cm^{-1}$ and 1440 $cm^{-1}$. Cycasin shows similar absorption at 1367 $cm^{-1}$ and 1430 $cm^{-1}$.

Ultraviolet Absorption Spectrum of:

(A) Macrozamin

(B) Cycasin

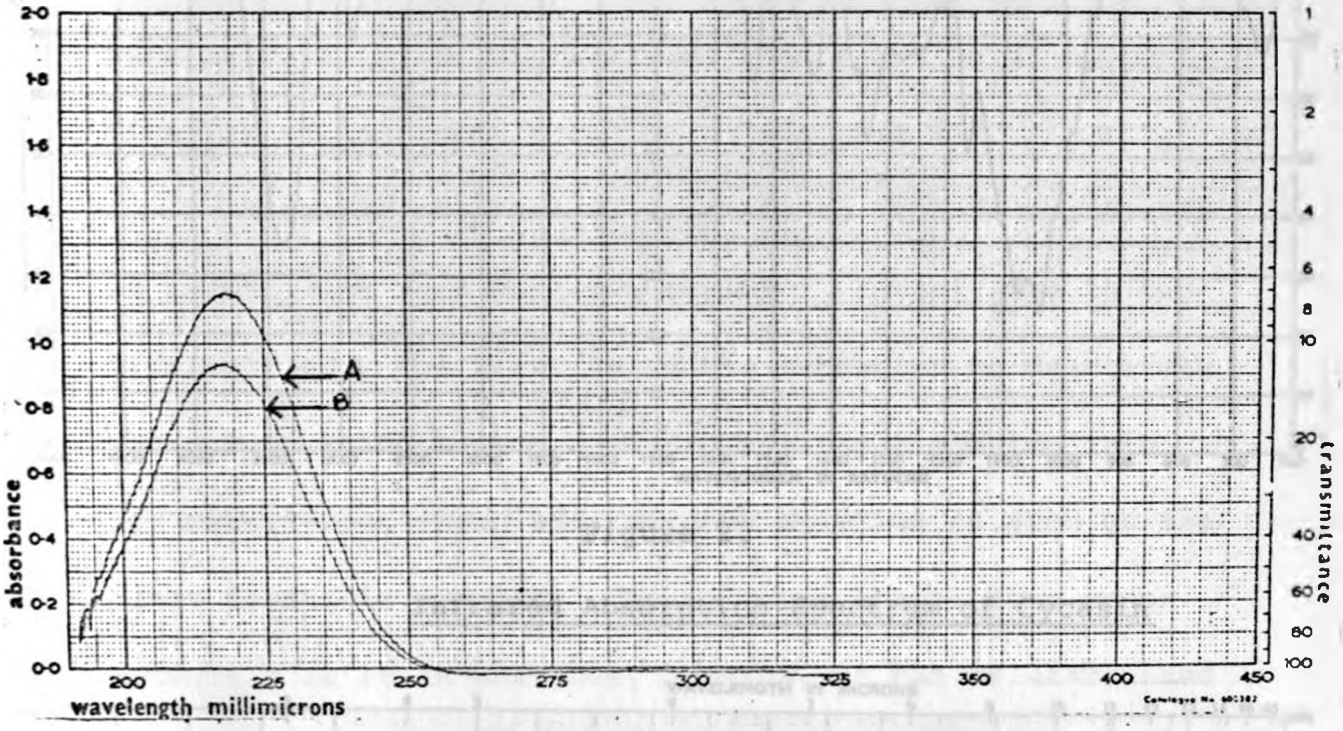



Figure 1.

Infrared Absorption Spectrum of Macrozamin

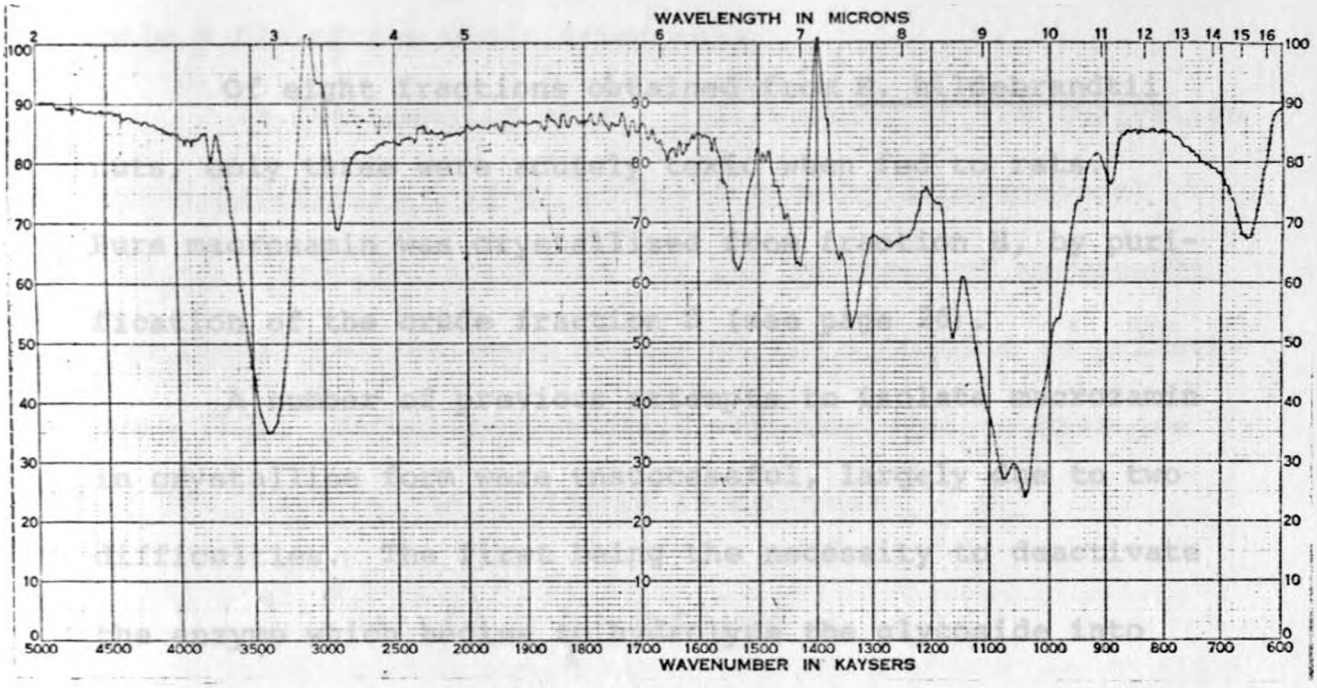



Figure 2.

Infrared Absorption Spectrum of Cycasin

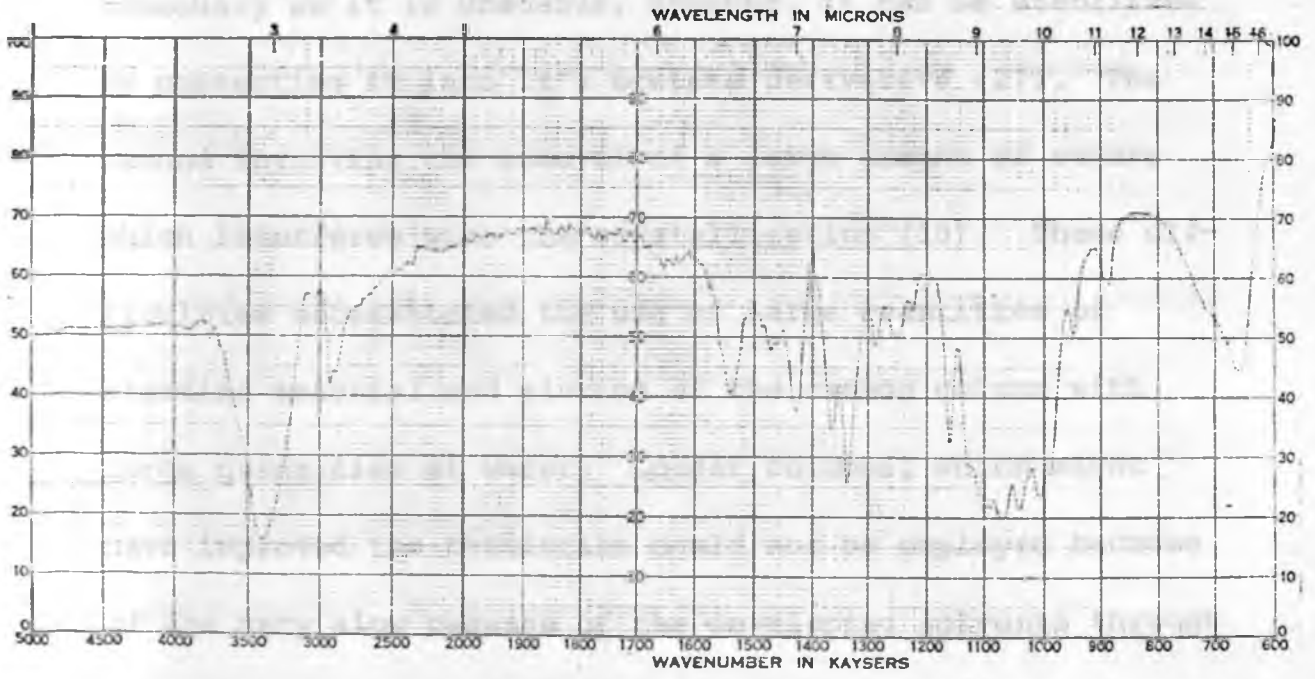



Figure 3.

## DISCUSSION

Of eight fractions obtained from E. hildebrandtii nuts, only three were acutely toxic when fed to rats. Pure macrozamin was crystallized from fraction H, by purification of the crude fraction D (see page 26).

A number of previous attempts to isolate macrozamin in crystalline form were unsuccessful, largely due to two difficulties. The first being the necessity to deactivate the enzyme which begins to hydrolyse the glycoside into its constituent sugar and aglycone moieties as soon as the plant tissue is damaged. The aglycone breaks down spontaneously as it is unstable, however, it can be stabilized by converting it into it's acetate derivative (27). The second involving the removal of a large amount of sugars which interferes with the crystallization (10). These difficulties necessitated the use of large quantities of starting material and elution of the carbon column with large quantities of water. Longer columns, which might have improved the resolution could not be employed because of the very slow passage of the developing solvents through the column. The amount of macrozamin separated was therefore

very small, and the final yield of pure crystals was only 0.01% of the whole dried nuts.

In 1951 Langley et al. (11) working on the Australian cycad Macrozamia reidlei proposed a methylazoxymethanol ($CH_3-\overset{\downarrow}{N}(O) = N-CH_2OH$) structure for the aglycone of macrozamin and identified the sugar moiety of the glycoside as primeverose (13). Methylazoxymethanol was concluded to have one or the other of the following structures:

(a) $CH_3 - \overset{\downarrow}{N}(O) = N-CH_2-OH$

(b) $CH_3 - N = \overset{\downarrow}{N}(O)-CH_2-OH$,

now believed to be structure (a) (42).

More recent toxicological studies have centered upon the azoxyglycosides isolated from species of Cycas, viz., C. revoluta (7), and several found therein were grouped under the name "cycasin". Further chemical studies on cycasin (32, 33, 34, 35) showed that extracts of the seed of C. revoluta contained glycosides variously called macrozamin, neocycasin A, neocycasin B, neocycasin C and neocycasin E. The glycosides may be for example, β-laminaribiosyloxyazoxymethane (neocycasin A) or

β-cellobiosyloxyazoxymethane (neocycasin E). Interconversion of the glycoside very likely occurs by transglycosylation in the plant *in vivo* (43). An important hydrolytic enzyme, cycad-β-glucosidase, isolated from *C. revoluta* (36) was found to hydrolyse all the glycosides irrespective of the nature of the sugar moeity.

The carbohydrate component of the macrozamin was first to be investigated (13). After hydrolysis of macrozamin with dilute HCl, and the removal of excess reagent, a gum remained from which D-glucose was isolated by crystallization of the monohydrate. Indications of the presence of another carbohydrate, probably a pentose, had been obtained at an earlier stage. Since furfuraldehyde was formed by the action of hot concentrated HCl on macrozamin, the other carbohydrate was identified as D-xylose. Since 6-(β-D-xylosido)-D-glucose (primeverose) is widely distributed in nature as the glycosidic component of numerous compounds, it was suggested that this disaccharide may be present in macrozamin. This later proved to be correct for when macrozamin was acetylated it gave a hexa-acetyl derivative in which all six groups are attached to the primeverosyl residue (13).

Taking into consideration the formation of a hexa-acetyl derivative, this establishes the following points:

(a) In macrozamin all six secondary alcoholic hydroxyl groups of primeverose are unsubstituted.

(b) The glycosidic centre of the glucose unit is the only position available for the attachment of the remainder of the molecule.

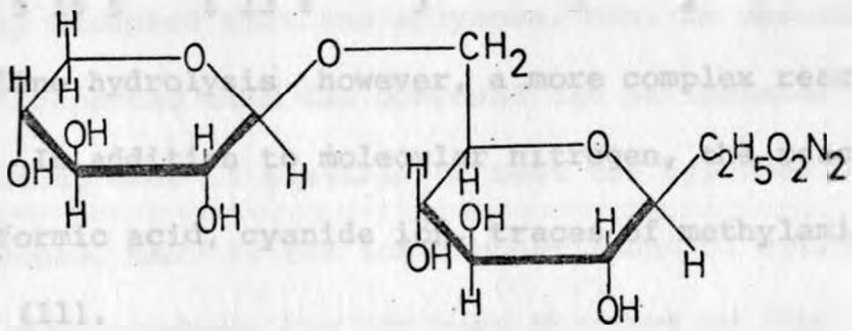
(c) The aglycone component contains no readily acetylatable function.

Once the carbohydrate component of macrozamin was characterized it was subsequently shown that the rest of the molecule is uniquely attached to C-1 of the glucose unit through a β-glucosidic linkage (11). Macrozamin is represented by the following partial structure as shown in figure 4.

The aglycone of macrozamin contains linked nitrogen atoms, common to all Cycad glycosides, a feature not previously encountered in natural products. On acid hydrolysis of the glycosidic bond, the aglycone decomposes spontaneously to produce nitrogen, methanol and formaldehyde.

MACROZAMIN : PARTIAL STRUCTURAL FORMULA

(Methylazoxymethanol - β - Primeverosyl )




Primeverosyl : 6-O-β-D-xylopyranosyl-D-glucopyranose

Methylazoxymethanol : $CH_3-\overset{\downarrow}{N}(\rightarrow O)=N-CH_2OH$

FIGURE 4.

Langley et al. (11) have given the quantitative determination of the liberation of the aglycone on acidic hydrolysis, having already demonstrated the quantitative formation of xylose and glucose (13). The equation for the hydrolysis is:

$$C_5H_9O_4.C_6H_{10}O_5.C_2H_5O_2N_2 + H_2O =$$

$$C_5H_{10}O_5 + C_6H_{12}O_6 + CH_3OH + CH_2O + N_2$$

On alkaline hydrolysis, however, a more complex reaction occurs. In addition to molecular nitrogen, the reaction yields formic acid, cyanide ion, traces of methylamine and ammonia (11).

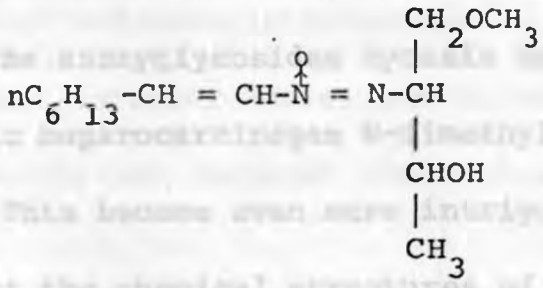
The parent aglycone of cycasin (15) methylazoxymethanol ($CH_3\underset{\downarrow}{N}{=}NCH_2OH$, O) has been shown to be identical with that of macrozamin (11). Polarographic methods have also been employed by Nishida et al. (12) (14) for the determination of cycasin and macrozamin. There was scarcely any difference in the polarograms of both the glycosides due to the identical aliphatic azoxy structure of the aglycone.

Evidence of carcinogenicity of crude cycad material was obtained early in 1962 from experiments with rats on

a cycad meal diet (28). Cycasin was shown to be non-toxic when administered intraperitoneally to rats but to be toxic when administered orally (29). Thus it was assumed that the azoxyglycoside was cleaved, probably by an intestinal microbial enzyme, and the toxic component was thought to be the aglycone, methylazoxymethanol (MAM), or a metabolic product of this compound. Although it was generally accepted that the aglycone, MAM, is unstable it has been reported that the compound can be isolated (7). This finding made it possible to test the hypothesis that the aglycone, MAM, is the toxic constituent of cycasin. Experimental evidence has now been obtained to show that indeed cycasin was carcinogenic only after passage through the gastro-intestinal tract whereas its aglycone, MAM, induced tumours independent of the route of administration. MAM is, therefore, the proximate carcinogen (30) (31).

A salient feature of these azoxy-compounds is their limited distribution in nature and their almost unique chemical structure. The unique features responsible for this chemical structure are:

(A) None of the very numerous nitrogen containing natural organic compounds are known to contain two nitrogen atoms directly linked to each other, in striking contrast to the large number of important synthetic substances showing this feature (azo-dyes, phenylhydrazine, pyrazolone drugs, diazomethane, to name only a few). It is true that in the years since 1951, a small number of other natural N-N compounds have been obtained from a variety of ogranisms, for example the isolation of Elaiomycin as cited by Schoental (16),



a compound having structural relationship to the azoxyglycosides.

(B) The instability of the aglycone and the nature of its cleavage products is also unique. Innumerable glycosides known to occur in nature are usually cleaved by acids or enzymes into sugars and fairly "well-behaved" molecules. Azoxyglycosides, however, break down completely

on acid treatment, forming two one-carbon fragments and elementary nitrogen. Free nitrogen is quite unique among the cleavage products of natural glycosides.

(C) Langley et al. (11) mention briefly that macrozamin decomposes liberating primeverose on treatment with cold dilute ammonia. Such sensitivity to a mild base is very uncommon among glycosides. Other consequences of the unique chemistry of azoxyglycosides may be of great value when analytical control of the work on Cycads is needed.

Lately there have been reports regarding the similarities between the chemical, biochemical and biological actions of the azoxyglycosides cycasin and macrozamin and the synthetic hepatocarcinogen N-dimethylnitrosamine (17) (18) (19). This became even more intriguing when it was realized that the chemical structures of these carcinogens were also very similar (see fig. 5).

Cycasin $CH_3\overset{+}{N}(O^-) = N - CH_2O$ - glucoside

Macrozamin $CH_3\overset{+}{N}(O^-) = N - CH_2O$ - primeveroside

Dimethylnitrosamine

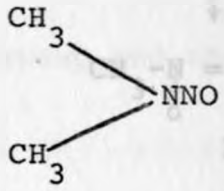
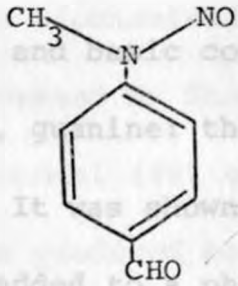


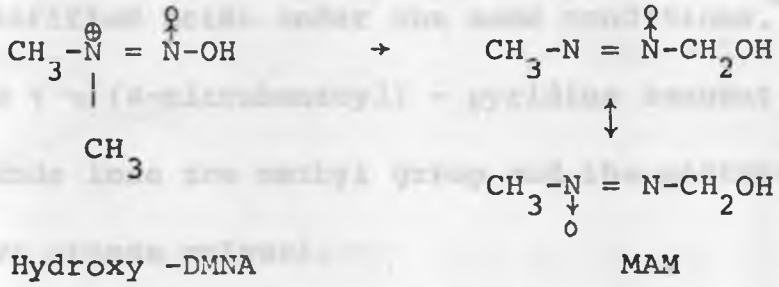

Figure 5.

Until recently N-nitrosamines were not known to occur naturally. However, Du Plessis et al. (20) have now shown the presence of N-dimethylnitrosamine in Solanum incanum of the Transkei in South Africa and in another interesting work Herrmann (21) has isolated 4-methylnitrosaminobenzaldehyde




from a common edible mushroom Clitocybe suaveolens.

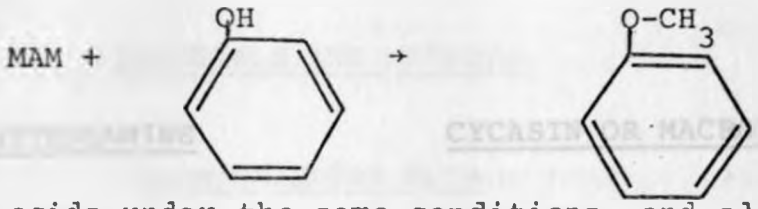
In 1965, MAM was, in fact, shown to be isomeric with hydroxydimethylnitrosamine (hydroxy - DMNA),



Hydroxy -DMNA MAM

and, thus, like N-dimethylnitrosamine, could be transformed into diazomethane, the very reactive methylating agent (37). The treatment of aqueous solutions of nucleic acids (ribosomal and transfer RNA) with ethereal diazomethane resulted in the methylation and disruption of the nucleic acids, this suggested a possible explanation for observed mutagenic effects in animals so treated (38).

It was confirmed that MAM was a good methylating agent (39) even under mild conditions it methylated phenolic, carboxylic acid, and basic compounds such as the nucleotide purine base, guanine: the product in this case was 7-methyl-guanine. It was shown in 1966 (39) that MAM produced anisole when added to a phenol solution at pH 7.0 and 37°,




esterified acids under the same conditions, and alkylated the γ - (4-nitrobenzoyl) - pyridine reagent (N-methyl compounds lose the methyl group and the mixture gives a positive orange colour).

Finally, the methylating action of cycasin itself was shown by Riggs, again the production of anisole from an acidified solution of phenol (37). Miller (22) proposed the following comparative mechanism (fig. 6) for the methylation reactions involving N-dimethylnitrosamine and cycasin (compounds in brackets are hypothetical intermediates which have not been isolated; both mechanisms envision diazomethane as an intermediate). This suggestion by Miller that cycasin and N-dimethylnitrosamine are metabolized to the same biochemically active product diazomethane has been discussed by Shank and Magee (23).

The work of Schoental (40) established that diazomethane itself may be produced metabolically by animals in vivo, and that of Friedman et al. (41), showed that this compound can methylate many bio-organic molecules (nucleic acids, nucleotides, nucleosides and constituent bases).

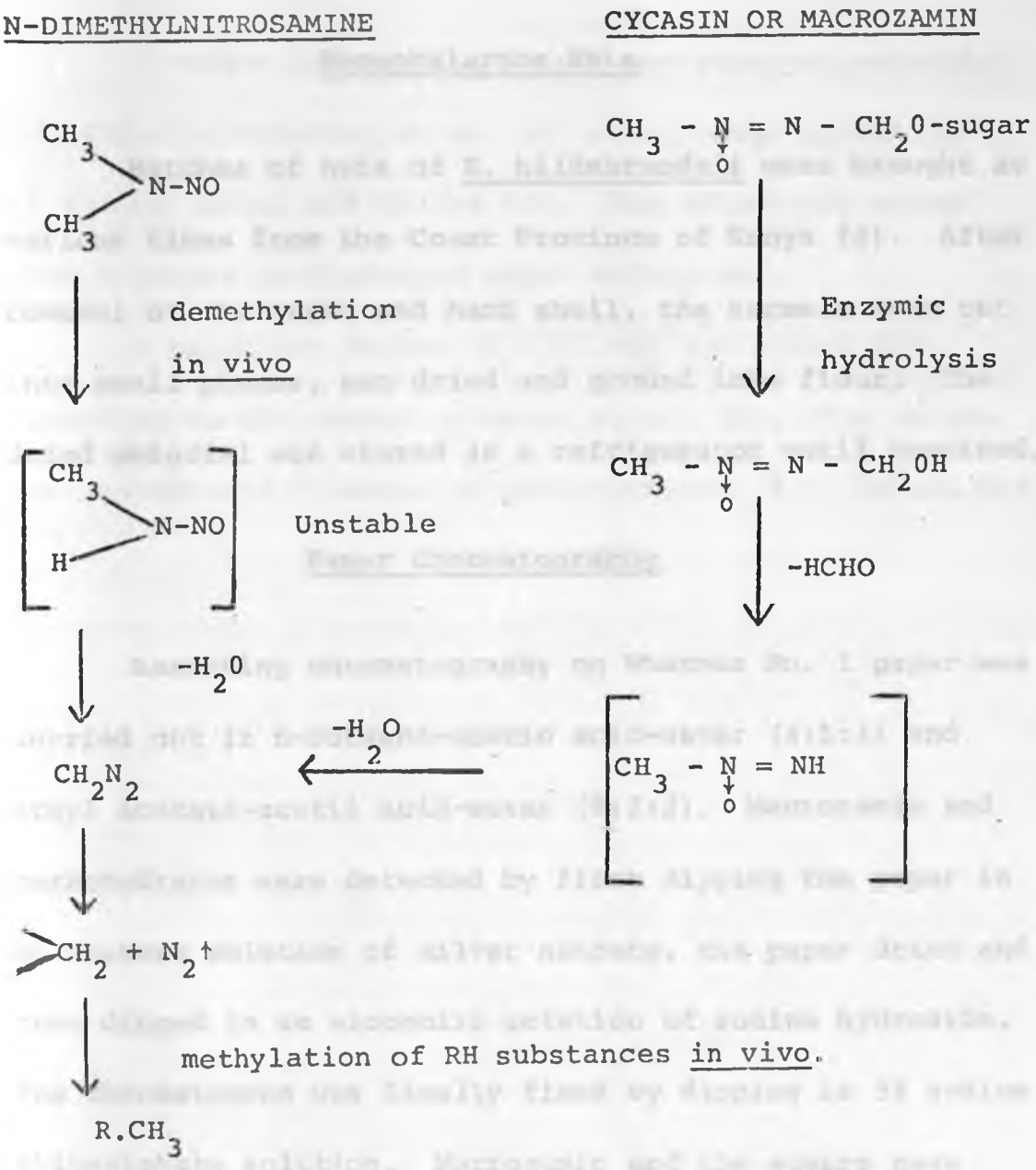

N-DIMETHYLNITROSAMINE
CYCASIN OR MACROZAMIN
CH3 - N = N - CH2O-sugar
O
demethylation in vivo
Enzymic hydrolysis
CH3 - N = N - CH2OH
O
Unstable
-HCHO
-H2O
-H2O2
CH2N2
CH3 - N = NH
O
CH2 + N2↑
methylation of RH substances in vivo.
R.CH3

Figure 6

## MATERIALS AND METHODS

### Encephalartos Nuts

Batches of nuts of E. hildebrandtii were brought at various times from the Coast Province of Kenya (3). After removal of the husks and hard shell, the kernels were cut into small pieces, sun dried and ground into flour. The dried material was stored in a refrigerator until required.

### Paper Chromatography

Ascending chromatography on Whatman No. 1 paper was carried out in n-butanol-acetic acid-water (4:1:1) and ethyl acetate-acetic acid-water (9:2:2). Macrozamin and carbohydrates were detected by first dipping the paper in an acetone solution of silver nitrate, the paper dried and then dipped in an alcoholic solution of sodium hydroxide. The chromatogram was finally fixed by dipping in 5% sodium thiosulphate solution. Macrozamin and the sugars gave black spots. The location of macrozamin was checked by simultaneous chromatography with an authentic sample. In doubtful cases the unknown solution was co-chromatographed with authentic samples of macrozamin and cycasin.

## Column Chromatography

A carbon column (5 x 40 cm) was prepared according to method of Whistler et al. (8) using equal quantities of active Carbon and Celite 545. The column was washed with 2 litres of distilled water before use.

A cellulose column (6 x 30 cm) was packed dry according to the method of Hough et al. (9). The column was washed with 2 litres of acetone-water (9:1) before use.

FLOW SHEET OF MACROZAMIN ISOLATION

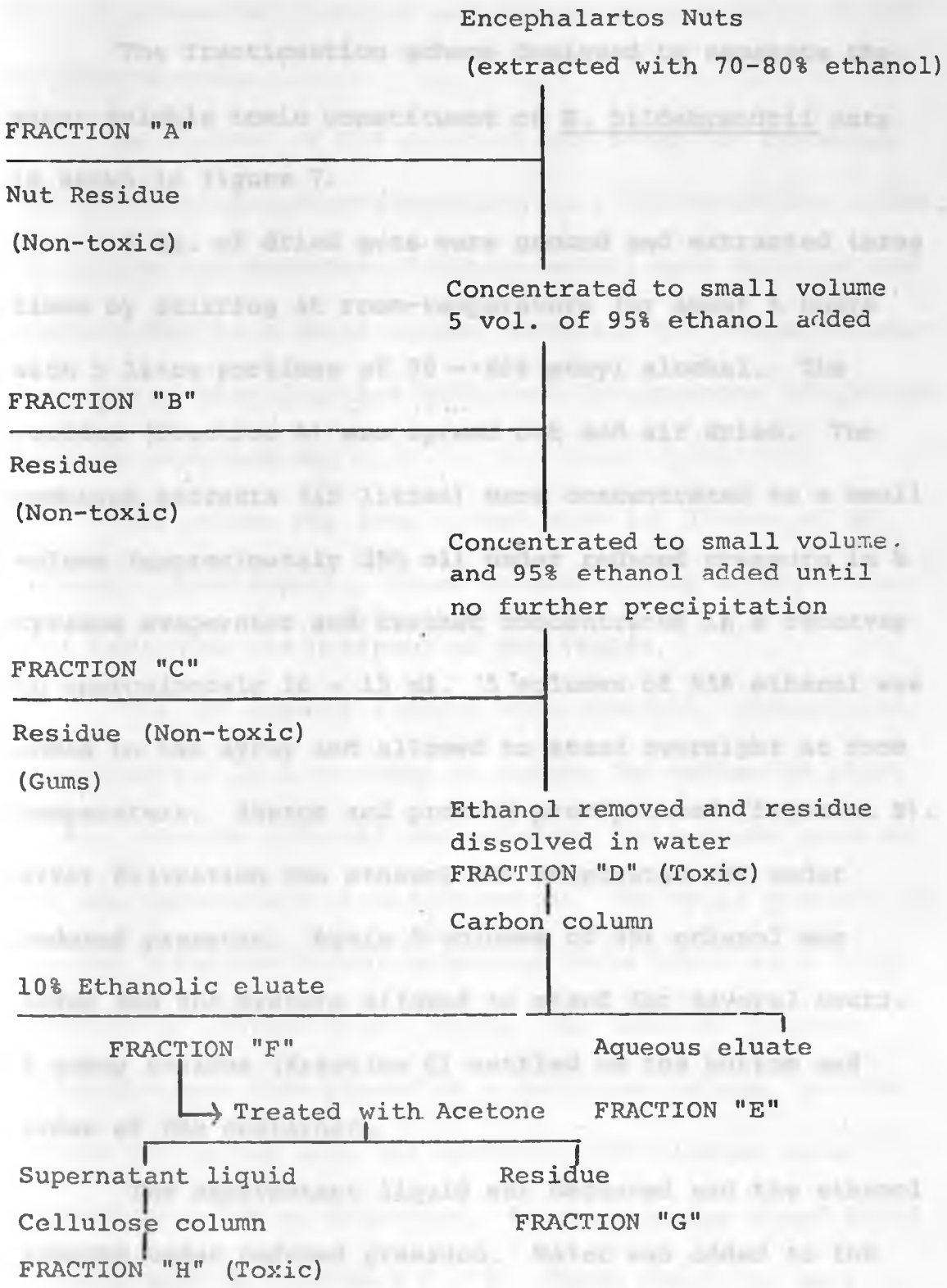



Figure 7

## EXPERIMENTAL PROCEDURE

The fractionation scheme designed to separate the water soluble toxic constituent of *E. hildebrandtii* nuts is shown in figure 7.

5 Kg. of dried nuts were ground and extracted three times by stirring at room-temperature for about 5 hours with 5 litre portions of 70 - 80% ethyl alcohol. The residue (fraction A) was spread out and air dried. The combined extracts (15 litres) were concentrated to a small volume (approximately 250 ml) under reduced pressure in a cyclone evaporator and further concentrated in a rotorvap to approximately 10 - 15 ml. 5 volumes of 95% ethanol was added to the syrup and allowed to stand overnight at room temperature. Starch and protein precipitated (fraction B). After filtration the ethanol was evaporated off under reduced pressure. Again 5 volumes of 95% ethanol was added and the mixture allowed to stand for several hours. A gummy residue (fraction C) settled on the bottom and sides of the container.

The supernatant liquid was decanted and the ethanol removed under reduced pressure. Water was added to the

syrupy mass and the mixture again concentrated. The residue was dissolved in water and the solution made up to 250 ml (fraction D).

An aliquot of the solution was saved for bioassay, and the remainder chromatographed on a Carbon-Celite column. The eluate and washings (2 litres water) were combined and concentrated to a small volume (fraction E). Paper chromatography of this fraction indicated the presence of glucose, fructose and sucrose.

The column was then eluted with 1.5 litres of 10% ethanol, (fraction F). Paper chromatography of this fraction indicated the presence of macrozamin.

The 10% ethanol eluates were combined, ethanol and water removed in a rotorvap to reduce the volume to about 25 ml. Acetone (100 ml) was added to the aqueous solution and the supernatant liquid decanted. The small quantity of residue (fraction G) was extracted three times with 25 ml portions of acetone-water (4:1). The combined acetone fractions were then placed on a cellulose column, and the column was eluted with 80% acetone. The eluates were collected in 100 ml fractions. Macrozamin was found to be concentrated in fractions 6 - 8. These fractions were

pooled and acetone removed in a rotorvap. The residue (fraction H) was dissolved in 50% ethanol and allowed to stand in a refrigerator. Macrozamin crystallized out as thin plates.

Crystallization proved difficult and was only successful after a number of attempts. This may be due to traces of unremoved sugars. Elution of the carbon column with large quantities of water gave satisfactory results. The overall yield was 0.01% of the whole dried nuts.

## SUMMARY

An attempt has been made in this chapter to summarize the important aspects of Cycad toxicity. These include the biochemical studies which led to the isolation and elucidation of the chemical structure of the azoxyglycoside, macrozamin. It also deals with the carcinogenic properties of crude E. hildebrandtii preparations and describes the carcinogenic properties of macrozamin, cycasin and synthetic dimethylnitrosamine. The conversion of macrozamin to methylazoxymethanol in vivo is also covered, including possible mechanisms by which macrozamin and cycasin may become toxic and carcinogenic in the living animal. The chapter also deals with certain aspects of the chemistry of azoxyglycosides.

Probably the most important contribution of research on Cycads has been the characterization of azoxyglycosides and the recognition of their potent toxic and carcinogenic effects. However, the most essential area which warrants attention in future investigations is the biosynthesis of azoxyglycosides within the Cycad plant.

The present work has provided additional evidence of a relationship between the use of Cycads as human and livestock food and a high incidence of carcinogencity amongst the Wasanya of Kenya. Cycasin and macrozamin had already been shown to be potent toxins and carcinogens and the present finding of macrozamin in E. hildebrandtii nuts is a further link in this chain of evidence.

## REFERENCES

1. Whiting, M.G., Econ. Botany, 17, 269 (1963).

2. Cooper, J.M., Proc. Roy. Soc. N. S. Wales, 74, 450 (1941).

3. Mugera, G.M. and Nderito, P.C., Brit. J. Cancer, 22, 563 (1968).

4. Mugera, G.M. and Nderito, P.C., Brit. J. Cancer, 23, 755 (1969).

5. Riggs, N.V., Aust. J. Chem., 7, 123 (1954).

6. Nishida, K., Kobayashi, A. and Nagahama, T., Bull. Agri. Chem. Soc. (Japan) 19, 77 (1955).

7. Matsumoto, H. and Strong, F.M., Arch. Biochem. Biophys. 101, 299 (1963).

8. Whistler, R.L. and Durso, D.F., J. Am. Chem. Soc., 72, 677 (1950).

9. Hough, L., Jones, J.K. and Wadman, W.H., J. Chem. Soc., 2511 (1949).

10. Personal Communication with Dr. Akira Kobayashi, (1969).

11. Langley, B.W., Lythgoe, B. and Riggs, N.V., J. Chem. Soc., 2309 (1951).

12. Kobayashi, A. and Nagahama, T., Chem. Abs., 53, 7827 (1959).

13. Lythgoe, B. and Riggs, N.V., J. Chem. Soc., 2716 (1949).

14. Kobayashi, A., Nagahama, T. and Nawata, J., Chem. Abs., 53, 7826 (1959).

15. Kobayashi, A. and Matsumoto, H., Arch. Biochem. Biophys., 110, 373 (1965).

16. Schoental, R., Nature, 221, 765 (1969).

17. Heath, D.F. and Magee, P.N., Brit. J. Industr. Med. 19, 276 (1962).

18. Magee, P.N. and Schoental, R., Brit. Med. Bull., 20, 102 (1964).

19. Magee, P.N. and Barnes, J.M., Advan. Cancer Res., 10, 163 (1967).

20. DuPlessis, L.S., Nunn, J.R. and Roach, W.A., Nature, 222, 1198 (1969).

21. Herrmann, H., Z. Physiol. Chem., 326, 13 (1961).

22. Miller, J.A., Fedn. Proc., 23, 1361 (1964).

23. Shank, R.C. and Magee, P.N., Biochem. J., 105, 521 (1967).

24. Green, D.F. and Hechet, S.S., Tetrahedron letters, 7, 575 (1969).

25. Langley, B.W., Lythgoe, B. and Rayner, L.S., J. Chem. Soc., 4191 (1952).

26. Riggs, N.V., Chem. Ind. (London), 926 (1956).

27. Matsumoto, H., Nagahama, T. and Larson, H.O., Biochem. J., 95, 13C (1965).

28. Laqueur, G.L., Mickelsen, O., Whiting, M.G. and Kurland, L.T., J. Natl. Cancer Inst. 31, 919 (1963).

29. Nishida, K., Kobayashi, A., Nagahama, T., Kojima, K. and Yamane, M., Seikagaku, 28, 218 (1956).

30. Laqueur, G.L. and Matsumoto, H., J. Natl. Cancer Inst., 37, 217 (1966).

31. Laqueur, G.L., McDaniel, E.G. and Matsumoto, H., J. Natl. Cancer Inst., 39, 355 (1967).

32. Nishida, K., Kobayashi, A., Nagahama, T. and Numata, T., Bull. Agri. Chem. Soc. (Japan), 23, 460 (1959).

33. Nagahama, T., Numata, T. and Nishida, K., Bull. Agri. Chem. Soc. (Japan), 23, 556 (1959).

34. Nagahama, T., Nishida, K. and Numata, T., Bull. Agri. Chem. Soc. (Japan), 24, 536 (1960).

35. Nagahama, T., Nishida, K. and Numata, T., Agr. Biol. Chem., 25, 937 (1961).

36. Kobayashi, A., Agr. Biol. Chem. 26, 203 (1962).

37. Riggs, N.V., Nature, 207, 632 (1965).

38. Kreik, E. and Emmelot, P., Biochemistry, 2, 733 (1963).

39. Whiting, M.G., Econ. Botany, 20, 98 (1966).

40. Schoental, R., Nature, 188, 420 (1960).

41. Friedman, O.M., Mahapatra, G.N., Dash, B., and Stevenson, R., Biochim. Biophys. Acta, 103, 286 (1965).

42. Korsch, B.H. and Riggs, N.V., Tetrahedron Letters, 10, 523 (1964).

43. Nagahama, T., Nishida, K. and Numata, T., Bull. Agri. Chem. Soc. (Japan), 24, 322 (1960).

## BIBLIOGRAPHY

Cooper, J.M., Isolation of a Toxic Principle from the Seeds of Macrozamia Spiralis. Proc. Roy. Soc. New South Wales, 74, 450 (1941).

DuPlessis, L.S., Nunn, J.R. and Roach, W.A., Carcinogen in a Transkeian Bantu Food Additive. Nature, 222, 1198 (1969).

Friedman, O. M., Mahapatra, G.N., Dash, B. and Stevenson, R., Studies on the Action of Diazomethane on Deoxyribonucleic Acid. Biochim. Biophys. Acta., 103, 286 (1965).

Green, D.F. and Hechet, S.S., Cyclic Azoxy Compounds - Relation of Structural Considerations to NMR Spectra. Tetrahedron Letters, 7, 575 (1969).

Herrmann, H., Identification of 4-methylnitrosaminobenzaldehyde as a Metabolic Product of Clitocybe suaveolens. Z. Physiol. Chem., 326, 13 (1961).

Hough, L., Jones, J.K. and Wadman, W.H., Quantitative Analysis of Mixtures of Sugars by the Method of Partition Chromatography Part IV. The Separation of the Sugars and their Methylated Derivates on

Columns of Powdered Cellulose. J. Chem. Soc., 2511 (1949).

Kobayashi, A., Nagahama, T. and Nawata, J., Polarography of Macrozamin Compared with that of Cycasin. Chem. Abs., 53, 7826 (1959).

Kobayashi, A., Nagahama, T. and Nawata, J., Polarographically Determined Velocity Constants of the Alkali or Acid Decomposition of Cycasin and Macrozamin. Chem. Abs., 53, 7287 (1959).

Kobayashi, A., Biochemical Studies in Cycasin, Part I. Purification and Properties of Cycad β-Glucosidase. Agr. Biol. Chem., 26, 203 (1962).

Kobayashi, A. and Matsumoto, H., Studies on Methylazoxymethanol, the Aglycone of Cycasin. Arch. Biochem. Biophys., 110, 373 (1965).

Korsch, B.H. and Riggs, N.V., Proton Magnetic Resonance Spectra of Aliphatic Azoxy Compounds and the Structure of Cycasin. Tetrahedron Letters, 10, 523 (1964).

Kreik, E. and Emmelot, P., Methylation and Breakdown of Microsomal and Soluble Ribonucleic Acid from Rat Liver by Diazomethane. Biochemistry, 2, 733 (1963).

Langley, B.W., Lythgoe, B. and Riggs, N.V., The Aliphatic Azoxy Structure of the Aglycone Part. J. Chem. Soc., 2309 (1951).

Langley, B.W., Lythgoe, B. and Rayner, L.S., Macrozamin Part III. Azoxymethane and Some Simple Analogues. J. Chem. Soc., 4191 (1952).

Laqueur, G.L. and Matsumoto, H., Neoplasms in Female Fischer Rats Following Intraperitoneal Injection of Methylazoxymethanol. J. Natl. Cancer Inst., 37, 217 (1966).

Laqueur, G.L., McDaniel, E.G. and Matsumoto, H., Tumour Induction in Germ Free Rats with Methylazoxymethanol (MAM) and Synthetic MAM Acetate. J. Natl. Cancer Inst., 39, 355 (1967).

Laqueur, G.L., Mickelsen, O., Whiting, M.G. and Kurland, L.T., Carcinogenic Properties of Nuts from Cycas circinalis Indigenous to Guam. J. Natl. Cancer Inst., 31, 919 (1963).

Lythgoe, B. and Riggs, N.V., Macrozamin Part 1. The identity of the Carbohydrate Component. J. Chem. Soc., 2716 (1949).

Magee, P. N. and Schoental, R., Carcinogenesis by Nitroso-compounds. Brit. Med. Bull., 20, 102 (1964).

Magee, P.N. and Barnes, J.M., Carcinogenic Nitroso-compounds. Advan. Cancer Res., 10, 163 (1967).

Matsumoto, H. and Strong, F.M., The Occurrence of Methyl-azoxymethanol in Cycas circinalis. Arch. Bio-chem. Biophys., 101, 299 (1963).

Matsumoto, H., Nagahama, T. and Larson, H.O., Studies on Methylazoxymethanol, the Aglycone of Cycasin: A Synthesis of Methylazoxymethyl Acetate. Biochem. J., 95, 13C (1964).

Miller, J.A., Comments on Chemistry of Cycads. Fedn. Proc., 23, 1361 (1964).

Mugera, G.M. and Nderito, P.C., Tumours of the Liver, Kidney and Lungs in Rats fed Encephalartos hildebrandtii. Brit. J. Cancer, 22, 563 (1968).

Mugera, G.M. and Nderito, P.C., Induction of Kidney Tumours in Rats by Feeding Encephalartos hildebrandtii for Short Periods. Brit. J. Cancer, 23, 755 (1969).

Nagahama, T., Numata, T. and Nishida, K., Neocycasin B. and Macrozamin. Bull. Agri. Chem. Soc., (Japan),

23, 556 (1959).

Nagahama, T., Nishida, K. and Numata, T., The Structure of Neocycasin C formed by Transglycosylation with Cycad Emulsion. Bull. Agri. Chem. Soc. (Japan), 24, 536 (1960).

Nagahama, T., Nishida, K. and Numata, T., Neocycasin E, β-Cellobiosyloxyazoxymethane. Agri. Biol. Chem., 25, 937 (1961).

Nagahama, T., Nishida, K. and Numata, T., Interconversion between the Azoxyglycosides by Cycad Emulsion. Bull. Agri. Chem. Soc., 24. 322 (1960).

Nishida, K., Kobayashi, A. and Nagahama, T., Isolation and the Structure of Cycasin. Bull. Agri. Chem. Soc. (Japan), 19, 77 (1955).

Nishida, K., Kobayashi, A., Nagahama, T. and Numata, T., Studies on Some New Azoxy Glycosides of Cycas revoluta, Part I. On Neocycasin A, β-Laminaribiosyloxyazoxymethane. Bull. Agri. Chem. Soc. (Japan), 23, 460 (1959).

Riggs, N.V., The Occurrence of Macrozamin in the Seeds of Cycads. Aust. J. Chem., 7, 123 (1954).

Riggs, N.V., Decomposition and Carcinogenic Activity of Azoxyglycosides. Nature, 207, 632 (1965).

Schoental, R., Carcinogenic Action of Elaiomycin in Rats. Nature, 221, 765 (1969).

Schoental, R., Carcinogenic Action of Diazomethane and Nitroso-N-methyl Urethane. Nature, 188, 420 (1960).

Shank, R.C. and Magee, P.N., Similarities between the Biochemical Actions of Cycasin and Dimethylnitrosamine. Biochem. J., 105, 521 (1967).

Whistler, R.L. and Durso, D.F., Chromatographic Separation of Sugars on Charcoal. J. Am. Chem. Soc., 72, 677 (1950).

Whiting, M.G., Toxicity of Cycads. Econ. Botany, 17, 269 (1963).

# C H A P T E R  I I

## ABSTRACT

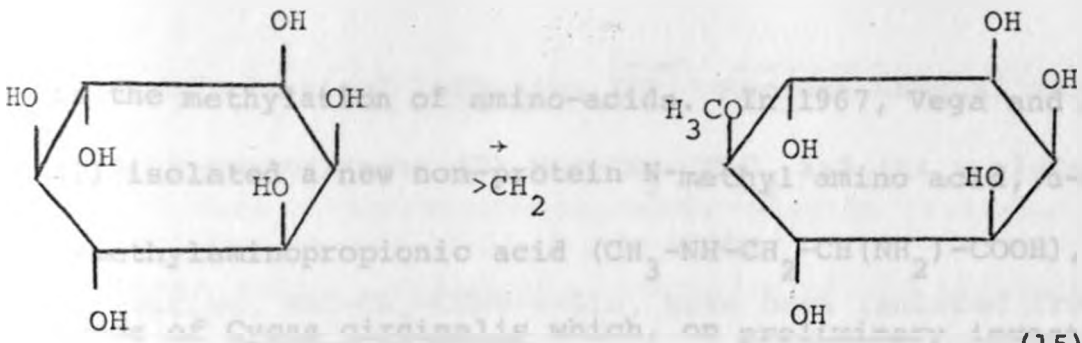
α-Amino-β-methylaminopropionic acid ($CH_3$-NH-$CH_2$-CH($NH_2$)-COOH), a non-protein amino acid reported to be present in the seeds of Cycas circinalis (Cycadaceae) by Vega and Bell (1), was not detected in the seeds of Encephalartos hildebrandtii (Zamiaceae), or in the leaves of Cycas thuarsii (Cycadaceae).

## INTRODUCTION

The azoxyglycosides, cycasin and macrozamin, which show hepato-toxic and carcinogenic properties when fed orally to experimental animals have been isolated from the seeds of several species of Cycads (3). In addition to the carcinogenic effects, however, the ingestion of leaves of species of four genera have been reported to produce a neurological disorder in cattle involving the irreversible paralysis of the hindquarters (2). Whiting has reviewed those papers pertinent to this neurological disorder in the Toxicity of Cycads (3).

The fact that carcinogenic and neurological effects do not necessarily occur simultaneously after the ingestion of cycad material suggested that a toxic compound other than a methylazoxymethanol derivative, might be responsible for the neurological symptoms.

In 1949 Riggs discovered a compound in Macrozamia reidlei with possible neutrotoxic relevance--"sequoyitol" --a mono methyl ether of myo-inositol.



(15)

Presumably, sequoyitol is biosynthesized from myo-inositol by methylation. A common method of synthesizing mono-methyl ether-- heme -acetals--is by heating carbohydrates with slightly acidic methanol (16). Methylazoxymethanol which decomposes into the more powerful methylating agent, diazomethane, could be the agent in this polyalcohol to ether conversion, though it is unclear why only one methylated product is known. (Presumably, the reaction is not spontaneous, but catalyses enzymatically, which would explain the specificity of the methylation reaction.) Such a carbohydrate derivative acting as an anti-metabolite of inositol, an abundant constituent of brain tissue, could conceivably lead to the neurological lesions observed from ingestion of some species of Cycads.

If methylazoxymethanol methylates, both actually and theoretically, such a wide variety of bio-organic molecules (nucleic acids, carbohydrates, etc.), it may be implicated

in the methylation of amino-acids. In 1967, Vega and Bell (1) isolated a new non-protein N-methyl amino acid, α-amino β-methylaminopropionic acid ($CH_3$-NH-$CH_2$-CH($NH_2$)-COOH), from seeds of Cycas circinalis which, on preliminary investigation, was found to be neurotoxic in chicks. They synthesized this compound which was as potent as the extracted natural compound (1).

The description of the paralysis, due to the ingestion of Cycads, is remarkably similar to that of "classical lathyrism" in man and higher animals, a disease produced by eating seeds of certain species of Lathyrus and Vicia. The toxic factors of various species of Lathyrus and Vicia have been isolated and individually characterized.

γ-glutamyl-β-aminopropionitrile (N≡C-$CH_2$-$CH_2$NH-γ-Glu) was found to be present in four of the Lathyrus species (4) (5). Seeds of other species are known to contain 2,4-diaminobutyric acid ($NH_2CH_2$-$CH_2$-CHN$H_2$ with COOH) and α-amino-β-oxalylaminopropionic acid (NHC$H_2$-CHN$H_2$ with CO and COOH and COOH) both of which produce

signs of classical lathyrism (6). Another unusual amino acid, β-cyanoalanine (7) $N\equiv C-CH_2-CHNH_2$ (COOH) and its γ-glutamyl derivative, $N\equiv C-CH_2-CHNH-\gamma-Glu$ (COOH), have been isolated from Vicia species (8). Both these compounds are toxic to animals and are found in at least sixteen species (9).

The distribution of similar azoxyglycosides in the Cycads suggested a fair possibility of the presence of the non-protein amino acid, α-amino-β-methylaminopropionic acid, in E. hildebrandtii and C. thuarsii.

## RESULTS AND DISCUSSION

None of the fractions, after elution from the ion-exchange column, showed any indication of the presence of the non-protein amino acid, α-amino-β-methylaminopropionic acid, either in the case of the seeds of E. hildebrandtii or the leaves of C. thuarsii. This was ascertained by the use of paper chromatography employing different solvent systems as described on page 50.

The fact that C. circinalis has been shown to contain this non-protein amino acid (1), and members of the same genus frequently contain chemical compounds which are common to all species of that genus, suggested the possibility that this non-protein amino acid might be present in those Cycads indigenous to Kenya, although it should be noted that no neurological disorder due to the ingestion of E. hildebrandtii seeds and C. thuarsii leaves have so far been reported. Moreover, Mugera et al. (10) do not report any neurological effects during their feeding experiments with the crude meal of E. hildebrandtii nuts.

However, negative findings may be related to differences between species, age of the plant material examined,

part of the plant employed and also the nutritional difference of the soil.

The spasmodic nature of outbreaks and the varied conditions under which paralysis occurs have led to a considerable controversy over the circumstances under which animals are affected. Well nourished as well as poorly fed stock suffer. Stewart (11) observed that many animals were not afflicted at a time when they were in poor condition but were when they were thriving. Plants of the same species of Cycad growing in different soils appear to vary in their toxicity. Lamb (12) reported a higher incidence among cattle that were grazing on Cycads growing on swampy land than those which were consuming Cycads on the ridges. There is evidence that old roots and leaves that have been on the ground for as long as two years retain their toxicity and produce paralysis (13).

Lastly there has been a recent communication in which a neurological disorder consisting of hind-leg paralysis was observed in 80% of newborn mice, following a subcutaneous injection of 0.5 mg. of cycasin per gm. of body weight (14). Mature mice of the same strain did not develop a

paralysis when equivalent amounts of cycasin were given by stomach tube.

## EXPERIMENTAL TECHNIQUES

### EXTRACTION

The fresh white kernels of E. hildebrandtii were macerated with 50% ethanol (1.5 litres) and the suspension homogenized for 17 hours at room temperature before filtering. The filtrate, after dilution with water, was applied to a strongly acidic cation exchange resin column (AMBERLITE IR-120, 100 x 3 cm.) in hydrogen form. The column was washed with water and the amino acids subsequently eluted with 1.25 N HCl and the eluates collected as a series of 50 ml fractions. The "common" amino acids, aspartic acid, glutalmic acid, serine, alanine, valine and glycine were detected in the first fifty fractions, along with certain other ninhydrin reacting compounds. The eluant was then changed to 1.5 N HCl and 25 fractions were collected. These fractions showed the presence of more basic amino acids, especially lysine.

A similar extraction procedure was carried out with fresh leaves of Cycas thuarsii.

## PAPER CHROMATOGRAPHY

Solvents used for paper chromatography on Whatman No. 1 paper were:

(1) butanol-acetic acid-water (12:3:5), migration time (8 inches) 10 hours, drying time 10 hours.

(2) phenol-water (8:2), migration time 10 hours, drying time 24 hours;

(3) ethanol-ammonia-water (18:1:1), migration time 4 hours, drying time 12 hours;

(4) methanol-pyridine-water (20:1:5), migration time 10 hours, drying time 12 hours.

Paper chromatography was used for a variety of applications: (1) two-dimensional separations with first butanol-acetic acid-water, then phenol-water. Crystals of KCN were added to the chromatographic chamber to suppress the oxidation of the phenol. A few ml. of concentrated $NH_4OH$ were poured down the sides of the chromatographic tank to provide a basic atmosphere, aiding the separation of basic components; (2) one-dimensional chromatograms in each of the above solvents with accompanying standard samples for comparison of $R_f$ values.

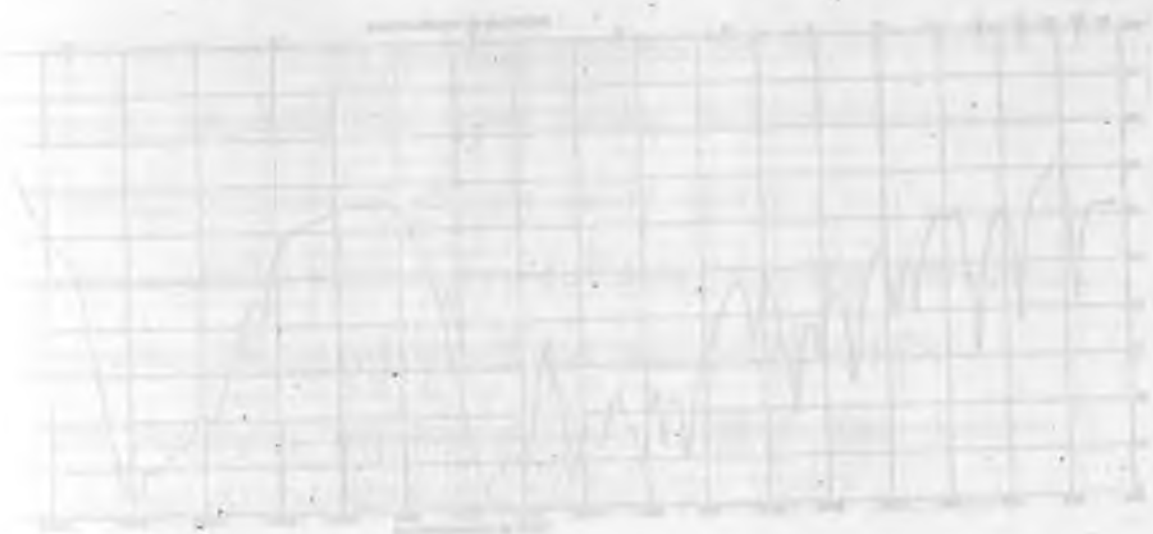
All of the above paper chromatograms were developed by spraying with 0.2 percent ninhydrin in acetone, followed by heating in an oven at 105° for 10 minutes.

SYNTHESIS OF α-AMINO-β-METHYLAMINOPROPIONIC ACID (1)

α-Acetamidoacrylic acid (5 g) was dissolved in 50 ml of a solution of approximately 30% aqueous methylamine and allowed to stand at 40° for 72 hours. Excess amine was removed by taking the reaction mixture to dryness at room temperature. The residue was boiled for 2 hours with 2N HCl (25 ml), and the solution again taken to dryness at room temperature and the residue recrystallized from aqueous alcohol (1:1) to give colourless crystals, m.p. 166° - 168° (dec.).

The synthetic material gave no absorption in the U.V. region of the spectrum. The infrared absorption spectrum (fig. 8) determined as a KBr wafer using a Beckman IR 4 spectrophotometer showed the following prominent bands at 3000 $cm^{-1}$ (-OH stretching frequency of carboxylic group); 1610 $cm^{-1}$, 1410 $cm^{-1}$ (antisymmetrical ionized carboxyl group of the dipolar ions); 2090 $cm^{-1}$ (N-H stretching frequency of $-NH_3^+$ ion). 3420 $cm^{-1}$ (N-H stretching frequency

of -NH - group); 1375 $cm^{-1}$ (symmetrical $CH_3$ stretching frequency). These assignments are made from the data of Vega and Bell (1).



Infrared Absorption Spectrum of α-amino-β-methylaminopropionic acid

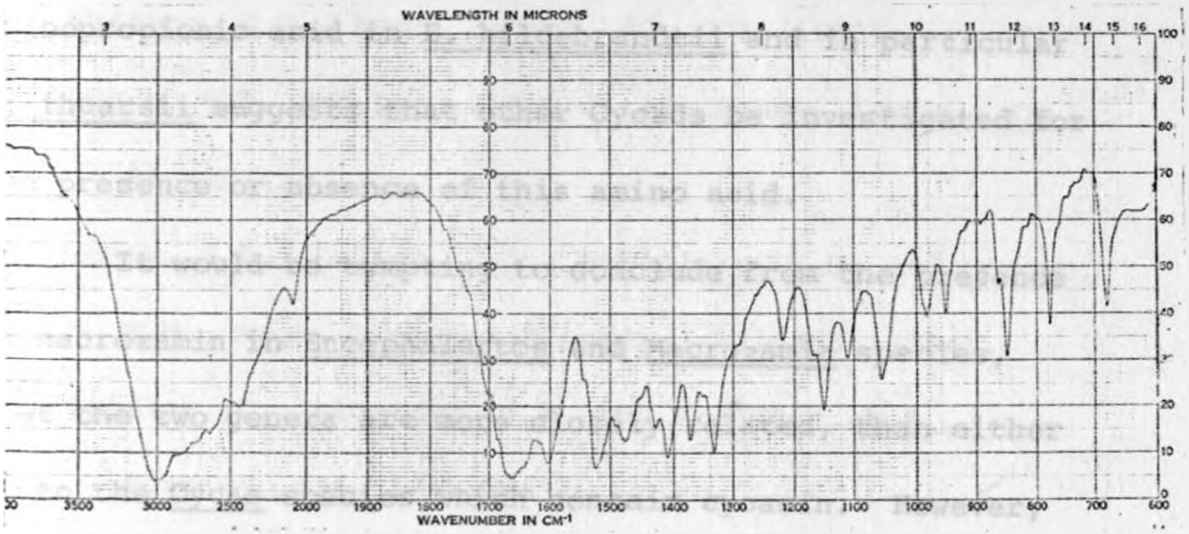



Figure 8.

## CONCLUDING COMMENT

All Cycads so far investigated (3), including *E. hildebrandtii* have been shown to contain the azoxyglycosides, cycasin or macrozamin, regardless of their geographical locations. However, the absence of α-amino-β-methylaminopropionic acid in *E. hildebrandtii* and in particular *C. thuarsii* suggests that other Cycads be investigated for the presence or absence of this amino acid.

It would be tempting to conclude from the presence of macrozamin in *Encephalartos* and *Macrozamia* species, that the two genera are more closely related, than either is to the *Cycas* species which contain cycasin. However, the presence of α-amino-β-methylaminopropionic acid in *C. circinalis* underlines the hazard of attempting to relate botanical species on the basis of selected chemical characters.

## REFERENCES


1. Vega, A. and Bell, E.A., Phytochem., 6, 759 (1967).
2. Mason, M.M. and Whiting, M.G., Fedn. Proc., 25, 533 (1966).
3. Whiting, M.G., Econ. Botany, 17, 271 (1963).
4. Dupuy, H.P. and Lee, J.G., J. Am. Pharm. Assoc., 43, 61 (1954).
5. McKay, G.F., Lalich, J.J., Schilling, E.D. and Strong, F.M., Arch. Biochem. Biophys., 52, 313 (1954).
6. Ressler, C., Redstone, P.A. and Erenberg, R.H., Science, 134, 188 (1961).
7. Ressler, C., J. Biol. Chem., 237, 733 (1962).
8. Ressler, C., Nigam, S.N., Giza, Y.H. and Nelson, J., J. Am. Chem. Soc., 85, 3311 (1963).
9. Bell, E.A., Biochem. J., 97, 104 (1965).
10. Mugera, G.M. and Nderito, P., Brit. J. Cancer, 22, 563 (1968).
11. Stewart, J.D., Agr. Gaz. New South Wales, 10, 1205 (1899).
12. Lamb, S., Agr. Gaz. New South Wales, 6, 505 (1895).

13. Maiden, J.H., Agr. Gaz. New South Wales, 10, 125 (1899).

14. Hirono, I. and Shibuya Ch., Nature, 216, 1311 (1967).

15. Riggs, N.V., J. Chem. Soc., 3199 (1949).

16. Morrison, R.F. and Boyd, R.N., Organic Chemistry, 2nd ed. Allyn and Bacon, Boston, pp. 735, 1009 (1966).

## BIBLIOGRAPHY

Bell, E.A., Association of Amino Acids and Related Compounds in the Seeds of 47 species of Vicia: their Taxonomic and Nutritional Significance. Biochem. J., 97, 104 (1965).

Hirono, I. and Shibuya Ch., Induction of a Neurological Disorder by Cycasin in Mice. Nature, 216; 1311-1312 (1967).

Lamb, S., Plants Injurous to Stock. Agr. Gaz. New South Wales, 6, 505 (1895).

Maiden, J.H., Effects on Cattle of Eating Macrozamia Roots. Agr. Gaz. New South Wales, 10, 1259 (1899).

Mason, M.M. and Whiting, G.M., Demyelination in the Bovine Spinal Cord caused by Zamia Neurotoxicity. Federation Proc., 25, 533 (1966).

Mugera, G.M. and Nderito, P., Induction of Tumours in Liver, Kidney and Lungs Following Chronic Feeding of Crude Meal Prepared from Nuts of Encephalartos hildebrandtii. Brit. J. Cancer, 22, 563 (1968).

Ressler, C., Redstone, P.A. and Erenberg, R.H., Isolation and Identification of a Neuroactive Factor from

Lathyrus latifolios. Science, 134, 188 (1961).

Ressler, C., Isolation and Identification from Common Vetch of the Neurotoxin β-Cyano-L-alanine, a possible factor in Neurolathyrism. J. Biol. Chem., 237, 733 (1962).

Ressler, C., Nigam, S.N., Giza, Y.H. and Nelson, J., Isolation and Identification from Common Vetch of γ-L-Glutamyl-β-cyano-L-alanine, a Bound Form of the Neurotoxin β-Cyano-L-alanine. J. Am. Chem. Soc., 85, 3311 (1963).

Stewart, J.D., A report on the disease affecting cattle in the Moraya district. Agr. Gaz. New South Wales, 10, 1205 (1899)

Vega, A. and Bell, E.A., α-Amino-β-methylamino propionic Acid, a New Amino Acid from seeds of Cycas circinalis. Phytochem., 6, 759-(1967).

Whiting, M.G., Toxicity of Cycads, Econ. Botany, 17, 271 (1963).

# C H A P T E R  I I I

## ABSTRACT

A high incidence of rumenal cancer of the oesophagus in Kenyan cattle in the valley of "Nasampolai" was reported by Dr. F.G. Peers and Dr. C.A. Linsell working at the International Agency for Research on Cancer, Regional Centre, Nairobi. All plants possibly implicated as causative agents were chemically screened for the presence of N-nitrosamines. In view of the fact that secondary amines might be present, which under appropriate conditions could give rise to N-nitrosamines, the plant extracts were subjected to conditions under which secondary amines are known to be nitrosated. A thin-layer chromatographic method, similar to that of Du Plessis et al. (13), was used in an attempt to detect naturally occurring or artifically produced N-nitrosamines.

## INTRODUCTION

In 1955 Plowright (1) reported that the residents of a remote valley in the Sakutiek area of the Masai district of Kenya recognized that a number of their cattle died from a disease which they claimed to diagnose clinically six to thirty six months prior to death or slaughter. The disease was sufficiently common to cause grave economic loss and the annual incidence was said to be as high as 10% of the cattle population, causing some owners with grazing lands elsewhere to remove their cattle from the valley. The clinical judgement of the Masai farmers was confirmed by finding squamous cell carcinoma of the rumen and oesophagus in three cases following field autopsies (2). No further investigation took place until September 1968, when, during a single preliminary visit, four clinical cases were presented and retrospective enquiry indicated that the incident had not greatly changed. The immediate slaughter of one of these cases and subsequent post-mortem examination again showed a carcinoma of the rumen with secondary deposits in the regional lymph nodes (2). Since February, 1969 a Masai field worker has gathered data on the cattle population and grazing areas.

The valley of "Nasampolai" is somewhat remote and its name in the local Masai dialect, means "the valley where cattle salivate". It is situated on the south-western slopes of the Mau Escarpment approximately 36° 07'E, 0° 50's. The valley lies at altitudes between 9,000 - 10,000 feet and runs into the forested Mau hills. The valley floor is approximately five square miles in area and a small stream runs through it. The sides of the valley are steep and a dense bamboo forest is present only 200' above the valley floor in some places. Large scale maps were constructed from existing aerial photographs to enable the study area to be defined topographically and to allow detailed examination of the relationships of "bomas" (enclosed homesteads) to cattle grazing areas.

Domestic animals are taken from the "bomas" to their grazing every morning and returned each evening, calves being retained near the "bomas" for the first six months or so and then accompanying the herds to more distant grazing. As the population of goats and sheep is essentially high, forest grazing of cattle is often a necessity but is recognized as an unsatisfactory source of pasture.

The rumenal cancer is sufficiently common to have acquired a vernacular name "Embonget" which is related to the clinical sign of rumenal tympany. The disease has been recognized by the farmers since about 1935 and the incidence, they say, has increased over the years, particularly since 1942. The fact that the disease has a vernacular name and the name of the valley itself, "Nasmapolai", means "where the cattle salivate" indicates a long standing and a clear recognition of the disease.

Rumenal cancer of the cattle is probably associated with the unusual type of forest grazing, which the farmers are forced to use as their land rights are limited. The farmers stated that this forest grazing comprises about thirty broad leaved plants and grasses.

Several natural products of plant and microbial origin that have been discovered in the past twenty years have proved to be carcinogenic in experimental animals. These products include certain pyrrolizidine alkaloids, aflatoxins, azoxyglycosides, amines, polycyclic hydrocarbons, etc. Magee and Barnes (4) commenting on Plowright's original observation (1) speculated that a N-nitroso-compound

could be present in one of the plants consumed by the cattle grazing in the forest area.

Recent studies have shown marked variation in the mortality rates and frequency ratios for oesophageal cancer throughout the world (21). The highest risks are reported in the Transkei of South Africa, in Central Asia and parts of China.

Since the discovery of the carcinogenic action of N-dimethylnitrosamine, the simplest N-dialkylnitrosamine by Magee and Barnes in 1956 (3), a large number of other N-nitroso-compounds are reported to be carcinogenic (4). Various aspects of carcinogenesis by N-nitroso-compounds has also been discussed recently (20) (18), although some of the nitrosamines screened for carcinogenic activity have been shown to be inactive. Nevertheless, as a group, these compounds must be regarded as among the most powerful of the known carcinogens, quite comparable in efficiency with the polycyclic hydrocarbons (19). The diversity of carcinogenic activity of the N-nitroso-compounds has recently been reviewed by Druckery et al. (5). A most interesting factor of carcinogenic nitrosamines is their potency, irrespective of the route of administration.

Although the reasons for the apparent selective action of some N-nitroso-compounds on specific organs is not yet clear, it is interesting to note that tumours have been induced in virtually every organ of the body by an appropriate N-nitroso-carcinogen (4). Herrmann (14) has isolated 4-methylnitrosaminobenzaldehyde from a common edible mushroom, Clitocybe suaveolens, and more recently Du Plessis et al. (13) have demonstrated the presence of N-dimethylnitrosamine in Solanum incanum occurring in the Transkei, South Africa. Prior to this the natural occurrence of compounds of this type in plant material, likely to be involved in human or animal diets, was apparently limited to cycasin and macrozamin (15).

There has also been an increasing interest in the possibility that nitrosamines can be formed in vivo and so give rise to tumours in individuals not apparently exposed to nitrosamines per se. Secondary amines have recently been shown to react with nitrite to form nitrosamines in conditions of defined pH and in other conditions similar to those in the mammalian stomach (6). Rats given nitrite and a secondary amine, N-methylbenzylamine, together in the diet developed oesophageal tumours (7), which

are also induced by N-nitrosomethylbenzylamine, the corresponding nitrosamine. Nitrates are widely distributed in nature, particularly in plants and forage, and nitrites are readily formed from them. The limiting factor then in the formation of nitrosamines seems to be the availability of nitrosable secondary amines.

About thirty plants from "Nasampolai" were chemically screened for the presence of nitrosamines as natural metabolites, and secondary amines, which are implicated as precursors of nitrosamines, in an attempt to account for some epidemiological aspects of the rumenal cancer among the cattle of this area.

## RESULTS AND DISCUSSION

In view of the magnitude of the task involved of systematically testing all plant materials which could possibly be implicated in this outbreak of oesophageal cancer of the cattle in the valley, there was a need for judicial selection of priority problems for study. Magee and Barnes (4) commenting on Plowright's (1) original observation in 1955, speculated that a N-nitroso--compound could be present in one or more of the plants consumed by the cattle grazing in the forest area.

This hypothesis was tentatively put forward by Dr. F.G. Peers of the International Agency for Research on Cancer, Regional Centre, Nairobi, who suggested that the potent carcinogen could either be ingested *per se* from the forest grazing of "Nasampolai" or rapidly produced *in vivo* from a precursor. This hypothesis was put to the test by chemically screening crude plant extracts for the presence of nitrosamines and secondary amines in conjunction with a toxicity testing program on rats carried out by the I.A.R.C., Regional Centre, Nairobi.

Nitrosamines as natural plant metabolites were not detected. However, there remained the possibility of a naturally occurring innocuous compound being metabolized in vivo to produce the active carcinogen. Ender et al. (11) have reported an acute outbreak of toxic hepatitis associated with feeding sheep nitrite preserved fish meal, which was subsequently demonstrated to contain N-dimethylnitrosamine (23). A report that rats fed on a diet incorporating both a secondary amine and nitrite developed oesophageal tumours (7) suggested the need to examine the plant extracts for the presence of naturally occurring secondary amines. Because secondary amines have seldom been sought in the environment, information about their distribution is scanty. Methods of identifying secondary amines from natural products are not well developed and those amines usually identified are dimethylamine and diethylamine, the remaining bases being conventionally grouped as unidentified amines. However, all secondary amines are converted to their corresponding nitrosamines by treatment with nitrous acid (HONO), and the latter are readily detected by means of their colour reaction with ninhydrin (24).

Each plant extract was therefore treated with a solution of sodium nitrite - dil. HCl (HONO) to nitrosate any secondary amines present, and the solutions chromatographed as described on page 72. Of the thirty plant extracts so treated, eleven gave a colour reaction with ninhydrin suggesting the possibility that secondary amines might be present. However, the unknowns gave a reddish colouration whereas the authentic nitrosamines produced violet colourations. The $R_f$ values of the unidentified spots varied from 0.2 to 0.7. Owing to their presence in extremely low concentrations, satisfactory infrared absorption spectra could not be obtained, and therefore, after initial separation by thin-layer chromatography, the unidentified compounds were examined by gas-liquid chromatography (GLC) as described on page 78. The standards, N-dimethylnitrosamine, N-diethylnitrosamine, N-dipropylnitrosamine, N-dibutylnitrosamine, N-nitrosomethylurea and N-nitrosopiperidine gave prominent peaks with retention times varying from 2.0 to 69.0 min. Gas chromatography of the samples obtained by preparative thin-layer separation showed no significant peaks, and those very minor peaks which were recorded did not have retention times identical with any of

the standards. The use of maximum voltage sensitivity (1750 volts on the Pye Argon Chromatograph) and shorter columns of 2% Apeizon L. operating at 200°C also gave negative results. The behaviour of unknowns when developed on thin-layer plates using hexane, ether and chloroform as individual solvents, suggested that they were not very polar, and hence, unless they were macro-molecules, they would be expected to give measurable peaks on gas-liquid chromatographic analysis.

Although no firm conclusions could be reached as to the nature of the carcinogen, a number of points warrant discussion. The carcinogen might conceivably be one of the several products of plant origin already demonstrated to be carcinogenic during the past few decades. Alkaloids of Crotolaria ardicola (8) are believed to be the cause of oesophageal tumours in horses, a condition known locally as "chillagoe" disease. Also, certain strained or α,β-unsaturated lactones, e.g. β-propiolactone, which have been shown to be carcinogenic, are widely distributed among natural products (9) (10). The botanical survey of the "Nasampolai" forest flora is not yet complete, and there remains the possibility that some insight into the nature

of the carcinogen might be gained when those species not yet identified have been botanically authenticated.

A close association has been demonstrated recently between molybdenum deficiency in garden plants and the occurrence of oesophageal cancer in localized areas of the Transkei; N-dimethylnitrosamine has been isolated from food plants in these areas (12) (13). Analyses of soil samples from "Nasampolai" might throw some light on the possibility of a similar situation existing in the valley.

Mimulopsis solmsii (17), which grows in the valley, flowers every seven years. This fact, coupled with a report by the Masai that during the second season after flowering, a heavy mortality occurs among the cattle, suggests the need for an intensive investigation of this particular plant.

It might also be born in mind that ingestion of a combination of plant materials may be necessary to generate the carcinogen in its active form. Another possibility is that the oesophageal cancer may be the direct result of a continuous irritation by exposure to plants such as the Masai "stinging nettle" (Utrica massaica) which the cattle

are known to eat, and which raises painful weals on the skin. A further possibility is that ingress of the active carcinogen takes place through lesions in the oesophagus brought about by such an irritant.

Clearly then, much further work is necessary before the cause of the malignant neoplasia among the cattle of "Nasampolai" can be established.

## MATERIALS AND EXPERIMENTAL METHODS

### PLANT SPECIMENS

The Masai identified about thirty broad leaved plants and grasses, which the cattle eat, by their vernacular name (see table 1), and a botanical survey of this forest flora is in progress (16) as a more authoritative identification of the plant specimens at all stages of growth is required. The survey is not yet complete however, and the final results will be reported elsewhere. These plants were collected periodically for extraction purposes by a Masai field worker.

### THIN-LAYER CHROMATOGRAPHY (TLC)

Methods for the separation of nitrosamines by TLC and their detection by colour reactions after photolytic dissociation have been described by Preussmann et al. (22), Kröller (24) and more recently by Sen et al. (25). Thin-layer chromatography was carried out on 5 X 20 cm. and 20 X 20 cm. silica-gel G plates (purchased commercially as Chromastrips) developed with mixtures of hexane, diethyl ether and methylene chloride in different compositions.

TABLE I

PLANTS SCREENED FOR NITROSAMINES AND SECONDARY AMINES

| NATIVE NAME | BOTANICAL NAME |
|---|---|
| 1. ENKATUNUTE | Pilea johnsonii. Oliv. |
| 2. OLOSIDA | Hypestes triflora. (Forsk). Roem and Schultes. |
| 3. OLKEKEYIET | Mimulopsis solmsii. Schweinf. |
| 4. OLKEDONGO | Cyathula cylindrica. Moq. |
| 5. OLKIKUEI LOOSORKON | Cardus chamoecephalus. Oliv. and Hiem. |
| 6. OLNGERIANDUS | Galium hamatum. A. Rich. |
| 7. ENKONGU EMBUAA | Impatiens cubromaculata. warb. |
| 8. NARERUK "A" | Usnia. |
| 9. NARERUD "B" | Anaptychia. |
| 10. ENTAMEJOI | Utrica massaica. Milde. |
| 11. OSUPULIAI | Dombeya goetzenii. K. Shun. |
| 12. OLOLAA | Bromus leptoclades. Mees. |
| 13. OLOIROPIJI LOOLGUYIAN | Haplocardium abyssinica. Hochst. |
| 14. OLKELELIT | - |
| 15. ESISIYIAN | - |
| 16. OLOLAAI LOLTEYANI | - |
| 17. ENAINGURRA ENKOP | Guaphalium declinatum. L.f. |

TABLE I (continued)

| NATIVE NAME | BOTANICAL NAME |
|---|---|
| 18. OLKEREMBORI | - |
| 19. EIIKOM OSUPUKO | - |
| 20. ENDULE OOLKIKU | - |
| 21. ENDULE OOLRUBAT | - |
| 22. ENKOGUMATI OSUPUKO | - |
| 23. OLPIRON | - |
| 24. EMBALAKAI ENTIM | - |
| 25. NO NAME | - |
| 26. OLAN TERERAI | - |
| 27. OLOREPIREP | - |
| 28. OLAIMURUNYAI | Maytenus Senegalensis |
| 29. OLOPURU DORROP | - |
| 30. NO NAME | - |
| 31. - | Solanum incanum. |

Pure samples of nitrosamines (N-dimethylnitrosamine, N-diethylnitrosamine, N-dipropylnitrosamine, N-dibutylnitrosamine, N-nitrosomethylurea and N-nitrosopiperidine) and crude plant extracts were applied with suitable microliter pipettes 2 cm. from the lower edge of the plate and placed in a glass chamber 10 X 22 X 21 cm. containing 200 ml. of solvent. When the solvent front had travelled 15 cm. from the starting line, the development was interrupted and the plates were dried at room temperature.

Solvents used for thin-layer chromatography were:

(1) Symmetrical dialkylnitrosamines:-

Hexane: diethyl ether: methylene chloride (4:3:2).

(2) Methyl - alkyl - nitrosamines:-

Hexane: diethyl ether: methylene chloride (4:3:2).

(3) Cyclic nitrosamines:-

Hexane: diethyl ether: methylene chloride (5:7:10).

(4) Aryl and diaryl nitrosamines:-

Hexane: diethyl ether: methylene chloride (10:3:2).

## SPRAYING REAGENTS

Ninhydrin and Greiss reagents (24) (25) have been found to be more specific for the detection of N-nitrosamines than the diphenylamine spray. Thin-layer plates of extracts when sprayed with diphenylamine reagent gave many false-positive spots for N-nitrosamines. The compounds responsible for these spots failed to give colour reactions with either of the other two reagents. Therefore it seemed that the ninhydrin spray techniques of Kröller (24) and the Greiss reagent were more specific for N-nitrosamines than the diphenylamine spray. The minimum detection limit for various N-nitrosamine standards was approximately 0.3-0.6 µg. with the Greiss reagent and slightly more sensitive 0.2 µg with the ninhydrin reagent. The spots were usually stable for 30 minutes and then either gradually disappeared or were overshadowed by the background colour.

After developing the plates in appropriate solvents, they were irradiated under UV light (without filter) for 5-10 min. The plates were then sprayed with ninhydrin reagent and heated in the oven at 80°C for 5-10 min. N-nitrosamines were located by their characteristic red-purple

colour. This particular spraying method was so marked, that it became the detecting system of choice, not only for satisfactory identification but also for quantitative purposes.

## GAS-LIQUID CHROMATOGRAPHIC ANALYSIS

Authenticate samples of N-dimethylnitrosamine, N-diethylnitrosamine, N-dipropylnitrosamine, N-dibutylnitrosamine, N-nitrosomethylurea, N-nitrosopiperidine were dissolved in ether and absorbed on Celite (80 - 100 mesh) by allowing the ether to evaporate off. The purpose of adsorption on Celite was to avoid using a solvent such as ether which would have "blanketed" the initial peaks.

Aliquots of plant extracts which gave a positive test with ninhydrin were spotted as streaks on thin-layer plates (20 X 20 cm) and developed in a suitable solvent. The unknown bands after separation by preparative thin-layer chromatography, were scrapped off the plates between $R_f$ 0.2 - 0.7 and extracted with ether. After removal of silica-gel by filtration, the extracts were dried and adsorbed on Celite (80 - 100 mesh) in the manner described above.

The analyses were carried out on a Pye Argon Chromatograph equipped with a glass column packed with Celite (80 - 100 mesh) which had been coated with 20% silicone grease.

The length of the column was 50 cm. and it was maintained at 83°C, with an Argon flow rate of 40 ml/min.

The standards and unknowns were applied by interrupting the gas flow and dropping the Celite directly on to the column. Analyses of unknowns at maximum detector sensitivity failed to show any significant peaks corresponding to the standards. The retention times of the standards were as follows:

(1) N-dimethylnitrosamine : 2.0 min.

(2) N-diethylnitrosamine : 5.5 min.

(3) N-nitrosomethylurea : 10.0 min.

(4) N-dipropylnitrosamine : 19.0 min.

(5) N-nitrosopiperidine : 21.5 min.

(6) N-dibutylnitrosamine : 69.0 min.

EXTRACTS OF SOLANUM INCANUM FRUITS (RIPE AND UNRIPE)

Reports of the presence of N-dimethylnitrosamine in the fruits of S. incanum (13) of the Transkei, South Africa, possibly related to molybdenum deficient soil, focused our attention on the S. incanum species present in "Nasampolai" valley.

S. incanum, ripe and unripe fruits (wet weight 1 Kg.), were macerated with ethyl alcohol (5 litres) in a high speed macerator, the mixture filtered and the filtrate concentrated to a small volume (approximately 250 ml) under reduced pressure in a cyclone evaporator. This solution was separated on thin-layer plates as described above and sprayed with ninhydrin. No colour reaction was obtained.

However, to check the efficacy of the above technique for further work on plant extracts as a means of detecting low concentrations of N-nitrosamines, another kilogram of the fruits was macerated and N-dimethylnitrosamine incorporated as an internal standard. This extract was divided into two portions "A" and "B", each containing 1 mg. of N-dimethylnitrosamine. "A" was evaporated under reduced pressure and concentrated to a small volume and

"B" was concentrated at atmospheric pressure (630 mm of Hg). Both fractions were subjected to thin-layer chromatography and N-dimethylnitrosamine identified by ninhydrin. However when N-dimethylnitrosamine from both "A" and "B" was recovered after dry column chromatography on alumina with methylene chloride as eluant, it was found that the recovery from fraction "A" was reduced. This difference was attributed to the use of vaccum distillation for the removal of excess alcohol in the case of fraction "A". Seventy percent recovery was obtained by distillation at atmospheric pressure in the case of fraction "B".

## EXTRACTION OF THE OTHER PLANTS

Thirty plant extracts were prepared in the laboratory in the following way:

Fresh plants (1 Kg.) were macerated with 95% ethyl alcohol (5 litres) in a high speed macerator, the mixture filtered and the filtrate concentrated to a small volume (250 ml) by distilling off the bulk of the alcohol at atmospheric pressure (630 mm of Hg).

## SCREENING FOR NITROSAMINES

Aliquots of the respective extracts were then chromatographed on thin-layer plates, using different solvent systems as described on page 72.

## SCREENING FOR SECONDARY AMINES

The most conventional method for identifying secondary amines in the laboratory is to convert them into their corresponding N-nitroso-compounds by treatment with excess nitrous acid ($NaNO_2$/HCl).

The plant extracts (100 ml) were subjected to this artificial treatment with excess $NaNO_2$/HCl solution, so that any secondary amines - if present, - would be nitrosated to the corresponding nitrosamines. The extracts so treated were then shaken with ether to remove any nitrosamines present and the ether layer separated and chromatographed on thin-layer plates in the manner described on page 72.

## CONCLUDING COMMENT

An attempt has been made in the foregoing section to deal with certain aspects of N-nitrosocarcinogens and their possible relation to the rumenal cancer amongst the livestock in the valley of "Nasampolai" in Kenya. However, it is too early to be able to suggest with any confidence the part N-nitroso-compounds or naturally occurring related compounds might play in this outbreak of malignant neoplasia. Answers to this complex problem lie in the future and require the participation of many workers with different scientific backgrounds and interests, eventually narrowing down the number of plant suspects with the help of cytotoxicity tests, which could later be substituted by a gross toxicity testing program.

## REFERENCES

1. Plowright, W., J. Comp. Path. Therap., 65, 108 (1955).

2. Peers, F.G., Linslell, C.A. and Plowright, W., In press (1970).

3. Magee, P.N. and Barnes, J.M., Brit. J. Cancer, 10, 114 (1956).

4. Magee, P.N. and Barnes, J.M., Advan. Cancer Res., 10, 163 (1967).

5. Druckery, H., Preussmann, R., Ivankovic, S. and Schmähl, D., Z. Krebsforsch, 69, 103 (1967).

6. Sander, J.V., Hoppe-Seyler's Z. Physiol. Chem., 349, 429 (1968).

7. Sander, J.V., Schweinsberg, F. and Menz, P.H., Hoppe-Seyler's Z. Physiol. Chem., 349, 1691 (1968).

8. Culvenor, C.C. and Smith, L.W., Aust. J. Chem., 15, 121 (1962).

9. Dickens, F. and Jones, H.E., Brit. J. Cancer, 15, 85 (1961); Brit. J. Cancer, 17, 100 (1963); Brit. J. Cancer, 19, 392 (1965).

10. Walpole, A.L., Roberts, D.C., Rose, F.L., Hendry, J.A., and Homer, R.F., Brit. J. Pharmacol., 9, 306 (1954).

11. Ender, F., Harve, G., Helgebostad, A., Koppang, N., Madsen, R. and Ceh, L., Naturwissenschaften, 51, 637 (1964).

12. Burrell, R.J., Roach, W.A. and Shadwell, A., J. Natl. Cancer Inst., 36, 201 (1966).

13. Du Plessis, L.S., Nunn, J.R. and Roach, W.A., Nature, 222, 1198 (1969).

14. Herrmann, H., Z. Physiol. Chem., 326, 13 (1961).

15. Whiting, M.G., Econ. Botany, 17, 269 (1963).

16. Greenway, P.J., East African Agriculture and Forestry Research Organization, East African Herbarium, Nairobi. (Personal communication).

17. Glover, P.E., Stewart, J., and Gwynne, M.D., E. A. Agr. and For. J., 2, 184 (1966).

18. Heath, D.F., and Magee, P.N., Brit. J. Industr. Med., 19, 276 (1962).

19. Hoffmann, F. and Graffi, A., Arch. Geswulstforsch, 23, 274 (1964).

20. Magee, P.N. and Schoental, R., Brit. Med. Bull., 20, 102 (1964).

21. Dunham, L.J. and Bailar, T., J.C. World Maps of Cancer Mortality Rates and Frequency Ratios. J. Natl. Cancer Inst., 41, 155 (1968).

22. Preussmann, R., Neurath, G., Wulf-Lorentzen, G., Daiber, D. and Hengy, H., Z. Anal. Chem., 202, 187 (1964).

23. Sakshaug, J., Sögen, E., Hansen, M.A. and Koppang, N., Nature, 206, 1261 (1965).

24. Kröller, E., Deut. Lebensm. Rundschau., 10, 303 (1967).

25. Sen, N.P., Smith, C.D., Schwinghamer, L. and Marleau, J.J., J. Assoc. Offic. Anal. Chem., 52, 47 (1969).

## BIBLIOGRAPHY

Burrell, R.J., Roach, W.A. and Shadwell, A., Esophageal Cancer in the Bantu of the Trankei Associated with Mineral Deficiency in Garden Plants. J. Natl. Cancer Inst., 36, 201 (1966).

Culvenor, C.C. and Smith, L.W., Alkaloids of Crotolaria trifoliastrum. Willd. and C. ardicola Domin. 1. Methyl Ethers of Supinidine and Retronecine. Aust. J. Chem., 15, 121 (1962).

Dickens, F. and Jones, H.E., Carcinogenic Activity of a Series of Reactive Lactones and Related Substances. Brit. J. Cancer, 15, 85 (1961).

Dickens, F. and Jones, H.E., Further Studies in the Carcinogenic and Growth Inhibitory Activity of Lactones and Related Substances. Brit. J. Cancer, 17, 100 (1963).

Dickens, F. and Jones, H.E., Further Studies on the Carcinogenic Action of Certain Lactones and Related Substances in the Rat and Mouse. Brit. J. Cancer, 19, 392 (1965).

Druckery, H., Preussmann, R., Ivankovic, S. and Schmähl, D., Organotropic Carcinogenic Effects of 65 Different N-nitrosc Compounds on BD-Rats. Z. Krebsforch, 69, 103 (1967).

Dunham, L.J. and Bailar, T., J.C. World Maps of Cancer Mortality Rates and Frequency Ratios. J. Natl. Cancer Inst., 41, 155 (1968).

Du Plessis, L.S., Nunn, J.R. and Roach, W.A., Carcinogen in a Transkeian Bantu Food Additive. Nature, 222, 1198 (1969).

Ender, F., Harve, G., Helgebostad, A., Koppang, N., Madsen, R. and Ceh, L., Isolation and Identification of a Hepato-toxic factor in Herring Meal Produced from Sodium Nitrite Preserved Herring. Naturwissenschaften, 51, 637 (1964).

Glover, P.E., Stewart, J., Gwynne, M.D., Masai and Kipsigis Notes on East African Plants Part I - Grazing, Browse, Animal Associated and Poisonous Plants. E. A. Agri. and Fors. J., 32, 184 (1966).

Herrmann, H., Identification of 4-methylnitrosaminobenzaldehyde as Metabolite Product of Clitocybe suaveolens, Z. Physiol. Chem., 326, 13 (1961).

Kröller, E., Detection of Nitrosamines in Tobacco Smoke and Food. Deut. Lebensm. Rundschau., 10, 303 (1967).

Magee, P.N. and Barnes, J.M., The Production of Malignant Primary Hepatic Tumours in the Rat by Feeding Dimethylnitrosamine. Brit. J. Cancer, 10, 114 (1956).

Magee, P.N. and Barnes, J.M., Carcinogenic Nitroso-Compounds. Advan. Cancer Res., 10, 163 (1967).

Magee, P.N. and Schoental, R., Carcinogenesis by Nitroso-compounds. Brit. Med. Bull., 20, 102 (1964).

Plowright, W.J., Malignant Neoplasia of the Oesophagus and Rumen of Cattle in Kenya. J. Comp. Path. Therap., 65, 108 (1955).

Preussmann, R., Neurath, G., Wulf-Lorentzen, G., Daiber, D. and Hengy, H., Spraying Methods and T.L.C. of Organic Nitroso-compounds. Z. Anal. Chem., 202, 187-192 (1964).

Sakshaug, J., Sögen, E., Hansen, M.A. and Koppang, N., Dimethylnitrosamine, its Effect in Sheep and its Occurrence in Toxic Batches of Herring Meal. Nature, 206, 1261 (1965).

Sander, J.V., Nitrosaminsynthese durch Bakterien. Hoppe Seyler's Z. Physiol. Chem., 349, 429 (1968).

Sander, J.V., Schweinsberg, F. and Menz, P.H., Untersuchungen über die Entstehung Cancerogener Nitrosamine im Magen. Hoppe-Seyler's Z. Physiol. Chem., 349 1691 (1968).

Sen, P.N., Smith, C.D., Schwinghamer, L. and Marleau, J.J., Diethylnitrosamine and Other N-Nitrosamines in Foods. J. Assoc. Offic. Anal. Chem., 52, 47 (1969).

Whiting, M.G., Econ. Botany, 17, 269 (1963).