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METABOLISM AND TRANSLOCATION OF LINAMARIN IN CASSAVA (*MANIHOT ESCULENTA* CRANTZ)

A Thesis Presented in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Biochemistry at Massey University, New Zealand.

Badu Michael Kofi Bediako

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ABSTRACT

The metabolism of linamarin in cassava (*Manihot esculenta* Crantz) has been investigated. Information on the biosynthetic pathway, synthetic sites, translocation and turnover of the cyanoglucoside has been obtained by precursor administrations to various parts of cassava plants grown under partially controlled conditions in the glasshouse.

Three volatile ^{14}C-labelled precursors of linamarin isobutyronitrile, isobutyraldoxime and 2-hydroxyisobutyronitrile were prepared, purified and administered to cassava leaves by a new technique in which the leaves were allowed to take up precursor vapour in an enclosed glass chamber. The incorporation of these precursors, and of L-valine administered by solution uptake, was consistent with a pattern of linamarin biosynthesis in cassava involving the reaction sequence through valine, isobutyraldoxime, isobutyronitrile and 2-hydroxyisobutyronitrile established for other plants.

The solution administration of L-[U-^{14}C] valine to various organs of the plant indicated that the leaves and the shoot apex synthesised linamarin more efficiently than the woody stem and the roots and tubers. More detailed investigations of leaf biosynthesis showed much higher incorporation of ^{14}C-valine into linamarin by the petioles and midribs (45-62% ^{14}C incorporation by petioles and 20% by midribs) than the leaf blades (2%). There was no direct relationship between endogenous linamarin content (which was higher in the blades than the petioles) and the apparent ability to synthesise

linamarin from exogenous valine. However, the low ability of the blade tissue to incorporate valine into linamarin could be due to more active competing pathways removing the exogenously administered valine. In further investigations with tuber peels and the edible cores, similar competing pathways have been implicated for an apparently low biosynthetic efficiency of linamarin.

The translocation of linamarin was demonstrated by specifically labelling ^{14}C-linamarin in attached leaves with 2-hydroxy[1-^{14}C] isobutyronitrile vapour and following the change in labelled linamarin content in the leaf and the distribution of linamarin to other parts of the plant. In both non-tuberous and tuberous plants there was a rapid loss of ^{14}C-linamarin due to translocation from the fully expanded leaves up to 69 hours after synthesis. However a residual component of the ^{14}C-linamarin (25-37% of that initially synthesised) remained in the leaves. A compartmentation of synthesised linamarin in cassava leaf tissues into a readily mobile and partially immobile fraction would account for these observations. In senescing leaves a continuous loss of both ^{14}C-labelled and endogenous linamarin occurred leaving almost no residual component although this was attributed to both translocation and turnover.

Translocated linamarin was distributed to all parts of the plant but the general pattern of translocate flow differed between non-tuberous and tuberous plants. An apical direction of linamarin distribution existed in

the non-tuberous plants while tuber-directed linamarin translocation prevailed in the tuberous plants. Leaf senescence apparently enhances linamarin translocation to the tubers.

There was little turnover of freshly synthesised ^{14}C-linamarin in detached leaves and tuber tissues over a period of 1 to 3 days. However the low recoveries of ^{14}C-linamarin in the whole plant translocation experiments suggest that active turnover may be occurring during translocation or in certain sink tissues.

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CONTENTS

Page number

ABSTRACT ii

ACKNOWLEDGEMENTS v

LIST OF CONTENTS vi

LIST OF FIGURES ix

LIST OF TABLES xi

SECTION 1 - INTRODUCTION

1.1 Hydrolysis of Cyanogenic glycosides 1

1.2 Toxicity associated with cyanogenic glycosides 4

1.3 Biosynthesis of cyanogenic glycosides 5

1.4 Cyanoglycosides of cassava and their importance 9

1.4.1 Cassava toxicity 10

1.5 Aims of the present study 12

SECTION 2 - MATERIALS AND METHODS

2.1 Source of plant materials 14

2.2 Plant culture

2.2.1 Propagation 14

2.2.1.1 Propagation from seeds

2.2.1.2 Propagation from shoot and stem cuttings 14

2.2.2 Growth of cassava in solution culture 16

2.2.3 Growth of cassava in gravel culture 18

2.2.4 Growth and maintenance of cassava plants in the glasshouse 18

2.3 Chemicals and solvents 21

2.4 Source and preparation of ^{14}C-labelled compounds 22

2.4.1 Some labelled compounds 22

2.4.2 [1-^{14}C] isobutyronitrile 22

2.4.3 2-hydroxy[1-^{14}C] isobutyronitrile 25

2.4.4 [U-^{14}C]isobutyraldoxime 26

2.4.5 [1-^{14}C] Linamarin 26

2.5 Preparation of linamarase 27

2.6 Extraction of plant material 28

Page number

2.7 Analytical methods 28

2.7.1 Linamarin estimation 28

2.7.1.1 Enzymic hydrolysis method 29

2.7.1.2 Gas chromatographic method 29

2.7.2 Paper chromatography 30

2.7.3 Thin layer electrophoresis 31

2.7.4 Detection of cyanogenic glucosides 31

2.7.5 Measurement of radioactivity 32

SECTION 3 - ADMINISTRATION OF LINAMARIN PRECURSORS TO CASSAVA LEAVES 34

3.1 Administration of ^{14}C precursors 35

3.1.1 Valine administration to leaves 35

3.1.2 Administration of ^{14}C volatile precursors to leaves 36

3.2 Results 39

3.2.1 Assimilation of L-[U-^{14}C] valine 39

3.2.2 Assimilation of 2-hydroxy[1-^{14}C] isobutyronitrile 41

3.2.3 Assimilation of [1-^{14}C] isobutyronitrile 41

3.2.4 Assimilation of [U-^{14}C] isobutyraldoxime 45

SECTION 4 - SITES OF LINAMARIN SYNTHESIS IN CASSAVA

4.1 Linamarin content of cassava tissues 48

4.2 Biosynthesis of linamarin from valine by detached organs of cassava 53

4.2.1 Methods for valine administration to the different tissues 53

4.2.2 Results 55

4.3 Linamarin synthesis by detached leaves 61

4.3.1 Introduction 61

4.3.2 Experimental procedure 62

4.3.2.1 Preliminary experiment to assess effect of carrier addition 62

4.3.2.2 Linamarin synthesis in detached leaves harvested at different times after precursor addition 65

4.4 Linamarin synthesis in cassava tubers 72

4.4.1 Uptake of labelled valine by tuber cores 73

4.4.2 Linamarin synthesis by tuber cores 75

4.4.3 Linamarin synthesis by tuber peels 79

Page number

SECTION 5 - TRANSLOCATION OF LINAMARIN IN CASSAVA

5.1 Introduction 83

5.2 Preliminary experiments on translocation 84

5.2.1 Translocation following administration of precursor to the mature leaf 84

5.2.2 Translocation following administration of precursor to the shoot apex 88

5.3 Linamarin translocation in immature non-tuberous cassava plants 90

5.3.1 Linamarin turnover in the source leaf 91

5.3.2 Translocation of linamarin from leaves 93

5.4 Linamarin translocation in mature tuberous cassava plants 100

5.4.1 Preliminary experiment to measure incorporation after 4 hours 102

5.4.2 Translocation from a mature green leaf over a 7 day period 104

5.4.3 Translocation from a senescing leaf over a 7 day period 106

5.4.4 Changes in linamarin content in leaves during senescence 111

SECTION 6 - DISCUSSION 114

6.1 General method of approach used in the study 115

6.2 Pathway of linamarin biosynthesis in cassava 117

6.3 Sites of linamarin synthesis in cassava 121

6.3.1 Biosynthesis in the petiolate leaf system 124

6.3.2 Biosynthesis by tuber tissues 127

6.4 Translocation of linamarin in cassava 131

6.4.1 Kinetics of linamarin translocation from leaves 132

6.4.2 Distribution of translocated linamarin in cassava 136

6.4.2.1 Distribution of translocated linamarin in non-tuberous plants 137

6.4.2.2 Distribution of translocated linamarin in tuberous cassava plants 138

6.5 Turnover of linamarin in cassava 141

REFERENCES 146

LIST OF FIGURES

Figure No.	Title	Page Number
1.1	Hydrolysis of linamarin in plants	2
1.2	Pathways for the biosynthesis of cyanogenic glycosides	8
1.4.1	A mature cassava plant growing in peat culture	9b
1.4.2	Cassava tubers	9b
2.2.2	Immature cassava plants growing in solution culture	19
2.4.2	Preparation of [1-^{14}C]isobutyronitrile	24
3.1	Administration of ^{14}C volatile precursors to attached cassava leaf	37
3	Incorporation of radioactivity from 2-hydroxy-[1-^{14}C]isobutyronitrile, [1-^{14}C]isobutyronitrile and [U-^{14}C] isobutyraldoxime in cassava leaves	42
4	Diagram of immature tuberous cassava plant	49
4.1.2	Linamarin estimation by gas chromatographic technique	51
4.2.1	Incorporation of radioactivity from L-[U-^{14}C] valine into ^{14}C-linamarin by cassava tissues	57
4.3.2.2	Biosynthesis of linamarin by petiolate leaf	68
4.4.3	Radioactivity recovered from tuber peels 4 hours after uptake of ^{14}C-valine	81
5.2.1	Radioactivity recovered from shoot apex, lower stem and source leaf of immature plants 7 days after uptake of 2-hydroxy[1-^{14}C]-isobutyronitrile by the source leaf	86
5.3.1	Changes in ^{14}C-linamarin and endogenous linamarin in detached leaves of variety manioke	92
5.3.2	Kinetics of ^{14}C-linamarin translocation out of leaves of immature cassava plants. ^{14}C-linamarin levels in leaf discs	95
5.3.3	Kinetics of ^{14}C-linamarin translocation out of leaves of immature cassava plants. Time-course of specific activity of linamarin in leaf discs	96

Figure No.	Title	Page Number
5.3.4	Diagram illustrating the distribution of ^{14}C-linamarin from lower to upper leaves with respect to phyllotactic configuration	101
5.4.2.1	Linamarin translocation in mature cassava	105
5.4.3.2	Linamarin translocation in mature cassava. Time-course of changes in linamarin in senescing source leaf discs on a tuberous plant	110
5.4.4	Effect of senescence on linamarin content of attached and detached leaves of cassava	112
6.2	Pathway of linamarin biosynthesis in cassava	119
6	Transverse section of a young storage cassava root	129

LIST OF TABLES

Table No.	Title	Page Number
2.2.1	Composition of stock nutrient solution for cassava plants growing in peat beds	15
2.2.2	Composition of nutrient solution for solution and gravel cultures	17
3.2.1.1.	Incorporation of valine into linamarin	40
3.2.1.2	Incorporation of valine into linamarin in other species	40
3.2.2.1	Incorporation of 2-hydroxyisobutyronitrile into linamarin	43
3.2.2.2	Incorporation of 2-hydroxyisobutyronitrile into linamarin in other species	43
3.2.3.1	Incorporation of isobutyronitrile into linamarin	44
3.2.3.2	Incorporation of isobutyronitrile into linamarin in other species	44
3.2.4.1	Incorporation of isobutyraldoxime into linamarin	46
3.2.4.2	Incorporation of isobutyraldoxime into linamarin in other species	46
4.1	Linamarin distribution in cassava	52
4.2.1	Incorporation of L-[U-^{14}C] valine into linamarin by cassava tissues	56
4.3.2.1	Effect of carrier valine on ^{14}C distribution and incorporation into linamarin by detached leaf	64
4.3.2.2	Distribution of ^{14}C in L-[U-^{14}C] valine fed leaf	69
4.3.2.3	Incorporation of ^{14}C-valine into linamarin by leaf tissues	70
4.4.1	Uptake of labelled valine by tuber cores	76
4.4.2	L-[U-^{14}C] valine administration to edible tuber cores	77
4.4.3	L-[U-^{14}C] valine administration to peels	80
5.2.1	Distribution of residual linamarin synthesised in cassava leaves	87
5.2.2	Distribution of linamarin synthesised in shoot apex	89
5.3.1	Distribution of ^{14}C-linamarin in non-tuberous plants 7 days after synthesis in upper source leaves	98

Table No.	Title	Page Number
5.3.2	Distribution of ^{14}C-linamarin in non-tuberous plants 7 days after synthesis in lower leaves	99
5.4.1	Distribution of linamarin activity in tuberous plant 4 hours after synthesis	103
5.4.2.2	Distribution of linamarin activity in tuberous plant 7 days after synthesis in leaf	107
5.4.3.1	Linamarin translocation in tuberous plants - Distribution during leaf senescence	109
6.2	Summary of L-[U-^{14}C] valine incorporation into ^{14}C-linamarin by cassava tissues	122
6.4.2.2	Distribution of translocated linamarin in tuberous plants	140

SECTION 1

INTRODUCTION

1. INTRODUCTION

Cyanogenic glycosides are glycosides of 2-hydroxy-nitriles linked to the sugar moiety in the β-configuration. They are widely distributed in higher plants and occur most commonly in the families, Rosaceae, Passifloraceae, Graminaceae and Euphorbiaceae. Linamarin (2-hydroxy-isobutyronitrile-β-D-glucopyranoside) for instance occurs in a number of taxonomically unrelated genera such as *Linum usitatissimum* (Linaceae), *Dimorphotheca* (Compositae), *Trifolium repens* (Leguminosae) and *Manihot esculenta* (Euphorbiaceae). It has been shown (Butler, 1965) that several of the plants, including *Manihot* spp., that contain linamarin also contain its homologue lotaustralin.

1.1 Hydrolysis of Cyanogenic Glycosides:

The cyanogenic glucosides of plants usually undergo hydrolysis liberating HCN when the tissues are crushed or damaged. Where the glucosides are simple β-D-glucosides as linamarin is, a specific endogenous β-glucosidase releases the 2-hydroxynitrile from the sugar moiety. The decomposition of the 2-hydroxynitrile to liberate cyanide may occur spontaneously, but, at least in some plants, a 2-hydroxynitrile lyase is present to catalyse the reaction (figure 1.1).

Where the glucosidic moiety is a disaccharide, for example gentiobiose in the case of amygdalin, then other specific hydrolytic enzymes first cleave the saccharide linkage to yield the simple β-glucoside. The emulsin system isolated from bitter almonds (*Prunus* sp.) is a mixture of enzymes whose action on amygdalin occurs in three steps (Haisman and Knight, 1967; Haisman *et al.*,

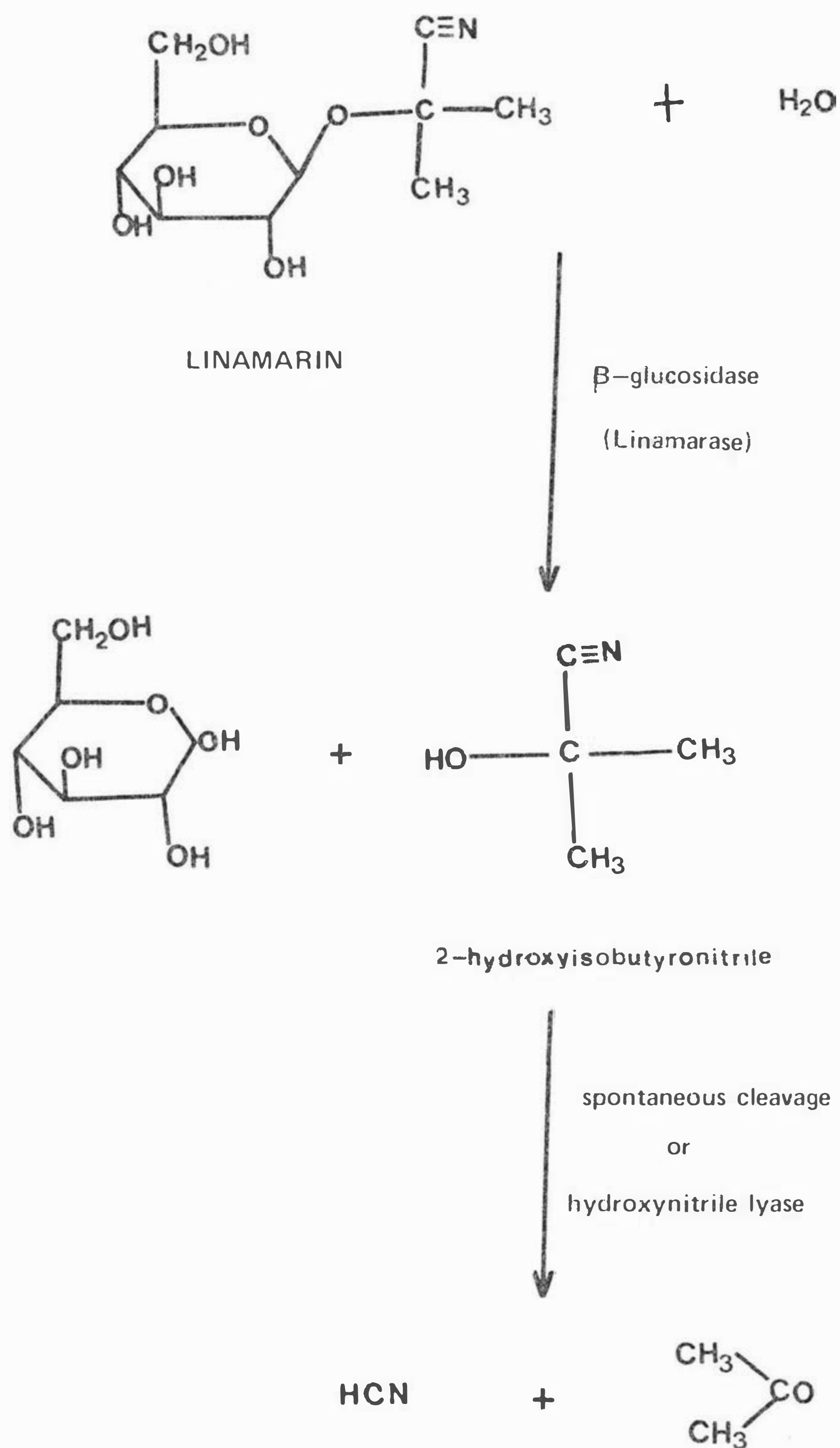


FIGURE 1.1. HYDROLYSIS OF LINAMARIN IN PLANTS

1967): Amygdalin hydrolase first cleaves the β, (1-6') bond of the gentiobiose portion of amygdalin to yield prunasin and glucose; prunasin is then hydrolysed by prunasin hydrolase to D-mandelonitrile and glucose; the mandelonitrile is finally cleaved by the action of hydroxynitrile lyase into benzaldehyde and HCN.

Within the intact plant itself cyanogenic glucosides appear to be metabolically active rather than the inert end products as was once thought to be the case. The work of Abrol *et al* (1966) indicated an active turnover of cyanogenic glycoside in *Nandina domestica*. Vicianin breakdown has also been associated with protein synthesis in *Vicia angustifolia* (Tschiersch, 1966). The turnover of cyanogenic glycosides in cyanogenic plants would result in the release of HCN into the tissues. However cyanogenic plants appear to be equipped with some form of HCN assimilation or detoxification system since free HCN that may result from a possible cyanogenic glucoside turnover is barely detectable in the intact plants. Feeding of [^{14}C]-HCN to plants result in the incorporation of ^{14}C into β-cyanoalanine and asparagine as the major products (Blumenthal-Goldshmidt *et al*., 1963; Fowden and Bell, 1965). Etiolated cassava seedlings have also been shown to convert [^{14}C]-HCN into asparagine (Nartey, 1969). A second mechanism of HCN detoxification may involve the enzymic conversion of the free cyanide into thiocyanate. The two enzymic activities required for this conversion, rhodanese and 3-mercaptopyruvate, sulfurtransferase, have been detected in plants including cassava (Boey

et al., 1976; Chew and Boey, 1972; Fiedler and Wood, 1956; Sorbo, 1953).

1.2 Toxicity Associated with Cyanogenic Glycosides

While cyanogenic glycosides and their breakdown products do not exert any detectable toxic effects on cyanogenic plants, the animals and humans which feed on such plants are known to show various symptoms of cyanide toxicity. Livestock poisoning due to ingestion of fresh cyanogenic plant material like *Sorghum sp*. (Rose, 1941) and *Trifolium repens* (Coop and Blakely, 1949) has been reported. The early literature on cyanide poisoning of humans due to ingestion of cyanogenic plants was reviewed by Montgomery (1969). Eating of *Phaseolus lunatus* was reported to have caused deaths to humans in Mauritius and Puerto Rico (Viehoever, 1940) and depression of growth in chicks fed on linseed meal have also been reported (MacGregor and McGinnis, 1948). Carmody (1900) and Clarke (1936) have reported that acute cyanide poisoning due to cassava was common in Trinidad and West Africa.

Toleration of sublethal doses of HCN is associated with a number of detoxification systems in animals and humans. As in some cyanogenic plants, the principal reaction involves the formation of thiocyanate by the enzyme rhodanese (Fiedler and Wood, 1956). The main site for this detoxification reaction is in the liver, although the enzyme is widely distributed in other tissues (Rosenthal, 1948). Mushet *et al* (1952) have proposed a second detoxification system involving the formation of cyanocobalamin (Vitamin B_{12}) from the

reaction between HCN and hydroxycobalamin in the liver.

Thiocyanate, a cyanide detoxification product, is a reversible competitive inhibitor of the accumulation of iodide by the thyroid gland. Increased thiocyanate levels in the blood leads to thiocyanate uptake by the thyroid gland and thus prevents thyroxine formation resulting in the enlargement of the thyroid gland. However, no clear evidence of a goitrogenic effect was obtained when white clover was fed to sheep, though an elevation of thiocyanate level in the blood plasma was observed (Butler *et al.*, 1957; Flux *et al.*, 1960).

The literature offers only circumstantial evidence to implicate chronic cyanide ingestion in human diseases. Several reports describe significant correlation between Cassava diets and the etiology of endemic goitre (Nwokolo *et al.*, 1966; Ekpechi, 1967). Delange *et al.* (1973) have confirmed the antithyroid properties of Cassava and showed that in Idjwi Island (Zaire, Africa), an endemic goitre area, Cassava contributes a dietary goitrogen responsible for the endemic goitre. The prolonged intake of Cassava has been associated with the occurrence of Tropical Ataxic Neuropathy in Southern Nigeria (Osuntokun, 1973). The prevalence of the disease in one high Cassava-eating village was 3% of the population while it was 8% of the people in the 50-60 year-old age group.

1.3 Biosynthesis of Cyanogenic Glycosides

Early attempts to elucidate the biosynthesis of cyanogenic glucosides investigated the possibility of the reversal of the hydrolytic reaction using the

breakdown products as precursors. Conn and coworkers showed that neither [^{14}C]-HCN nor [^{14}C]-acetone was significantly incorporated into cyanogenic glucosides (Blumenthal-Goldschmidt et al., 1963; Butler and Conn, 1964). However experiments using ^{14}C labelled amino acids demonstrated good incorporations of ^{14}C into cyanogenic glycosides in Sorghum and Prunus persica (Gander, 1958; Ben-Jehoshua and Conn, 1964). The conversion of valine into linamarin and isoleucine into lotaustralin was first demonstrated in Trifolium repens by Butler and Butler (1960). This has been confirmed in other plants (Abrol et al., 1965) including etiolated Cassava seedlings (Nartey, 1968).

Several experiments using ^{14}C and ^{15}N labelled amino acids showed that the nitrogen of cyanogenic glucosides is derived from the precursor amino acids. The possible intermediates between amino acids and cyanogenic glucosides have been investigated primarily by administering labelled compounds to determine their effectiveness as precursors of the glucoside aglycone. Hahlbrock et al (1968) showed that linen flax seedlings efficiently convert [1-^{14}C] isobutyronitrile and 2-hydroxy[1-^{14}C] isobutyronitrile into linamarin. Isobutyraldoxime was also shown to be significantly converted to linamarin by linen flax. Corresponding nitriles, hydroxynitriles and oximes were significantly incorporated into prunasin in Cherry laurel shoots (Hahlbrock et al., 1968; Tapper and Butler, 1971; Farnden et al, 1972). A general pathway of cyanogenic glucoside biosynthesis from amino acids via aldoximes, nitriles and 2-hydroxynitriles has been formulated and

is presented in figure 1.2. The intermediates involved in the biosynthesis of cyanogenic glucosides have not been detected from extracts of untreated plants. Nevertheless there is good evidence to support the existence of this pathway. By carrying out trapping experiments using unlabelled isobutyraldoxime and isobutyronitrile administered together with L[U-^{14}C] valine, Tapper and Butler (1972) isolated the corresponding ^{14}C labelled oxime and nitrile. In a similar set of experiments the presence of inhibitors of linamarin biosynthesis (D, L-allo-O-methylthreonine; D,L-2-methoxypropionaldoxime; D,L-O-methylthreonine) caused the accumulation of radioactive isobutyraldoxime.

On the enzymic level the enzyme UDP-glucose-ketone cyanohydrin β-glucosyltransferase, the enzyme responsible for the final glycosylation step in linamarin and lotaustralin biosynthesis has been isolated from linen flax (Hahlbrock and Conn, 1970). The corresponding enzyme, UDP-glucose-aldehyde-cyanohydrin β-glucosyltransferase has also been isolated from sorghum (Reay and Conn, 1969). Recently McFarlane _et al_ (1975) isolated an enzyme complex from Sorghum that converts tyrosine to p-hydroxymandelonitrile via p-hydroxyacetaldoxime.

Administration of other possible intermediates and precursors to plants has suggested some alternative pathways for biosynthesis of cyanogenic glycosides. Tapper _et al_ (1967) and Tapper and Butler (1971) have demonstrated that α-keto-isovaleric acid oxime was converted into linamarin by linen flax and 2-oximino-3-propionic acid to prunasin in cherry laurel leaves (_Prunus laurocerasus_). The involvement of an oximino acid

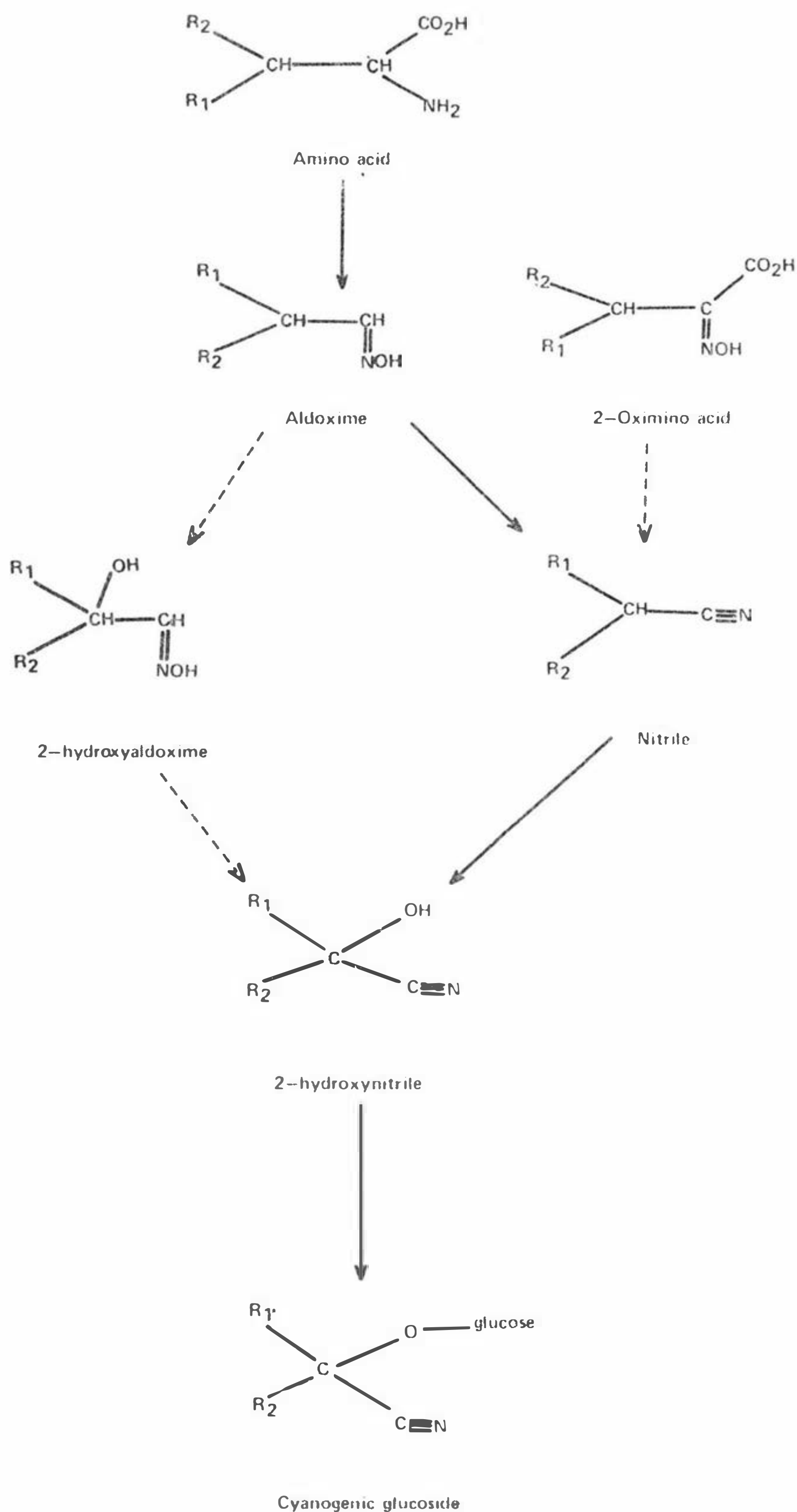


FIGURE 1.2. PATHWAYS FOR THE BIOSYNTHESIS OF CYANOGENIC GLUCOSIDES. For linamarin, $R_1=R_2=CH_3-$, and the amino acid is valine. For lotaustralin, $R_1=C_2H_5-$, $R2 = CH_3-$, and the amino acid is isoleucine. Alternative pathways shown in dotted lines.

in the biosynthetic pathway is however doubtful because of the possibility of its non-enzymic conversion into nitrile (Ahmed and Spencer, 1961).

An alternative pathway involving the conversion of hydroxyaldoximes into cyanogenic glycosides was considered as a possibility. Tapper *et al* (1972) demonstrated the conversion of 2-hydroxyisobutyraldoxime into linamarin in linen flax and DL-2-hydroxyphenylacetaldoxime into prunasin in cherry laurel leaves. However the microsomal enzyme system of McFarlane (1975) was used by Shimada and Conn (1977) to demonstrate *in vitro* that the general pathway involving the nitrile but not the 2-hydroxyaldoxime appears to be the most likely *in vivo* pathway of dhurrin biosynthesis in sorghum.

1.4 Cyanogenic glycosides of cassava, and their importance

Cassava, *Manihot esculenta* Crantz (Euphorbiaceae), is a root crop grown in tropical regions where its tubers are of great importance as a source of carbohydrate. Cassava (Plates 1.4.1 and 1.4.2) has various names around the world: Mandioca (Brazil), Yuca (Carribean), Cassava (English-speaking Africa), Tapioca (East Indies) and Manioc (French). It is a shrubby perennial species, 5-12 feet high, with enlarged tuberous roots. The stems may be branched or unbranched bearing 5 or 7-lobed palmate leaves on long slender petioles (Plate 1). It is grown mainly in lowland areas often giving a biannual crop. It is propagated by means of stem cuttings buried 3-7 cm below the soil surface. Africa alone produces 30 million tons of cassava tubers accounting for 35% of

PLATE 1.4.1. A MATURE CASSAVA PLANT GROWING IN PEAT CULTURE (var. Manioke)

PLATE 1.4.2. CASSAVA TUBERS (var. Manioke) grown in peat culture.

the world production (Nestel, 1973). The global average of cassava yields is quoted as about 9.4 tons per hectare though yields as high as 40 to 60 tons/ha have been reported (Nestel, 1973). Traditionally cassava is grown as a subsistence crop on a shifting cultivation basis.

1.4.1 Cassava Toxicity

Consumers of cassava have been familiar with the toxic nature of certain varieties for a long time. Clausius, in 1605, made the first known written record of the poisonous nature of cassava tubers (Montgomery, 1964). Dunstan (1906) identified the toxic principle as linamarin and more recently Butler (1965) demonstrated the additional presence of lotaustralin as a minor component in *Manihot* spp.

Cyanogenic glucosides occur in all cassava varieties although there is considerable difference in content between different varieties. Such varietal differences led to earlier attempts to classify cassava into 'sweet' and 'bitter' categories. The major limitation of such a taste classification is that cyanogenic glycoside content is sensitive to various environmental conditions (Rogers, 1963). Environmental factors that have been implicated in the variation of cyanogenic glucosides in cassava are plant age, water stress, mineral nutrition and shading. Higher cyanogenic glucoside contents have been reported in young leaves compared to older leaves (de Bruijn, 1971). Different effects of age on cyanogenic glucoside contents of tubers have been reported (Bolhuis, 1954;

Sinha and Nair, 1968). The roots of young cassava plants under extreme water stress were observed to double their cyanogenic glucoside contents (de Bruijn, 1971). As with other cyanogenic plants application of nitrogen fertilizers tends to increase, whilst potassium application results in a decrease, of cyanogenic glucoside levels in leaves and tubers (de Bruijn, 1971). In the same report de Bruijn stated that shading of young plants increased the cyanogenic glucoside concentration of leaves by as much as 40% and that of roots by 30%.

The only previous investigation concerning cyanogenic glucoside biosynthesis in cassava reported the significant conversion of valine and isoleucine into linamarin and lotaustralin, respectively, by etiolated cassava seedlings (Nartey, 1968). The actual biosynthetic pathways have not as yet been elucidated. Post-harvest handling, deterioration or damage of cassava tubers or tissues releases the enzyme linamarase (Montgomery, 1969) which quickly hydrolyses linamarin and lotaustralin to yield HCN as the toxic product. Etiolated cassava seedlings have been shown to convert HCN into asparagine (Nartey, 1970). The enzyme rhodanese which has been detected in cassava (Chew and Boey, 1972; Boey *et al*., 1976) may also be important in removal of HCN from cassava tissues.

Processing methods aid the removal of cyanogenic glucosides by leaching or hydrolysis followed by evaporation of released HCN. Soaking, boiling, fermentation and toasting are some of the methods that are used to prepare and detoxify cassava for use as food.

Despite these techniques long term ingestion of residual HCN and cyanogenic glucosides in Cassava diets still constitute a health hazard to consumers. The acute and chronic effects of cassava cyanogenic glucosides have been discussed in section 1.2. Earlier reports of deaths due to acute poisoning have been recorded by Carmody (1900) and Clark (1936) while tropical ataxic neuropathy and endemic goitre (Osuntokun, 1973; Delange *et al.*, 1973) are among the pathological cases implicating chronic cyanide toxicity.

Cassava has a great potential in tropical agriculture. Not only does it provide food for over 300 million people but its use as animal feed is on the increase in both tropical and temperate countries (Nestel, 1974). There is also growing interest in the use of cassava leaves as vegetables and for extraction of leaf protein concentrates (Terra, 1964; Adrian and Peyrot, 1970; Yeoh and Chew, 1976).

1.5 Aims of the Present Study

The increasing role of cassava as food and feed makes it of great importance to understand something of the metabolism of cyanogenic glucosides in the cassava plant, the changes which occur during processing of the edible tissues and the fate of the glucoside in the body of the consumer. The need for further investigation of the physiology of cassava with respect to the metabolism of its cyanogenic glucoside was emphasised in the discussions of the conference of Chronic Cassava Toxicity (Nestel, 1973). It was of the opinion that such investigations might facilitate screening and breeding experiments for

acyanogenic or low cyanogenic cassava. The present study has concentrated on identifying the major sites of linamarin biosynthesis in the plant and investigating the possibility of translocation of linamarin from synthetic sites to other parts of the plant.

SECTION 2

MATERIALS AND METHODS

2.1 Source of Plant Materials

Cassava seeds of unspecified varieties were obtained from the Centro Internacional de Agricultura Tropical, Cali, Colombia, while stem cuttings of varieties Beqa, Manioke and Navolau were obtained from the Ministry of Agriculture, Koronovia Research Station, Nansori, Fiji.

2.2 Plant Culture

2.2.1 Propagation

2.2.1.1 Propagation from Seeds

Cassava seeds were germinated in peat beds contained in 2.5 litre plastic pots under glasshouse temperatures of 18-35°C and 75-90% Relative Humidity. Each pot was filled with a mixture of Irish Peat moss, lime (6g per pot) and Dolomite (3.8g per pot). The peat beds were drenched with 0.04% solution of the fungicide Dexon (Fenaminosulf; Sodium 4-dimethylaminobenzenediazo-sulphonate, Bayer 5072) and kept moist by subsequent drenching with tap water as required. After sprouting seedlings were nourished with the half strength NPK solution described in Table 2.2.1, followed, four weeks later, by full strength nutrient application.

2.2.1.2 Propagation from Shoot and Stem Cuttings

The cassava stem cuttings of the seed-grown plants and cuttings of the Fiji varieties were propagated in plastic troughs containing the peat bed formulation as described in section 2.2.1.1. Each trough (73 x 21 x 26 cm) was provided with drainage holes and filled with the free draining moss mixture in which 5-10 cm lengths

Table 2.2.1

Composition of stock nutrient solution for cassava plants growing in peat beds

CHEMICALS	CONCENTRATION[1] g/litre
Urea, $CO(NH_2)_2$	13.8
KNO_3	26.0
$Na_4P_2O_7.10H_2O$	2.5
Fe (sequestrene) (6% Fe)	1.0

[1] : Diluted x10 before application

of cassava stems were struck.

Rooted sections of cassava for solution or gravel culture were obtained in a number of ways. Shoot tip cuttings were rooted by planting in a bed of polythene beads kept under mist similar to the method later published by Wholey and Cock (1975). Some tips tended to rot and better results were obtained using sections of more mature stems. Rooting also occurred when cuttings were held in baskets of polythene beads partially suspended in aerated water. Some cuttings used for gravel culture were rooted in free draining sand under mist. The particular method chosen was one of convenience for the subsequent growth conditions.

2.2.2 <u>Growth of cassava in Solution Culture</u>

Shoots or stems that had been struck in polythene or sand beds were placed in support baskets and suspended through the cover of a 4-litre plastic pot containing half strength nutrient solution as described by Forno *et al* (1973) Table 2.2.2 . Fresh nutrient was applied every 14 days and topped up in between with tap water and at 8-week intervals the pots were thoroughly cleaned out and nutrient solution re-applied. Nutrient solution was shielded from light by a covering of aluminium foil, laminated building paper around the pots and the solution itself aerated by a constant stream of air bubbled through it.

Growth was vigorous with the development of large leaves and numerous primary roots. No tubers were produced after 8 months of growth though some woody secondary thickening occurred in some of the Beqa plants and one of the Colombian varieties. Plants growing in

Table 2.2.2

Composition of Nutrient Solution for Solution and Gravel Cultures

CHEMICALS	CONCENTRATION of Stock Solution (g/litre)[1]	Nutrient Application ml/litre
$Ca(NO_3)_2.4H_2O$	236.0	4.0
KNO_3	101.0	4.0
KH_2PO_4	13.6	4.0
$MgSO_4.7H_2O$	246.0	1.6
Fe (Sequestrene, 6% Fe)	5.0	0.8
H_3BO_3	2.86	0.8
$MnSO_4.H_2O$	4.22	0.2
$ZnSO_4.7H_2O$	2.30	0.2
$CuCl_2.2H_2O$	0.51	0.2
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.04	0.2

[1] The major nutrients were stored in 1 litre plastic bottles while $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $CuCl_2.2H_2O$ and $(NH_4)_6Mo_7O_{24}.4H_2O$ were made up in 1-litre solution and applied at 0.2 ml/litre.

solution culture are shown in Plate 2.2.2.

2.2.3 Growth of Cassava in Gravel Cultures

The failure of solution cultures to produce tubers and a need for solution cultured tuberous plants for experimental work prompted the development of the gravel culture technique. This involves the periodic nutrient circulation around the cassava root system supported in loose gravel. The circulation of nutrient afforded a means of root aeration while at the same time avoiding water-logging around the root system. Sprouted stems were supported by loose gravel in free-draining pots. Six of such pots were arranged in large plastic troughs and nutrient solution pumped from beneath at 30 minute intervals such that the solution percolated the gravel cultures and drained back. In each cycle the gravel remained saturated for about 10 minutes.

Nutrient solution (Table 2.2.2) was made up to mark daily with tap water and replaced at 14 day intervals with fresh nutrient solution. Cassava growth in the gravel culture was vigorous and tubers developed readily with yields of up to 800 gm per plant within 5 months of growth.

2.2.4 Growth and Maintenance of Cassava Plants in the Glasshouse

Growth was vigorous in both the solution and gravel cultured plants. Manioke and Variety A grew faster than all other varieties but tuber production occurred earlier in the Beqa variety. Tuberization however occurred only in the peat and gravel cultured plants. Tuber development in cassava has been associated

PLATE 2.2.2. IMMATURE CASSAVA PLANTS GROWING IN SOLUTION CULTURE.

with the onset of cambial activity within the primary roots (Williams, 1974). The active cambium produces xylem parenchyma cells which are filled with starch granules. In both Beqa and variety A secondary thickening of the roots in solution culture occurred after 8 months when the container pots became excessively crowded with large woody roots.

Under temperate New Zealand conditions, growth of cassava to maturity in the glasshouse requires control of temperature, humidity and nutrient supply. Plants grew well under the glasshouse temperatures of 18°C (in winter) to about 35°C (in summer) and relative humidities above 75%. During winter period it became increasingly difficult to maintain good growth patterns probably due to light and temperature effect.

At the early stages of this project before optimal growth conditions were developed the plants exhibited several symptoms of possible disease or nutrient deficiency. In the variety Beqa white leaf spots often developed in the young leaves at the shoot apex region usually after about 6 weeks of growth. Fresh normal leaves soon developed and there was no recurrence of the symptom for the rest of the life of the plant. The survey of cassava diseases by Lozano and Booth (1974) does not describe this symptom and its cause is unknown.

Mite infection was a problem common to all the varieties. Mites generally attacked the lower surfaces of the older leaves and if left uncontrolled continued infection spread upwards towards the younger leaves. A heavy infection leads to discolouration of the leaf

followed by premature wilting and leaf drop. Early control attempts included spraying with 0.1% Kelthane (Yates). However this miticide temporarily arrested apical growth and furthermore the mites soon developed resistance to the chemical. The release of Tedion V-18 (Dupher, Holland) smoke bomb also provided only a temporary control. Spraying with 0.03% Plictran (active ingredient, 50% tricyclohexyltin hydroxide) in the presence of a wetting agent, Multi Film X 77 (2 ml/litre) at 7 day intervals effectively controlled the mites. The growing tips had to be avoided during spraying with this chemical since, like Kelthane, it leads to temporary arrest of apical growth.

Slugs were an occasional pest and were controlled by the use of Bysol (2% methiocarb; Bayer) or Blitzem (3% metaldehyde; Yates). In one instance a heavy caterpillar infection occurred and was controlled by spraying with cabaryl 80 (1-naphthyl-carbamate, Yates) administered at 1.55 g/litre.

2.3 Chemicals and Solvents

Valine, sodium cyanide, picric acid and ninhydrin were obtained from British Drug House Ltd. (BDH), Poole, England.

Hydrindantin, methylcellosolve and Tri-Sil were procured from Pierce Chemical Company Rockford, Illinois, USA.

Linamarin was obtained from Calbiochem (San Diego, USA) while prunasin was prepared in this laboratory by Mr W.D. Bennett by extracting peach shoots with ethanol, concentrated and extracted with ethyl acetate. It was

purified by repeat crystallization from ethyl acetate.

Cab-O-Sil (thixotropic gel powder for liquid scintillation counting suspensions) was purchased from Packard Instrument Company, Illinois, USA.

Triton X-100 (alkyl phenoxy polyethoxyethanol) and Omniflour (98% 2,5-diphenyloxazole (PPO) and 2% p-bis-(o-Methylstyryl)-benzene, Bis-MSB) were both obtained from New England Nuclear, Boston, USA.

Isobutyronitrile came from Aldrich Chemical Company (Milwaukee, USA) and Dimethyl sulphoxide from Hopkins and Williams Ltd (Chadwell Heath, England).

Acetone and ethanol were the products of Ajax Chemicals Ltd., Sydney, Australia. Toluene, butanol, Butan-2-one, acetic acid and propanol came from BDH, Poole, England.

2.4 Source and preparation of ^{14}C-labelled Compounds

2.4.1 L[U-^{14}C]valine, [^{14}C]-NaCN and [1-^{14}C]hexadecane were obtained from the Radiochemical Centre, Amersham, England.

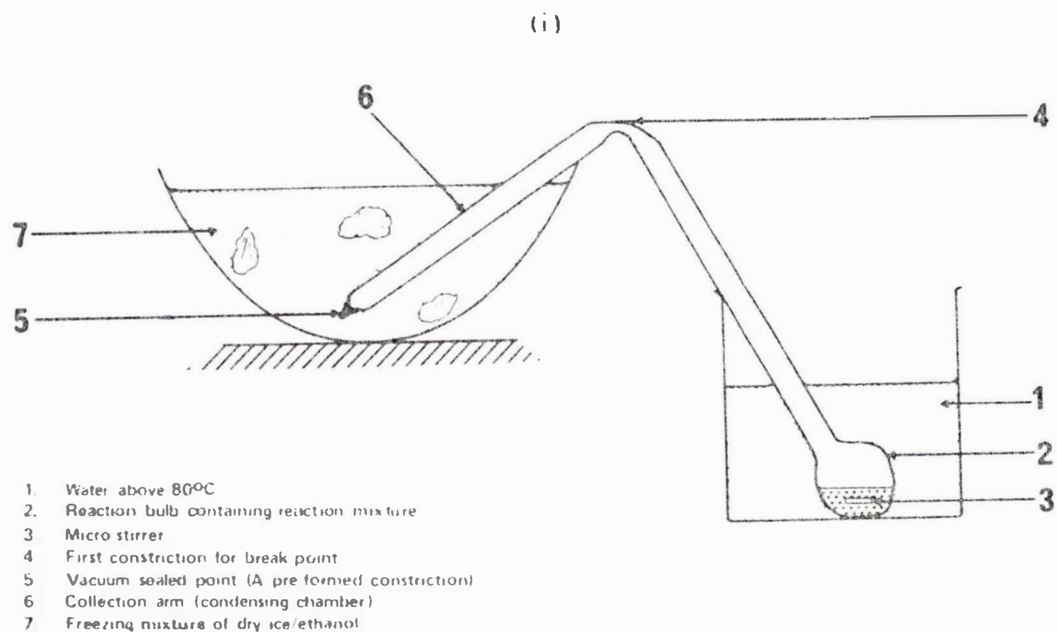
2.4.2 [1-^{14}C] Isobutyronitrile

^{14}C-Isobutyronitrile was prepared by a modification of Smiley and Arnold's (1960) method for the formation of aliphatic nitriles from secondary alkyl halides and sodium cyanide in dimethylsulphoxide solvent.

A specially made thick-walled glass reaction tube was used for this purpose. A piece of thick-walled pyrex glass tubing (0.5 cm internal diameter x 25 cm long) was sealed at one end over a flame and formed into a 1.7 cm diameter bulb that served as the reaction tube. A small magnetic bar was inserted into the bulb after

which two constrictions were made about 7 cm and 13 cm from the bulb. Dry sodium cyanide (104 mg) and isopropyl chloride (366 μl) were transferred into the reaction tube followed by 500 μCi [^{14}C] NaCN (55.5 mCi/mmol) dissolved in 568 μl dimethylsulphoxide. While the reaction mixture was frozen in dry ice the tube was bent over a hot flame and the open end sealed off under vacuum (figure 2.4.2). The sealed tube was incubated at 100°C for 24 hours accompanied by continuous stirring of the reaction mixture. With the tube inclined at the angle shown in figure 2.4.2 the isobutyronitrile formed was distilled into the collection tube by placing the reaction bulb in hot water with the collection arm immersed in dry ice-ethanol mixture.

After allowing the tube to come to room temperature it was snapped open at the vacuum sealed point and the total distillate quickly transferred onto a GLC column (25% DEGS 60cm x 12mm; detector and collector temperatures 80°C, Oven temperature 78°C, Nitrogen gas flow rate 110 ml/min, elution time 12 minutes). The outlet of the GLC was connected to a trapping system consisting of a modified Aerograph prep collector connected to a U-shaped stainless steel tube (3mm diameter; figure 2.4.2). The trapped [1-^{14}C]isobutyronitrile was concentrated into the sample collection tube by brief centrifugation of the Aerograph prep tube section at low speed. The collection tube was detached at the break-off point and inverted over a Reacti-Vial (0.3 ml; Pierce Chemical Company, Rockford, Illinois, USA) and the nitrile transferred into the vial by brief



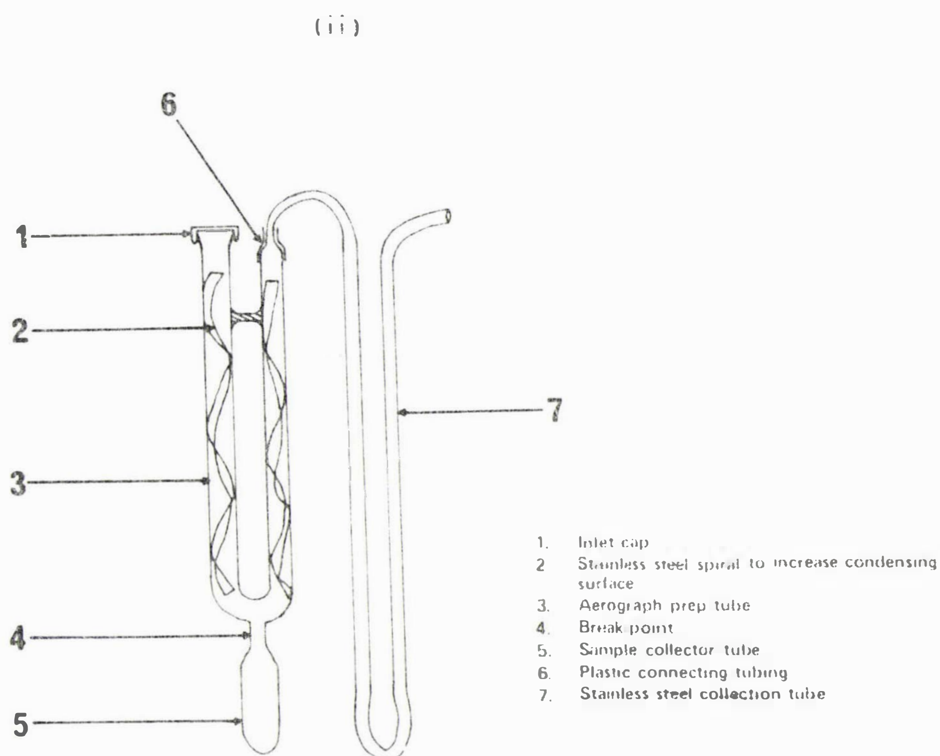


FIGURE 2.4.2. PREPARATION OF [1-^{14}C] ISOBUTYRONITRILE. (i) Preparative apparatus for distillation; Upright for reaction. (ii) A two stage collection system which is surrounded with dry ice/ethanol mixture during collection of isobutyronitrile vapour from glc. column.

centrifugation. From 2.02 mmol [^{14}C]-NaCN at specific activity 247.5 μCi/mmol, 955 μmol of isobutyronitrile (specific activity 336 μCi/mmol) were obtained giving a radiochemical yield of about 64%.

2.4.3 2-Hydroxy[1-^{14}C] isobutyronitrile

2-Hydroxy[1-^{14}C] isobutyronitrile was prepared by a modification of the method of Cox and Stormont (1943) where hydrogen cyanide is added to acetone. About 0.8 mg dry NaCN was transferred into a Reacti-vial containing a small magnetic stirrer and to this was added 500 μCi [^{14}C] NaCN dissolved in 150 μl distilled water. The vial was sealed, stirred to dissolve the NaCN, 24 μl acetone added and cooled to 10°C in a water bath. Four 10 μl aliquots of 3M H_3PO_4 were then added over a period of 15 minutes while maintaining the bath temperature below 15°C. After standing for 1 hour, 6 μl bromocresol blue indicator solution was added followed by 6 μl of 5M H_3PO_4 which shifted the pH of the reaction mixture to below pH 3.6 as indicated by the yellow colour of the indicator. After a further hour, a gentle stream of dry nitrogen gas was bubbled through the mixture for 7 minutes to remove unreacted cyanide and other readily volatile products which were trapped in 1M NaOH solution.

The chemical yield of 2-hydroxyisobutyronitrile prepared by the above method was estimated by a separate preparation using unlabelled NaCN followed by assay of the product on a Varian Model T-60 NMR Spectrometer using dimethyl sulphoxide as an internal standard. The various methyl protons could be easily identified and measured. The conditions described gave a yield of approximately 85% (chemical yield). 2-hydroxy[1-^{14}C]

isobutyronitrile prepared and purified by this method and stored at pH below 3.6 was stable at 2°C for several months.

2.4.4 <u>[U-^{14}C] Isobutyraldoxime</u>

[U-^{14}C] isobutyraldoxime was prepared by the method of Tapper <u>et</u> <u>al</u>. (1967) by the action of hydroxylamine on isobutyraldehyde prepared from valine.

L-[U-^{14}C] Valine (50 μCi; specific activity 102 mCi/mmol) was dissolved in 2 ml distilled water and transferred into a reaction flask containing 11.7 mg carrier L-Valine, 100 mg ninhydrin and 2 ml of 1M citrate buffer (pH 2.5). The mixture was heated on a boiling water bath for 5 minutes and isobutyraldehyde steam distilled into a vessel containing 139 mg hydroxylamine hydrochloride and 164 mg sodium bicarbonate in 10 ml of distilled water. The distillate collected (about 18 ml) was allowed to stand overnight, 10 μl of pure isobutyraldoxime was added as a carrier and the mixture extracted five times with a total of 25 ml diethyl ether. The combined ether extract was concentrated to 150 μl and injected on a GLC column (Silicone Oil DC 550 on Chromosorb W column 2m x 9.5mm OD) at 110°C. Isobutyraldoxime peak was trapped and transferred to a septum sealed vial. About 8 μl of [1-^{14}C] isobutyraldoxime of specific activity 0.848 μCi/mg (74 μCi/mmol) was recovered which represented radiochemical yield of 12%.

2.4.5 <u>[1-^{14}C] Linamarin</u>

[1-^{14}C] Linamarin was prepared in this laboratory by Dr B.A. Tapper by the administration of 2-hydroxyisobutyronitrile to linen flax seedlings (<u>Linum</u>

usitatissimum. 2-Hydroxy [1-^{14}C] isobutyronitrile was freshly prepared under conditions similar to that described in section 2.4.3, and administered to flax seedlings packed in 1 ml beakers. After standing overnight in illuminated growth chambers (21°C) the seedlings were rinsed and extracted with boiling 80% ethanol. The combined extracts were concentrated and chromatographed on Whatman No 3mm paper using butanol saturated with water as solvent. The zone corresponding to linamarin was eluted and frozen. Rechromatography of the eluate in propanol:water (70:30) and Butan-2-one: Acetone:Water (15:5:3) followed by elution and counting showed that the extract was radiochemically 87% pure. The specific activity of the ^{14}C linamarin was 144.9 μCi/mmol.

2.5 Preparation of Linamarase

Linamarase was prepared from linseed by the method of Coop (1940). Linseed (*Linum usitatissimum*, Redwood variety) was ground in a Waring blender and defatted with ether. About 100 gm of the defatted meal was vigorously shaken for about two minutes in three portions with 2 litres of water and immediately centrifuged. To the extract was added 200 ml of 0.2M acetate buffer (100 ml of 0.2M sodium acetate plus 100 ml of 0.2M acetic acid) and left to stand. The white precipitate which formed was centrifuged off and discarded. The supernatant was cooled to 10°C and one half its volume of cold ethanol added slowly to give a precipitate which was again centrifuged off and discarded. More cold ethanol was added to the supernatant to give an ethanol concentration

of 60% (v/v) and the precipitate formed was recovered by centrifugation (1000g for 10 minutes) and subsequently dissolved in 250 ml distilled water. The enzyme solution adjusted to pH 7 with dilute NaOH was stored at 5°C under a few drops of toluene. The crude enzyme prepared in this way was diluted with 0.1M phosphate buffer pH 5.6 before use.

2.6 Extraction of Plant Material

Small plant samples (less than about 4g) were extracted in boiling 80% ethanol maintained for 20 minutes in a hot water bath. The insoluble residue from this first extraction was re-extracted three times with about 15-20 ml 80% boiling ethanol after blending in a Sorvall Omnimixer, the shaft of which was adapted for sample blending in a boiling tube. The extracts were combined.

Larger plant samples were cut into smaller pieces, extracted for 30 minutes in boiling 95% ethanol and subsequently soaked in the extracts overnight at room temperature. Extracts were decanted and the residue blended in small aliquots in fresh 80% ethanol with a Sorvall Omnimixer. Blended samples were further extracted twice under reflux. Combined extracts were stored in the dark at room temperature or reduced to dryness under vacuum at 50°C, the residue taken up in a small volume of 20% propanol and stored at 0°C.

2.7 Analytical Methods

2.7.1 Linamarin Estimation

2.7.1.1 Enzymic hydrolysis Method

The method involves the enzymic hydrolysis of linamarin and trapping of the evolved HCN in alkaline solution and its assay by a colorimetric or potentiometric method. Enzymic hydrolysis of samples was performed in diffusion flasks fitted with a centre well. To the main compartment of the flask was introduced 0.5 - 1.0 ml of phosphate buffer (0.02M, pH 5.8), 0.5 - 1 ml of linamarase (2 μ moles/min/mg protein) solution and 0.3 - 0.5 ml of concentrated plant extract and mixed by slight agitation while the centre well contained 0.5 ml of 1M-NaOH. The flasks were tightly sealed and agitated at 50-100 r.p.m. on a rotary shaker at 25°C for 18 hours. HCN released from the hydrolytic mixture was trapped in the alkaline solution. The trapped HCN diluted to appropriate concentration was initially assayed colorimetrically by the method of Aldridge (1944) but in subsequent estimations trapped cyanide was assayed directly by a potentiometric method with a Cyanide Ion Activity Electrode (Orion Research, Model 94-06) calibrated by means of a set of standards (10^{-5} - 4 x10^{-4}M in 0.1 M NaOH).

2.7.1.2 Gas Chromatographic Method

A gas chromatographic technique was developed for linamarin assay in this laboratory for the estimation of smaller samples of linamarin. The technique also enabled the estimation of linamarin in crude plant extracts without interference from accompanying lotaustralin as is the case when the enzymic method is used in the crude extracts. The trimethylsilyl derivatives of linamarin and lotaustralin were assayed

on a Varian Aerograph 1440 connected to an electronic digital integrator (Varian Model 485) and a recorder (Unicorder U-225M). About 100-150 μl portions of ethanolic extracts containing 0.04 and 0.2 μmoles of linamarin were taken to dryness at 50°C in a Reacti-Vial under a stream of dry nitrogen gas. An internal standard consisting of 100 μl solution of prunasin (1.0 mg/ml) was added, again dried down and the final traces of water removed in a vacuum desiccator over a silica gel. To the dried sample was added 100 μl Tri-Sil and the mixture was incubated for 1 hour at 100°C under a teflon seal. A 1 μl aliquot of the TMS extract was injected into a glass column (2.5m x 2.2mm internal diameter) packed with 1% fluorosilicone (QF1) on Gas Chrom Q (100-120 mesh). The N_2 carrier gas flow-rate was 20 ml/min while the gas flow rates for the flame ionization detectors were 20 ml/min for Hydrogen and 300 ml/min for Air. A temperature programme was used from 170°C to 220°C with a rate of increase of 2°C/min. Injection and detection temperatures were both 200°C. Prunasin standards were used to evaluate the linamarin content in each extract*.

2.7.2 Paper Chromatography

Paper chromatography of concentrated ethanol extracts was by descending chromatography using Whatman No 3MM paper. The following solvent systems were employed, the numbers in the brackets being the ratio of the solvents by volume: Butanol saturated with water; butan-2-one: acetone:water (15:5:3); propanol:water (7:3); butanol:acetic acid:water (12:3:5), butanol:pyridine:water

(6:4:3) and phenol:water (500:125) equilibrated with borate buffer of pH 8.4 (McFarren, 1951).

2.7.3 Thin Layer Electrophoresis

Thin layer electrophoresis followed by chromatography was performed according to the method of Bieleski and Turner (1966) with minor modifications. Cellulose thin layer plates used were either Polygram Cell 300 MM (Macherey-Nagel & Co., Germany) or glass plates (20 x 20cm) with a 250 μm cellulose (MN300) powder layer. Plant extract was applied as a 2 cm streak near one edge of the plate and the cellulose layer uniformly sprayed with formic acid-acetic acid buffer at pH 1.9. Electrophoresis was carried out for 40 minutes at 1000V (55 V/cm) and 20-30 mA using a Warner-Chilcott Model E8002B electrophoresis apparatus provided with a circulating ice-water cooling system. The plates were dried in air, placed on edge in about 1cm water at right angles to the partially separated bands so that the bands were eluted into compact spots above the original baseline, dried again, and then developed chromatographically in Methanol:Pyridine:Acetic Acid:Water (80:4:1:20).

2.7.4 Detection of Cyanogenic Glucosides

Cyanogenic glucosides were detected on paper or thin layer chromatograms by a method adapted from Butler and Butler (1960). The technique involves the enzymic hydrolysis of linamarin spots on chromatograms and the simultaneous reaction of the HCN evolved with a picric acid soaked paper overlay to produce brownish coloured spots. Chromatograms were lightly sprayed with linamarase solution (0.5 gm/ml in 0.02M phosphate buffer

at pH 5.8), laid on a glass plate and covered with plastic mesh. A sheet of chromatographic paper sprayed damp with alkaline picrate solution (1% w/v picric acid plus 10% w/v Na_2CO_3) was superimposed on the mesh and weighed down with a heavy glass slab placed on top of the picrate paper. HCN released by the enzyme action diffused through the netting and reacted with the picric acid to give brownish spots over a yellowish background after one hour.

2.7.5 Measurement of Radioactivity

^{14}C-labelled compounds on paper chromatographic strips were located by scanning with either a Nuclear-Chicago Actigraph III, 4π thin window counter, or a Packard 7200, 4π windowless radiochromatogram scanner. Radioactive compounds were also detected after one or two dimensional chromatographic and electrophoretic separations by radioautography using Kodak medical X-ray (no-screen) film. The exposure time required was up to 4 weeks depending upon the ^{14}C activity in the separated spots or bands.

Radioactive spots or bands on paper chromatograms were eluted into scintillation vials according to a technique described by Tapper (1968). The bands were excised from the paper as rectangular strips which were then stood in a Petri-dish containing a shallow layer of water so that all radioactive substances on the paper were washed to the upper end of the strip. The wet paper was rolled in 'Parafilm' (Gallenkamp, London), inverted and held tightly in the neck of a 10 ml conical centrifuge tube with the conical tip cut off at the 0.5 ml mark and

fitted through a bung into a scintillation vial. The tube was lightly centrifuged and additional water was then added to the paper strip which was recentrifuged to give a quantitative elution of ^{14}C from the strip into the vial. The sample was made up to 1 ml with distilled water and 10 ml scintillation mixture (1:2 Triton-toluene solution containing 6 gm Omniflour per litre) added.

^{14}C activity was determined using either a Packard Tricarb liquid scintillation spectrometer (Model 2002) with a wide and narrow window or a Searle Isocarp/300 liquid scintillation system with a Teletype ASC 11 paper punch connection. Quenched ^{14}C standards prepared by mixing aliquots of [1-^{14}C] hexadecane standards with increasing amounts of chloroform were counted for quench correction. The channels ratio method was employed for counting on the Tricarb scintillation counter whilst the external standard ratio method was used for Isocarb counting. Manual or computer processing of data from the quenched standards enabled "quench curves" to be plotted and used to deduce true sample activities. Counting efficiency for both scintillation counters ranged from 70-80% for most samples depending on the contents of each scintillation vial.

SECTION 3

ADMINISTRATION OF LINAMARIN PRECURSORS TO CASSAVA LEAVES

ADMINISTRATION OF LINAMARIN PRECURSORS TO CASSAVA LEAVES.

Several of the radioactive precursors that have been employed in biosynthetic studies of linamarin in other plants were prepared in the course of this study as described under section 2. The precursors included valine, isobutyronitrile, isobutyraldoxime and 2-hydroxyisobutyronitrile. Valine has been extensively used in investigating linamarin biosynthesis in plants by allowing the plants to take up ^{14}C valine solutions. Solution uptake techniques have also been employed in administering the biosynthetic intermediates, isobutyraldoxime, 2-hydroxyisobutyronitrile, and isobutyronitrile to plants. These intermediates are rather volatile and one of them, isobutyronitrile is not readily water soluble. It is likely that significant amounts of these precursors may be lost from the feeding solution due to volatilization. The volatile property of the precursors has been used in this study to administer ^{14}C labelled substances to Cassava leaves by allowing the leaves to take up the precursor vapours through the stomata. This section describes the results of feeding experiments in which ^{14}C labelled valine, isobutyronitrile, isobutyraldoxime and 2-hydroxyisobutyronitrile were administered to excised Cassava leaves. The precursors were metabolised by the leaf tissues and linamarin was subsequently isolated and the incorporation of ^{14}C from the precursor determined. The experiments described here were carried out primarily to ascertain the suitability of the various precursors for subsequent studies on translocation and the biosynthetic activity of different

tissues. They were carried out at widely different times but the data have been collected together in this section since the results are of interest in connection with the pathway of linamarin biosynthesis in Cassava.

3.1 Administration of ^{14}C precursors:

For the work described in this section matured cassava leaves were excised from plants grown in solution culture as described in section 2.2.2. The leaves used were fully expanded but taken from well above the region of leaf senescence. Leaf petioles were bent under water and detached while still submerged as close to the stem end as possible and the cut ends rinsed repeatedly to remove latex exudate.

3.1.1 Valine administration to leaves:

Valine was fed to the leaf by allowing solution to be taken up through the cut end of the petiole. Leaf petioles were trimmed down while submerged in water to leave about 2-3 cm length attached to the leaf blade. The feeding solution contained 0.7 - 1.0 μc L-[U-^{14}C] valine with unlabelled valine added as a carrier to bring the final specific activity to between 316 and 1000 μCi/mmol. The leaves were placed in the solution in a temperature controlled growth chamber at 21°C. The administered solution was generally taken up within 20 to 30 minutes after which leaves were transferred into 20 ml beakers filled with water. The leaves were then covered with a large bell-jar to prevent excessive moisture losses and wilting and allowed to metabolise the valine for 24 hours. Labelled leaves were extracted in boiling 80% ethanol and ^{14}C-linamarin determined on the concentrated extracts by the

enzyme hydrolysis method followed by alkaline trapping of evolved $H-^{14}CN$ (Section 2.7).

3.1.2 Administration of ^{14}C Volatile precursors to leaves:

The volatile precursors, [1-^{14}C] isobutyronitrile, 2-hydroxy [1-^{14}C] isobutyronitrile and [U-^{14}C] isobutyraldoxime were administered to leaves by vapour uptake in a glass feeding chamber. The lid of the chamber, shown in plate 3.1, consists of a thick-walled circular glass dish (21 cm diameter x 2 cm deep) with two 0.7 cm diameter apertures on opposite sides of its wall clamped to a flat glass plate. The rim of the dish was ground and a groove large enough to accommodate the leaf petiole introduced at one point on the rim. The blade of a detached leaf was spread out on the glass plate tightly clamped to give a horizontal surface. A magnetic bar was placed on a clear area of the plate beside one of the leaflets and stirred by means of a magnetic stirrer clamped under the suspended plate; this enabled the air to be stirred throughout the period of vapour feeding. The ground rim of the glass chamber lid was lined with a thin layer of silicone gum (SE-30, General Electric, N.Y.) and then inverted over the leaf blade so that the petiole was loosely accommodated in the side groove. Where necessary extra gum was added at the dish-glass plate junction and around the point of insertion of the petiole to create an airtight chamber around the leaf blade. Finally the dish was tightly clamped down on the glass plates with screw clamps. Precursor solution was fed through the inlet septum by means of a syringe on to the inner glass plate with care being taken

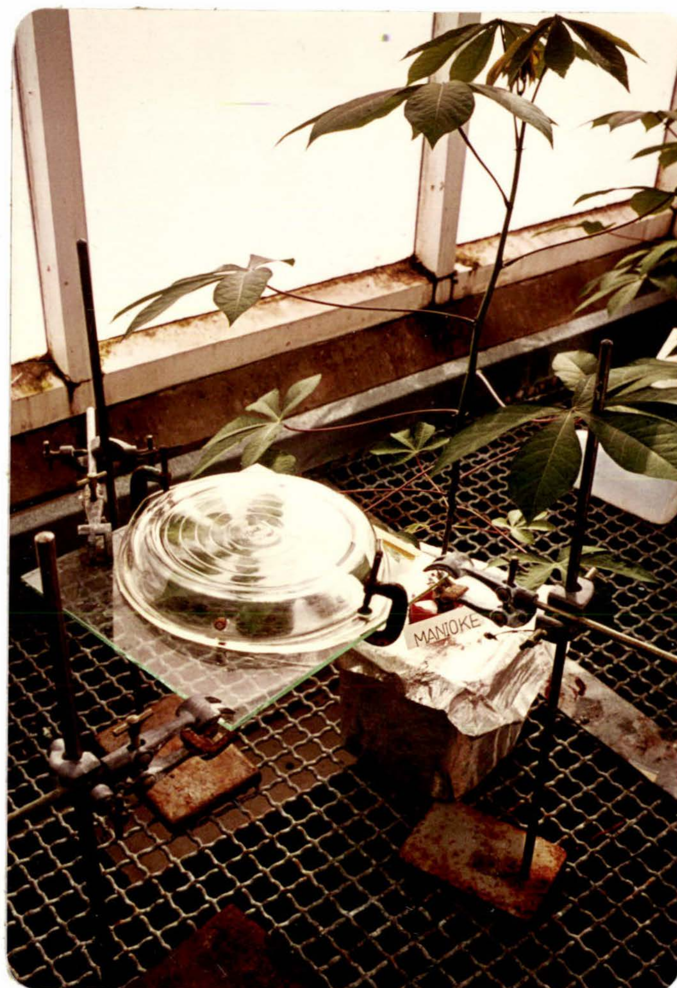


PLATE 3.1. ADMINISTRATION OF ^{14}C VOLATILE PRECURSORS TO ATTACHED CASSAVA LEAF. Vapour uptake by lower leaf of an immature cassava plant.

to avoid depositing the sample too near the leaf blade. In the case of 2-hydroxy [1-^{14}C] isobutyronitrile rapid vapourisation was achieved by warming the droplet by means of a heated rod placed in close contact with the glass surface underneath the droplet. Heating the rod with warm water quickly warmed the droplet resulting in its vapourization within 10 to 20 minutes. Heat treatment was not required for vapourization of ^{14}C isobutyraldoxime and isobutyronitrile as these are very volatile at room temperature. Plastic bags partially filled with water at room temperature were sealed and laid over the top of the feeding chamber to prevent excessive heat generation within the chamber.

All experiments were carried out in a glasshouse at 21°C with each leaf exposure lasting 3 hours. Residual vapours were drawn out from the chamber and trapped in 1M-NaOH solution by admitting water from a reservoir at a slow rate controlled by an aspirator. When the chamber was about half filled with water all apertures were sealed off and the support plate slightly agitated to rinse off moisture condensates on the inner chamber walls. All washings were recovered and after dismantling the chamber the leaf surface was dried with absorbent paper. Leaves were generally extracted in boiling ethanol within one hour after removal from feeding chamber thus giving a maximum of 4 hours metabolism from time of initial exposure to precursor vapours. Determination of ^{14}C linamarin activity and linamarin content was performed on concentrated extracts as described in section 2.7.

3.2 Results

3.2.1 Assimilation of L-[U-^{14}C] Valine

The ^{14}C distribution pattern obtained by scanning a one-dimensional paper chromatogram of the ethanol soluble extracts indicated the presence of ^{14}C-linamarin as a major product. The presence of other labelled substances showed that extensive metabolism of the administered ^{14}C valine had taken place. The results of the valine feeding experiments are given in Table 3.2.1.1 together with some values from the literature (Table 3.2.1.2). In all ^{14}C valine feeding experiments equal labelling of 5C atoms of valine has been assumed. Since the conversion of valine into linamarin involves a decarboxylation of valine at 1 C position (Butler and Butler, 1960) linamarin activities have been adjusted by 5/4 to give a true indication of the percent of ^{14}C valine incorporation and dilution of specific activity. The incorporation of ^{14}C into ^{14}C-linamarin was between 11 and 17%. In this valine feeding experiment the enzymic hydrolysis method was employed to measure ^{14}C-linamarin content in the eluate from the corresponding linamarin band of one-dimensional paper chromatogram. Some amount of the ^{14}C-acetone evolved may have been trapped along with ^{14}C-HCN. However the present incorporation values are just slightly higher than the 9-13% incorporations recorded in subsequent ^{14}C valine feeding to leaves employing improved analytical techniques. The results are consistent with the percent incorporation obtained in etiolated cassava seedlings (Nartey, 1969). The results are higher than was obtained in white clover, *Trifolium repens*

Table 3.2.1.1

Incorporation of Valine into Linamarin

Cassava variety	Fresh weight gm	Valine administered			Metabolic Time hrs	Linamarin		Percent ^{14}C Incorporation[a]	^{14}C Dilution[a]
		μCi	μmol	Uptake % total ^{14}C fed		μCi	S.A.[b] μCi/mmol		
Manioke	4.6	0.954	3.0	99.7	24	0.164	5.2	17.2	61
Navolau	2.0	0.700	1.0	99.8	24	0.070	2.0	12.5	350
Variety A	2.3	1.000	1.0	98.2	24	0.091	3.3	11.6	303

[a] Corrected for loss of carboxyl carbon

[b] S.A. = specific activity (μCi/mmol)

Table 3.2.1.2

Incorporation of Valine into Linamarin in other Species

Plant Material	Valine administered		Metabolic Time Hrs	Percent ^{14}C Incorporation into ^{14}C linamarin	^{14}C Dilution	References
	μCi	μmol				
Linum usitatissimum	1.09	1.2	7	23.0	43	Tapper and Butler(1971)
Linum usitatissimum	0.10	1.0	7	48.0	62	Hahlbrock *et al*.(1968)
Trifolium repens	0.92	4.6×10^{-3}	4-7	6.6	14200	Hughes and Conn(1976)
Manihot utilissima	1.41	0.01	48	13.2	-	Nartey(1969)

but generally lower than in linen flax (Linum usitatissimum (Table 3.2.1.2).

3.2.2 Assimilation of 2-hydroxy[1-^{14}C] isobutyronitrile

Leaves fed with 2-hydroxyisobutyronitrile took up most of the precursor vapour. A radiochromatogram scan of the ethanol extracts (figure 3) shows a linamarin peat at Rf 0.4; the identity of this peak was confirmed in other solvent systems (2-Butanone-acetone:Water and propanol:water; Section 2.7.2). Further resolution of the ^{14}C labelled compounds was achieved by TLC-electrophoresis of the ethanol extracts followed by autoradiography (section 2.7.3) which showed a heavily labelled ^{14}C-linamarin spot plus several labelled amino acids. Asparagine was the major amino acid labelled and lesser amounts of ^{14}C were present in spots corresponding to β-cyanoalanine, glutamine, glutamic acid, aspartic acid, glycine and histidine. The pattern of ^{14}C incorporation was similar to that observed when flax seedlings were solution fed with ^{14}C-2-hydroxyisobutyronitrile (Tapper, 1968). Like the flax seedlings the separated extracts showed the presence of other neutral substances.

The results given in Table 3.2.2.1 show that incorporation of ^{14}C activity into ^{14}C linamarin was 20%, a level somewhat less than that obtained for linen flax (Table 3.2.2.2).

3.2.3 Assimilation of [1-^{14}C] isobutyronitrile

The uptake of ^{14}C-isobutyronitrile was relatively low (Table 3.2.3.1). The maximum ^{14}C activity

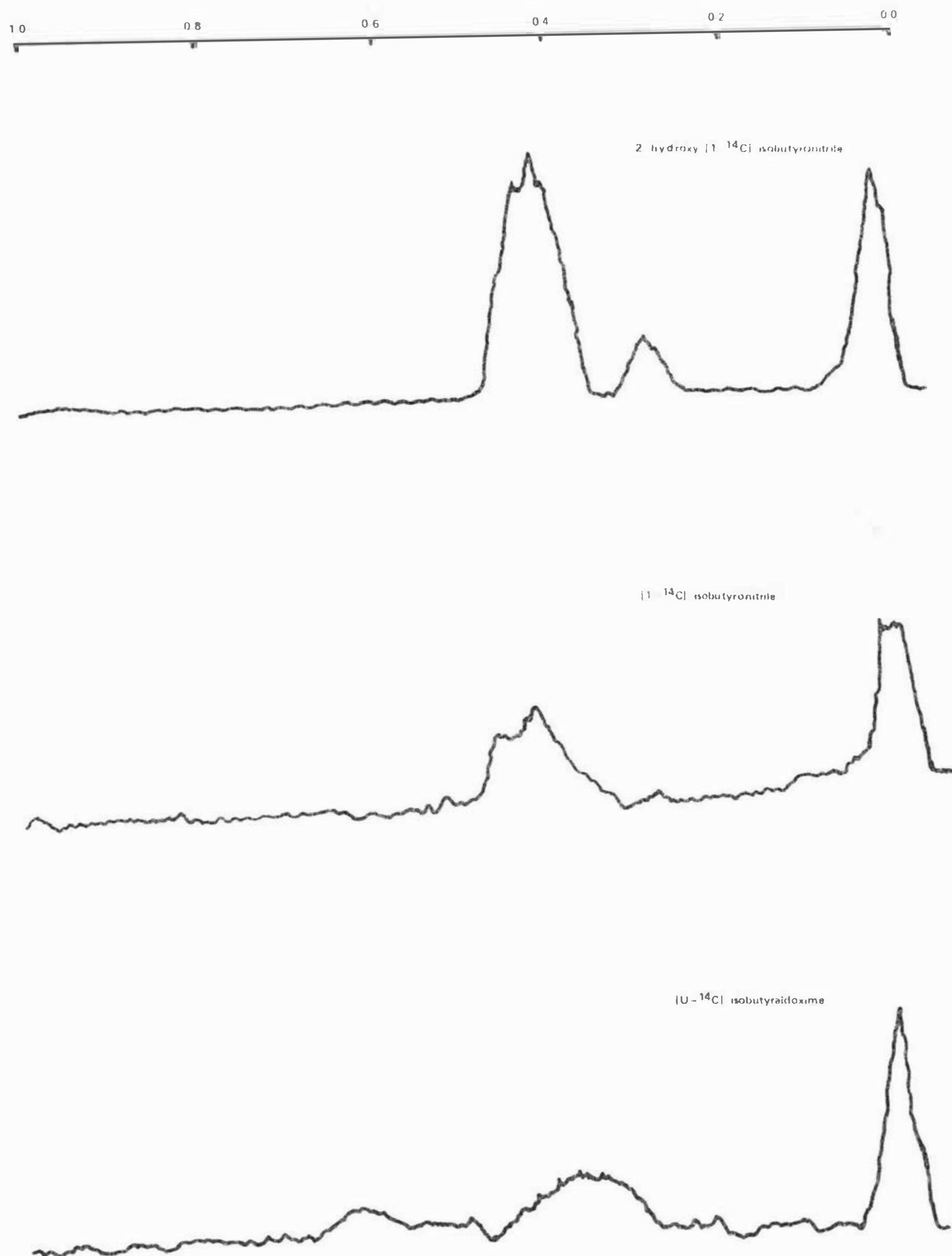


FIGURE 3 INCORPORATION OF RADIOACTIVITY FROM 2 HYDROXY [1 ^{14}C] ISOBUTYRONITRILE, [1-^{14}C] ISOBUTYRONITRILE AND [U ^{14}C] ISOBUTYRALDOXIME IN CASSAVA LEAVES Linear scans of radioactivity on paper chromatogram strips developed with Butanol Water Detector sensitivity, 10^3 Linamarin has an Rf of 0 40

Table 3.2.2.1

Incorporation of 2-hydroxyisobutyronitrile into Linamarin

Cassava variety	Fresh weight gm	2-hydroxyisobutyronitrile			Metabolic Time (hrs)	Linamarin		Percent ^{14}C Incorporation	^{14}C Dilution
		µCi	S.A. µCi/mmol	Uptake % ^{14}C fed		µCi	S.A. µCi/mmol		
Manioke[a]	3.1	2.58	262	95.3	4	0.44	11.8	11.3	22.0
Beqa	4.1	3.82	262	96.0	4	0.79	13.6	20.6	19.3

[a]: Leaf partially wilted in course of experiment.

Table 3.2.2.2

Incorporation of 2-hydroxyisobutyronitrile into Linamarin in other Species

Pl nt Material	2-hydroxyisobutyronitrile		Metabolic Time (Hrs)	Percent ^{14}C Incorporation	^{14}C Dilution	References
	µCi	µmol				
Linum usitatissimum	4.59	2.5	7	28	17.5	Tapper and Butler(1971)
Linum usitatissimum	4.57	2.5	7	28	17.0	Hahlbrock *et al*.(1968)

Table 3.2.3.1

Incorporation of Isobutyronitrile into Linamarin

Cassava variety	Fresh weight	Isobutyronitrile			Metabolic Time Hrs	Linamarin		Per Cent ^{14}C Incorporation	^{14}C Dilution
		μCi	μmol	Uptake % ^{14}C fed		μCi	S.A. μCi/mmol		
Manioke	2.4	11.3	33.6	3.7	4	0.052	1.9	12.4	177
Manioke	2.0	11.3	33.6	2.4	4	0.029	1.1	10.7	305
Manioke	2.3	11.3	33.6	10.6	24	0.190	7.9	15.9	43

Table 3.2.3.2

Incorporation of Isobutyronitrile into Linamarin in other Species

Plant Material	Isobutyronitrile		Metabolic Time Hrs	Percent ^{14}C Incorporation	^{14}C Dilution	References
	μCi	μmol				
Linum usitatissimum	0.239	10.0	7	9	-	Tapper and Butler (1971)
Linum usitatissimum	0.066	10.1	7	11	10	Hahlbrock *et al.* (1968)

taken up by the leaf in 24 hours of exposure was just over 10% of the original ^{14}C activity, although the estimation of uptake was subject to a slight error. After dismantling the feeding chamber there was a rapid loss of volatile ^{14}C activity from the leaf surface as measured by surface scanning with a hand monitor (Mini-Monitor, Mullard MX 168, London, England). Volatile ^{14}C in the ethanol extracts measured as the difference in ^{14}C activity after taking the extract to dryness under a stream of dry nitrogen gas indicated that 27% of the soluble extract was due to volatile ^{14}C activity. The strip scans of one dimensional chromatograms showed besides linamarin one other major peak at the origin. The range of ^{14}C incorporation into ^{14}C-linamarin was comparable to that obtained for linen flax shoots (Table 3.2.3.1 and 3.2.3.2) assuming that the uptake figures were accurate.

3.2.4 Assimilation of [U-^{14}C] isobutyraldoxime

A nearly quantitative uptake of ^{14}C-isobutyraldoxime vapour was obtained. A radiochromatographic scan of the ethanol extracts showed one other major peak besides linamarin at the origin (figure 3). Measurement of ^{14}C-linamarin activity gave an incorporation of 24% of the ^{14}C activity in the oxime into ^{14}C-linamarin. The level of ^{14}C incorporation was comparable to that obtained in linen flax (*Linum usitatissimum*) but much higher than in white clover (*Trifolium repens*), (Tables 3.2.4.1 and 3.2.4.2).

Table 3.2.4.1

Incorporation of Isobutyraldoxime into Linamarin

Cassava variety	Fresh weight gm	Isobutyraldoxime			Metabolic Time Hrs	Linamarin		Percent ^{14}C Incorporation	^{14}C Dilution
		μCi	μmol	Uptake % ^{14}C fed		μCi	S.A. μCi/mmol		
Manioke	2.3	0.61	8.22	98.8	4	0.150	6.0	24.9	12

Table 3.2.4.2

Incorporation of Isobutyraldoxime into Linamarin in other species

Plant Material	Isobutyraldoxime		Metabolic Time Hrs	Percent ^{14}C Incorporation	^{14}C Dilution	References
	μCi	μmol				
Linum usitatissimum	1.33	3.40	7	21	21.1	Tapper *et al.*(1967)
Linum usitatissimum	3.74	20.0	7	24	20.5	Tapper(1968)
Trifolium repens	0.90	1.22	-	2.6	15.2	Hughes and Conn(1976)

The administration of volatile precursors to cassava leaves by the vapour uptake technique has facilitated the direct feeding of precursors to cassava blade tissues for endogenous ^{14}C-linamarin synthesis. The failure of the leaves to take up significant amounts of ^{14}C-isobutyronitrile was probably due to the low solubility of the nitrile resulting in its low diffusion in the leaf sap. The loss of ^{14}C from leaves fed with ^{14}C-isobutyronitrile was probably due to loss from volatile ^{14}C-isobutyronitrile fraction trapped in leaf interstitial spaces.

The chromatographic scans obtained from extracts of leaves fed with volatile precursors show similarities in ^{14}C labelling patterns namely the presence of linamarin and a major peak at the origin. The ^{14}C amino acid pattern was similar to the general pattern obtained when ^{14}C-HCN is fed to plants including cassava (Tapper, 1968; Nartey, 1969; Blumenthal-Goldschmidt, et al., 1963) although free ^{14}C-HCN could have entered the fed leaf as an impurity or a breakdown product in the case of ^{14}C-2-hydroxy-isobutyronitrile administration. ^{14}C-HCN may also have been generated as a result of endogenous breakdown of ^{14}C-2-hydroxyisobutyronitrile or a turnover of the synthesised ^{14}C-linamarin.

Although none of the fed leaves showed visible toxicity symptoms during precursor administration it is possible that the precursors themselves or their breakdown products may exert some effect on the general metabolic pattern in the leaves. However, all the ^{14}C labelled precursors were significantly incorporated into ^{14}C-linamarin with slightly higher values obtained for ^{14}C-isobutyraldoxime and ^{14}C-2-hydroxyisobutyronitrile.

SECTION 4

SITES OF LINAMARIN SYNTHESIS IN CASSAVA

4.1 Linamarin Content of Cassava Tissues.

One of the main aims of the present study was to ascertain which tissues of the cassava plant were active in linamarin biosynthesis. The linamarin content of various parts of the plant could give some indication of the relative biosynthetic activities of different organs and tissues. Data are presented here for the linamarin content of various tissues of the cassava varieties. The linamarin content of Manioke and variety A was assayed at the commencement of this study using the enzymic hydrolysis method as described under section 2.7.1. Linamarin determinations in Beqa were carried out at a later date and used the GLC method described under section 2.7.2.

The various organs and sections were detached from approximately 2-month old plants grown in peat culture. The categories used are as illustrated in figure 4. The 'Shoot Apex' consisted of the 3-cm length of the growing tip region. The 'Upper Leaf' was the first fully expanded leaf blade near the apex region while a 'lower leaf' was selected from the lowest non-senescing blades on the stem. The 'upper' and 'lower' petioles were the petioles of upper and lower leaves. The 2-3 cm sections next to the shoot apex represented the upper stem and a similar length of stem midway between the lowest leaf and the root system represented the lower stem. The "primary roots" comprised the small normal roots of the plant as distinct from those bearing swollen tubers. Tuber cores were 2 x 1.5 cm diameter cores punched from the inner part of the swollen tuberous roots, while the tuber peels were

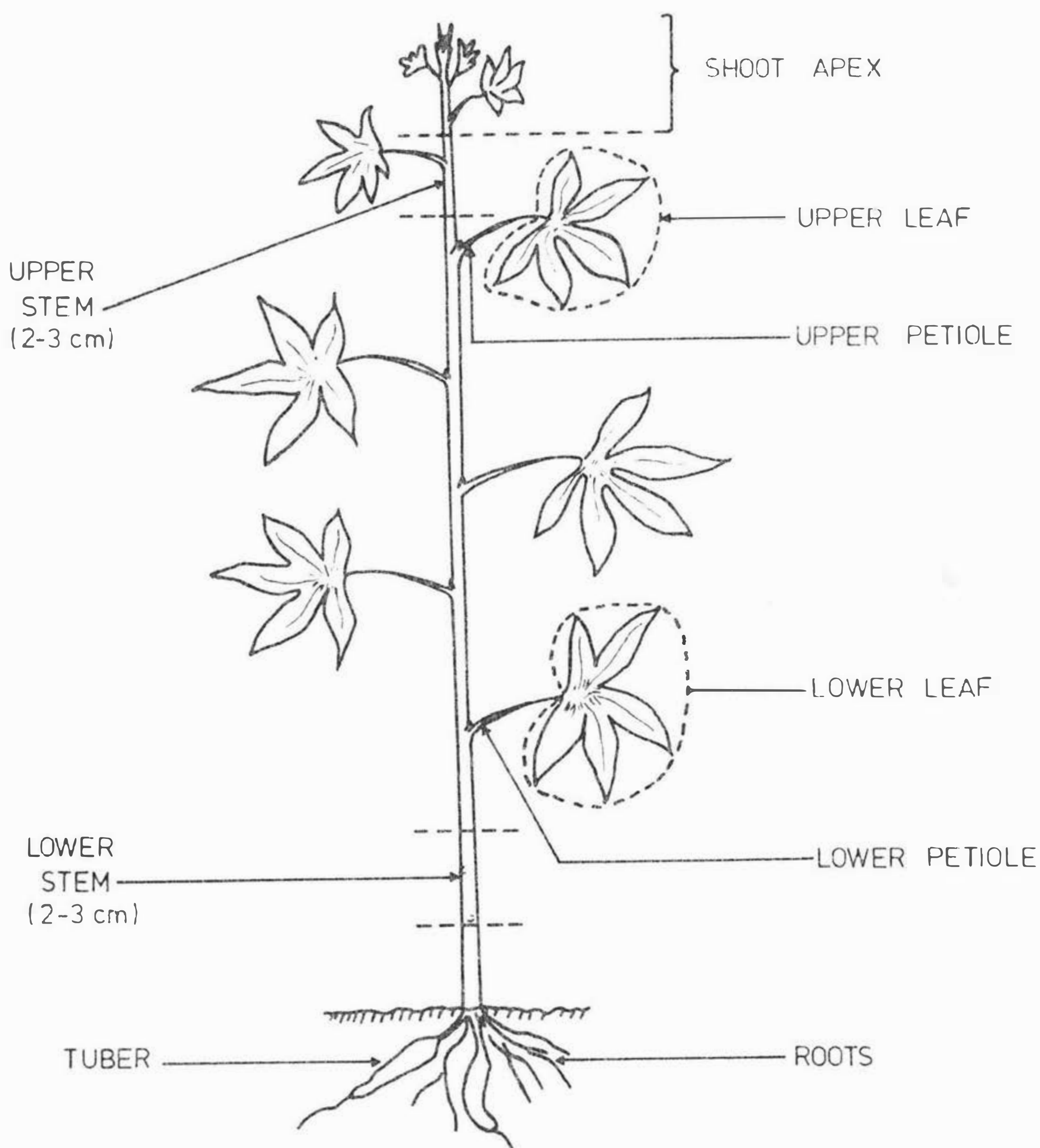


FIGURE 4 DIAGRAM OF AN IMMATURE TUBEROUS CASSAVA PLANT. Organs and tissues employed for linamarin estimations and for ^{14}C–valine administration are indicated.

the outer covering readily detachable from the 'edible' tuber.

Plant sections were extracted in boiling aqueous ethanol and assayed for linamarin. Figure 4.1.2 illustrates a representative G.L.C. separation of the TMS ether of linamarin in the ethanol extract. Linamarin is shown separated from lotaustralin which was present at a concentration of 4-6% of that of linamarin in all samples. Linamarin content of various plant sections together with some values reported in the literature are presented in Table 4.1. The results represent the means of determinations from at least two plants.

Apart from the tuber cores there was a general similarity in the distribution of linamarin in the three plant varieties. The Manioke tubers had about three times as much linamarin as the two other varieties. Due to developmental differences between varieties the tubers assayed were not all the same age and size. The higher level of linamarin in Manioke tubers may therefore be due to a difference in tuber maturity rather than a distinctive feature of this variety. The general distribution patterns compared fairly well with the reported values for Tabouca and Ta25 except for the roots. The nature of the roots used for the estimations reported in the literature were not clearly defined. Since peel tissue covering older roots would have a high linamarin content, old roots would tend to give higher values than the relatively young roots employed in the present analysis.

Within each plant variety the highest linamarin content was recorded in the shoot apex. Generally the tissues like

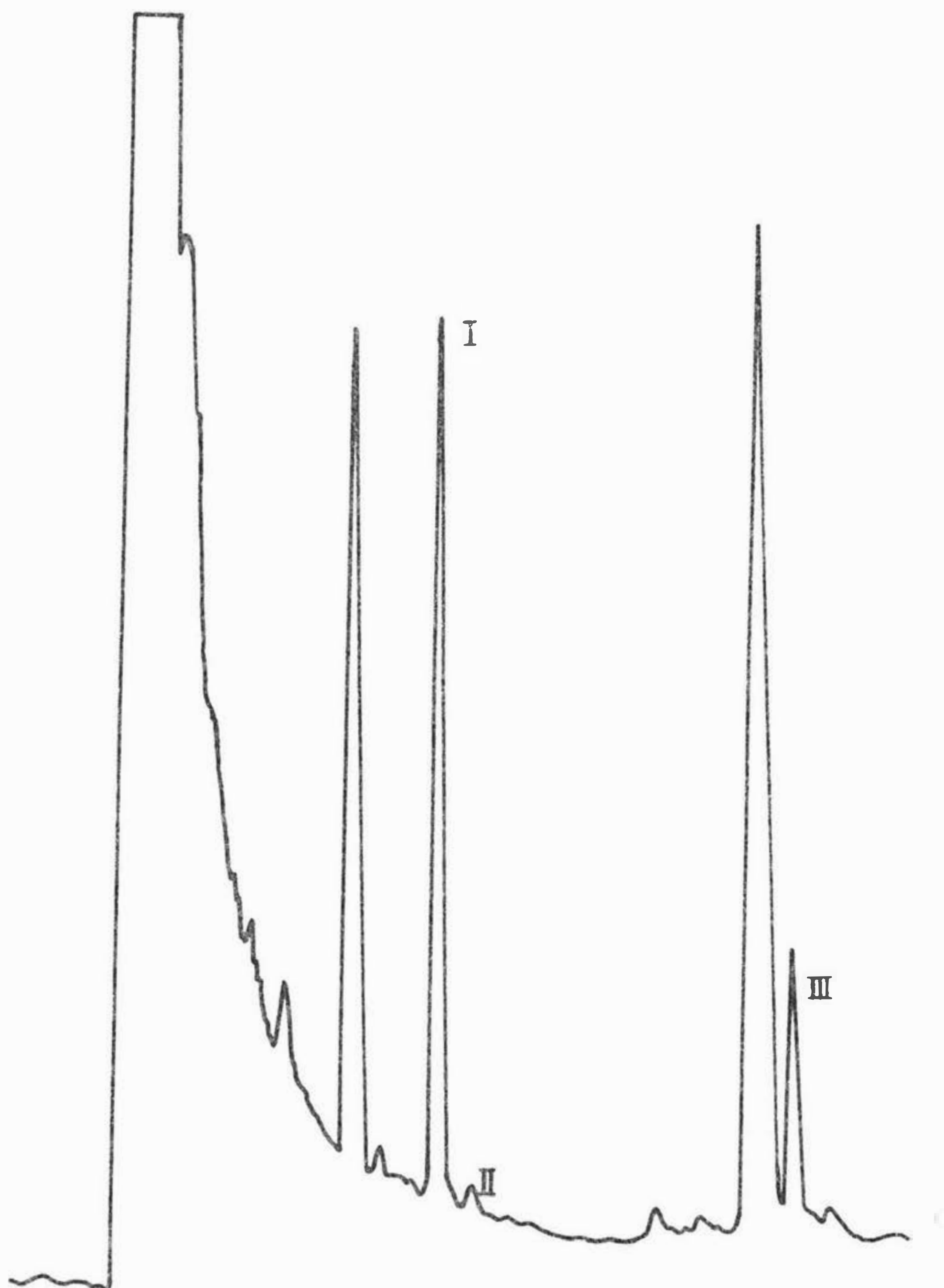


FIGURE 4.1.2. LINAMARIN ESTIMATION BY GAS CHROMATOGRAPHIC TECHNIQUE. Separation of TMS derivatives of ethanolic extracts from leaves on Glass column packed with 1% flourosilicone (QF1) on Gas Chrom Q. I: Linamarin; II: Lotaustralin; III: Prunasin added as internal standard.

Table 4.1

Linamarin Distribution in Cassava

PLANT ORGAN	LINAMARIN CONCENTRATION (μmol/gm)					
	Manioke	Beqa[c]	Variety A	Literature values[1]		
				Tabouca	Ta_{25}	Others
Shoot apex	32.7	29.7	26.0	-	-	-
Upper leaves	12.0	16.3	17.1	15.6	21.1	-
Lower leaves	17.7	17.6	12.4	9.3	11.9	40.3[a]
Leaf petioles	6.9	5.7	3.3	4.4	6.3	-
Upper stem	18.6	18.3	15.1	-	-	-
Lower stem	6.4	6.1	5.0	-	-	-
Primary roots	0.9	0.6	0.6	7.4	11.1	-
Tubers cores	4.5	1.8	1.5	1.3	7.8	7.8[b]
Tuber peels	17.8	14.5	14.0	14.8	33.0	31.1[a]

[1]: Tabouca and Ta_{25} values adapted from de Bruijn (1971);

[a]: Variety Kokotou, Office de la Recherche Scientifique et Technique Outre-Mer (O.R.S.T.M.), cited by de Bruijn (1971)

[b]: Variety S.P.P., Bolhuis (1954)

[c]: Beqa analysis by GLC

the upper stem, leaves and peels with higher contents of active living cells had higher linamarin content than more woody tissues like the petioles and stems or typical storage tissues such as the inner tubers.

While linamarin is present in all tissues of Cassava, linamarin content differs considerably in different tissues. The differences between various parts of the plant are fairly consistent between different plant varieties. The distribution pattern by itself however does not indicate which organs are most actively synthesising linamarin in the plants.

4.2 Biosynthesis of Linamarin from Valine by Detached Organs of Cassava.

The results presented in section 4.1 indicate large differences in the content of linamarin in different parts of the plant. The differences may be due to different rates of linamarin degradation by the tissues or the translocation of linamarin from sites of synthesis to other parts of the plant. The different capacities for biosynthesis of linamarin by the different tissues may also be playing a significant role in the observed differences. In this section the various tissues were investigated for their ability to synthesise linamarin from administered valine.

4.2.1 Methods for Valine administration to the different tissues:

The organs and tissues employed were the same as those described in the previous section (section 4.1). ^{14}C-valine administration was conducted in a growth chamber at 21°C and 80% R.H. All samples were allowed to

metabolise the precursor for 24 hours. ^{14}C-valine was administered to the detached organs as follows:

(a) the shoot apex was detached under water and the exuding latex rinsed off. Accompanying young leaves that exceeded $\frac{1}{4}$ the size of a fully expanded leaf were trimmed off. The valine solution administered (100 μl, specific activity as shown in Table 4.2.1) was almost completely taken up after 5 hours and the apex was then supplied with distilled water for a further 19 hours.

(b) The upper and lower leaves had about 1-2 cm lengths of the petiole tissue still attached to the blade. ^{14}C valine was fed through the cut end of the petiole, using the solution uptake technique described in section 3.1.1. Solution uptake was complete within 10 minutes and the leaves were then supplied with distilled water for the rest of the 24 hours.

(c) Upper and lower stems. The cut surfaces of detached stem sections were rinsed, weighed and fed with labelled valine of specific activity as given in Table 4.2.1. Feeding was achieved by standing the basal ends of the cut surface in 5 ml beakers containing about 100 μl of the label. Solution uptake was slow and it took about 6 hours for the initial volume to be taken up.

(d) Petioles were selected as the upper and lower petioles of the corresponding leaves. Labelled valine was administered as micro-droplets on the cut distal ends i.e. the ends nearest the leaf-blades.

(e) Primary roots were excised from solution cultured plants and subdivided into 1-2 cm sections.

Root sections were packed into flat-bottomed 1.5 cm diameter tubes and the labelled solution administered. Solution uptake was extremely slow and was not complete after 24 hours.

(f) Tubers were harvested from about 2-month old peat cultured plants. Tubers (with intact peels) of approximately 2 cm diameter were cut into sections of 1.5 cm thickness, rinsed with cold water to remove latex and dried with adsorbent paper. Labelled valine solution was spread on the cut surface as a thin layer, allowed to soak into the tuber tissue and was followed by 50 μl distilled water applied in two successive aliquots as a wash in. Tuber discs were covered with beakers and harvested after 24 hours.

All labelled samples were extracted in boiling 80% ethanol, concentrated to dryness and the residue taken up in aqueous propanol for chromatography as described under section 2.6.

4.2.2 Results

Table 4.2.1 presents the quantitative data on incorporation of ^{14}C from valine into linamarin. ^{14}C-linamarin was assayed by the enzymic hydrolysis method after chromatography in Butanol-Water solvent (2.7.2). The separation of ^{14}C labelled substances in the extracts of some of the tissues are illustrated in figure 4.2.1.

Shoot apex. The radiochromatogram shows a peak at Rf 0.4 which was confirmed to be due to ^{14}C-linamarin after rechromatography in two other solvents in both of which it co-chromatographed with authentic linamarin. The average ^{14}C incorporation into ^{14}C-linamarin by the

Table 4.2.1

Incorporation of L-[U-^{14}C] valine into linamarin by cassava tissues

Plant Section	Cassava variety	Fresh Weight (gm)	L-[U-^{14}C] valine administered			^{14}C-linamarin recovered			Incorporation %	^{14}C Dilution
			µmol	S.A. (µCi/mmol)	Percent Uptake	µCi[a]	µmol	S.A. (µCi/mmol)		
Shoot apex	A	1.3	1.0	993	99.3	0.160	37.3	4.3	16.1	231
	Manioke	1.0	1.0	1930	98.5	0.262	24.3	10.8	13.4	184
	Beqa	2.1	2.0	500	99.9	0.172	40.3	4.3	17.2	116
Upper leaves	A	2.0	2.0	496	99.9	0.088	34.8	2.5	8.9	198
	Manioke	1.8	1.0	993	99.3	0.116	18.0	6.4	11.1	155
	Beqa	1.5	3.0	333	98.3	0.157	20.6	7.6	16.0	44
Lower leaves	A	2.3	2.0	496	100.0	0.067	28.0	2.4	6.8	207
	Manioke	0.9	1.0	993	99.5	0.104	12.8	8.1	10.5	123
	Beqa	1.8	3.0	333	99.5	0.150	31.0	4.8	15.1	66
Upper leaf petioles	Manioke	1.0	3.0	333	100.0	0.623	6.4	97.3	62.3	3.4
	Beqa	0.8	3.0	333	100.0	0.646	3.3	196.0	64.6	1.7
Lower leaf petioles	Manioke	1.2	3.0	333	100.0	0.487	8.8	55.3	48.7	6.0
	Beqa	2.0	3.0	333	100.0	0.559	11.1	50.4	55.9	6.6
Upper stem	A	2.0	1.0	993	99.5	0.137	31.0	4.4	13.1	226
	Manioke	0.8	1.0	993	98.6	0.124	10.7	11.5	12.7	86
	Beqa	1.7	3.0	333	99.8	0.170	26.5	6.4	17.0	52
Lower stem	A	4.2	1.0	993	99.8	0.009	22.5	0.4	0.9	2483
	Manioke	4.5	1.0	993	98.5	0.084	31.1	2.7	8.6	368
	Beqa	4.8	3.0	333	98.9	0.044	32.1	1.4	4.5	238
Roots	Manioke	0.7	1.0	2000	83.0	0.040	0.56	71.4	2.4	28
	Beqa	0.7	1.0	333	58.0	0.002	0.43	5.1	0.4	189
Tubers (+ peels)	Manioke	4.8	3.0	333	100.0	0.063	58.2	1.1	6.3*	303
	Beqa	5.0	3.0	333	100.0	0.049	49.1	1.0	4.9*	333

a: corrected for an assumed loss of ^{14}COOH from L-[U-^{14}C] valine

* maximum value, see pp 59-60

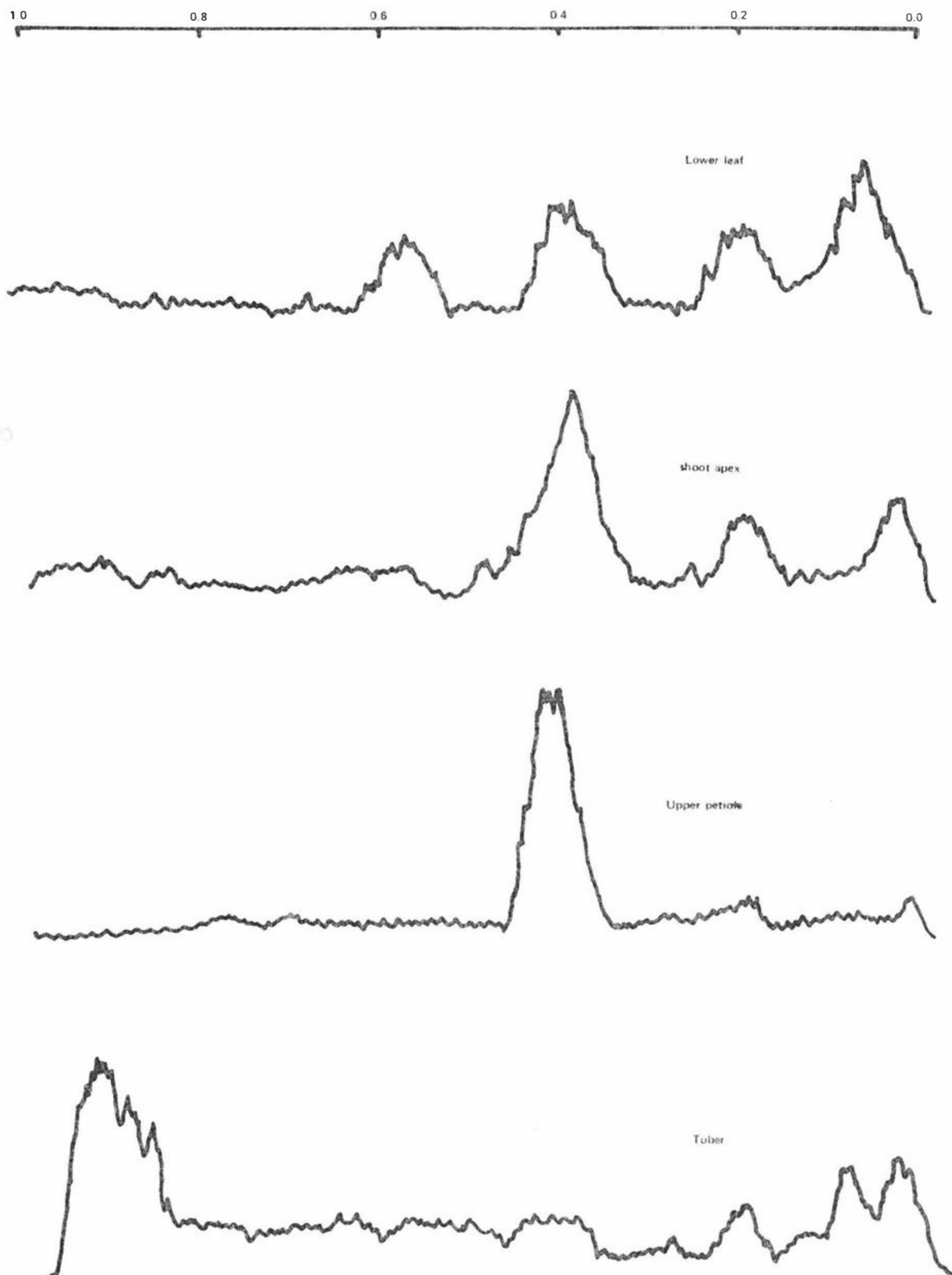


FIGURE 4.2.1 INCORPORATION OF RADIOACTIVITY FROM L-[u-^{14}C] VALINE INTO ^{14}C LINAMARIN BY CASSAVA TISSUES. Linear scans of radioactivity on paper chromatogram strips developed with Butanol: Water. Detector sensitivity for petiole 3×10^3, all other scans at 1×10^3 range.

three varieties was 15.6%. The shoot apex of Manioke showed a somewhat lower incorporation than the Beqa and variety A.

Leaves. Both the upper and lower leaf types gave similar labelling patterns (figure 4.2.1) and the peaks at Rf 0.4 were confirmed to be mostly ^{14}C-linamarin. All three varieties show that the incorporation by upper leaves is slightly higher than the lower leaves. Mean incorporation values were 10.8% and 12.0% for lower and upper leaves respectively.

Petioles. The chromatographic scans of the petiole extracts show that ^{14}C-linamarin is almost the sole labelled product of valine metabolism apart from some material near the origin. This was confirmed by re-chromatography of the zone at Rf 0.4 in two separate solvents. The results indicate that the upper petioles give slightly higher incorporation of ^{14}C into ^{14}C-linamarin than the lower petioles. The incorporation rates were high compared to other tissues with correspondingly lower dilutions especially in the upper petiole tissue. Subsequent investigations have confirmed this high level of ^{14}C valine incorporation into ^{14}C linamarin.

Stems. The ^{14}C activity distribution in the soluble extracts of the upper stem was similar to that of the shoot apex. This was to be expected since they were adjacent tissues on the plant differing only slightly in degree of maturity. Incorporation values were also similar but the dilution of the specific activity was less for the upper stem compared to the apex. ^{14}C-

linamarin peak was however poorly defined in the radiochromatograms of the lower stem extracts and rechromatography in two other solvents revealed that the linamarin was only a minor component of the original peak. The lower stems of all three varieties had considerably lower incorporation rates and higher dilutions than the upper stems.

Roots. The low rate of uptake of fed valine by the roots was reflected in a large peak nearer the origin on the chromatographic scans. At least part of this peak will be residual valine retained in metabolically inactive regions e.g. xylem vessels and root "free space". However much activity was present in unidentified products in the solvent front. Linamarin activity as assayed by the enzyme hydrolysis method gave 2.4% and 0.4% incorporations for Manioke and Bega respectively. The enzymic hydrolysis method used with [U-^{14}C] linamarin was later found to give a slight overestimation of ^{14}C-linamarin because of the probability of co-trapping labelled acetone as well as [^{14}C]-HCN for counting, so, the true incorporation may be even lower. Rechromatography of the primary root extracts in other solvents failed to establish the presence of a linamarin peak.

Tubers. The chromatographic scans of the tuber extracts show that most of the radioactivity was present at the solvent front. Again no clearly defined ^{14}C-linamarin peak was present on a one-dimensional radiochromatogram. The ^{14}C-linamarin zone was eluted and rechromatographed in two further solvent systems. A small radioactive peak was revealed in the ^{14}C-linamarin position. The incorporation of ^{14}C into ^{14}C-linamarin by Manioke and

Beqa tubers averaged 5.6%. This was assumed to be a maximum figure in view of the low ^{14}C-linamarin levels detected chromatographically.

The chromatographic scans of the ethanol extracts of all three varieties indicate that administered ^{14}C-valine was extensively metabolised in all tissues. The petiole tissues gave by far the highest percentage incorporation of ^{14}C into linamarin. The leaves, upper stem and shoot apex gave similar incorporation values which were considerably higher than those for the lower stem and the underground tissues - the tubers and roots.

While the results indicate marked differences in the ability of different tissues to convert valine into linamarin, these differences are not necessarily a reflection of the different rates of biosynthesis. Radiochromatogram scans indicate that ^{14}C-valine is incorporated into compounds other than linamarin. The ^{14}C-valine is thus being metabolised by competing metabolic pathways and these pathways may be much more active in some tissues than others. It is also possible that in some tissues exogenously fed valine may have a greater or less access to competing pathways than endogenously synthesised valine. Thus the high percent incorporation in petiole tissue may be due to low activity of competing pathways. Furthermore the ratio of linamarin turnover in the different tissues may be very different with the result that a low percent incorporation at the end of a 24 hour feeding period could be due to a high rate of linamarin degradation.

A more detailed investigation of the metabolism of administered valine to linamarin was therefore undertaken. Two tissue systems showing large differences in percentage incorporation were selected - the petiolate leaf and the tuber.

4.3 Linamarin Synthesis by Detached Leaves

4.3.1 Introduction

The experiments in section 4.2 have indicated that the leaves, petioles and shoot apices are much more active in converting valine to linamarin than the mature stem and underground parts. Since the leaves, including the petioles, make up much of the weight of the total shoot system, this would indicate that they account for the greatest proportion of total linamarin biosynthesis in the plant. Therefore a more detailed study of the conversion of valine to linamarin by the petiolate leaf system was undertaken.

A notable finding from the survey of biosynthetic activities reported in section 4.2 was the very high percent incorporation in petioles. Since the upper and lower leaves used in that survey included a 2-3 cm length of petiole attached to the leaf lamina it is possible that this may have contributed considerably to the total linamarin synthesis by the leaf tissues. One possible approach to studying the relative biosynthetic activity of the leaf lamina tissue, the petiole and the midrib tissue would be to examine linamarin biosynthesis from valine in small excised pieces, e.g. leaf discs, midrib and petiole sections. This involves considerable

damage to the tissues and problems of ensuring that differences in valine metabolism were not due to differences in uptake by the cells of these two very different experimental systems. Therefore it was decided to study valine metabolism in the entire petiolate leaf system allowing the valine to be taken up via the transpiration stream through the petiole. By ascertaining the distribution of total ^{14}C after different time intervals in the different component tissues (petiole, midribs and blade) and also the residual valine and the linamarin formed it was hoped to obtain an assessment of the relative ability of the different tissues to utilise valine for linamarin biosynthesis.

4.3.2. Experimental Procedure

4.3.2.1 Preliminary Experiment to Assess effect of Carrier Addition.

In the previous valine feeding experiment (section 4.2) about 1-3.0 μmol of carrier valine was added to 0.011 μm of the labelled valine so that valine was administered at a level of about 1-2 μmol/gm tissue. Increasing the amount of carrier added resulted in significant lowering of the specific activity of the recovered linamarin within each tissue. Added carrier may lead to a significant alteration of normal biosynthetic activity in the leaf; for example, it could result in precursor saturation of the linamarin biosynthetic pathway.

A preliminary study was carried out to compare the general ^{14}C distribution and incorporation into linamarin in tissues of the leaf system in the presence (1 μmol/gm fresh weight) and absence of added carrier. The leaves

were detached at the base of the petiole and the cut end quickly submerged under water. While still submerged, about 0.5 cm was trimmed off the cut end and rinsed repeatedly to remove exuding latex. The weighed leaves were each fed with about 1.24 μCi L[U-^{14}C] valine with or without 1.0 μmol of carrier valine added. Leaf feeding was performed in a growth chamber at 80% relative humidity at 21°C. Solution uptake was complete in about 20 minutes after which leaves were supplied with water for the rest of the 4-hour period, harvested, and quickly dissected into the following sections:

Midribs: The central midrib of each leaflet was excised to leave as little lamina tissue as possible attached.

Blade: The remainder of the lamina including vein tissue.

Petiole: The leaf petiole.

Each tissue was extracted in boiling ethanol and ^{14}C linamarin determined on the concentrated extracts by chromatography in two solvents followed by elution of corresponding ^{14}C-linamarin band and counting in a scintillation counter. The results are presented in Table 4.3.2.1. The presence of carrier valine at the level used did not cause any major difference in the ^{14}C distribution to the different tissues. The addition of carrier slightly increased the proportion of total ^{14}C found in the blades and has considerably decreased the incorporation of ^{14}C into ethanol insoluble compounds in the petiole and midrib tissues. While the presence of carrier has increased the ethanol soluble ^{14}C in the petiole and midrib tissue, the ^{14}C incorporation into linamarin was lower in the same tissues of carrier-fed

Table 4.3.2.1

Effect of Carrier Valine on ^{14}C Distribution and Incorporation into Linamarin by Detached Leaf[1].

Leaf Tissue	Fresh weight (gm)	Valine added μmol/g F.wt.	^{14}C Distribution in tissues[2]			Linamarin	
			Total μCi in tissues	μCi/g Fr.wt.	% of total ^{14}C taken up by leaf	μCi[4]	% of total ^{14}C in tissue
Petiole	0.200		0.229	1.145	21.1	0.101	44.1
Midribs	0.562		0.312	0.555	28.8	0.033	10.6
Blade	2.600		0.543	0.209	50.1	0.011	2.0
Petiole	0.205	1.0	0.187	0.912	16.2	0.069	37.0
Midribs	0.555	1.0	0.294	0.530	25.4	0.015	5.1
Blade	2.500	1.0	0.675	0.270	58.4	0.015	2.2

1: Leaves were fed with 1.24 μCi valine at specific activities of 107 mCi/mmol (carrier-free leaf) and 1.226 μCi/mmol (carrier-fed leaf) for 4 hours

2: Total ^{14}C uptakes were 87.4% (carrier-free leaf) and 93.2% (carrier-fed leaf)

4: Corrected for an assumed loss of $-^{14}COOH$ in the conversion of L-[U-^{14}C] valine into ^{14}C-linamarin.

leaf. About 13.3% and 8.6% ^{14}C was incorporated into ^{14}C-linamarin by the carrier-free and carried fed leaves.

A significant aspect of ^{14}C incorporation into linamarin was the high incorporation attained in the petioles as compared to the blade tissues; this confirms the finding reported in section 4.2. The total ^{14}C-linamarin found in petiole and midrib tissue is much higher than that in the blades even though 50% of the total ^{14}C administered was recovered from the blade tissue.

4.3.2.2 Linamarin Synthesis in Detached Leaves Harvested at Different Times after Precursor Addition.

The preliminary experiment (4.3.2.1) has shown that the presence of carrier valine lowers ^{14}C incorporation into linamarin by the petiolate leaf. The added carrier probably alters the physiological conditions of the tissues much more than is the case with carrier-free administration. Furthermore since the ^{14}C distribution between the lamina, midrib and petiole was not greatly different in the presence or absence of carrier valine the following experiment was carried out without the presence of added carrier. An experiment was designed to investigate the incorporation ^{14}C into linamarin and changes in the synthesised ^{14}C-linamarin in the various leaf tissues over a 24 hour period.

In preliminary experiments on leaf feeding by solution uptake through the petiole, some leaves were found to wilt badly during the experiment even though the solution level was maintained. A microscopic

examination of the cut surface of the leaf showed that damaged latex vessels were closely associated with the vascular bundles. Latex exudate therefore easily floods the xylem vessels and stops solution uptake. Although the cut surface was repeatedly rinsed to wash off latex it was apparent that some of the vessels could still be plugged by condensed latex at or near the exposed petiole surface. Solution uptake by all xylem vessels was necessary for uniform ^{14}C distribution within the leaf system. To avoid latex plugging the terminal 1 or 2 cm of the petiole (the cut end) was scalded in water above $90^{o}C$ for about 10-15 seconds. The scalded end was cooled in water at room temperature and about 0.5 cm of the heated section trimmed off with a sharp razor blade while still immersed in water. The heating coagulates latex within the latex vessels at the treated zone and arrests further exudate. Subsequent cuts within the scalded section therefore leaves the xylem exposed for solution uptake.

Matured leaves of solution-cultured Beqa plants were excised at the stem end of the petiole and the cut surface submerged in cold water. The cut ends were quickly scalded and cooled as described. About 0.5 cm sections were excised off the heated zone and the leaf allowed to take up 50 μl valine solutions of specific activity 107 μCi/mmol. Solution uptake was complete within one minute and each leaf was subsequently fed with water for the rest of the 24 hour period. The labelled leaves were harvested at $\frac{1}{2}$, 4 and 24 hour intervals and dissected into petioles, midribs and blade sections as described in 4.3.2.1.

The petioles were subdivided into a basal section (the lowest half of the petiole nearest the stem) and a distal section. Each tissue was weighed and extracted three times in boiling 80% ethanol for 15-20 minutes (for each extraction). Radioactivity was determined in the total ethanol soluble and insoluble fractions. ^{14}C valine and ^{14}C-linamarin were determined after separation by paper chromatography in two solvent systems followed by elution of the corresponding zones. Linamarin was estimated in the ethanolic extracts by the GLC method as described under section 2.7.1.2. Valine was separated by chromatography in two solvent systems, eluted and estimated by the modified ninhydrin-hydrindantin method of Matheson _et al_. (1961). The ^{14}C content and distribution in the various fractions and leaf tissues is presented in figure 4.3.2.2 and in Tables 4.3.2.2 and 4.3.2.3.

The total ^{14}C recoveries (Table 4.3.2.2) show very little change over the 24-hour period. The 4% loss in recovered ^{14}C activity in 24 hours may be ascribed to ^{14}C losses due to $^{14}CO_2$ and other volatile products. The results therefore indicate that very little of the administered valine was being lost as volatile products from the leaf in 24 hours.

The pattern of distribution of administered ^{14}C was largely established after 4 hours (Figure 4.3.2.2 and Table 4.3.2.2) with little further change between 4 and 24 hours. From $\frac{1}{2}$ to 4 hours there was some redistribution of total leaf ^{14}C as exemplified by the fall in total ^{14}C in the basal petiole accompanied by a corresponding rise

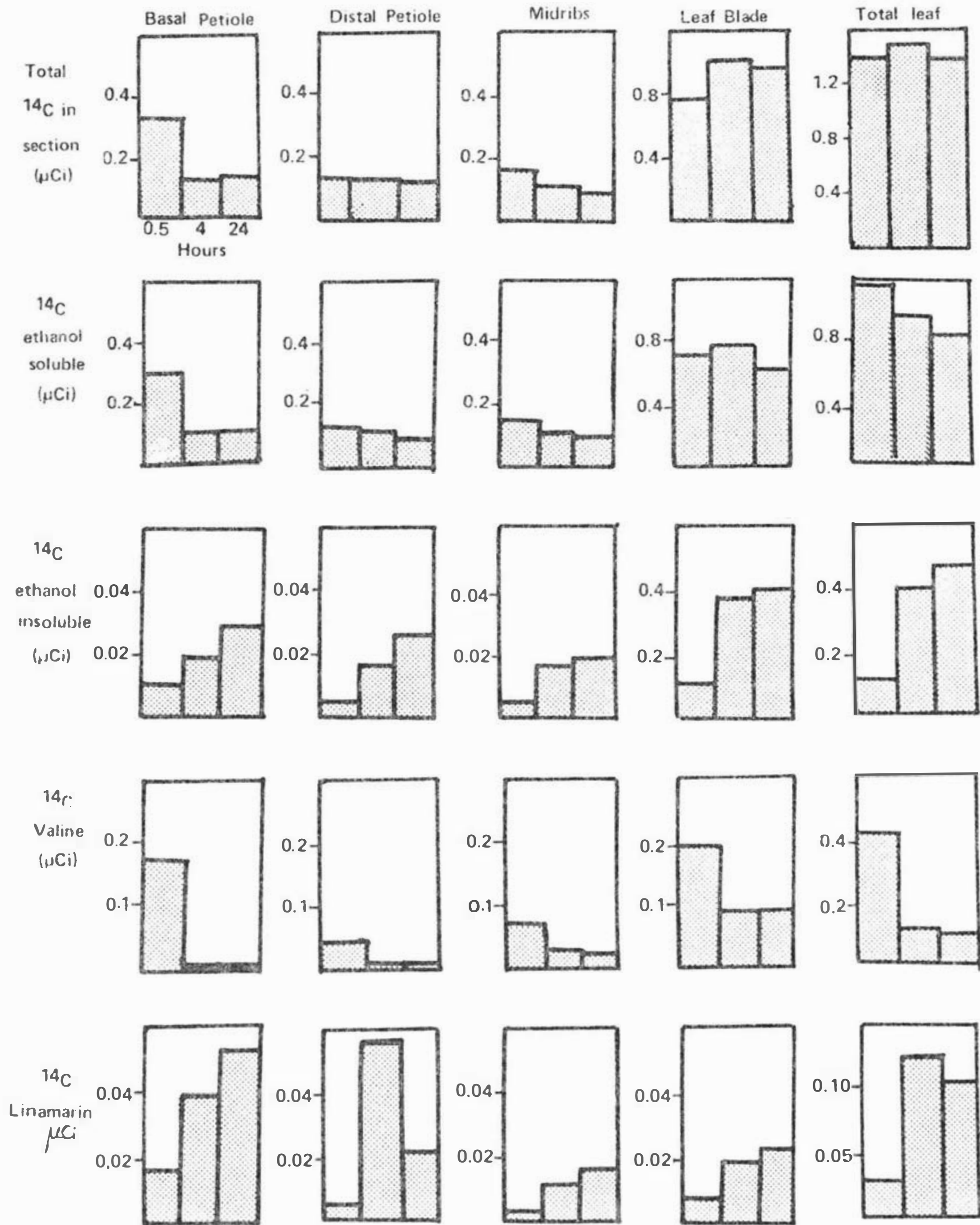


FIGURE 4.3.2.2. BIOSYNTHESIS OF LINAMARIN BY PETIOLATE LEAF. Histograms of ^{14}C incorporation from ^{14}C-valine into linamarin, ethanol soluble and insoluble fractions. The first, second and third columns of each histogram represents, respectively, samples taken at 0.5, 4 and 24 hour

Table 4.3.2.2

Distribution of ^{14}C in L-[U-^{14}C] Valine fed Leaf.

Time after ^{14}C Administration (Hrs)	Leaf Section	Fresh weight of section	^{14}C Distribution in Tissues		% of Total ^{14}C in tissue		Total ^{14}C Recoveries (% of ^{14}C fed)
			μCi/g F.wt.	% of total ^{14}C taken up by leaf	Ethanol soluble %	Ethanol insoluble %	
0.5	Basal petiole	0.509	0.610	23.6	97.2	2.8	99.6
	Distal petiole	0.444	0.260	8.8	96.5	3.5	
	Midribs	0.560	0.221	9.5	96.0	4.0	
	Blade	2.058	0.362	56.8	86.7	13.3	
4.0	Basal petiole	0.471	0.244	8.7	83.5	16.5	99.0
	Distal petiole	0.434	0.237	7.7	85.1	14.9	
	Midribs	0.419	0.209	6.6	82.5	17.5	
	Blade	1.908	0.550	79.1	64.2	35.8	
24.0	Basal petiole	0.400	0.310	9.3	77.1	22.9	96.5
	Distal petiole	0.340	0.297	7.6	75.0	25.0	
	Midribs	0.390	0.194	5.8	89.9	10.1	
	Blade	1.831	0.540	74.9	59.7	40.3	

Table 4.3.2.3

Incorporation of ^{14}C-Valine into Linamarin by Leaf Tissues

Time after ^{14}C Adminis-tration (Hrs)	Leaf Section	Residual Valine			Linamarin		
		μmol/gm	S.A. μCi/mmol	% Total ^{14}C in section	μmol/gm	S.A. μCi/mmol	% Total ^{14}C in section
0.5	Basal petiole	14.3	21.0	49.0	8.65	3.1	4.4
	Distal petiole	16.0	5.2	32.0	14.00	0.9	4.8
	Midribs	10.7	9.4	46.0	33.00	0.2	2.0
	Blade	16.9	5.4	25.0	15.20	0.2	0.8
4.0	Basal petiole	12.5	1.0	5.0	6.8	12.5	34.9
	Distal petiole	13.1	0.8	4.0	6.9	19.7	57.1
	Midribs	18.9	2.4	21.6	31.1	0.8	12.3
	Blade	14.8	2.9	8.4	14.0	0.6	1.8
24.0	Basal petiole	18.3	0.8	4.7	7.6	16.6	41.6
	Distal petiole	13.2	0.4	1.6	8.2	7.9	21.9
	Midribs	21.3	1.2	13.0	32.8	1.2	20.2
	Blade	17.7	2.6	8.5	17.0	0.7	2.1

in ^{14}C in the blade. Within all tissues a fall in ethanol soluble ^{14}C was accompanied by an increase in ethanol insoluble ^{14}C. Ethanol insoluble ^{14}C activity did not change much after 4 hours. The Blade tissues generally converted more of the ^{14}C into insoluble ^{14}C than the midribs and petioles.

There was a rapid fall in specific activity of valine over the first 4 hours in all tissues even though endogenous valine concentration remained relatively constant (Table 4.3.2.3). This reflects rapid metabolism of the administered valine which is largely complete by 4 hours. There was little further decline in the residual ^{14}C valine between 4 and 24 hours (Figure 4.3.2.2). This residual valine is possibly due to a minor valine fraction trapped in some inert pool or valine adsorbed onto cell wall exchange sites.

A significant proportion of the ethanol-soluble ^{14}C in each tissue was not accounted for by the sum of valine and linamarin radioactivities. There were no major labelled components on the chromatographic scans of the ethanol extracts which would account for the difference. The ^{14}C activity unaccounted for may be due to volatile ^{14}C products lost on concentration of ethanol extracts or to the presence of a large number of weakly labelled substances which are not distinguishable on the chromatogram scans.

Incorporation of ^{14}C valine into linamarin occurred largely over the first 4 hours, a pattern similar to that of the rise of insoluble ^{14}C activities. These trends are to be expected since the fed precursor was largely

exhausted by then. Over the period from 4 to 24 hours basal petiole, midribs and blade tissues showed slight increases in total linamarin while in the distal petiole there was a considerable fall in ^{14}C linamarin. These changes may represent some redistribution of the freshly synthesised linamarin within the leaf tissues although leaf to leaf variation in the ability of the distal petiole to incorporate exogenous ^{14}C valine into linamarin may be implicated. The total leaf ^{14}C linamarin does not decline greatly between 4 and 24 hours indicating relatively little linamarin degradation and turnover within the 24 hour period.

There was much higher ^{14}C incorporation into linamarin in the petiole tissues than in the blade tissue both on a percentage and an absolute basis. The 35 and 57% ^{14}C incorporation by the petioles (in 4 hours) correspond with similar incorporation values recorded in the petioles of the experiments in sections 4.2 and 4.3.1.1. The much lower ^{14}C incorporation into linamarin achieved in the blade tissue cannot be due to failure of ^{14}C to reach the blade lamina tissue since over 50% of the total ^{14}C administered was recovered from the blades after only 30 minutes and 25 per cent of this ^{14}C was still residual valine. Possible reasons for the higher incorporation by the petiole relative to the leaf blade will be discussed in section 6.

4.4 <u>Linamarin Synthesis in Cassava Tubers</u>

The preliminary valine feeding experiments reported in Section 4.2 indicate that tuber tissue had a relatively low ability to convert administered valine

into linamarin compared to the petiole, leaf and young stem tissue. However cassava tubers have a high content of linamarin particularly in the peel tissue and it is important to ascertain how far this linamarin comes from the biosynthetic activity of the tuber itself. Furthermore the very different linamarin contents of the peel and core tissue (table 4.1) may reflect different biosynthetic efficiencies of these two parts of the intact tuber.

In this section the ability of the tuber core tissue (the edible tuber) and tuber peel to convert exogenous ^{14}C-valine to linamarin has been more thoroughly investigated. Particular attention has been paid to the quantitative estimation of the labelled linamarin synthesised since the 'linamarin peak' obtained on chromatography of ethanol extracts may have been incompletely resolved from other labelled products in the preliminary experiments of section 4.1.

4.4.1 <u>Uptake of labelled valine by Tuber Cores</u>

In the preliminary tuber feeding experiments valine was administered as a layer of solution spread over the surface of the cut tuberous tissue. The limitation of this method of administration lies in the low penetration of tissue material by the labelled compound and possible saturation of the cells on the surface with precursor solution. Apart from adversely disturbing the normal physiological conditions at the site of application the technique does not afford a uniform distribution of the labelled compound within the tissue.

The ideal method would be to administer the precursor

by the most appropriate physiological method probably involving distribution of the precursor in tuber tissues in the same manner as other metabolites are translocated within the tissues. Such a method would require very large amounts of precursor and most of the precursor would in any case be metabolised close to the point of entry. Vacuum infiltration of valine into excised tuber cores is another alternative technique that would enable administered solution to penetrate the "free space" in the tuberous tissues. The conditions likely to affect the uptake of label during vacuum infiltration include time of vacuum application and incubation period after application. The effect of time of incubation was investigated by administering labelled valine to tuber cores by vacuum infiltration.

Tuber cores (1.2 x 1 cm) were punched by means of a cork borer from the tuber of an 8-month old Beqa plant grown in gravel culture (section 2.3). Each core was rinsed with distilled water and dried between adsorbent papers. Meanwhile aliquots of labelled valine solutions were transferred into flat-bottomed micro-test tubes selected so as to provide a close fitting for the tuber cores. Weighed cores were immersed in the valine solution and about 50 μl of distilled water added, just enough to flood the cores. Each core was weighed down by a glass rod, the tubes placed in a vacuum dessicator and vacuum gently applied until air bubbles ceased to be evolved from the core tissue (about 1-2 minutes). The vacuum was then released and the feeding vials containing the cores were incubated at 21°C in a water bath. Samples

were removed after 10 minutes, ½ hour and 4 hours by means of a glass loop, rinsed thoroughly with distilled water, extracted in boiling aqueous ethanol and aliquots counted for ^{14}C activity. Residual ^{14}C in the feeding vial was also determined. The total ^{14}C uptake and ^{14}C activity of ethanol soluble extracts is given in table 4.4.1.

The results show that over 65% of the administered precursor had penetrated the tuber by half an hour. Much of this might possibly still be external to the tuber cells; however, the results of subsequent experiments show that the labelled valine is extensively metabolised by the tuber cells after quite short periods of time when administered by this procedure.

4.4.2 Linamarin synthesis by tuber cores

Linamarin synthesis was investigated in tuber cores by the solution uptake technique described in section 4.4.1. Labelled valine (4.26 μCi of specific activity 107 mCi/mmol) was fed to tuber cores punched from the edible portion of the tuber. After infiltration sample vials were incubated at 21°C for 0.5, 4, 10 and 24 hours. Duplicate samples were taken at each of these times and the ethanol soluble substances extracted.

The ^{14}C distribution in the tuber cores is shown in Table 4.4.2. There is a drop in total ^{14}C recoveries in the soluble and insoluble fractions to about 70% of total taken up after 10 hours. These low recoveries are possibly due to losses of ^{14}C labelled volatile substances during extraction. Ethanol insoluble ^{14}C activity increased steadily to 11% of the total ^{14}C taken up during 24 hours.

Table 4.4.1

Uptake of Labelled Valine by Tuber Cores.

Incubation Time (Hrs)	L-[U-^{14}C] Valine administered (μCi x 10^{-3})	^{14}C uptake		Ethanol soluble ^{14}C
		μCi x 10^{-3}	% ^{14}C fed	% ^{14}C uptake
0.17	2.68	1.39	52.0	80.0
0.17	4.01	1.99	49.6	88.6
0.17	4.01	2.04	50.9	85.3
0.50	0.90	0.76	84.4	97.4
0.50	2.68	1.75	65.3	73.5
0.50	4.01	3.10	77.3	80.3
4.00	0.90	0.73	81.1	96.2

Table 4.4.2

L-[U-^{14}C] Valine Administration to Edible Tuber Cores

Tuber samples		Precursor uptake		Total	^{14}C distribution in cores		Residual valine		Linamarin
Time (Hrs)	Fresh Weight (gm)	^{14}C in Tuber Core (μCi)	% of Total ^{14}C fed	^{14}C Recovered (μCi)	Ethanol soluble ^{14}C as % of total uptake	Ethanol insoluble ^{14}C as % of total uptake	μCi	% ^{14}C uptake	μmol/gm F.W.
0.5	0.930	2.64	61.9	98.1	96.6	1.5	0.407	15.4	1.50
	0.865	2.34	55.0	97.2	95.4	1.8	0.283	12.1	1.40
4	0.907	3.33	78.2	78.0	73.9	4.1	0.362	10.9	1.40
	0.937	2.65	62.2	76.0	72.8	3.1	0.361	13.6	1.40
10	0.925	3.07	72.0	75.0	71.8	3.2	0.455	14.8	1.50
	0.902	2.50	58.7	67.1	65.0	2.1	0.303	12.1	1.50
24	0.913	3.88	91.1	68.0	57.0	11.0	0.497	12.8	1.44
	0.917	3.25	76.3	70.1	58.5	11.5	0.291	8.0	1.43

Residual valine radioactivity was only 15% of the total taken up at 0.5 hour. This residual valine pool would appear to be inaccessible to metabolism since it remained virtually constant between 0.5 and 24 hours. A scan of a one-dimensional chromatogram of the 4-hour ethanol extract indicated considerable metabolism of the administered valine into various substances much of which ran towards the solvent front. There was a distinct peak at the R_f value corresponding to linamarin, but rechromatography of this peak in two other solvent systems, Butanol:Water (50:9) and Propanol:Water (7:3), showed that the major component of this peak was not due to linamarin. The zone corresponding to linamarin on the chromatogram of the 24 hour sample in the second solvent was eluted and hydrolysed with linamarase in a sealed tube. The released acetone was recovered as acetone semicarbazone crystals and counted for radioactivity. The recovered ^{14}C was converted to take account of the loss of one carbon atom from linamarin on conversion to acetone. A very low maximum incorporation value of 0.11% was obtained from the 24 hour sample by this procedure. Since the endogenous linamarin contents of the cores did not decrease during the 24 hours (Table 4.4.2.2) the low level of ^{14}C linamarin could not be due to breakdown of the synthesised linamarin in the tubers unless newly synthesised linamarin is in a distinct metabolic pool. The result suggests that edible tuber tissue does not convert exogenous valine to linamarin to a significant extent.

4.4.3 Linamarin synthesis by tuber peels

The ability of tuber peel to convert valine into linamarin was investigated by vacuum infiltration of peel strips with labelled valine. Peels were removed from the mid section of a tuber harvested from a 9-month old tuberous Beqa plant grown in gravel culture. The cork layer was removed from the outer surface. Peels were sectioned into strips of about 15 x 1.5 mm, rinsed with distilled water and packed tightly in a flat-bottomed feeding vial. The peel strips were covered with labelled valine solution and subjected to vacuum infiltration for about 5-6 minutes according to the method described in 4.4.1. Metabolism was terminated after 0.5, 10, and 24 hours. The results are shown in table 4.4.3. Again the total recoveries were low and residual valine accounted for only a small proportion of the total ^{14}C even after 0.5 hour. The scans of chromatograms of the ethanol extracts show high levels of ^{14}C labelled substances at the solvent front and in the valine zone (figure 4.4.3). The scans also showed a significant peak in the linamarin zone but rechromatography of this zone in a second solvent showed that most of the activity was not due to linamarin. In a third solvent system a minor peak at the R_f corresponding to linamarin activity was detected but it was largely overlapped by a substance of higher activity and slightly greater R_f value. It was possible therefore that some linamarin was being synthesised but the presence of other labelled metabolites prevented the quantitative determination of its radioactivity by counting the eluate from the linamarin zone. The linamarin zone was eluted from the

Table 4.4.3

L-[U-^{14}C] Valine Administration to Peels

Peel sample		Precursor uptake		^{14}C distribution in major fractions		Residual valine		Linamarin		
Time Hrs	Fresh weight gm	µCi	% Uptake	Ethanol soluble (%)	Ethanol Insoluble (%)	µCi	% ^{14}C administered	µol/gm	µCi	% Incorporation
0.5	0.255	1.110	52.1	69.0	0.54	0.096	8.6	13.7	0.009	0.8
10	0.232	1.471	69.1	66.0	6.73	0.037	2.5	13.1	0.032	2.2
24	0.216	1.476	69.3	60.4	8.90	0.021	1.4	12.0	0.019	1.3

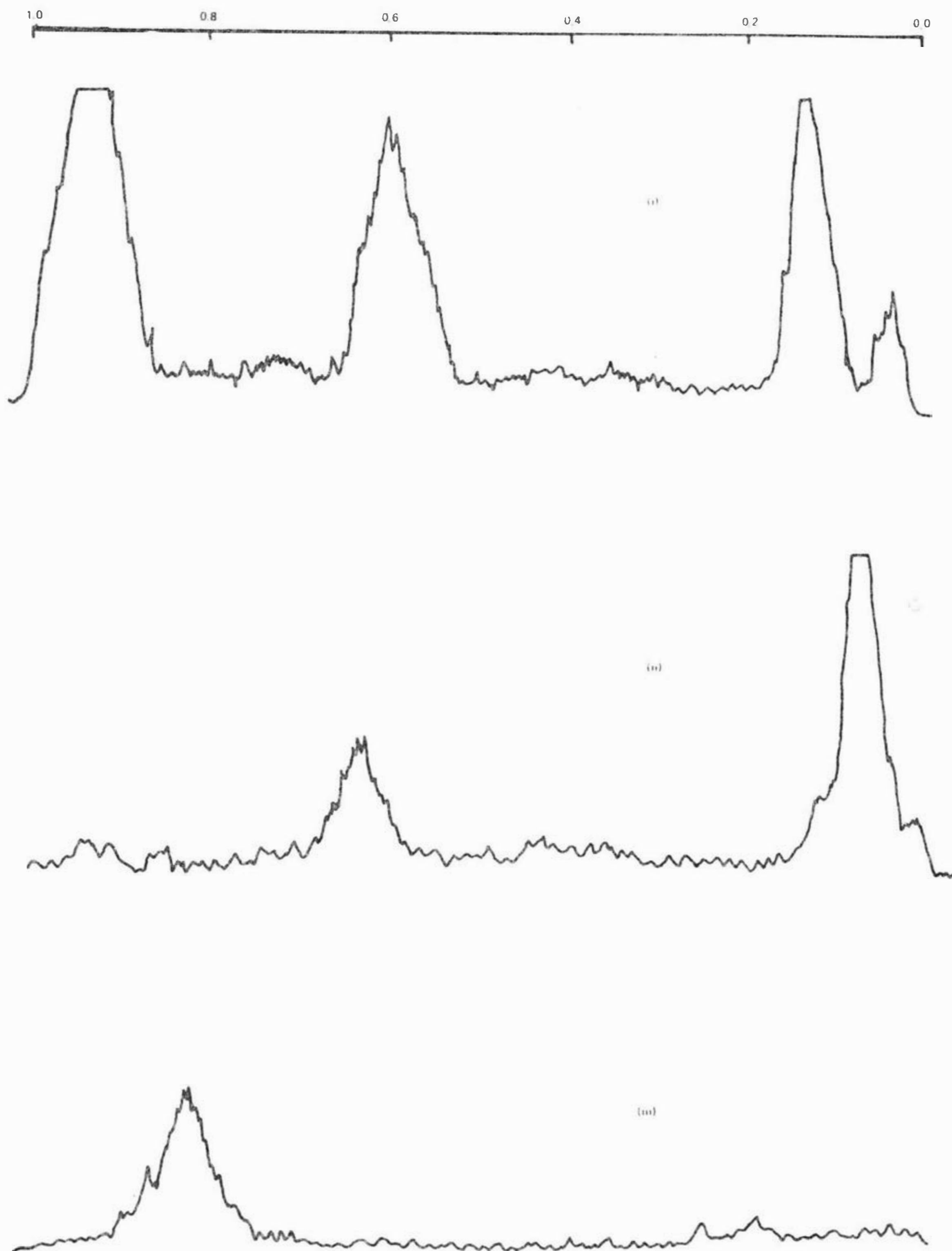


FIGURE 4.4.3 RADIOACTIVITY RECOVERED FROM TUBER PEELS 4 HOURS AFTER UPTAKE OF ^{14}C VALINE
Linear scans of radioactivity on paper chromatogram strips (i) Chromatogram of ethanol extracts in 2–Butanone Acetone Water solvent, Detector sensitivity (DS) 1×10^4, Collimator slit width (csw) 5mm, Rf of Linamarin, 0.6 (ii) Rechromatography of corresponding linamarin zone eluted from (i) in Butanol Water solvent, DS, 3×10^3, csw, 5mm, Rf of linamarin, 0.4 (iii) Rechromatography of corresponding linamarin zone eluted from (ii) in Propanol Water D.S., 300, csw, 2mm, Rf of Linamarin, 0.72.

chromatogram developed in the second solvent and linamarin activity estimated after enzymic hydrolysis by assaying ^{14}C activity in the released acetone-semicarbazone crystals. The results given in Table 4.4.3 show a maximum incorporation in the 10-hour peel sample of 2.2%. The lower value of 1.3% incorporation for the 24 hour sample and the decrease in specific activity between 10 and 24 hours may have been due to partial breakdown or turnover of synthesised linamarin.

SECTION 5

TRANSLOCATION OF LINAMARIN IN CASSAVA

5.1 Introduction

The valine feeding experiments described in section 4 have shown that the cassava tuber system does not readily convert exogenous valine to linamarin. Assuming that this is indicative of a low *in vivo* rate of linamarin synthesis in the tuber then most of the linamarin synthesis must take place in the shoot tissues of the plant. The low biosynthetic activity of the tubers contrasts with their relatively high linamarin content. This suggests that the tuber linamarin probably arises from translocation and accumulation of linamarin previously synthesised by some other parts of the plant. The mobilization of linamarin in Cassava has scarcely been investigated. de Bruijn (1971) carried out stem ringing experiments on Cassava and reported accumulation of cyanogenic glucosides above the point of incision. This was interpreted as evidence for movement of cyanogenic glucosides towards the lower section of the plant. Further evidence for translocation of linamarin in Cassava is lacking in the literature. In this section the results of experiments designed to investigate linamarin translocation in Cassava are presented. Specifically labelled linamarin was synthesised *in situ* in the leaf system. The ability of Cassava leaves to synthesise linamarin from volatile precursors (section 3) made it possible to administer 2-hydroxy [1-^{14}C] isobutyronitrile directly to the physically undamaged leaf while still attached to the plant. The disappearance of the labelled linamarin from the fed leaf and its appearance in other tissues could then be followed with minimum disturbance to the plant.

5.2 Preliminary Experiments on Translocation

The survey of the ability of different cassava tissues to convert ^{14}C valine to linamarin indicated that the mature petiolate leaf and shoot apex were active biosynthetic sites. A preliminary experiment was carried out to investigate the feasibility of determining ^{14}C linamarin translocation from these two tissues. The shoot apex and petiolate leaf blade of two-month old Manioke plants were fed in separate experiments with ^{14}C labelled 2-hydroxyisobutyronitrile* by the vapour uptake technique (section 3). After a brief labelling period (3 hours) the labelling chamber was removed and the plants maintained under normal glasshouse conditions for a seven day period. At the end of the period the plant was divided into various parts and the ^{14}C-linamarin extracted and determined.

5.2.1 Translocation following administration of precursor to the mature leaf.

The intact leaf was selected midway within the canopy of a 75 cm plant grown in solution culture. The plant itself had an actively growing apex and a root system consisting essentially of a proliferation of non-tuberous small primary roots. The leaf was administered with 17.8 μCi of freshly prepared precursor. The feeding system and details of the feeding procedure have been described in section 3. About 96% of the precursor was taken up during a 3-hour exposure time after which the leaf was exposed to the normal glasshouse conditions for 7 days. The plant was then harvested and sectioned into the following parts: The upper leaves consisted of all leaves and their petioles above the

source leaf; the upper stem was the stem section bearing the upper leaves; the lower leaves and lower stems were the respective sections below the labelled leaf and the root system consisted of the primary roots. All sections were weighed and extracted in boiling aqueous ethanol. As well as isolating the linamarin by paper chromatography other labelled substances were separated by two dimensional TLC-autoradiographic techniques as described in section 2.

Autoradiographs of the ethanol extracts indicated the presence of several labelled substances other than linamarin in most tissues containing detectable ^{14}C. The pattern of other labelled products in the fed leaf was similar to that obtained in the detached leaf experiment (section 3). Asparagine was the major ^{14}C amino acid with minor radioactive spots corresponding to glutamine, glutamic acid, and aspartic acid in the source leaf. Labelled asparagine and linamarin were present in all the major sections, although only at very low levels in the roots.

The strip scans of one dimensional paper chromatograms are shown in figure 5.2.1 and linamarin recoveries from the extracts presented in Table 5.2.1. About 74% of the total recovered linamarin activity was still located in the source leaf plus the petiole. Most of the labelled linamarin recovered beyond the source leaf was located in the stem and the shoot apex. Only a very small amount of labelled linamarin was recovered from the primary roots. The total ^{14}C-linamarin recovery represents about 20% of the expected amount of linamarin originally synthesised in

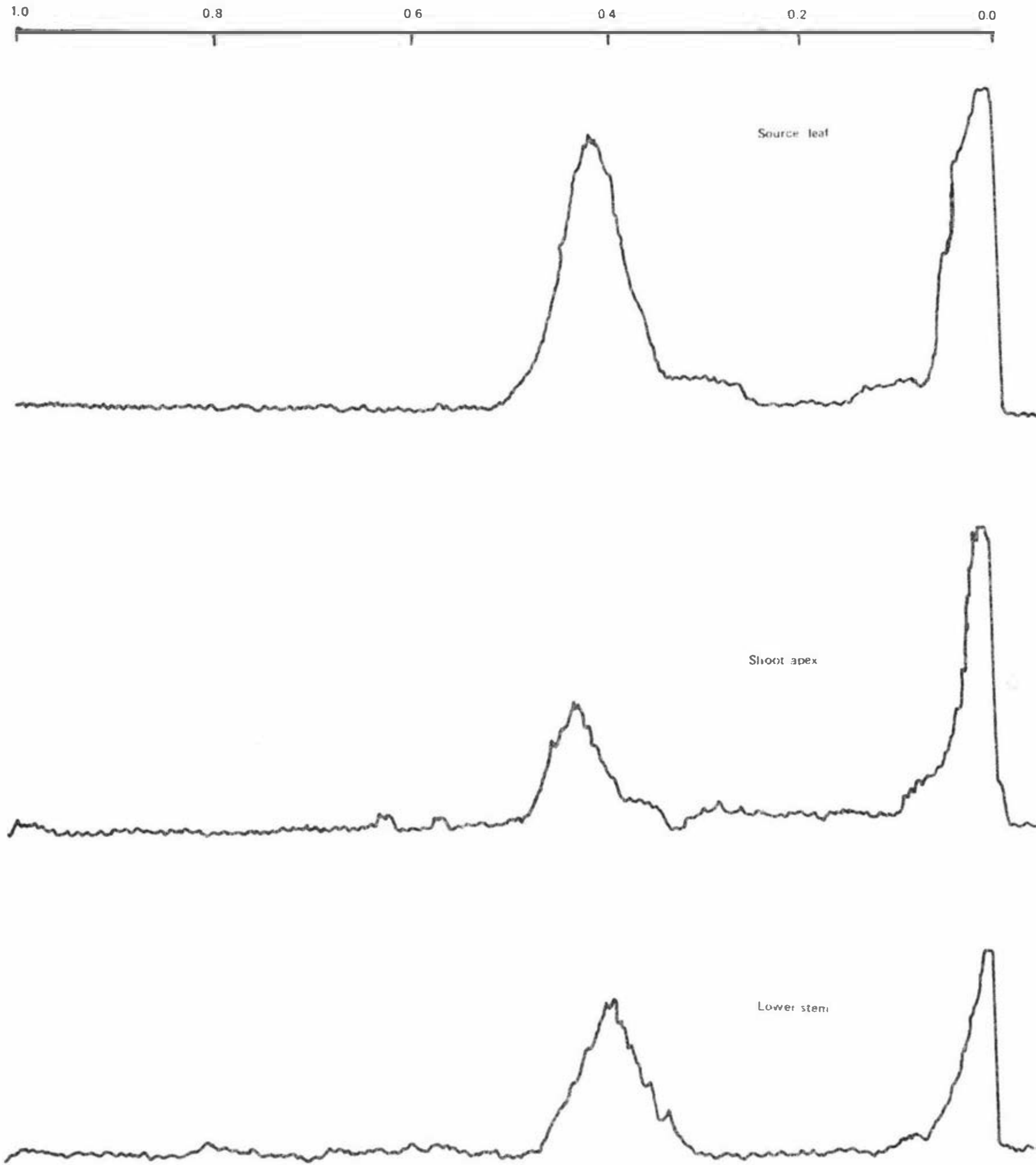


FIGURE 5.2.1. RADIOACTIVITY RECOVERED FROM SHOOT APEX , LOWER STEM AND SOURCE LEAF OF IMMATURE PLANTS 7–DAYS AFTER UPTAKE OF 2–HYDROXY[1–^{14}C] ISOBUTYRONITRILE BY THE SOURCE LEAF Linear scans of radioactivity on paper chromatogram strips developed in Butanol Water. Linamarin has Rf. 0 4

Table 5.2.1

Distribution of Residual Linamarin Synthesised in Cassava Leaves

PLANT SECTION		LINAMARIN		TRANSLOCATION
Sample	Weight(gm)	μCi($x10^{-2}$)	S.A.(μCi/mmol)	% of Recovered linamarin
Source leaf blade	2.0	47.67	14.45	70.1
Source leaf petiole	1.2	2.65	3.08	3.9
Shoot apex	3.0	5.00	0.60	7.4
Upper stem	1.8	4.00	1.29	5.9
Lower stem	28.0	7.60	0.54	11.2
Upper leaves	9.7	0.75	0.12	1.1
Lower leaves	1.0	0.04	0.03	0.1
Roots (primary roots)	40.0	0.30	0.08	0.4

the source leaf. This figure is based on the ^{14}C-linamarin level obtained in detached leaf feeding experiments (section 3)*. The low recovery of total ^{14}C-linamarin presumably represents turnover within the tissues over the 7-day period.

5.2.2 Translocation following Administration of Precursor to the Shoot Apex.

Linamarin was specifically labelled in the shoot apex region by vapour administration, of 2-hydrox[1-^{14}C] isobutyronitrile using the same procedure as described in the previous experiment. The 'apex' consisted of the growing tip region with young leaves of less than $\frac{1}{3}$ matured leaf area. Precursor uptake was achieved by exposing the apex to the vapour in the enclosed feeding chamber. About 92% of the precursor was taken up and after 7 days various organs and tissues were extracted and analysed for ^{14}C-linamarin.

^{14}C linamarin was present at a high level in the shoot apex while the other sections apart from the stem failed to indicate significant ^{14}C linamarin above the background level. The quantitative data for linamarin distribution in the plant presented in Table 5.2.2 indicate that very little linamarin was translocated from the labelled shoot apex. About 96% of the total linamarin activity in the plant was present in the tissues of the fed apex.

The shoot apex experiment confirms earlier findings

* subsequently confirmed for an attached leaf (see p.102)

Table 5.2.2

Distribution of Linamarin Synthesised in Shoot Apex

PLANT SECTION		LINAMARIN		TRANSLOCATION % Recovered Linamarin
Sample	Weight(gm)	μCi($x10^{-2}$)	S.A.(μCi/mmol)	
Shoot apex	2.0	51.80	9.40	96.0
Stem	17.5	1.70	2.00	3.2
Roots	48.0	0.30	0.10	0.5
Leaves	8.2	0.20	0.03	0.3

of high biosynthetic activity in this tissue when fed with ^{14}C-valine (section 4.2.2). The present results indicate, however, that there was little linamarin translocation from this source to other parts of the plant, although a significant proportion of the linamarin translocated from the leaf was recovered from the apex.

The mature leaf, on the other hand, does appear to export a measurable proportion of the linamarin synthesised. While the major proportion of recovered linamarin was still present in the source leaf, the preliminary experiment demonstrates the feasibility of studying linamarin translocation and distribution in cassava following vapour labelling of the leaf linamarin. A more detailed study was subsequently undertaken using plants at different levels of maturity.

5.3 Linamarin Translocation in Immature Non-Tuberous Cassava Plants

Initial translocation experiments were carried out on young cassava plants lacking tubers. This was partly because tuber-bearing, solution-cultured plants were not available until a late stage in the present study. Secondly it was thought to be of interest to compare the translocation pattern in a young actively growing non-tuberous plant with that of a mature tuber-bearing plant since one might expect the development of underground storage organs to have a marked effect on

the direction of net translocation from the leaves. In this section results are presented for the rate of disappearance of ^{14}C labelled linamarin from a mature leaf and its distribution to other parts of the plant. The possibility that the translocation pattern may differ according to the position of the 'source leaf' on the plant was also investigated.

5.3.1 Linamarin Turnover in the Source Leaf.

One of the problems inherent in investigating translocation of linamarin by following the disappearance of ^{14}C-linamarin from a source leaf is to distinguish between loss due to linamarin breakdown in the fed leaf and loss due to translocation to other parts of the plant. The extent to which breakdown occurs in the source leaf was investigated by a time-course analysis of changes in freshly synthesised ^{14}C linamarin within a detached leaf. Excised mature leaves were administered with 2-hydroxy [1-^{14}C] isobutyronitrile for 3 hours under experimental conditions as previously described (section 3.1.2). At various time intervals discs of about 6 mm diameter were punched from the blade section using a cork-borer. Discs were then quickly weighed and extracted in boiling ethanol. In the results illustrated in figure 5.3.1 each point is the average of duplicate samples (taken from two separate leaves).

Endogenous linamarin level showed only a very small decrease throughout the 60 hour period of investigation. Labelled linamarin decreased by only about 5% with the

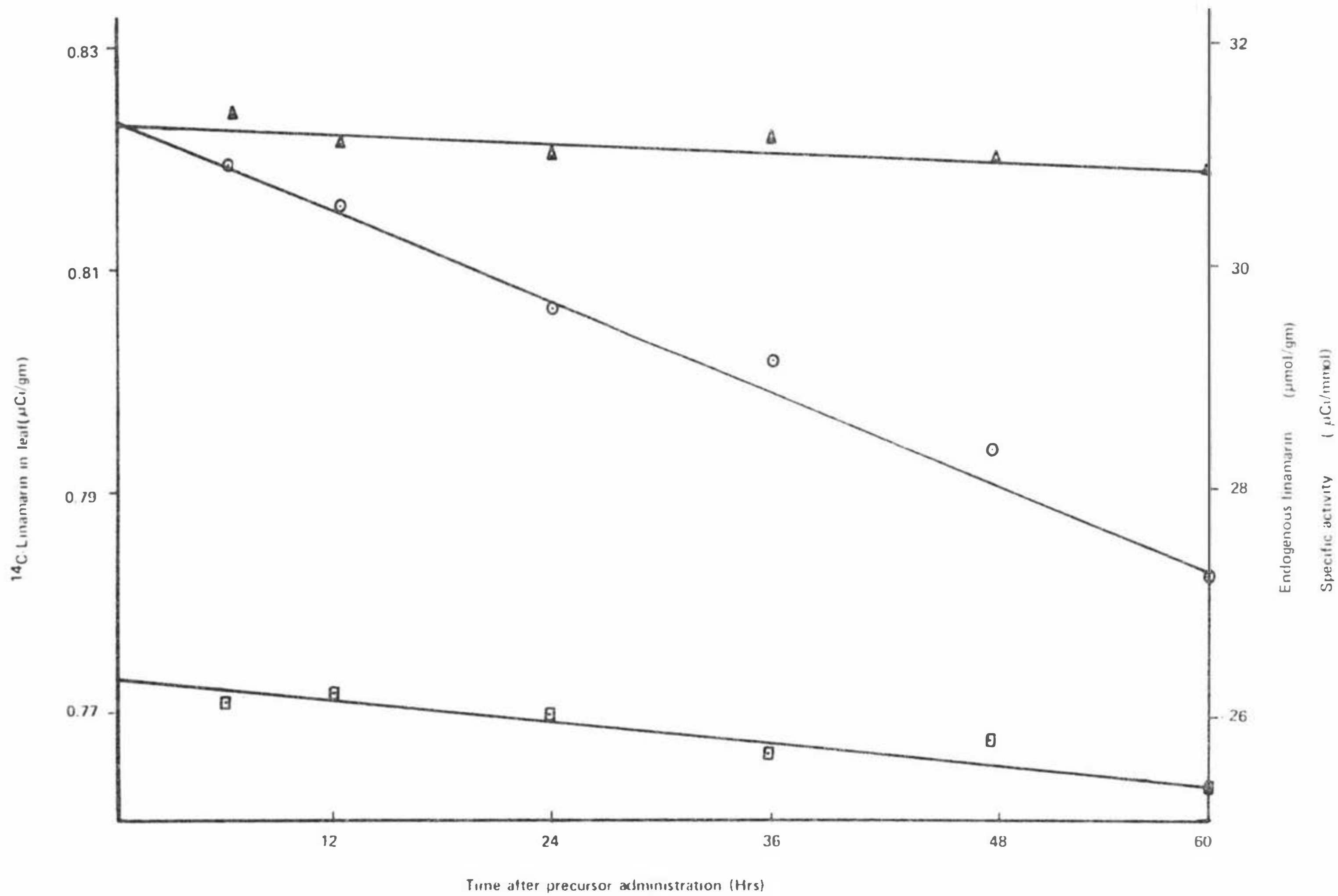


FIGURE 5.3.1 CHANGES IN ^{14}C LINAMARIN AND ENDOGENOUS LINAMARIN IN DETACHED LEAVES OF VARIETY MANIOKE. ⊙ ^{14}C-Linamarin in leaf Δ: Specific activity ⊡: Endogenous linamarin

result that there was little major change in the specific activity of linamarin. There was no significant loss of ^{14}C into the nutrient solution in which the petiole was immersed suggesting that there was no major translocation out of the blades. The very small decrease in ^{14}C-linamarin may therefore be due to metabolic breakdown in the fed leaf.

Due to possible disturbance of normal metabolic patterns the changes in linamarin content in detached leaves may not necessarily reflect conditions in the attached leaf. However it seems unlikely that degradation would be more active in attached leaf than a detached leaf. Therefore disappearance of ^{14}C-linamarin from an attached leaf may be used as a reasonable indication of translocation.

5.3.2 Translocation of Linamarin from Leaves.

Translocation was investigated in 2-month old solution-cultured non-tuberous Manioke plants. Linamarin was specifically labelled by 2-hydroxy[1-^{14}C] isobutyronitrile administration to either the upper or lower leaves and translocation and distribution in the plant was investigated through 7 days. The upper leaf consisted of the first fully expanded leaf at the apex end of the plant; one of the leaves approximately a third of the distance up the stem was selected as the lower source leaf. After the normal 3-hour uptake of the precursor, leaf

discs were periodically punched from the blades and analysed for linamarin. The leaf punching avoided the main veins so as to minimise interference of injury with subsequent translocation. After 7 days the plants were divided into major sections and analysed for ^{14}C-linamarin activity. The experiments were conducted in triplicate for both upper and lower leaf labelling i.e. the data presented are averages of separate values derived from three different plants.

Labelled linamarin recovered in the leaf discs after various time intervals was expressed as a percentage of the original linamarin in the initial punched discs. Residual ^{14}C-linamarin and the specific activity of linamarin recovered are illustrated in figures 5.3.2 and 5.3.3 for both the upper and lower labelled leaves (source leaves). In both cases ^{14}C linamarin content decreased rapidly in the leaf over the first 21 hours. After 69 hours the ^{14}C linamarin had fallen to about 30% of the initial activity in the case of the upper leaf and to about 24% of the initial ^{14}C level for the lower source leaf. After 93 hours there was little further decrease in ^{14}C linamarin content. Throughout the 7 days total endogenous linamarin levels in the discs remained virtually constant; the result was that the specific activities of recovered linamarin decreased along a similar time course as total ^{14}C linamarin in both leaf types (figure 5.3.3). The time taken for 50% of the initial ^{14}C linamarin to disappear ($t_{\frac{1}{2}}$) was 14 hours for the lower leaves and 31 hours for the upper leaves.

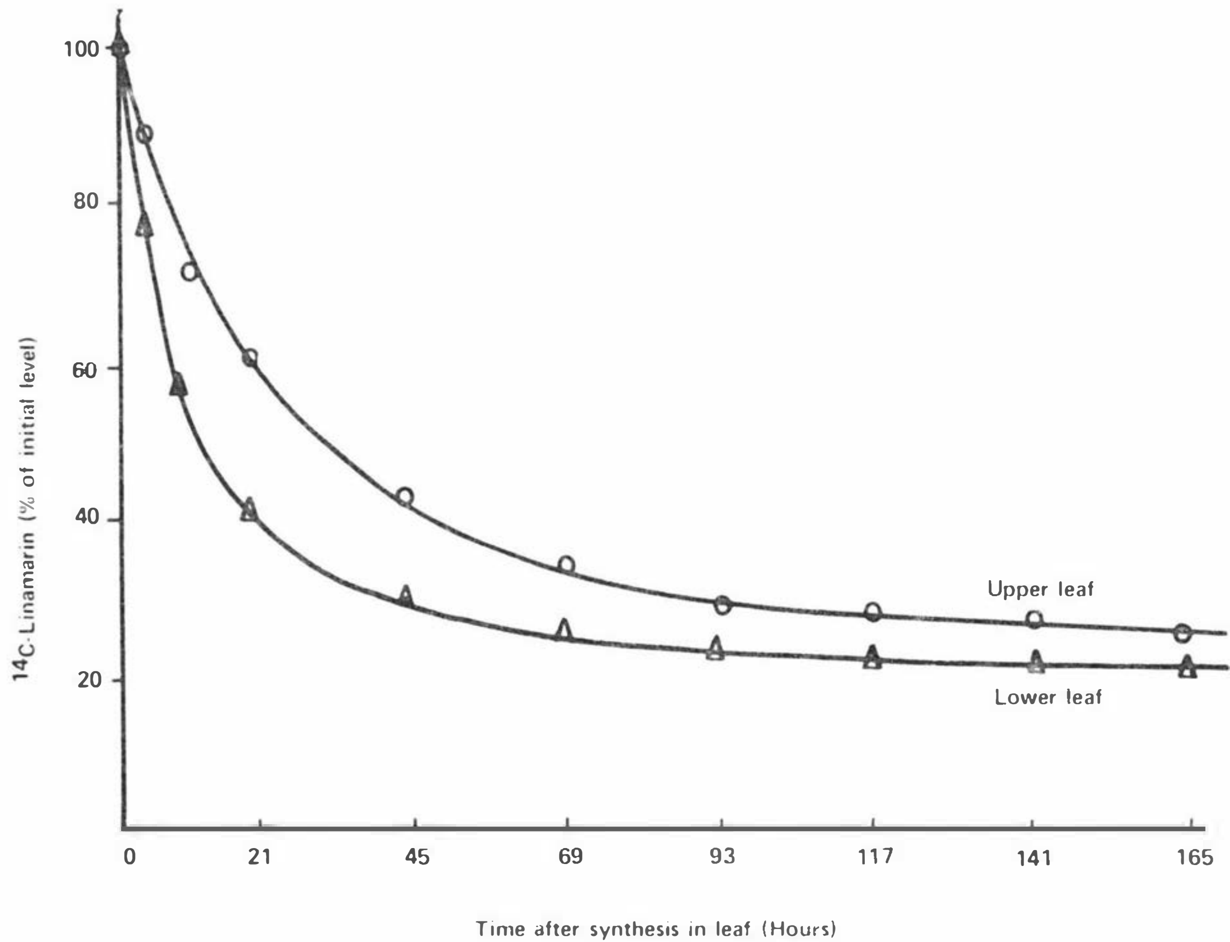


FIGURE 5.3.2 KINETICS OF ^{14}C-LINAMARIN TRANSLOCATION OUT OF LEAVES OF IMMATURE CASSAVA PLANTS. ^{14}C-linamarin level in leaf discs taken various times after precursor administration. Each point represents the mean of samples from 3 separate plants of variety Manioke.

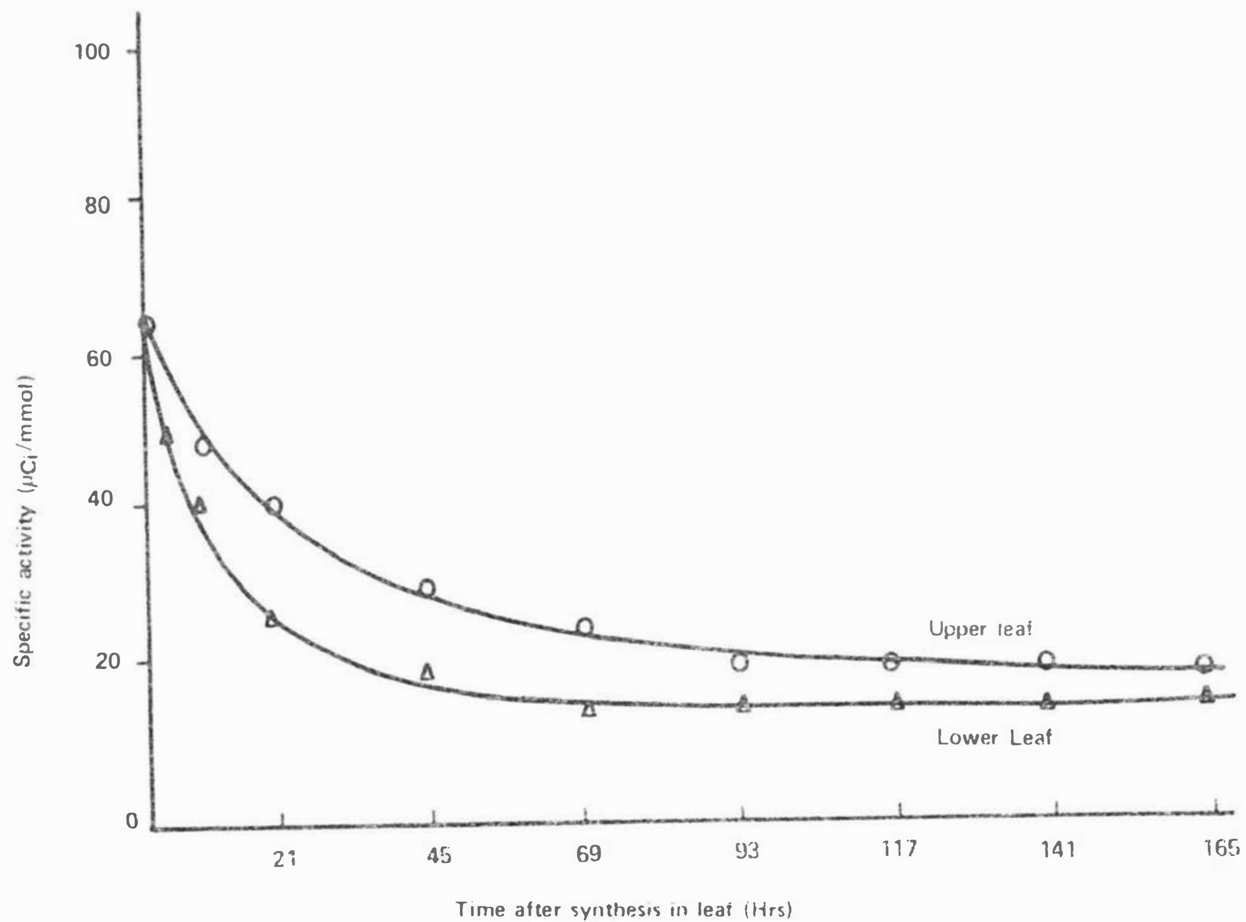


FIGURE 5.3.3. KINETICS OF ^{14}C-LINAMARIN TRANSLOCATION OUT OF LEAVES OF IMMATURE CASSAVA PLANTS. Time course of specific activity of linamarin in leaf discs. Each point represents the mean of 3 samples from 3 separate plants of variety Manioke.

Loss of linamarin from discs may be brought about by linamarin breakdown within the leaf or translocation out of the leaf. The time-course analysis of detached leaves (figure 5.3.1) suggest that linamarin breakdown alone could not account for the decreases observed in the intact leaves. The observed losses are therefore probably due mainly to linamarin translocation out of the leaves.

The average overall recovery of ^{14}C-linamarin was about 16% of anticipated initial synthesis in both upper and lower leaf-fed plants based on a value determined from the ^{14}C linamarin synthesised in detached leaves (section 3.2.2). The distribution of recovered ^{14}C linamarin in different organs of the plants (Tables 5.3.1 and 5.3.2) indicates that most of the ^{14}C linamarin present after 7 days was recovered from the source leaves. Of the ^{14}C linamarin recovered from outside the source leaf about 53% and 36% was recovered from the upper and lower source leaf petioles respectively, an indication that the petioles of these young plants retain an appreciable proportion of the translocated fraction of leaf-synthesised linamarin.

Translocated ^{14}C linamarin arriving in the stem was distributed in both apical and basal directions. The stem itself accounted for about 3% of the linamarin recovered when the upper leaf was source and 4.4% when the lower leaf was the source. Basal translocation from the lower leaves was somewhat greater than from the upper leaves. A small amount of linamarin was recovered

Table 5.3.1

Distribution of ^{14}C-Linamarin in non-tuberous plants 7 days after Synthesis in Upper Source Leaf

Plant Organ	Fresh weight (gm)	^{14}C-linamarin recoveries: % of initial ^{14}C-linamarin in source leaf	^{14}C-linamarin recoveries: % of total ^{14}C-linamarin recovered
Primary roots	73.0	0.1	0.6
Lower stem	22.0	0.2	1.5
Lower leaves	23.4	0.0	0.1
Upper stem	5.3	0.3	1.6
Upper leaves	12.7	0.3	1.6
Source leaf petiole	2.2	1.0	6.1
Source leaf	2.8	14.1	88.1

Table 5.3.2

Distribution of ^{14}C-linamarin in non-tuberous plants 7 days after synthesis in lower leaves.

Plant Organ	Fresh weight (gm)	^{14}C-linamarin recoveries	
		% of initial ^{14}C-linamarin in source leaf	% of total ^{14}C-linamarin recovered
Primary roots	50.0	0.2	1.2
Lower stem	13.0	0.3	1.9
Lower leaves	5.6	0.0	0.1
Upper stem	17.4	0.2	2.5
Upper leaves	33.1	0.3	2.0
Source leaf petiole	0.8	0.7	4.3
Source leaf	1.6	13.8	86.4

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from the root system but the level was higher in the case of the plants translocating ^{14}C from the lower leaves.

The leaves below each labelled leaf received only small amounts of translocated linamarin while significant activities were recovered from the leaves above the source leaves. The distribution of linamarin to individual upper leaves followed the pattern of leaf arrangement on the plant (figure 5.3.4). Leaves on the same orthostichy as the source leaf received the highest translocated activity and leaves on neighbouring orthostichies also received higher translocated ^{14}C-linamarin than more distant leaves. Within the same orthostichy younger leaves imported more ^{14}C translocated linamarin than older leaves.

5.4 Linamarin translocation in Mature Tuberous Cassava Plants

Translocation of leaf-synthesised ^{14}C-linamarin was investigated in mature tuberous plants by feeding the leaf with 2-hydroxy[1-^{14}C] isobutyronitrile. Preliminary experiments were carried out to measure incorporation and the extent of short term translocation in the plant. This was followed by two long-term experiments in which disappearance of linamarin from the fed leaf was investigated by means of leaf discs punched from the blade and analysed for ^{14}C-linamarin. Eventually plants were harvested after 7 days when distribution of ^{14}C-linamarin in various tissues was determined. The long-term

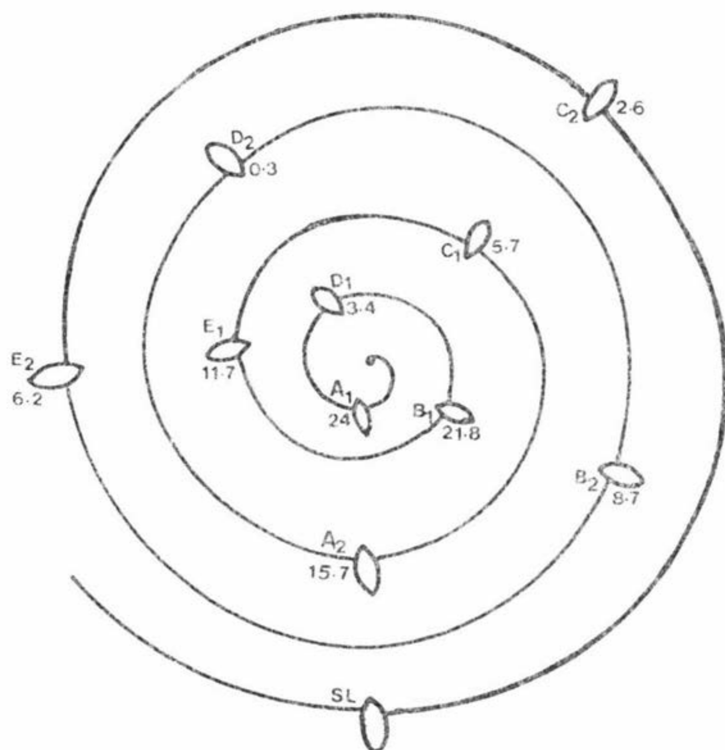

FIGURE 5.3.4. DIAGRAM ILLUSTRATING THE DISTRIBUTION OF ^{14}C LINAMARIN FROM LOWER TO UPPER LEAVES WITH RESPECT TO PHYLLOTACTIC CONFIGURATION The figure besides each leaf represents ^{14}C linamarin distribution to the leaf expressed as a percentage of total ^{14}C linamarin recovered from the upper leaves. The Manioke variety has a 2/5 phyllotaxis. SL: Source leaf. A–E: Orthostichies.

experiments were intended as duplicate experiments to investigate reproducibility of results but in the second experiment the fed leaf entered a senescent yellowing phase during the course of the 7 day experiment. It thus inadvertently gave a comparison between translocation from a mature green leaf and a senescing leaf. The second experiment is therefore reported separately.

For all experiments described in this section tuberous 8-month old Beqa clones grown in gravel culture (section 2) were used. Each plant had branched at about 70 cm up the stem. A leaf was selected midway up the primary stem and fed with 2-hydroxy[1-^{14}C] isobutyronitrile vapour in the same way as in previous experiments (section 3.1.2).

5.4.1 Preliminary experiment to measure Incorporation after 4 hours

In this experiment the fed leaf was removed from the chamber and leaf discs punched out of the blade section to provide a comparison of ^{14}C linamarin in the leaf lamina and the combined blade tissues. The rest of the plant was harvested, divided into various sections and extracted in boiling ethanol. The results (Table 5.4.1) indicate a 19.5% incorporation of the precursor ^{14}C into linamarin. This value was comparable to the usual 20% incorporation previously obtained for detached leaves (section 3.2.2).

The linamarin in punched discs had considerably lower specific activity than in the combined source leaf.

Table 5.4.1

Distribution of Linamarin Activity in Tuberous Plant 4 hours after Synthesis

PLANT ORGAN		LINAMARIN			
Sample	Fresh weight(gm)	μmol/gm	μCi	% Recovery	μCi/mmol
Tubers	264.0	8.64	N.D.[1]	N.D.	N.D.
Primary roots	142.0	1.65	N.D.	N.D.	N.D.
Lower stem	136.0	7.40	0.0736	1.5	0.07
Upper stem	78.6	9.8	N.D.	N.D.	N.D.
Upper leaves	164.1	27.3	N.D.	N.D.	N.D.
Source leaf petiole	2.1	5.7	0.109	2.1	9.00
Source leaf	4.27	25.1	4.904	96.4	46.00
Punched discs	0.051	25.5	0.034	0.67	26.20

[1]: (N.D.); Not detectable

Also the content of ^{14}C-linamarin was lower in the discs (0.67 μCi/gm.fr.Wt.) than in the source leaf as a whole (1.10 μCi/gm.Fr.Wt.). This difference may be accounted for possibly by a rapid loss of ^{14}C-linamarin from the blade tissue to the main veins and midribs. The presence of a separate pool of unlabelled linamarin in the leaf mesophyll tissue may also contribute to the observed difference in specific activity. It is also possible that the tissues associated with the veins and midribs may be more actively synthesising ^{14}C linamarin than the punched sections; such a tendency would be consistent with greater incorporation of ^{14}C valine into linamarin by the midribs tissue than the blade tissue (section 4.3.2.2).

About 3.6% of the ^{14}C-linamarin was recovered beyond the fed leaf after only 4 hours, an indication of early ^{14}C linamarin translocation. Translocated linamarin was recovered mainly from the petiole and lower stem section. Basipetal ^{14}C-linamarin translocation was therefore the predominating direction of translocate flow at this early stage.

5.4.2. Translocation from a Mature Green Leaf over a 7 day period.

Linamarin was specifically labelled in the attached leaf of the tuberous plant as in previous experiments and the time-course of its disappearance from the leaf was followed by measuring linamarin activities in the punched discs. The time-course of ^{14}C linamarin loss from the fed leaf blade is shown in figure 5.4.2.1. The results indicate a decrease up to

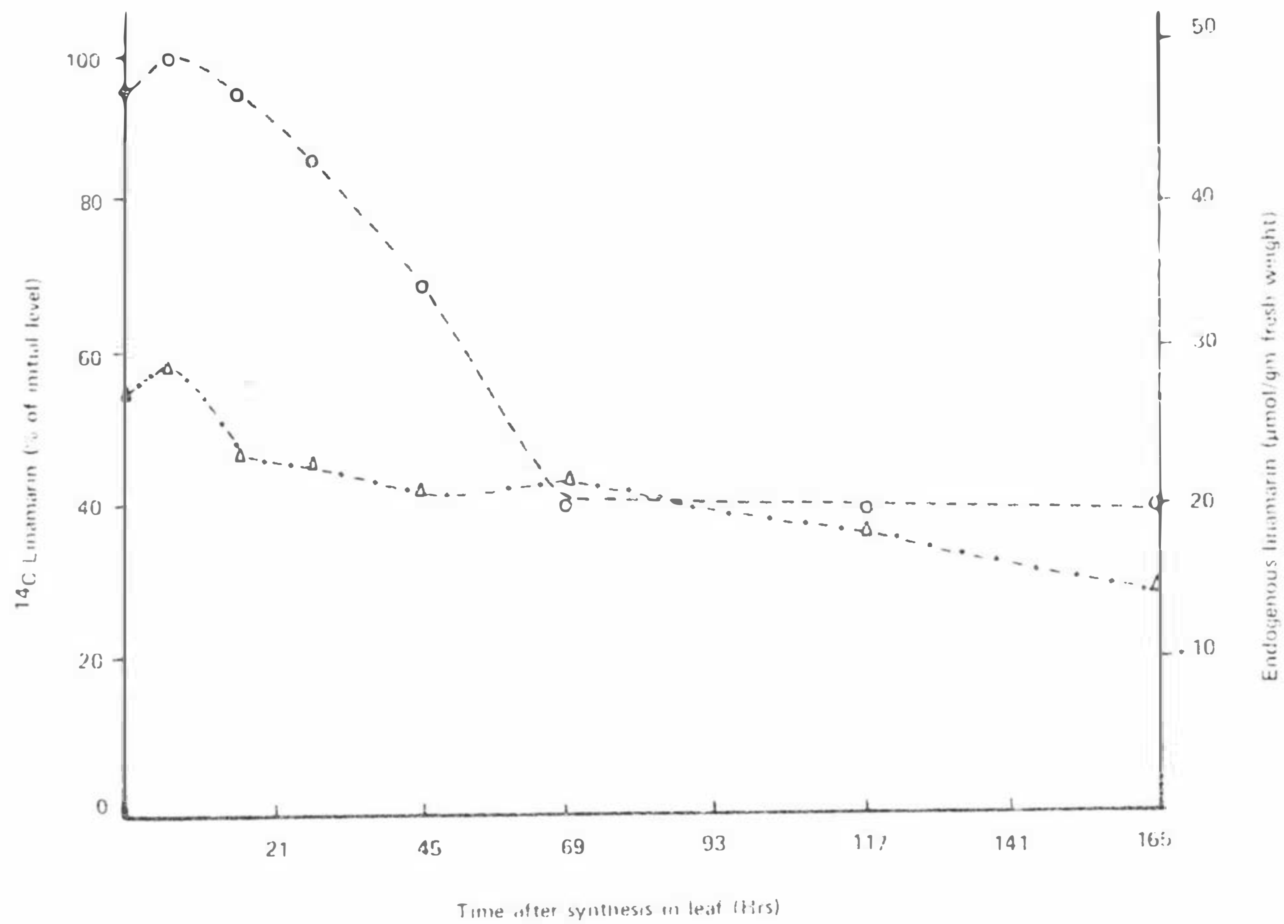

FIGURE 5.4.2.1 LINAMARIN TRANSLOCATION IN MATURE CASSAVA (Var. BEQA) Trend Lines indicating the time course of changes in linamarin in source leaf discs of mature tuberous plant.

O: ^{14}C Linamarin (% of initial level) ; Δ : Endogenous linamarin (µmol/gm fresh weight)

69 hours after synthesis after which time the level of ^{14}C-linamarin remained virtually constant between 69 hours and 165 hours. There was a decrease to a lesser extent in endogenous disc linamarin. Thus in contrast to the findings with immature, non-tuberous plants, the specific activity of the linamarin did not change significantly over the seven day period.

The distribution of linamarin in separate organs is shown in Table 5.4.2.2. The total recovery of ^{14}C linamarin was 31.4% as based on initial conversion of 20% of precursor to linamarin (Section 5.4.1 and section 3). The source leaf blade contained the major proportion of the linamarin recovered after 7 days although the amount of ^{14}C-linamarin retained was only 25% of the initial ^{14}C linamarin. Contrary to findings in section 5.4.1, the residual ^{14}C-linamarin level in the discs (0.21 μCi/gm) after 7 days was similar to that in the combined leaf as a whole (0.26 μCi/gm). The source leaf petiole accounted for just 3% of the overall linamarin recovered outside the tissues of the fed leaf; the corresponding figure for petioles of immature source leaves was 36-53% (Tables 5.3.1 and 5.3.2). About 7% of the total recovered ^{14}C-linamarin was located in the tubers representing 37% of the recovered ^{14}C-linamarin as translocated ^{14}C-linamarin.

5.4.3 Translocation from a senescing leaf over a 7-day period

The source leaf was exposed, as a fully green leaf, to 2-hydroxy[1-^{14}C] isobutyronitrile vapour for 3 hours. The uptake of ^{14}C precursor was 81% giving

Table 5.4.2.2

Distribution of Linamarin Activity in Tuberous Plant 7 days after Synthesis in Leaf

Plant Organ	Fresh Weight (gm)	LINAMARIN				
		μmol/gm	μCi	% Initial Activity	% Recovered Activity	S.A. μCi/mmol
Tubers	611.8	5.13	0.11	2.4	7.1	0.04
Primary roots	285.5	0.84	0.03	0.7	1.9	0.13
Lower stem	176.2	4.22	0.10	2.2	6.4	0.13
Upper stem	120.9	8.45	0.03	0.7	1.9	0.03
Upper leaves	190.7	16.20	0.03	0.7	1.9	0.01
Source leaf petiole	2.41	1.90	0.01	0.2	0.6	2.18
Source leaf	5.83	14.20	1.25	24.5	80.1	15.05

an expected initial yield of 5.7 μCi based on the assumption of 20% incorporation (section 5.4.1). Leaf discs were punched from the blades as before and analysed for ^{14}C-linamarin over a 7-day period. The onset of senescence was observed on the third day by the typical colour change from green to yellow and by the seventh day the leaf had turned completely yellow. Total ^{14}C-linamarin recovered was 10.4% of the expected initial ^{14}C-linamarin, a low value compared to 31.4% in the previous 7-day experiment.

Distribution of ^{14}C-linamarin among the various organs is presented in Table 5.4.3.1. In this case only 3.7% of the total linamarin recovered from the plant was still present in the source leaf. The highest activities were recovered from the tubers and the basipetally recovered translocate was twice as much as the apical recoveries.

The changes in ^{14}C-linamarin in punched leaf discs are shown in figure 5.4.3.2. ^{14}C-linamarin activity in the leaf discs showed a steady drop up to 7 days. The decline in disc ^{14}C-linamarin was correlated with the drop in endogenous linamarin content resulting in only a small change of the specific activity in the discs. The depletion of ^{14}C-linamarin and endogenous linamarin during senescence could be attributed to both translocation and turnover. The recovery of ^{14}C-linamarin obtained beyond the source leaf was 0.75 μCi for the senescing leaf compared with 0.31 μCi for the non-senescing leaf.

Table 5.4.3.1

Linamarin Translocation in Tuberous Plants - Distribution during leaf Senescence

Plant Organ	Fresh Weight (gm)	LINAMARIN				
		µmol/gm	µCi	% Initial Activity	% Recovered Activity	S.A. µCi/mmol
Tubers	854.4	5.01	0.340	6.0	33.0	0.08
Primary roots	223.4	0.35	0.024	0.4	2.3	0.31
Lower stem	219.5	9.33	0.300	5.3	28.8	0.15
Upper stem	146.8	8.75	0.030	0.5	2.9	0.02
Upper leaves	296.5	15.20	0.028	4.9	26.8	0.06
Source leaf petiole	2.20	0.08	0.030	0.5	2.8	170.5
Source leaf blade	5.23	0.13	0.039	0.5	3.7	45.6

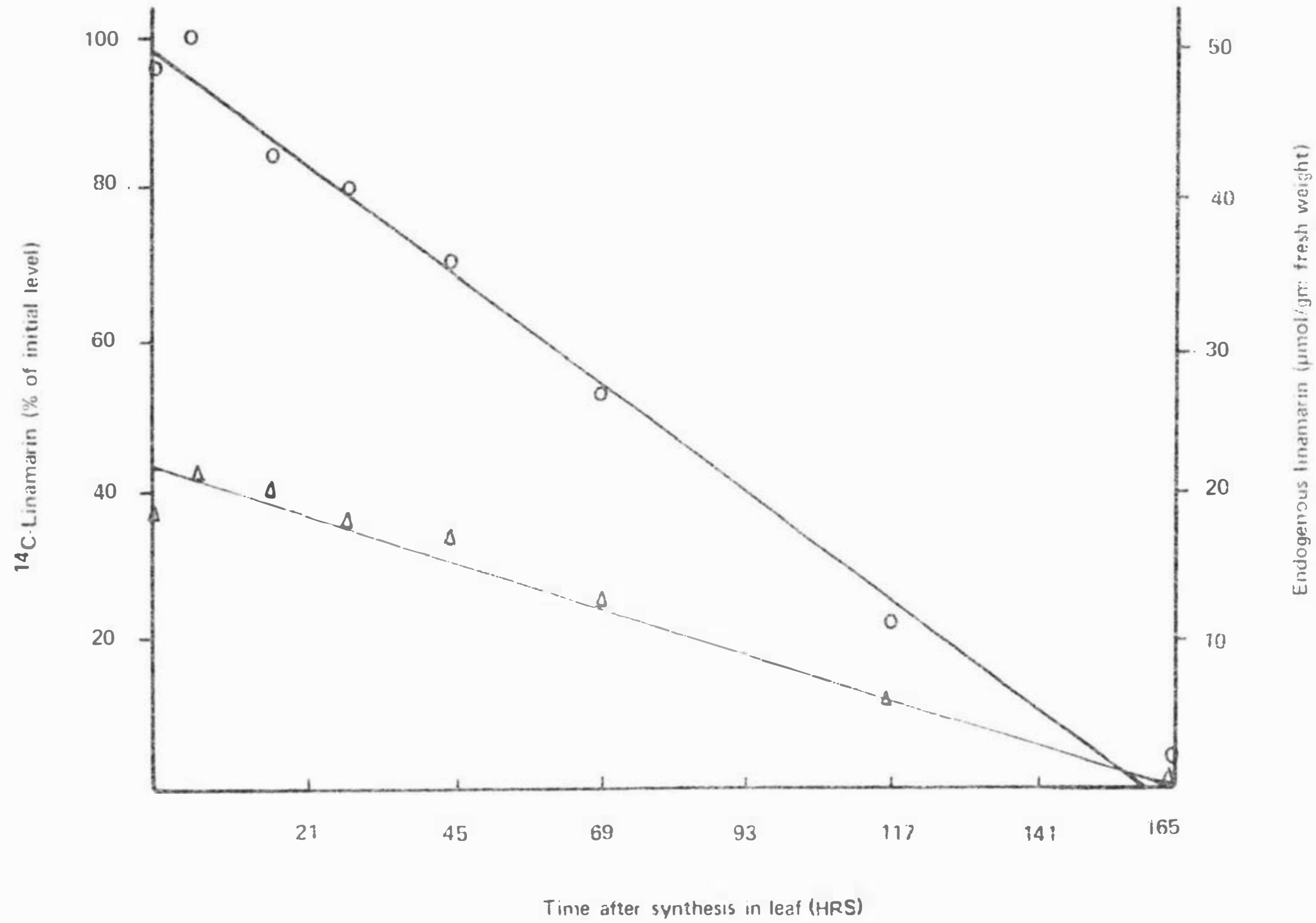

FIGURE 5.4.3.2. LINAMARIN TRANSLOCATION IN MATURE CASSAVA (Var BEQA). Time-course of changes in linamarin in seneseing source leaf discs on a tuberous plant. Δ : Endogenous linamarin (µmol/gm fresh weight)
O : ^{14}C-Linamarin (% of initial level)

5.4.4 Changes in Linamarin content in leaves during senescence

In view of the results obtained from the study of translocation from a senescent leaf a further brief study of the extent to which translocation and linamarin turnover in the leaf affects the endogenous linamarin levels during senescence was undertaken. By detaching a leaf at the first detectable signs of senescence, translocation out of the leaf was arrested. Subsequent changes in linamarin content of the detached leaf were measured to give some indication of linamarin breakdown. The combined effects of both translocation and breakdown were also investigated by measuring total linamarin contents in attached leaves at various stages of senescence. The commencement of senescing was taken as the onset of greenish-yellow colouration in the leaf. Leaves were detached at the base of the petiole and fed through the cut end with ½ strength nutrient solution (Table 2.2.2). Leaves at a comparable stage of senescence and at a similar location on the plant were used for following changes in linamarin while each leaf remained attached to the plant. At 24 hour intervals one (or two) of the 7 leaflets of each leaf was excised, weighed and quickly extracted in boiling ethanol. Each leaf experiment was performed in duplicate. Aliquots of combined extracts were measured for chlorophyll content (by determining absorbance at 652 nm) to give a quantitative indication of the extent of senescence. Figure 5.4.4 shows the change of linamarin content as a function of the extent of senescence (as measured by

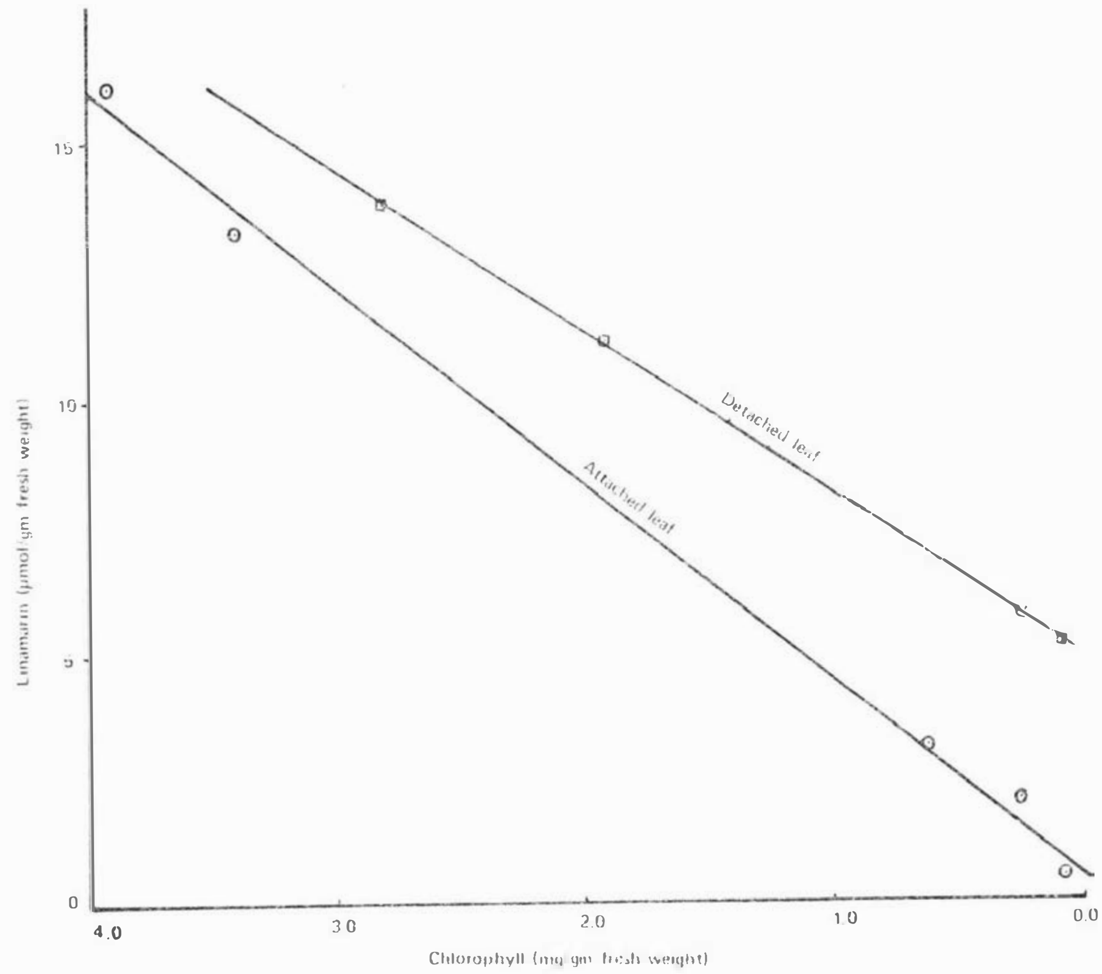

FIGURE 5.4.4. EFFECT OF SENESCENCE ON LINAMARIN CONTENT OF ATTACHED AND DETACHED LEAVES OF CASSAVA (var. BEQA). Each point represents the mean of samples from three separate leaves.

chlorophyll content) in the detached and attached senescing leaves. Although only a small number of data points could be collected, a fairly good correlation existed between degree of senescence and linamarin content in both attached and detached leaves. This trend is similar to the almost linear drop in ^{14}C-linamarin in the senescing source leaf of the previous experiment. The data clearly indicate considerable linamarin degradation during senescence. However at the stage of senescence where no chlorophyll was detectable the linamarin content of the attached leaf had dropped to an undetectable level while measurable linamarin still remained in the detached leaf.

SECTION 6

DISCUSSION

DISCUSSION

Much of the previous literature on cassava cyanogenic glucosides has dealt with the levels of the glucosides in the plant tissues, particularly in the edible tubers (Nestel, 1973). Butler (1965) found that linamarin accounts for about 93% of the cyanogenic glucosides in Manihot sp. The very low content of lotaustralin has been confirmed in the present study and consequently this investigation has concentrated on the major glucoside, linamarin. The only work on the metabolism of this glucoside in cassava was that by Nartey (1968) who demonstrated the incorporation of ^{14}C-valine into linamarin in etiolated cassava seedlings. Metabolic investigations of linamarin in cassava are desirable for a better understanding of its accumulation in the edible tubers. In order to obtain a full understanding of the factors controlling the level of linamarin in any particular tissue and the way that this is influenced by environmental factors answers to the following questions must be obtained.

1. Does linamarin biosynthesis occur in all parts of the plant or is it restricted to, or much more active in, certain tissues?

2. Can linamarin synthesised in one part of the plant (the source) be translocated to other parts (sinks) and if so, what are the major 'sources' and 'sinks' for such translocation.

3. Is linamarin a stable end product of biosynthesis or is it further metabolised in the plant. If there is a turnover further investigations should describe the abilities of different tissues to metabolise or degrade

linamarin.

The present study has made a beginning at trying to answer some of these questions.

6.1 General Method of Approach Used in the Study

The present investigations have approached these problems by administering ^{14}C labelled linamarin precursors to specific parts of the plant and following the fate of the ^{14}C label. Biosynthesis has been measured by determining the incorporation of precursor into linamarin while translocation and turnover were measured by following changes in ^{14}C-linamarin content subsequent to the labelling period. Precursor administration was considered to be preferable to the direct administration of ^{14}C-linamarin itself since, by labelling linamarin via *in vivo* biosynthesis, ^{14}C-linamarin turnover and translocation could be investigated directly at the site of synthesis itself. Furthermore a direct ^{14}C-linamarin administration could result in a substantial breakdown of the labelled substance before it can be distributed in the tissues.

The two types of procedures employed to administer precursors to cassava tissues were by solution uptake and vapour uptake. The solution uptake method involved the administration of ^{14}C-valine to detached organs or pieces of plant tissue through cut surfaces. The vapour uptake technique involved the exposure of the detached or attached organs, such as the leaf or shoot apex, to the vapour of the precursor and allowing the vapour to be taken up through the stomata. The uptake of precursor vapour offers a number of advantages over solution uptake.

By the vapour uptake method, the precursors are offered a different and more direct access to leaf mesophyll tissue compared to the longer route through the petiole and midrib tissue when solution uptake is employed. The vapour administration technique offers a more uniform distribution of labelled precursor over a wider surface area of leaf. It also affords a means of administering precursors to attached organs such that the technique of introducing ^{14}C-linamarin into such organs is analogous to the widely used technique of labelling photoassimilates in attached leaves by administration of $^{14}CO_2$. With such a technique, readily volatile precursors that could be lost by evaporation during solution feeding may easily be administered to plants. Since the volatile intermediates are more immediate precursors of linamarin than is valine, they may be assumed to label ^{14}C-linamarin more specifically since the intermediates are less likely to be metabolised by other pathways. In the case of 2-hydroxyisobutyronitrile, the precursor used in the translocation studies, there is the possibility of it being non-enzymically broken down to release $H-^{14}CN$ which can be metabolised. However at the precursor concentrations used in the present investigations it is unlikely that the free HCN would reach toxic levels.

In metabolic studies involving exogenous precursor administration there are several factors that need to be taken into account in interpreting the data. The process of precursor administration may lead to a disturbance of the normal course of metabolism, e.g.

changes in precursor pool size. The endogenous precursor may be present only in trace amounts and the administration of an exogenous precursor could lead to excessively high levels that may inhibit some enzyme along the biosynthetic pathway. This could be remedied in certain cases by administering very small amounts of precursor. Linamarin turnover may be occurring so that the level of ^{14}C linamarin present at any time does not represent the linamarin synthesised over the period. A more reliable indication of the biosynthetic activity and the existence of turnover can, however, be obtained from a time-course investigation. The ^{14}C precursor may fail to reach the synthetic sites or gain access to normal translocation routes due to compartmentation at the tissue or cellular level. The administered precursor may not be readily available for ^{14}C linamarin formation because of competing metabolic pathways within the plant tissue. The interpretation of the data presented in sections 3 to 5 will be discussed in relation to these problems.

6.2 Pathway of Linamarin Biosynthesis in Cassava

The investigation of the suitability of different precursors for ^{14}C linamarin synthesis in the leaf involved administration of the probable intermediates in the pathway of biosynthesis to the leaf tissues. The incorporation of these precursors into linamarin thus gives some information on the pathway of biosynthesis of this glucoside in cassava although this was not a primary aim of this investigation.

The incorporation of the volatile precursors,

isobutyronitrile, 2-hydroxyisobutyronitrile and isobutyraldoxime, into linamarin was similar to that obtained for incorporation of valine from solution into linamarin (Tables 3.2.1.1-3.2.4.1). The ^{14}C incorporation values for the different precursors using leaves from 3 different varieties were valine (12-17%), 2-hydroxisobutyronitrile (21%) isobutyronitrile (12-16%) and isobutyraldoxime (25%). The results indicate that these volatile substances were efficient precursors of linamarin in cassava leaves. The data obtained are consistent with the pathway of linamarin biosynthesis involving a reaction sequence from valine through isobutyraldoxime, isobutyronitrile, and 2-hydroxyisobutyronitrile to linamarin as illustrated in figure 6.2. This pathway has been demonstrated in *Linum usitatissimum* by solution administration of labelled precursors (Hahlbrock *et al.*, 1968; Tapper and Butler, 1971). Confirmation of this pathway by demonstration of the presence of the enzymes catalyzing the biosynthetic steps is available from the investigations of dhurrin biosynthesis in *Sorghum bicolor* by Conn and his coworkers (Reay and Conn, 1974; McFarlane *et al.*, 1975; Shimada and Conn, 1977). Reay and Conn reported an enzyme from *Sorghum* which catalyzed the conversion of hydroxymandelonitrile to dhurrin. McFarlane *et al.* (1975) isolated a 'high speed' microsomal fraction from seedlings of *Sorghum* which catalyzed the formation of p-hydroxymandelonitrile from L-tyrosine via p-hydroxyphenylacetaldoxime and p-hydroxyphenylacetonitrile. More recently Shimada and Conn (1977) used the same

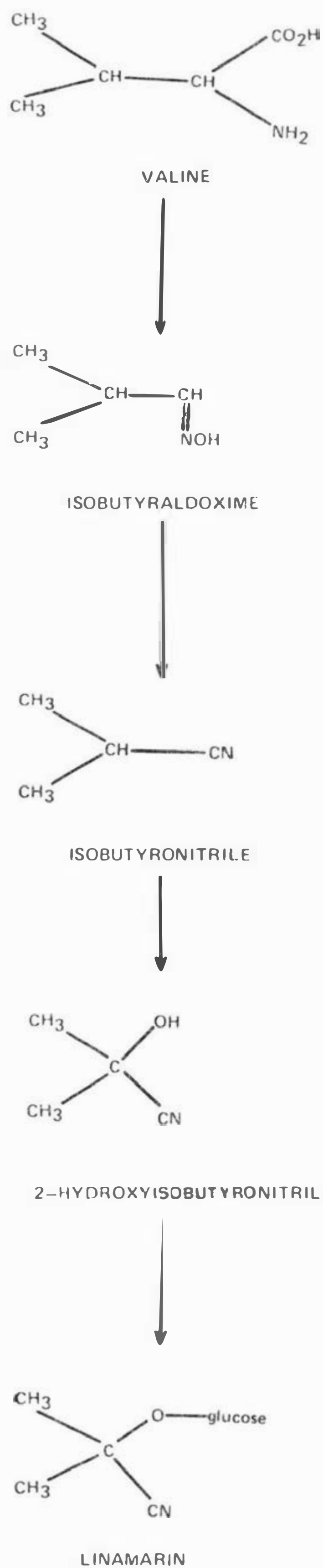

FIGURE 6.2 PATHWAY OF LINAMARIN BIOSYNTHESIS IN CASSAVA

microsomal preparation from *Sorghum* in conjunction with trapping experiments to show that 2-hydroxy(p-hydroxyphenyl) acetaldoxime was not a normal intermediate in dhurrin biosynthesis. Although no comparable enzyme studies have been carried out on linamarin biosynthesis, the involvement of analogous intermediates suggests that the general pathway of cyanogenic glucoside biosynthesis is common to most cyanogenic species including cassava.

The assimilation of the administered precursors by cassava leaves yielded products similar to those previously identified in other species (Tapper, 1968). The labelled products were investigated in detail for leaves fed with 2-hydroxy[1-^{14}C]isobutyronitrile. Apart from incorporation of ^{14}C into linamarin, several amino acids were labelled. These included asparagine (by far the major ^{14}C amino acid), β-cyanoalanine, glutamine, glutamic acid, aspartic acid, glycine and histidine. The pattern of labelled amino acids is similar to that obtained from [^{14}C]-HCN administration to linen flax and etiolated cassava seedlings (Tapper, 1968; Nartey, 1969). Thus it is quite probable that the labelled products were derived from [^{14}C]-HCN generated within the tissues from decomposition of 2-hydroxy[1-^{14}C]isobutyronitrile.

Although significant amounts of ^{14}C-linamarin were formed from all the administered volatile precursors, isobutyraldoxime and 2-hydroxyisobutyronitrile appeared to be more suitable than isobutyronitrile for this method of precursor administration. The uptake of isobutyronitrile vapour was about 10% which was low

compared with over 98% uptake of isobutyraldoxime and 2-hydroxyisobutyronitrile. This low uptake of isobutyronitrile could possibly be due to the low water solubility of the compound resulting in the poor diffusion of the nitrile into the cell. The incorporation of both the oxime and 2-hydroxynitrile into linamarin was also somewhat greater than the incorporation of the nitrile.

6.3 Sites of Linamarin Synthesis in Cassava

The investigation of linamarin content of cassava tissues showed that linamarin was present in all tissues. The levels found for three different varieties were consistent and similar to those reported by other workers (de Bruijn, 1971; Bolhius, 1954). The younger, metabolically more active tissues tend to have higher linamarin contents than older tissues with a higher percentage of woody fibrous tissue. The overall pattern of endogenous linamarin distribution, however, does not necessarily indicate which organs are responsible for the production of the cyanogenic glucoside.

Table 6.2 gives a summary of the amounts of ^{14}C valine incorporated into ^{14}C-linamarin by various detached organs and plant tissues. Certain tissues like the shoot apex, the petioles and the leaves appear to have a much higher linamarin biosynthetic ability than other tissues like the roots and tubers. In evaluating this data as an indication of the relative biosynthetic efficiency of the different organs and tissues a number of factors have to be taken into account.

1. In this preliminary survey an arbitrary time of 24 hours was set for termination of precursor metabolism

Table 6.2

Summary of L-[U-^{14}C] Valine Incorporation into ^{14}C-Linamarin by Cassava Tissues.

Cassava Tissue	LINAMARIN[1]	
	μmol/gm F.Wt.	(%) Incorporation
Shoot apex	23.2	15.6
Upper leaves	13.9	12.0
Lower leaves	14.4	10.8
Upper leaf petioles	5.4	63.5
Lower leaf petioles	6.2	52.3
Upper stem	15.2	14.3
Lower stem	6.4	4.7
Roots	0.9	1.4
Tubers (+ peels)	11.0	5.6

[1] = Average of results from two or three cassava varieties as presented in Table 4.2.1.

and the results expressed as the percent incorporation and the dilution factor. When a fixed quantity of isotope is added at the beginning of the experiment these parameters are time-dependent (Campbell, 1975). When an arbitrary single sampling time is chosen, as was the case in the preliminary experiment, the incorporation value may be examined either before or after exhaustion of the labelled precursor, or after the product has been broken down into other substances. A time-course examination of the incorporation is therefore necessary to obtain data which can be used to compare biosynthetic activity.

2. The ability of the precursor to reach the biosynthetic site may be very different for the different tissues. Thus the low incorporation by tuber tissue slices may be due to inability of the valine to enter the cells particularly those in interior of the slice. The method of valine administration in this preliminary experiment may have restricted valine metabolism to the damaged surface cells where any linamarin synthesised could have been broken down.

3. In those tissues where the incorporation of ^{14}C into linamarin is very low confirmation of the identity of the labelled material is necessary to establish the significance of the incorporation value.

In view of these problems in interpretation, two tissue systems were selected for a more thorough investigation of ^{14}C valine incorporation into linamarin, namely, the petiolate leaf system representing an apparently active synthetic site and the tuber tissues,

representing an apparently "poor" site of linamarin synthesis. In these experiments the tissues were allowed to metabolise valine for different intervals so that some idea of the time-course could be obtained. Because of possible tissue to tissue variations in endogenous linamarin contents and differences in biosynthetic activities, a large number of replicate samples would be necessary in order to establish a true time-course by investigating samples after different times of exposure to the labelled precursor. In the case of the leaf system each leaf was dissected into four component tissues and each tissue had to be extracted, concentrated and the ^{14}C determined in several different fractions. The problems involved in processing large numbers of samples in this way precluded experimental replication. Therefore differences between samples taken at different times cannot be used as a basis for quantitative calculations that could be obtained from a true time-course. However, the trends do reveal some important time-dependent features which must be taken into account in interpreting the incorporation data.

As well as determining the linamarin in samples harvested at different times, the total ^{14}C distribution and the distribution between ethanol soluble and insoluble ^{14}C and residual ^{14}C valine was determined to give information on the utilization of the precursor and its penetration to different parts of the system.

6.3.1 Biosynthesis in the Petiolate Leaf System

In the time-course experiment ^{14}C valine was administered to leaf petioles, midribs and leaf blades by allowing detached leaves to take up and

distribute the precursor through the transpiration stream.

The total ^{14}C activity per gram fresh weight attained a fairly constant level within each tissue 4 hours after precursor uptake. There was relatively little difference in ^{14}C distribution between the 4 and 24 hour samples. Similar levels of ^{14}C activity were obtained in the petiole and midrib tissues (0.2 μCi/gm fresh weight) whilst about twice as much ^{14}C (about 0.4 μCi/gm fresh weight) was distributed to the blades (Table 4.3.2.2). In a complex tissue system like the petiolate leaf the ^{14}C distributed to each tissue by solution uptake does not necessarily indicate the amount of ^{14}C valine which has reached each of the component tissues. If the valine is transported solely through the xylem, it is unlikely that it would undergo significant metabolic transformation since xylem vessels are essentially non-living. Metabolic transformation may, however, occur in parenchyma cells associated with the xylem and the products of these transformations could re-enter the xylem and be transported along with the valine. Between 25% (in the leaf blade) and 49% (in the basal petiole) of the total ^{14}C in the tissues was found to be due to ^{14}C-valine $\frac{1}{2}$ hour after uptake although a significant amount of ^{14}C activity had been converted into other products by that time. Thus a substantial proportion of ^{14}C activity arriving in the tissues consisted of ^{14}C-valine.

^{14}C valine arriving in the tissues was quickly metabolised leaving a small but constant residual ^{14}C valine from 4 to 24 hours. The percentage of ^{14}C

valine still present after 4 hours was about 4% in the petiole, 8% in the blades and 13-22% in the midribs (Table 4.3.2.3). The constancy of this residual valine level is in contrast to the rapid rate of initial ^{14}C-valine assimilation in the tissues up to 4 hours and suggests that this residual fraction was contained in some inactive pool not readily available for assimilation.

The time-course analyses show that all the tissues of the leaf system incorporate ^{14}C-valine into ^{14}C-linamarin although incorporation in the leaf blades was very low. A high level of incorporation (35-57%) was attained in the leaf petioles. This high incorporation was also found in the preliminary experiments in which 52-64% incorporations were recorded for the petioles (Table 6.2). Such high incorporations of ^{14}C-valine into ^{14}C-linamarin are comparable only to the 48% incorporation of ^{14}C-valine to linamarin by *Linum usitatissimum* found by Hahlbrock *et al* (1968). In the midribs about 20% of the total ^{14}C in this tissue was due to ^{14}C-linamarin after 24 hours, a lower incorporation than in the petioles but considerably higher than the 2% value attained in the leaf blades. The very low incorporation into linamarin in the leaf blade could be due to a low biosynthetic capacity of the mesophyll tissue or to the transformation of much of the precursor into other substances before reaching this tissue. When 2-hydroxyisobutyronitrile vapour was administered to an attached leaf, both the specific activity of the linamarin and the ^{14}C-linamarin per gram

of tissue in the leaf blade (i.e. in the blade discs excluding midribs) were much lower than the corresponding values for the leaf as a whole after 4 hours exposure to the precursor (section 5.4.1). Thus the specific activity of linamarin in the leaf discs was 26.2 μCi/mmol, and that in the combined leaf including midribs and 1-2 cm of the petiole was 46.0 μCi/mmol; on a weight basis, the discs contained 0.67 μCi/gm F. Weight and the whole leaf 1.10 μCi/gm F.W. Since leaf discs were taken from several different parts of the blade on each leaflet, it is unlikely that this difference is due to sampling error. The difference could indicate that tissues included in the midrib have a higher capacity for conversion of precursors to linamarin than the mesophyll tissues of the blade.

The endogenous linamarin content of the blade was 11-17 μmol/gm while that of the midribs was 31-33 μmol/gm f.wt. The blades and the midribs both had higher linamarin levels than the petioles which had about 6-8 μmol/gm. Thus there seems to be no simple relationship between linamarin levels and apparent biosynthetic ability. Physiological factors such as linamarin accumulation or breakdown within the leaf tissues may be contributing to the observed lack of relationship. The ability of each tissue to store endogenously synthesised linamarin may be greater within the blade and midrib tissues in comparison to the petioles.

6.3.2 Biosynthesis by Tuber Tissues

Administration of ^{14}C-valine to tuber tissue presented difficulties. The tissue is dense and lacks a suitable natural vascular system for administration

experiments. The transverse section of a young storage cassava tuber is illustrated in figure 6. The edible tuber core tissue consists of densely packed storage parenchyma cells filled with starch grains and interrupted by strands of xylem tissue radiating outwards from the centre. The tuber peel consists of an outer zone containing sclerenchyma and parenchyma tissues overlaid on the outside with a periderm and an inner zone containing the phloem tissue. If the precursor is simply applied to the cut surface it would not be uniformly distributed. To assist better distribution a vacuum infiltration technique was used to apply carrier free ^{14}C-valine.

Both the core and peel tissue metabolised ^{14}C-valine even more rapidly than the leaf system leaving about 10% of the initial activity as residual ^{14}C-valine after ½ hour (Tables 4.4.2 and 4.4.3). In the tuber cores this residual fraction does not further decrease between ½ hour and 24 hours. This fraction of residual ^{14}C-valine may be part of a relatively inaccessible or inactive pool in the tissues of the tuber cores possibly located in cell vacuoles or bound to sites external to the cell membrane. Within the peels there was a further slow assimilation of ^{14}C-valine after ½ hour resulting in a decrease of residual ^{14}C-valine from 9% (½ hour) to 1% (24 hours). A possible problem which should not be overlooked is that some microbial activity may have developed over the longer time period with the tuber and peel experiments. Although no special antibiotic precautions were taken microbial activity may be assumed to be insignificant for

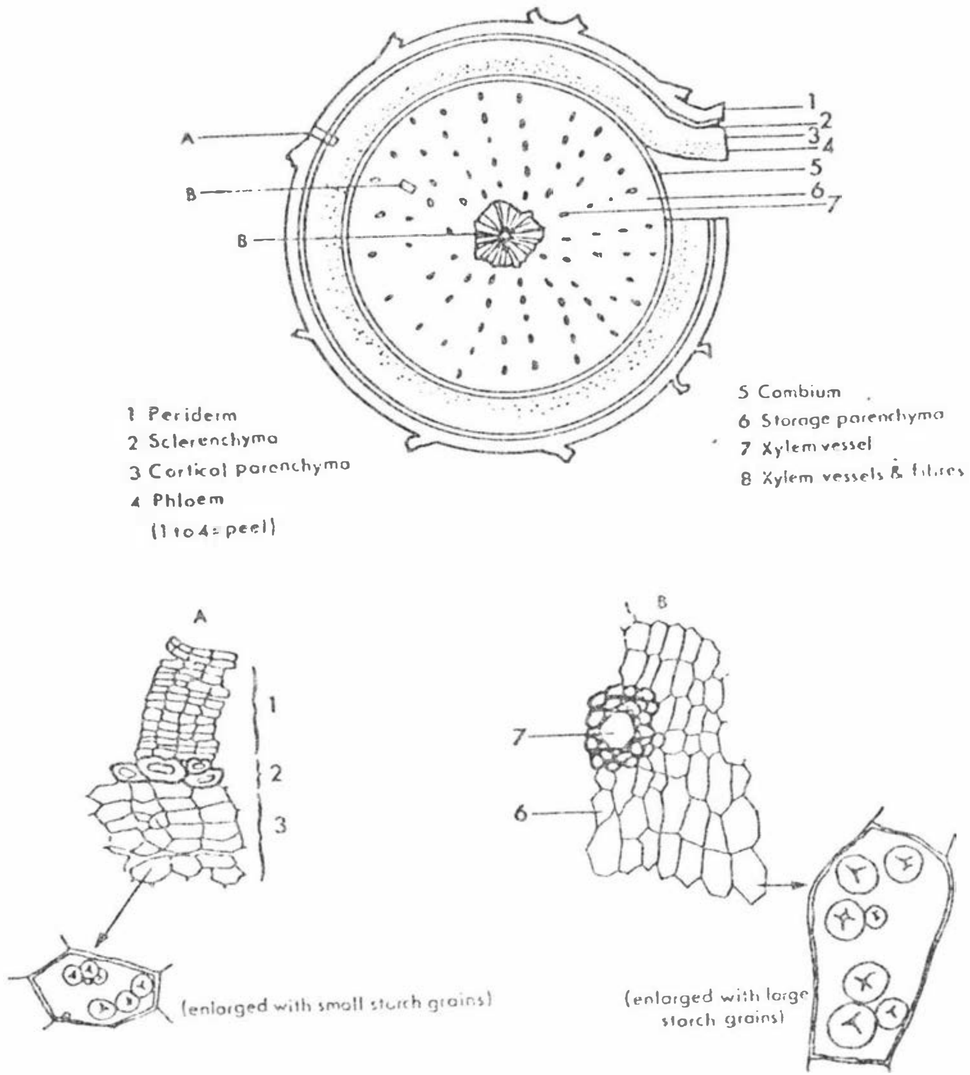

FIGURE 6 TRANSVERSE SECTION OF A YOUNG STORAGE CASSAVA ROOT (Hunt et al., 1977)

the shorter time periods and probably of unknown importance at 24 hours.

The maximum values for incorporation of ^{14}C-valine into linamarin by both the tuber core and tuber peels were low. The tuber cores incorporated about 0.1%, while the peels incorporated 1-2% of the administered ^{14}C into linamarin. Despite these low levels of incorporations the endogenous linamarin level in the peels was almost as high as that in the leaves. The peel, like the leaf blade may be accumulating much of the linamarin it synthesises. Nevertheless much of the tuber linamarin may come from aerial organs of the plant as is indicated by the translocation experiments (section 6.4).

While the tuber tissues and the leaf blades gave low incorporations of ^{14}C into linamarin as compared to the petioles and midribs the former tissues converted more ^{14}C into products other than linamarin. Thus the low conversion of ^{14}C-valine into ^{14}C-linamarin in the blade and tuber tissues may not necessarily be reflecting a true inability of these tissues to synthesise linamarin. It is apparent that within such tissues the administered ^{14}C-valine was being rapidly removed to form substances other than ^{14}C-linamarin. The presence of more actively competing biosynthetic pathways in the tuber and blade tissues probably accounts for the observed rapid removal of valine so that the precursor was not readily available for conversion into linamarin. How far this competition affects normal endogenous linamarin biosynthesis will have to be examined by further experiments. Estimation of the relative activities of the enzymes involved in the

biosynthetic pathway from both 'efficient' and 'poor' sites of synthesis should provide further confirmation of the relative linamarin biosynthetic abilities of these tissues.

6.4 Translocation of Linamarin in Cassava

Although tuber tissues of cassava contain fairly high linamarin levels the ability of these tissues to convert valine into linamarin was apparently low. The possibility that translocation of linamarin from active synthetic sites to the tuber may contribute to the endogenous linamarin in cassava tubers has therefore been investigated. Linamarin translocation from the petiolate leaf to other parts of the plant was investigated by labelling of linamarin in attached leaves using volatile precursors and determining the time-course of ^{14}C-linamarin decrease and the eventual distribution to different parts of the plant.

In translocation studies in plants cultural, morphological and physiological factors may influence data interpretation. Two plants growing under different moisture regimes may translocate metabolites at different rates. In a field experiment, for example, Hume (1975) noted a greater amount of ^{14}C-photoassimilate translocation from cassava leaves to tubers during the wet season than in the dry season. All the plants used in the present investigations were actively growing under conditions in which water was not limiting. Experiments with immature non-tuberous plants were performed in triplicate after careful plant selection with respect to plant height, age,

number of leaves on the plant and comparable primary root sizes. The data obtained from each of the three plants in these experiments agreed closely. Due to cultural and environmental problems it was not possible to produce a sufficient number of mature tuberous cassava plants with similar morphological characteristics to permit similar replication of the translocation experiments with these plants. The data for mature tuberous plants are therefore based on single plants.

6.4.1 Kinetics of Linamarin Translocation from leaves

The time-course of changes of ^{14}C-linamarin has been followed in attached source leaves of non-tuberous and tuberous plants by taking representative disc samples from the leaf blade tissue without damaging the major leaf veins and midrib tissues. Time-course analysis assumes that disc specific activity is representative of the whole leaf. The lower specific activities recorded for punched discs during short term experiments with intact leaves shows however that the specific activity of linamarin in the discs is considerably lower than that in the source leaf as a whole. The differences between blade, midrib and petiole have already been discussed (section 6.3.1). Disc analysis nevertheless provided a general indication of linamarin removal from leaves.

The extent of linamarin turnover within the leaf blade could influence the validity of the time-course data as an indication of translocation. ^{14}C-linamarin was found to undergo relatively little turnover in a

detached leaf. ^{14}C-linamarin labelled in detached leaves by the uptake of ^{14}C-valine showed very little turnover through a 24-hour period (figure 4.3.2.2). The relative stability of the ^{14}C-linamarin pool in detached leaves was also demonstrated when ^{14}C linamarin synthesised from 2-hydroxy [1-^{14}C] isobutyronitrile vapour was found to undergo very little change in level over 57 hours (figure 5.3.1). Under normal growth conditions there is no reason in assuming that linamarin turnover is greater in an attached leaf than a detached leaf. The observed kinetic data may therefore be used as a valid indication of translocation.

Typical time-course patterns obtained by analysis of punched discs indicate a high rate of ^{14}C-linamarin loss from the source leaf immediately after synthesis up to 69 hours after which the ^{14}C linamarin level remained almost constant (figures 5.3.2, 5.3.3 and 5.4.2.1). This pattern was found in discs from non-senescing intact leaves regardless of the location of the source leaf on the plant or the maturity of the plant. Similar translocation patterns in which the initial high rate was followed by a slow and almost constant phase have been observed for ^{14}C-photoassimilates in corn, *Zea mays* (Hofstra and Nelson, 1969), Sugar-beet, *Beta vulgaris* (Joy, 1964) and Soy-bean, *Glycine max* (Moorby *et al.*, 1963).

The rates of ^{14}C-linamarin translocation from the source leaves differed between leaves of tuberous and non-tuberous plants and also according to the age or position of the source leaf on the plant. The rate of

^{14}C loss from lower leaves as indicated by the time for ^{14}C-linamarin to drop to 50% of its initial level was twice as great as from an upper leaf on non-tuberous plants.

The level of ^{14}C-linamarin in the non-senescent leaf blades of both tuberous and non-tuberous plants did not significantly decrease after 69 hours although residual ^{14}C-linamarin was still over 25% of the initial level. The kinetic pattern suggests that some of the ^{14}C-linamarin synthesised during the labelling period becomes transferred into an 'immobile' pool from which it can be translocated only very slowly. This immobile pool could be in the cell vacuoles of the leaf mesophyll tissue.

The accumulation of cyanogenic glucosides in plant cell vacuoles was suggested by Stafford (1974) while Saunders et al. (1977) demonstrated that about 90% of dhurrin (p-hydroxymandelonitrile-β-D-glucopyranoside) synthesised in young *Sorghum bicolor* seedlings by administration of tritium-labelled p-hydroxyphenylacetaldoxime may be located in the cell vacuoles. A similar intracellular accumulation of ^{14}C-linamarin in cell vacuoles of cassava leaves probably accounts for the observed 'slow' phase while the initial 'fast' phase may be attributed to rapid loss from a more accessible pool in the plant cells. The lower specific activity of ^{14}C-linamarin in source leaf discs as compared to the whole leaf soon after synthesis may be due to a higher degree of linamarin synthesis in the midrib tissues (Table 5.4.1 and section 6.3). After 7 days of

translocation from the leaf the specific activity of linamarin in the discs and the whole leaf were similar (section 5.4.2 and Table 5.4.2.2). This may indicate that the 'accessible', readily translocated pool of ^{14}C-linamarin is, in part, that linamarin synthesised in vascular parenchyma tissue associated with the veins.

An interesting finding of the time-course study was the apparently higher rate of ^{14}C-linamarin translocated out of the leaf blades of immature non-tuberous plants than from the blades of tuberous plants. It appears that the presence of tubers, which might be expected to constitute an effective 'sink' does not increase the rate of linamarin translocated out of individual leaves. However, the differences between rates of translocation from tuberous and immature plants may be due to size, age or varietal differences.

Translocation of freshly synthesised ^{14}C-linamarin proceeded in normal green leaves while the endogenous linamarin levels remained virtually unchanged. This could be interpreted as indicating a steady state condition between synthesis and translocation in the leaf tissues. In contrast to the situation in non-senescing leaves, the senescing leaf blade shows a continuous loss of both endogenous and freshly synthesised ^{14}C-linamarin with the same initial rate (i.e. the specific activity of leaf disc linamarin does not change significantly over the 7 day period). Similar enhanced losses of ^{14}C-photoassimilates have been observed in senescing leaves of *Beta vulgaris* (Joy, 1964).

6.4.2 Distribution of Translocated Linamarin in Cassava.

The mature and immature cassava plants whose attached single leaves had been selectively labelled with ^{14}C-linamarin were harvested after 7 days, divided into various parts, extracted and analysed for ^{14}C-linamarin and endogenous linamarin.

Clearly the time at which the plant is harvested will have a considerable influence on the pattern of distribution of translocated linamarin. A relatively long period of seven days was chosen in the experiments conducted in the present study to enable measurable levels of ^{14}C-linamarin to be attained in the various organs. In the light of the kinetic patterns revealed by the leaf disc linamarin changes and the low recoveries found after seven days it would be advantageous to investigate distribution after different times of translocation although the work involved in such a study would be considerable.

Because of turnover factors the ^{14}C-linamarin recovered from the various organs and tissues represent the difference between ^{14}C-linamarin translocated to the tissue and ^{14}C-linamarin degraded within the tissue. Thus ^{14}C-linamarin recoveries do not provide a quantitative assessment of the total linamarin translocated from the source leaf but are indicative of the general pattern of 'source-sink' relationships between the leaves and other organs and tissues.

Although the shoot apex was shown to be an active biosynthetic site as indicated by the high incorporation

of valine (Table 6.2) the apical tissues are evidently not significant 'source' organs for translocation. Apart from the fact that they comprise a relatively small proportion of the total plant (ratio of total petiolate leaves to 'shoot apex' is about 30:1 on a weight basis in a mature plant), an experiment in which the linamarin in the attached shoot apex was labelled by 2-hydroxy[1-^{14}C] isobutyronitrile administration (section 5.2.2) showed that very little linamarin is translocated from this tissue to other parts of the plant. Subsequent translocation experiments were therefore confined to the investigation of the fully expanded leaves as source organs.

6.4.2.1 Distribution of translocated Linamarin in Non-tuberous Plants.

A high percentage (about 80%) of total ^{14}C-linamarin recovered from the whole plant was still located in the source leaves (Table 5.3.1). There was not much difference in ^{14}C-linamarin recoveries when upper and lower leaves were used as the source leaf. This relatively high retention of ^{14}C-linamarin in the source leaves may be ascribed to ^{14}C compartmented in source leaves as has already been discussed (section 6.4.1).

There were some general trends in the pattern of distribution to tissues beyond the source leaf. The source leaf petioles did accumulate substantial amounts (31-50%) of the translocated linamarin recovered, but this represented only a small percentage of the original ^{14}C-linamarin synthesised in the leaf. The ^{14}C-linamarin recovered from the petioles and stems after 7 days would

be due to ^{14}C-linamarin accumulated in these tissues.

The distribution of ^{14}C-linamarin to the primary root system of immature plants was very low as compared to other parts of the plant (Table 5.3.1). The distribution to the roots appears to be slightly greater for plants in which lower leaves were the source than those with upper source leaves as source. This difference may be ascribed to the closer proximity of the roots to lower leaves.

In these young non-tuberous plants, leaves above the source leaves contained a significant proportion (14-16%) of the translocated linamarin recovered. The pattern of ^{14}C-linamarin distribution to such leaves followed the leaf arrangement on the plant (figure 5.3.4). As has been observed for ^{14}C-photoassimilates in other plant species (Shiroya _et al_, 1961; Joy, 1964; Ho and Peel, 1969), the younger leaves received more translocated linamarin than older leaves while more ^{14}C-linamarin was distributed to leaves on nearer orthostichies than those on more distant orthostichies. On the other hand much less ^{14}C-linamarin was detected in leaves below the source leaf. Thus in young, non-tuberous plants there is a predominantly upward translocation from older leaves to young leaves and shoot apices.

6.4.2.2 Distribution of Translocated Linamarin in Tuberous Cassava Plants

Seven days after synthesis in the leaves, ^{14}C-linamarin was recovered from all parts of the mature tuberous plants. In the plant where a non-senescent leaf was the source leaf, the plant retained

much of the synthesised linamarin in the source leaf (Table 6.4.2.2). Although about 25% of the initial ^{14}C-linamarin was still present in the source leaf this fraction (as was the case with the non-tuberous plant) represented 80% of the overall ^{14}C-linamarin recovered. In marked contrast to this, the senescing source leaf retained only 0.5% of its initial ^{14}C-linamarin. By measuring total linamarin levels in attached and detached leaves undergoing senescence the extra loss of linamarin during senescence was shown to be due to both linamarin turnover and translocation out of the source leaf (figure 5.4.4). The enhanced translocation of ^{14}C-linamarin out of the senescing leaf appears to result in increased ^{14}C-linamarin recoveries in its sink tissues in comparison with the sink tissues of the plant with a non-senescing leaf as source (Table 6.4.2.2).

In contrast to young, non-tuberous plants, the distribution of translocated linamarin in the tuberous plant appears to be directed towards the basal organs of the plant. Only one hour after the labelling period, 1.5% of ^{14}C-linamarin was recovered from the lower stem while none of the other 'sink' tissues showed any detectable ^{14}C-linamarin. After 7 days of translocation the tuberous plants gave higher recoveries of ^{14}C-linamarin in the lower stem and tubers than in other organs of the plant. The ^{14}C-linamarin recovered from parts below the source leaf (tubers, roots and lower stem) was higher within the senescing source leaf plant (11.7% of initial ^{14}C-linamarin) than the plant with non-senescent source leaf (5.3%). The distribution of

Table 6.4.2.2

Distribution of Translocated Linamarin in Tuberous Plants

PLANT ORGAN	Per cent initial ^{14}C-Linamarin[1]		
	1-hr Trans- location from fresh leaf	7 days trans- location from fresh leaf	7 days trans- location from senescing leaf
Tubers	ND[2]	2.4	6.0
Primary roots	ND	0.7	0.4
Lower stem	1.5	2.2	5.3
Upper stem	ND	0.7	0.5
Upper leaves	ND	0.7	0.5
Upper leaves	ND	0.7	4.9
Petiole of source leaf	2.1	0.2	0.5
Source leaf	96.4	24.5	0.5
Percentage linamarin recovered	100	31.4	18.1

[1]: Percentage of initial ^{14}C-linamarin synthesised in source leaf assuming incorporation of 20% ^{14}C- 2-hydroxyisobutyronitrile into ^{14}C-linamarin.

[2]: Not detectable.

translocated ^{14}C-linamarin to the tuber system of the tuberous plants is high compared with the very small amount recovered from roots of non-tuberous plants. Thus the presence of tubers while it does not increase the rate of ^{14}C-linamarin translocation from an individual leaf, does influence the general distribution of translocated ^{14}C-linamarin in cassava plants.

In translocation experiments using $^{14}CO_2$ to label photoassimilates in tuberous cassava plants Hume (1975) reported that 4.4% of the initial ^{14}C photoassimilate was recovered from the tubers after 7 days of translocation while 38% was recovered in the labelled leaves. Although Hume was using a different variety of cassava grown in a different way to that used in the present study his values are not very different from the present observations and suggests that linamarin and products of $^{14}CO_2$ assimilation in cassava may be simultaneously translocated from leaves to the tubers. Further experiments comparing the translocation of ^{14}C labelled linamarin and photoassimilates in leaves under comparable conditions should throw more light on the apparent similarities.

6.5 Turnover of Linamarin in Cassava

As has already been pointed out, the interpretation of both biosynthesis and translocation data depends on the extent to which linamarin turnover occurs. Although linamarin turnover was not investigated by experiments specifically designed to determine this, indirect evidence of the extent of turnover has been obtained in the course of work described in this thesis.

The time-course of the valine feeding experiments showed that in both the detached leaves and tuber tissues ^{14}C-linamarin levels remained relatively stable after synthesis (figure 4.3.2.2; table 4.4.3). The time-course of ^{14}C-linamarin in detached leaves administered with 2-hydroxy[1-^{14}C] isobutyronitrile also showed this general ^{14}C-linamarin stability. Although the period examined could not be prolonged beyond 60 hours due to the sudden onset of wilting in the detached leaves, about 95% of the initial ^{14}C-linamarin was still present at the end of this time.

Compared with detached organs, recoveries of ^{14}C-linamarin from whole plants harvested 7 days after precursor labelling of linamarin were low. Recoveries from immature plants were 15-16% of the estimated initial ^{14}C-linamarin while 31.4% was recovered from the mature tuberous plant in which linamarin was labelled in a non-senescent leaf.

The finding that freshly synthesised linamarin was not significantly broken down in detached cassava leaf tissues while extensive linamarin turnovers were recorded when the source leaves were attached so that translocation could occur indicates that much of the linamarin transformation takes place outside the immediate site of synthesis. It is likely that the breakdown of translocated linamarin may be occurring in the phloem tissue during translocation or within the 'sink' tissues.

As already mentioned, linamarin turnover is apparently greatly enhanced during leaf senescence

(Table 6.4.2.2) since the total linamarin level falls steadily as chlorophyll content decreases in detached senescing leaves (figure 5.4.3.2).

The present study has sought to investigate the metabolism of linamarin in cassava. In the course of this study the general pathway of linamarin biosynthesis, the sites of linamarin synthesis, the translocation of synthesised linamarin and turnover of linamarin in cassava have been investigated. However certain findings from the present study need to be pursued in order to give a more complete picture of the pattern of linamarin metabolism and distribution in the cassava plant.

The general pathway of linamarin biosynthesis established from work in other plants has been demonstrated to be present in cassava leaves. It may be necessary in future investigations to examine the existence of possible alternative pathways of linamarin biosynthesis in cassava. Such a study could involve the administration of possible ^{14}C precursors such as 2-hydroxyisobutyraldoxime in conjunction with the detection of relevant biosynthetic enzymes. Enzymatic investigations carried out on both the 'active' and 'poor' linamarin synthetic sites may help to clarify the roles of the respective organs in endogenous linamarin synthesis.

An important finding of the present study was the rapid removal of administered valine from the metabolic pool to form products other than linamarin possibly due to competing pathways in the cassava tissues. Further investigations using other precursors may result in higher incorporation values and more specific labelling of linamarin which could give a more reliable estimation of *in vivo* biosynthetic rates between tissues. By virtue of its relative stability, solubility and volatility isobutyraldoxime may be a suitable precursor for such vapour administration studies.

The apparent similarity in the translocation patterns of linamarin and that of photoassimilates could be followed up to establish more clearly whether linamarin and photoassimilates are simultaneously translocated. Linamarin and a photoassimilate such as sucrose could be simultaneously labelled by exposing an attached leaf to $^{14}CO_2$ and [1-^{14}C] isobutyraldoxime.

Considerable turnover of linamarin has been shown to occur in cassava plants during translocation. This could be more effectively measured by designing translocation experiments using a large number of replicate plants. After leaf labelling plants could be harvested at various time intervals to evaluate the extent of linamarin turnover.

The finding that the leaf petiole is a very active synthetic site could be used as a basis for a rapid assay of biosynthetic ability which would be of value for breeding work on cassava. Screening of varieties differing in their cyanoglucoside content could be

carried out to assess how far these differences are due to differences in biosynthetic ability. The present investigations have also shown that translocation from the leaves contributes significantly to the generally high linamarin contents of the tubers. Future breeding experiments may need to consider the effects of linamarin translocation and turnover on the cyanogenic glucoside contents of the edible tubers and leaves of newly developed cassava strains.

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