BIOSYNTHESIS AND METABOLISM OF PLANT GLYCOSIDES

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SUMMARY

The biosynthesis of selected cyanogenic glucosides and glucosinolates was examined in higher plants. Linen flax seedling shoots (Linum usitatissimum L.) were used exclusively to study linamarin biosynthesis while prunasin biosynthesis was studied in both peach shoots (Prunus persica Batsch) and cherry laurel shoots (P. laurocerasus L.). Isopropylglucosinolate and benzylglucosinolate were studied in scurvy grass seedlings (Cochlearia officinalis L.) and garden cress seedling shoots (Lepidium sativum L.) respectively.

Altogether 9 isotopically labelled compounds were prepared as part of the study and 14 were administered to plant tissue. The quantity of cyanogenic glucoside was determined by measuring hydrogen cyanide following enzymic hydrolysis. The specific activity or dilution of the labelled compound after incorporation into the glucosides was determined and used to judge the effectiveness of the administered compound as a precursor of the glucosides. Benzaldehyde from prunasin was measured as its semicarbazone and the isothiocyanates, obtained by enzyme hydrolysis of the glucosinolates, were identified by conversion to thiourea derivatives. Paper and thin layer chromatography and electrophoresis were used to separate non-volatile radioactive compounds.

Isobutyraldoxime-U-^{14}C, 2-oximinoisovaleric acid-U-^{14}C, isobutyronitrile-1-^{14}C and 2-hydroxyisobutyronitrile-1-^{14}C were all incorporated into linamarin to extents comparable to that from L-valine-U-^{14}C. By the use of ^{15}N labelled compounds the C-N bond of isobutyraldoxime and 2-oximinoisovaleric acid was shown to remain intact during the conversion to linamarin. Isobutyramide-1-^{14}C and hydrogen cyanide-^{14}C were not significantly incorporated into linamarin.

Phenylacetaldoxime-U-^{14}C, 2-oximino-3-phenylpropionic acid-2-^{14}C and phenylacetonitrile-1-^{14}C were converted to prunasin to greater extents than was L-phenylalanine-U-^{14}C. Radioactivity from D,L-mandelonitrile-1-^{14}C and, to a

lesser extent, from hydrogen cyanide-^{14}C was also incorporated into the nitrile moiety of prunasin. Phenylacethydroxamic acid-1-^{14}C was not significantly converted to prunasin.

Linen flax seedling shoots were examined for both volatile and non-volatile intermediates. Radioactive precursors of linamarin were administered in the presence of other suspected intermediates or inhibitors of linamarin biosynthesis. Both isobutyraldoxime and isobutyronitrile were shown to be formed in the shoots from L-valine-U-^{14}C.

A non-volatile compound which accumulated in the presence of a few inhibitors of linamarin biosynthesis was studied in detail. Treatment with acid under mild conditions yielded isobutyraldehyde while emulsin gave isobutyraldoxime. It was resistant to linamarase. Isobutyronitrile was a product of pyrolysis. The proposed structure for this compound is isobutyraldoxime-O-glucoside.

Isobutyraldoxime-U-^{14}C and phenylacetaldoxime-U-^{14}C were both better precursors of the corresponding glucosinolates than were L-valine-U-^{14}C or L-phenylalanine-U-^{14}C. 2-Oximinovaleric acid-U-^{14}C was not significantly incorporated into isopropylglucosinolate.

It is concluded that aldoximes are intermediates in the biosynthesis of both cyanogenic glycosides and glucosinolates. Other intermediates proposed in cyanogenic glycoside biosynthesis are nitriles and 2-hydroxynitriles in that order. N-Hydroxyamino acids may be intermediates between amino acids and aldoximes. 2-Oximino acids may also be intermediates in cyanoglycoside biosynthesis although it is possible that the observed incorporation was by way of prior non-enzymic conversion to nitriles. The experiments with labelled administered compounds have outlined a pathway of cyanogenic glycoside biosynthesis which may now be profitably studied for confirmation at the enzymic level.

PREFACE

I would like to express my gratitude and appreciation to Dr G. W. Butler for his interest, encouragement, and guidance during this investigation and for his assistance in facilitating support from the Department of Scientific and Industrial Research. I wish to extend my appreciation to Prof. R. D. Batt for the opportunity to study in his department. I am indebted to many of my colleagues at Plant Chemistry Division, D.S.I.R. and Massey University who were always most helpful. In particular I would like to thank Dr L. J. Peterson for many useful comments during the preparation of this thesis. I am also grateful for occasional technical assistance from Mr W. D. Bennett and for his cooperation in developing a method of detecting cyanogenic glycosides. Some of the experiments in this investigation would not have been possible without the willing assistance of Dr J. C. Hawke, Massey University, with gas chromatography, or Dr J. R. Hulston, Institute of Nuclear Sciences, for ^{15}N analyses. I am grateful to Dr E. W. Underhill and Prof. A. Kjaer for gifts of ^{14}C labelled compounds and seeds.

Not least, I would like to thank Dr E. E. Conn for the interest and stimulation he has offered. It has been a pleasure to cooperate with Dr E. E. Conn, Dr G. W. Butler, Dr K. Hahlbrock and Mr W. D. Bennett in four short publications, copies of which are appended to this thesis.

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CHAPTER I

INTRODUCTION

1.1 Glycosides

The term "glycoside" as commonly used embraces a very wide class of compounds even within the confines of the plant kingdom. McIlroy (1951) narrowly defines glycosides as derivatives of the cyclic forms of sugars in which the reducing or potential aldehyde group of the sugar is substituted by condensation with an alcohol or phenol (the aglycone) to form a hemi-acetal. These are the O-glycosides. Subsequently in his monograph he discussed the nucleosides as examples of N-glycosides and thioglycosides (principally glucosinolates) as examples of S-glycosides and thereby implies an extension of the definition of "glycoside". In recent years a new class of C-glycosides has been recognised (Wagner, 1966) where the anomeric sugar C atom is directly linked to a C atom of the aglycone.

In almost all cases glycosides have the β-configuration although the α-glycosidic linkage does occur in a number of common polysaccharides. A few natural compounds where the linkage is not to the anomeric C atom are known and these would fall outside the above definitions.

McIlroy accepts that many oligosaccharides and polysaccharides are also glycosides but these are generally classified separately and are usually separately discussed. In most cases the natural process of carbohydrate polymerisation will have much in common with glycosylation during glycoside biosynthesis. Details of this step will be discussed later (Section 1.3). Both chemically and physiologically the natural glycosides are distinguished more by their aglycone portion than by their glycosyl portions and are therefore usually treated under separate headings such as flavonoids, phenolics and steroids (e.g. Robinson, 1963).

In this investigation particular attention is directed at a study of the biosynthesis of cyanogenic glycosides and glucosinolates as examples of two distinct but possibly related groups of glycosides. It will be shown that a particular relationship between these two classes lies principally in the nature and biogenesis of the aglycones.

1.2 General function of glycosides

The role of glycosides within plants is a subject open to much speculation. Well defined metabolic roles are known for only a few glycosides and most of these can be considered essential for the growth and metabolism of the plant. Examples include the nucleosides which occur in all living organisms, some phenolic glycosides such as coniferin and syringin which appear to be involved in lignin biosynthesis (Neish, 1965), or the glycolipids which may act in maintaining the integrity of higher plant chloroplast membranes. In contrast the restricted occurrence of a considerable number of glycosides indicates that many are not essential for the basic metabolism and growth of the plants. These are often considered to be secondary metabolites - a description which may be applied to many products that are not glycosides. Although the non-essential nature of these compounds may be demonstrated it is not denied that they may have a beneficial effect in the plant. Glycosides, in particular, may be formed from toxic, weakly soluble, or reactive compounds to permit accumulation or translocation in a form which may later be further metabolised. It has been noted frequently that some accumulated glycosides are hydrolysed when plant tissue is damaged either mechanically or by parasites. The aglycones then formed may be important factors in protecting the plant from further damage. It has been suggested that some glycosides represent waste products of metabolism in a form not toxic to the plant (Miller, 1942). Further examination may however show other distinct physiological roles for such glycosides.

1.3 Glycosylation

It is now widely accepted that the formation of O-glycosidic bonds generally involves the transfer of a monosaccharide unit from a nucleoside-5'-diphosphate sugar to a suitable acceptor (Hassid, 1967). This type of reaction applies not only to glycoside biosynthesis but also to the synthesis of many polysaccharides. A few examples are known where glycosidic bonds are formed by other transfer mechanisms such as glycogen formation in vitro from α-D-glucose-1-phosphate by the action of a phosphorylase (Cori and Cori, 1940) or stachyose biosynthesis from galactinol and raffinose (Hassid, 1967). These are exceptions to the general rule.

The first demonstration of what may now be recognised as normal glycoside biosynthesis was the formation of o-aminophenyl-D-glucuronoside from o-aminophenol by Dutton and Storey (1951). Since then a large number of glycosylation reactions have been studied which involve various nucleoside-5'-diphosphate sugars.

The formation of glycosides with disaccharide or even larger sugar groups follows the pattern of transfer of monosaccharide from a nucleoside-5'-diphosphate sugar. An example which may be analogous to the biosynthesis of amygdalin is the formation of phenyl-gentiobiose and uridine diphosphate (UDP) from UDP-D-glucose and phenyl-glucoside by the action of enzymes from wheat germ (Yamaha and Cardini, 1960). Similarly, rutin may be obtained from quercetin by the transfer of glucose from either UDP-D-glucose or thymidine diphosphate D-glucose followed by transfer of L-rhamnose from UDP-L-rhamnose or thymidine diphosphate L-rhamnose. The necessary enzymes have been obtained from mung bean leaves (Barber, 1962).

The formation of the N-glycosidic bonds of nucleotides differs from the type of mechanism described above in that 5-phosphoribosylpyrophosphate is the

source of the sugar moiety (Carter, 1956).

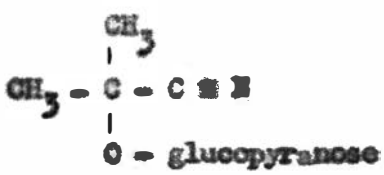
Very little study has been made of S-glycosylation. Gessner and Acara (1968) have demonstrated an enzyme system in some insects which transfers glucose from UDP-D-glucose to thiophenol and 5-mercaptouracil. A few thioglucosides and S-glucuronosides are known as metabolites of exogenous thiols in plants and animals but their formation has not been studied.

1.4 Cyanogenic glycosides

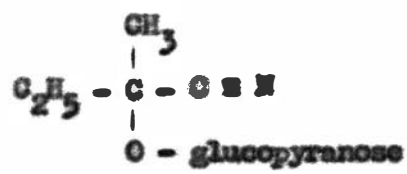
Chemistry. Cyanogenic glycosides form, for the most part, a well characterized group all capable of yielding hydrogen cyanide upon hydrolysis with suitable β-glycosidase preparations. All well studied cyanogenic glycosides may be considered as β-glycosyl derivatives of cyanohydrins of ketones or aldehydes. Table I lists most of the known cyanogenic glycosides, the aglycones and the sugar groups. Details on the properties and occurrence of individual glycosides has been reviewed by McIlroy (1951) and Dilleman (1958). A structure for the glucoside, gynocardin, was recently proposed by Coburn and Long (1966).

The most widely distributed cyanogenic glycosides are linamarin (I) and lotaustralin (II) which usually occur together (Butler, 1965), the derivatives of mandelonitrile especially prunasin (III), and the derivatives of p-hydroxymandelonitrile.

I. Linamarin



II. Lotaustralin



III. Prunasin

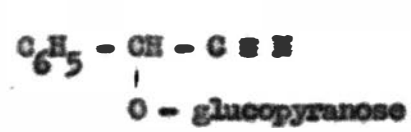


TABLE I CYANOGENIC GLYCOSIDES

Glycoside	Aglycone	Sugar
Acacipetalin	dimethylketen cyanohydrin	glucose
Amygdalin	D-mandelonitrile	6-β-glucosylglucose
Dhurrin (Phyllanthin)	L-p-hydroxymandelonitrile	glucose
Gynocardin	a cyclic cyanohydrin	glucose
Linamarin (Phaseolunatin)	acetone cyanohydrin	glucose
Lotaustralin	methylethylketone cyanohydrin	glucose
Prunasin	D-mandelonitrile	glucose
Sambunigrin	L-mandelonitrile	glucose
Taxiphyllin	D-p-hydroxymandelonitrile	glucose
Vicianin	D-mandelonitrile	6-α-L-Arabinosylglucose
Zierin	m-hydroxymandelonitrile	glucose

All the glycosides listed in Table I are substituted on the cyanhydrin hydroxyl group. However a p-hydroxymandelonitrile glucoside has been reported (Abrol et al., 1966) where substitution is on the p-hydroxyl group and as a consequence it releases hydrogen cyanide by normal cyanhydrin dissociation without enzymic treatment. Prulaurasin which is not included in Table I has been described as D, L-mandelonitrile-D-glucoside but should more properly be considered as an equimolar mixture of prunasin and sambunigrin. Prulaurasin was first reported as occurring in the leaves of cherry laurel (Prunus laurocerasus L.) (Bourquelot, 1905) but it has since been shown that prulaurasin may be formed by partial isomerisation of prunasin or sambunigrin under relatively mild conditions (Fleurier, 1935), and this may have occurred during the original extraction of cherry laurel leaves from which prunasin has since been isolated.

An unusual cyanogenic glycoside, lotusin, was reported by Dunstan and Henry (1901) to occur in the sap of Lotus arabicus and to yield maltose cyanohydrin and lotoflavin upon hydrolysis but recent work by Abrol and Conn (1965) has cast strong doubt upon its existence.

Distribution and function. No clear pattern of distribution of cyanogenic glycosides within mature higher plant organs has emerged. Seeds of some plants tend to accumulate the glycosides while in others the leaves have high concentrations of the glycosides. If more than one glycoside occurs then the relative proportions may vary between organs. In several Prunus species amygdalin is accumulated in seeds but prunasin predominates in leaves (Robinson, 1930). Where the glycosides occur in vegetative parts of the plant there is a tendency to higher concentrations in seedling shoots and other actively growing young shoot tips which also frequently appear to be the sites of most active biosynthesis of these compounds (Butler and Conn, 1964a).

The specific function of cyanogenic glycosides in higher plants are for

the most part unknown. It has been suggested that cyanogenic glycosides may be a soluble storage form of nitrogen - a role suggested from the occasional accumulation in seeds. Indeed, mechanisms which utilise the nitrogen from cyanogenic glycosides are known. However it is more likely that the role of cyanogenic glycosides is directly associated with the physiological properties of the hydrolysis products, particularly hydrogen cyanide. This compound which is toxic to most organisms at relatively low concentrations should be considered a most significant common factor in all attempts to understand the function of cyanogenic glycosides. A few scattered reports indicate that attention should be directed at the possibility of cyanogenic glycosides being major factors in the protection of the plants against a broad spectrum of parasites. Jones (1966) has shown that cyanogenesis in Lotus corniculatus is a significant factor in protecting the plant against attack from certain slugs, snails and voles but had little effect on attack by the larvae of two Zygaena species. It is notable that these moths are among the very few animals which are themselves cyanogenic (Jones et al., 1962). Some millipedes are also cyanogenic (Blum and Woodring, 1962; Pallares, 1946).

Besides occurring in several unrelated families of angiosperms, cyanogenesis has been reported with gymnosperms and pteridophytes (Hegnauer, 1959; Towers et al., 1964). Altogether, about 750 species from 60 families are known to be cyanogenic.

Cyanogenesis has been reported for several fungi, particularly basidiomycetes (Bach, 1956), but little is known as to the nature of the compounds involved. In one unidentified parasitic psychrophilic basidiomycete, which has been studied in some detail, invasion of host tissue was associated with an accumulation of hydrogen cyanide to concentrations highly toxic to the host (Lebeau and Dickson, 1955). This fungus contains linamarin (Stevens and

Strobel, 1968) and possibly other less stable cyanogenic compounds.

Metabolism. Many cyanogenic plants have been shown to contain enzymes for the hydrolysis of the glycosides. In some cases a sequence of enzymic steps for a particular glycoside have been demonstrated. "Emulsin" from almonds (Prunus amygdalus Batsch) contains at least two β-glucosidases (Helferich and Kleinschmidt, 1968) which have been crystallised. Complete hydrolysis involves cleavage of a disaccharide linkage to give D-glucose and prunasin, hydrolysis of prunasin to L-mandelonitrile and D-glucose, and finally cleavage of L-mandelonitrile to benzaldehyde and hydrogen cyanide (Haisman and Knight, 1967). This last reversible reaction is catalysed by a stereo-specific oxynitrilase which contains a flavin prosthetic group, FAD, (Becker, et al., 1963). Similarly the hydrolysis of dhurrin requires a β-glucosidase and the release of hydrogen cyanide is again catalysed by an oxynitrilase although this enzyme from sorghum seedlings does not contain a flavin nucleotide (Sealy et al., 1966).

Butler et al., (1965) studied the glucosidases of linen flax (Linum usitatissimum L.) and white clover (Trifolium repens L.) and found a β-glucosidase with partial specificity to linamarin and lotaustralin. However certain varieties of white clover lack this activity (Corkill, 1942). Emulsin and extracts from other higher plants gave only weak or no hydrolysis of these two cyanoglucosides. With β-glucosidase extracts from linseed, separation of activity towards linamarin or salicin in one case and amygdalin or cellobiose in the other was possible. The latter activity was attributed to a β-disaccharase.

Rapid hydrolysis of cyanogenic glycosides usually occurs when the plant tissue is crushed or otherwise damaged. This indicates a normal compartmentation of enzymes and glycosides within the plant tissue. Slow hydrolysis of the

cyanogenic glycosides may be inferred from observations on the subsequent metabolism of the glycosides, and particularly of hydrogen cyanide.

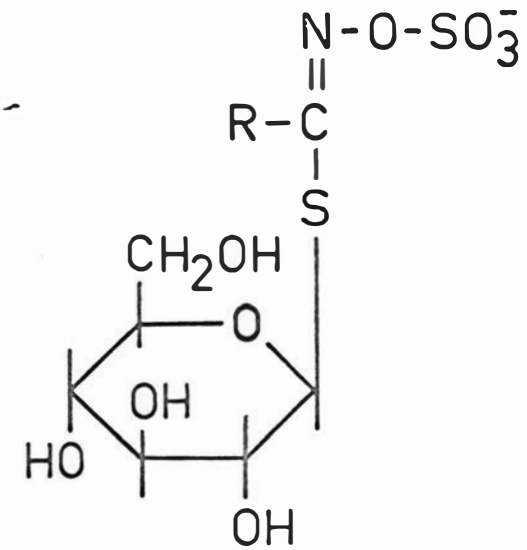
Interest in the metabolism of hydrogen cyanide in higher plants was stimulated by the finding that, although it was not incorporated into cyanogenic glycosides, it was extensively and specifically converted to the amide moiety of asparagine (Blumenthal-Goldschmidt et al., 1963; Tschiersch, 1964). Only in common vetch (Vicia sativa L.) was hydrogen cyanide converted to a compound other than asparagine. Subsequent studies (Ressler et al., 1963; Fowden, 1965) have shown that 3-cyanoalanine is an intermediate in the formation of asparagine and that the compound derived from hydrogen cyanide in common vetch is (5-glutamyl)-3-cyanoalanine. The metabolism of hydrogen cyanide according to these steps occurs in a number of plants, including Chlorella, not believed to be cyanogenic. The enzymes for 3-cyanoalanine biosynthesis have been studied from various plants by Floss et al., (1965), Dunhill and Fowden (1965), and Hendrickson (1968).

The metabolism of hydrogen cyanide in some fungi has been studied by Strobel (1966, 1967). An unidentified psychrophilic basidiomycete, which produces sufficient hydrogen cyanide to be toxic to host plants, was shown to be capable of incorporating hydrogen cyanide into 1-carboxyl groups of alanine and glutamic acid. The fungus contained enzymes capable of catalysing the Strecker synthesis of 2-aminopropionitrile and 4-amino-4-cyanobutyric acid from hydrogen cyanide, ammonia and acetaldehyde or succinic semialdehyde respectively. The nitriles were hydrolysed to the amino acids.

1.5 Glucosinolates

Chemistry. The glucosinolates may all be considered to be S-β-D-glucopyranosyl derivatives of O-sulphonated thiohydroximic acids as shown in Figure Ia. The aglycone derivatives vary in complexity from methyl to indole. Challenger (1959) and Kjaer (1960, 1963) have reviewed the chemistry and

1a General glucosinolate structure:



1b Gadamer's structure for sinigrin:

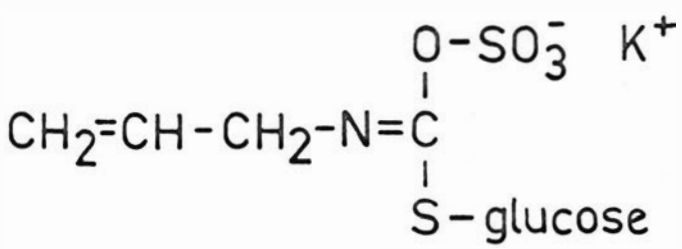


FIGURE 1 STRUCTURES PROPOSED FOR GLUCOSINOLATES

history of glucosinolates.

Until recently trivial names, e.g. sinigrin for allylglucosinolate or glucotropaeolin for benzylglucosinolate were in common usage. In the last few years a considerable number of these glucosides has been described. To simplify naming, the semisystematic term "glucosinolate" has been introduced by Ettlinger and Kjaer (1961) and will be used in this thesis. This group has also been referred to as "thioglucosides" or "mustard oil glucosides" based on the typical, although not universal occurrence of volatile isothiocyanates, i.e. mustard oils, as products of enzymic hydrolysis.

All glucosinolates occur as salts, usually of potassium but other bases are known. Sinalbin, isolated from white mustard seed (Sinapis alba), is the salt of p-hydroxybenzylglucosinolate and sinapin, the choline ester of 4-hydroxy-3,5-dimethoxycinnamic acid. Some glucosinolates have been crystallised as quaternary ammonium salts and are not of natural origin.

Gadamer (1897) originally proposed a structure for allylglucosinolate as illustrated in Figure Ib, chiefly on the basis that allyl isothiocyanate was a product of hydrolysis, and that silver ions cleave off glucose, indicating the thioglucoside linkage. This structure was surprising as allyl cyanide and sulphur had previously also been found as hydrolysis products of sinigrin - an observation not easily reconciled with Gadamer's structures.

Ettlinger and Lundeen (1956) proposed the revised structure for glucosinolates as in Figure Ia and explained the characteristic production of isothiocyanates by intramolecular rearrangement. The alternative production of nitriles and sulphur was also explained. Both reactions are illustrated in Figure 2.

Additional evidence for the revised structure in the case of allylglucosinolate was:

(i) Production of n-butylamine upon hydrogenation,

(ii) Acid hydrolysis gave vinylacetic acid,

(iii) Cold concentrated hydrochloric acid yielded hydroxylamine.

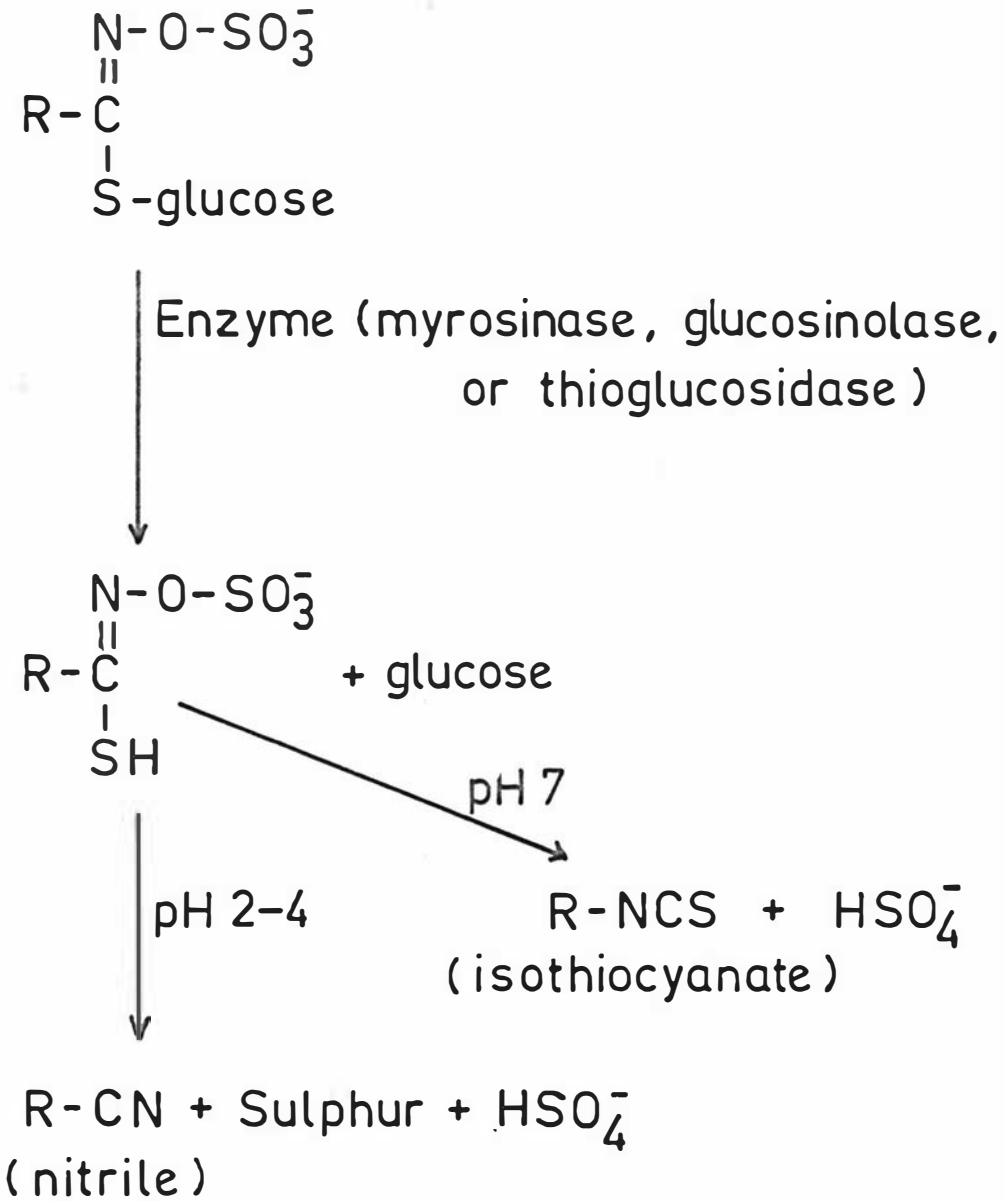


FIGURE 2 HYDROLYSIS OF GLUCOSINOLATES BY ONE ENZYME

The β-D-thioglucopyranosyl configuration was suggested by earlier work (Schneider et al., 1930) and the general revised structure has been confirmed by the synthesis of benzylglucosinolate (Ettlinger and Lundeen, 1957). X-Ray crystallography by Waser and Watson (1963) has demonstrated the anti configuration of the allyl group and the sulphate residue of allylglucosinolate.

Distribution. The diversity of aglycones and the distribution within families has been reviewed by Kjaer (1966). Approximately fifty different glucosinolates have been described, many of which are related, often in homologous series. These include compounds with simple and branched alkyl side chains and variously hydroxylated derivatives of these, monoketo-alkyls, ω-methylthioalkyls and the corresponding sulphoxides and sulphones, aralkyl groups and hydroxylated derivatives, and a few with heterocyclic substituents.

Glucosinolates occur in a restricted number of dicotyledonous families. They occur in most species of most families of the order Rhoeadales. This includes Cruciferae, Capparidaceae, Resedaceae and Moringaceae. Unrelated families, e.g. Tropaeolaceae and Euphorbiaceae also have some species containing glucosinolates.

As in the case of cyanogenic glycosides no generalisations are possible on the distribution of glucosinolates among plant organs. There is a frequent, but not universal, tendency for glucosinolates to accumulate to high concentration, and perhaps be formed at a high rate in young and actively growing tissue. Many species contain more than one glucosinolate and considerable differences in relative amounts may occur between organs of the one plant (Josefsson, 1967; Kjaer, 1960).

There is some evidence to indicate that nutrition and climatic factors influence the production and accumulation of glucosinolates just as other secondary metabolites may be affected. It has been shown that sulphate levels directly affect glucosinolate biosynthesis (Sedlak et al., 1963); however,

more investigation of these factors is required.

Function and metabolism. The diverse range of aglycones complicates a discussion on the possible roles of glucosinolates within the plant. Apart from speculation based only on comparison with the way in which other secondary metabolites act, there is little systematic evidence for specific roles of glucosinolates. There is no evidence to show that glucosinolates as such have a beneficial effect in the plant. Indeed it has been shown that certain parasitic insects have their feeding stimulated by the presence of glucosinolates - a situation where the glucosinolates appear to be having an undesirable effect.

The principal products of enzymic hydrolysis of glucosinolates beside sulphate and glucose are isothiocyanates, nitriles, and sulphur, and in a few cases organic thiocyanates. White mustard powder when mixed with water has also produced small amounts of N,S-di(p-hydroxybenzyl)dithiocarbamate, presumably from p-hydroxybenzylglucosinolate or its simpler hydrolysis products (Kawakishi et al., 1967). The dithiocarbamate can be further decomposed to di-(p-hydroxybenzyl) disulphide, p-hydroxybenzylamine, p-hydroxybenzyl alcohol and hydrogen sulphide. This complex degradation scheme may occur with other glucosinolates.

The isothiocyanates are generally reactive and may spontaneously undergo further reactions with amines, including free amino groups of proteins, to give substituted thioureas. Isothiocyanates with 2-hydroxyl or 3-hydroxyl groups may undergo cyclisation to give thiooxazolidones (Challenger, 1959). A few isothiocyanates, notably p-hydroxybenzyl isothiocyanate, 3-indolylmethyl isothiocyanate (from glucobrassicin) and its N-methoxy derivative (from neoglucobrassicin) spontaneously give thiocyanate and corresponding alcohols which may be involved in further reactions, for example, in the formation of ascorbigen (Virtanen, 1962).

Enzymic hydrolysis within the plant is widely considered to occur only

when the plant tissue is crushed. It has been claimed that the hydrolytic enzyme system known as myrosinase is isolated in specialised cells termed idioblasts (Guignard, 1890) and glucosinolates may accumulate in the vacuolar sap of other cells. There has been dispute over whether the classical myrosinase system is one enzyme, two, or more. Gaines and Gowring (1962), following earlier workers (Sandberg and Holly, 1932), have claimed that myrosinase from oriental mustard seed (Brassica juncea) could be prepared by ethanol precipitation followed by fractionation with ammonium sulphate and ion exchange chromatography, and that separate enzymic activity for a β-thioglucosidase and a "myrosulphatase" could be demonstrated. Further, they claimed that both activities were required for the complete hydrolysis of allylglucosinolate. Subsequent work by Calderon et al., (1966) has failed to substantiate these claims although a recent study of oriental mustard has indicated that myrosinase is a glycoprotein with both β-thioglucosidase and sulphatase activity (Thompson, 1966).

Ettlinger and Lundeen, when proposing the revised structure of glucosinolates, suggested that only one enzymic activity would be required (namely the β-thioglucosidase), and that nitriles or isothiocyanates would be formed according to the pH of the medium. Subsequently an enzyme was described from white mustard flour which was insoluble in salt solutions and which hydrolysed a range of glucosinolates by way of unstable intermediates detectable by U.V. spectrophotometry (Ettlinger et al., 1961; Ettlinger and [illegible], 1962). The intermediates were postulated as thiohydroximate-O-sulphonates and the enzyme, termed glucosinolase, was shown to require ascorbate as a cofactor. Other workers (Tsuruo and Hata, 1967; Kojima and Tamiya, 1964; Nagashima and Uchiyama, 1959) have also investigated the effect of ascorbate on what they consider to be myrosinase. It is apparent that there may be several different

plant enzymes with β-thioglucosidase activity and, possibly, sulphatase activity and that not all of these require ascorbate. MacGibbon (personal communication) has shown that there are at least two enzymes in rape leaves, (Brassica napus L.) which can be separated by acrylamide gel electrophoresis and detected by causing precipitation of barium sulphate in the gel when treated with barium chloride and allylglucosinolate in the absence of added ascorbate.

The systematic name Thioglucoside glucohydrolase E.C. 3.2.3.1 has been given to β-thioglucosidases. An enzyme fraction from mustard which was said to contain this activity has been shown to be capable of transferring glucose from p-nitrophenyl-β-1-D-glucopyranoside to glycerol (Howard and Gaines, 1968). This type of transfer is commonly observed with other β-glucosidases.

Organic thiocyanates are formed upon enzymic hydrolysis of glucosinolates in several plants. The production of phenylacetonitrile, benzyl thiocyanate and benzyl isothiocyanate has been studied by Virtanen and Saarivirta (1962) when finely ground seeds of garden cress (Lepidium sativum L.) were added to water at 0°. It was shown that if the reaction was stopped by rapid heating within about 30 seconds then benzyl isothiocyanate predominated. With longer periods benzyl thiocyanate and phenylacetonitrile were formed and only low concentrations of benzyl isothiocyanate were observed. The production of both benzyl thiocyanate and phenylacetonitrile required particular enzymes and it was suggested that an isomerase converted benzyl isothiocyanate to benzyl thiocyanate although no direct evidence was obtained. An alternative explanation of benzyl thiocyanate formation may involve an enzyme acting on an intermediate such as benzyl thiohydroxamate-O-sulphonate. The initial high concentration of benzyl isothiocyanate could be attributed to nonenzymic degradation of the accumulated intermediate after all enzymes had been destroyed at the end of the

short incubation period. Both possibilities are illustrated in Figure 3. Further experiments are required to settle this matter.

Not all cases of nitrile formation require specific enzymes. It has been shown that under acidic conditions several glucosinolates may give nitriles when treated with myrosinase. Of particular interest is the formation of 3-indolylacetonitrile from 3-indolylmethylglucosinolate (Gmelin and Virtanen, 1961). This nitrile has strong auxin activity in a wide range of plants and attracted considerable interest when it was first isolated from Brassica species. There have been some recent attempts to study the glucosinolate itself for auxin activity but no clear conclusions have emerged (Kutacek et al., 1966; Anderson and Muir, 1966).

The reactions described above occur when plant tissue or seeds are cut or ground with water and there is little evidence to show that these reactions occur in undamaged tissue. Amines, or their derivatives, related or derived from isothiocyanates have been detected in plants containing corresponding glucosinolates (Larsen, 1965; Challenger et al., 1955) and these findings may represent evidence of metabolism of the glucosinolates in intact plants.

Other examples of further metabolism of glucosinolates occur in microorganisms (Oginsky et al., 1965; Reese et al., 1958) and animals (Goodman et al., 1959). The decomposition of plant material containing glucosinolates may involve microorganisms as well as enzymes from the plant. It is therefore not possible to attribute to specific organisms the source of various degradation products including carbon disulphide, hydrogen sulphide, various disulphides (e.g. diallyl disulphide), amines and perhaps many other compounds, all derived from glucosinolates.

Although no beneficial effect to the plant has been ascribed to the glucosinolates as such, there is some evidence to show that the hydrolysis products

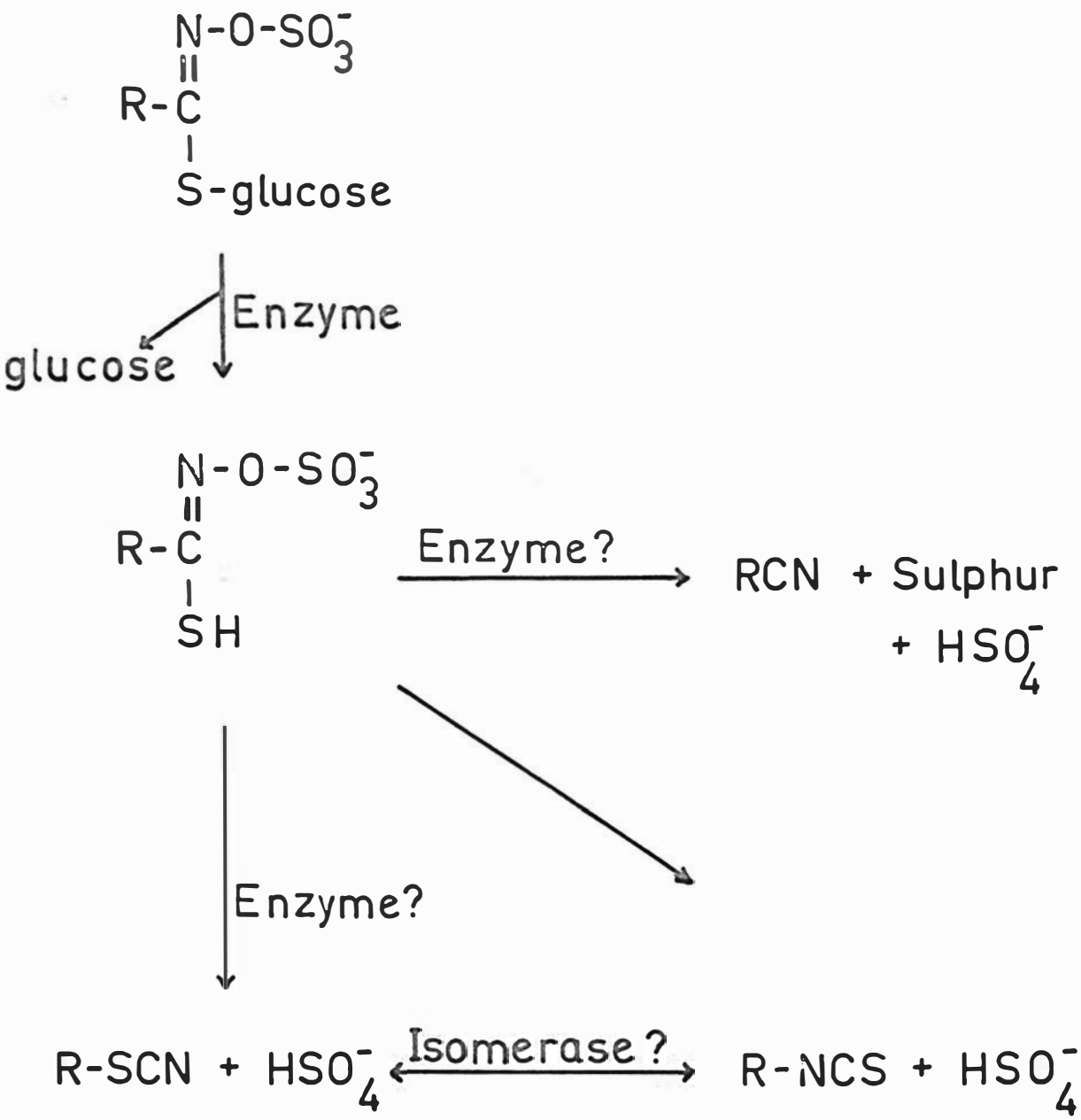


FIGURE 3 ENZYMIC HYDROLYSIS OF GLUCOSINOLATES TO GIVE THIOCYANATES, ISOTHIOCYANATES AND NITRILES.

may help protect the plant from some parasites. Isothiocyanates are known to be toxic to insects (Lichtenstein, 1966). Virtanen (1962) has shown isothiocyanates and thiocyanates have anti-bacterial or anti-fungal effects at low concentration. However it seems some parasites do not find glucosinolates or their hydrolysis products toxic and selectively attack plants containing glucosinolates (Wenser, 1962; Traynier, 1965; Hovanitz and Chang, 1963).

1.6 Biogenetic relationship between glucosinolates and cyanogenic glycosides

The biogenetic relationship between these two classes arises from the nature of the aglycones rather than from the fact that both classes are glycosides. Following the presentation of a revised structure for glucosinolates, it was recognised by Kjaer (1960) that many glucosinolates have a considerable portion of the carbon and nitrogen skeleton in common with known amino acids. In certain cases the aglycone may be directly related to an amino acid by assuming the loss of the amino acid carboxyl group and oxidation of the 2-carbon atom and amino group to a hydroxamic acid. This relationship has since been confirmed in a number of experiments.

Similarly most cyanogenic glycosides have aglycones which may be related to amino acids by assuming the loss of a carboxyl group and oxidation of the 2- and 3-carbon atoms as well as the amino group. Of those glycosides listed in Table I only gynocardin, acacipetalin and sierin do not bear such a direct relationship to common amino acids. Again numerous experiments have confirmed this relationship.

Cyanogenic glycosides and glucosinolates may thus both be derived from amino acids with loss of carboxyl groups and oxidation of the remaining fragment. If the retention of the C-N bond is accepted and if the carboxyl group is lost as carbon dioxide then in both cases a six electron oxidation is required which may be interpreted as three oxidation steps. The possibility arises that the

biosynthesis of each class may have certain intermediates in common. In theory these intermediates could include amines, N-hydroxyamines, N-hydroxyamino acids, oximes, 2-oximino acids, amides, nitriles, and hydroxamic acids. Experiments, as detailed below, have virtually eliminated some of these possibilities but have shown the C-N bond is generally retained.

1.7 Cyanogenic glycoside biosynthesis

The relationship between amino acids and cyanogenic glycosides was experimentally demonstrated by Gander (1958) and Conn and Akamatsu (1958) when significant incorporation of radioactivity from ^{14}C-labelled tyrosine was observed in the hydrolysis products of dhurrin from sorghum (Sorghum vulgare). Gander also observed an increase in content of cyanogenic glycoside when L-tyrosine, 3-(p-hydroxyphenyl)serine, L-serine, glycine or hydroxylamine was included in the nutrient for sorghum seedlings and he suggested that 3-(p-hydroxyphenyl)serine might be an intermediate between tyrosine and dhurrin.

Similarly Mentzer et al., (1963) showed the phenylalanine was converted to prunasin (although described as "prulaurasin") in cherry laurel shoots, and the same conversion was observed by Ben-Yehoshua and Conn (1964) in peach (Prunus persica, Batsch) seedlings. Butler and Butler (1960) showed white clover shoots synthesised linamarin and lotaustralin from L-valine and L-isoleucine respectively and these observations have been extended to other plants containing these glucosides (Butler and Conn, 1964a; Abrol, 1966; Abrol and Conn, 1966; Nartey, 1968). Recently taxiphyllin has also been shown to be formed from tyrosine (Bleichert et al., 1966).

Good evidence for demonstrating that the C-N bond of amino acids remains intact in the biosynthesis of dhurrin (Uribe and Conn, 1966), taxiphyllin, (Bleichert et al., 1966) and linamarin (Butler and Conn, 1964a) has been obtained by administering amino acids with both ^{14}C and ^{15}N labels and comparing the

degree of dilution of these two isotopes.

Attempts to obtain evidence for intermediates between amino acids and cyanogenic glycosides met with less success. Before it was shown that the C-N bond remained intact, attempts were made to test some non-nitrogenous compounds as precursors or intermediates. It was shown that acetone-2-^{14}C, 3-hydroxyisovaleric acid-3-^{14}C and 3,3-dimethylacrylic acid-4,4'-^{14}C were not efficiently incorporated into linamarin (Butler and Conn, 1964a). Neither were tyramine-B-^{14}C, p-hydroxyphenylacetic-B-^{14}C, p-coumaric acid-3-^{14}C, or p-hydroxybenzaldehyde-7-^{14}C efficient precursors of dhurrin although p-hydroxybenzaldehyde-7-^{14}C was incorporated into a glucoside which was initially thought to be dhurrin (Koukol et al., 1962; Libby, 1962; Gander, 1962). L-phenylalanine was not a precursor of dhurrin, eliminating the possibility of the phenolic group being introduced after the branching point in the biosynthetic pathways to tyrosine and phenylalanine. Further, there was no significant incorporation from hydrogen cyanide-^{14}C into linamarin or dhurrin (Ehrenthal-Goldschmidt et al., 1963) although it was extensively metabolised. This excluded any possibility of the biosynthesis being a reversal of cyanoglycoside degradation. Competitor experiments, where unlabelled compounds were administered with labelled amino acids in the hope of observing reduced incorporation of label into the glycosides, failed to indicate likely intermediates.

Following the experiments with ^{15}N labelled amino acids, attention was focused on possible intermediates containing nitrogen. The two classes of compounds which could be considered as free intermediates in nitrile formation are amides and oximes. Rearrangement of hydroxamic acids to 2-hydroxyl compounds is unlikely but cannot be excluded. A certain minimum number of definable steps may be postulated. These may include the loss of the carboxyl group, the introduction of a hydroxyl group possibly via an unsaturated intermediate prior to glycosylation, the oxidation of the amino group possibly in two steps to an

amide or oxime, and the dehydration of the amide or oxime to give a nitrile. Some of these steps may occur in concerted reactions, for example, the conversion of 2-oximino acids to nitriles is known to occur (Ahmad and Spenser, 1961) and would represent combined decarboxylation and dehydration reactions. Similarly amino acids can be oxidised to amides in one step with loss of carbon dioxide (Masalis and Ingraham, 1962). Because there are several combinations of the order in which these steps may occur there is, in theory, approximately fifteen to twenty compounds which could be intermediates. It is also possible that several of the necessary transformations occur with bound intermediates.

It has been suggested that the introduction of a 3-hydroxyl group on the amino acid precursor is the first step in the pathway to cyanogenic glycosides and evidence has been presented to show that 3-(p-hydroxyphenyl)serine may be formed from tyrosine in sorghum (Gander, 1959). However, subsequent experiments with labelled 3-hydroxy amino acids have failed to show significant incorporation into dhurrin (Uribe, 1965) or linamarin (Butler and Conn, 1964a), and tend to exclude the introduction of the 3-hydroxyl group as the first step.

Amides have been tested as precursors of cyanoglycosides but no good evidence of incorporation has been obtained. p-hydroxyphenylacetamide-U-^{14}C, which is formed from L-tyrosine-U-^{14}C by particulate enzymes from sorghum seedlings, was incorporated into dhurrin at only a small fraction of the rate of L-tyrosine-U-^{14}C (Uribe, 1965). In flax seedlings 2-hydroxyisobutyramide-β-1-D-glucopyranoside-^{14}C was not converted to linamarin although this amide has been identified in trace amounts in ethanolic extracts of linen flax seedlings (Conn and Butler, unpublished results, 1965). This observation may have been due to partial hydrolysis of linamarin during extraction and chromatography. While no experiments have excluded a pathway involving amides, there is little evidence to show that they are the principal compounds involved.

The other group of compounds which could be intermediates are the oximes. Many aldoximes (Sidgwick, 1966) and 2-oximino acids (Ahmad and Spenser, 1961) may be converted to nitriles by one or two simple chemical steps as illustrated in Figure 4. However there are only a few scattered reports of oximes occurring as natural products. A pathway to "prulaurasin" has been postulated by Mentzer et al., (1963), with 2-oximino-3-hydroxy-3-phenylpropionic acid as an intermediate. The suggestion that this oximino acid was formed from the corresponding keto acid and hydroxylamine was inconsistent with the later finding that the C-N bond remained intact. Other workers (Uribe, 1965; Butler and Conn, 1964a) have also suggested that oximes may be intermediates in cyanogenic glycoside biosynthesis.

Some attempts to directly observe possible intermediates have been made. Uribe (1965) systematically varied the conditions under which sorghum seedlings incorporated labelled L-tyrosine into dhurrin but could not detect an intermediate. Gander (1966) has reported that sorghum seedlings can make another phenolic glucoside from L-tyrosine which cannot be separated from dhurrin by paper chromatography but which may be distinguished by its reaction with 2,4-dinitrophenylhydrazine and its partial resistance to hydrolysis by β-glucosidase. No further information has been published on this glucoside. Recently Gander, in a personal communication, has indicated that it may have been an artifact.

In an attempt to induce the accumulation of intermediates in linamarin biosynthesis Butler and Conn (1964b) administered a range of analogues of valine and isoleucine together with L-valine-U-^{14}C. A number of the analogues inhibited linamarin biosynthesis and a few allowed the accumulation of an unidentified glucoside. These observations together with further studies on the unidentified glucoside will be discussed in detail in Section 3.3 of this thesis. Butler and Conn did not examine the plant tissue for volatile inter-

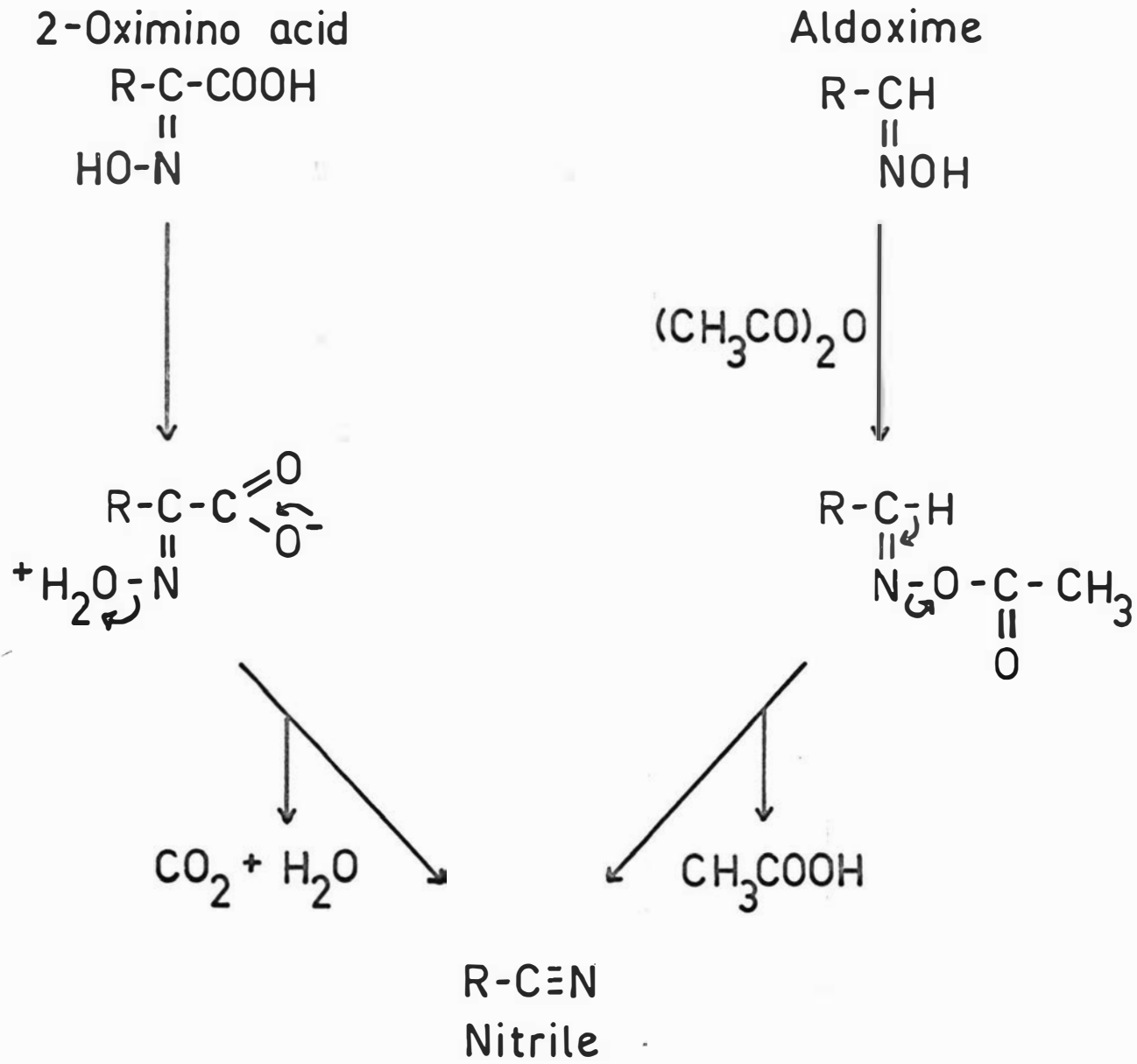


FIGURE 4 CONVERSION OF OXIMINO ACIDS AND ALDOXIMES TO NITRILES

mediates.

1.8 Glucosinolate biosynthesis

Although only a few glucosinolates have aglycones directly related to common amino acids these have been studied in most detail. It has been shown that benzyl-, isopropyl-, and 3-indolylmethylglucosinolates are efficiently formed from the common amino acids L-phenylalanine (Underhill and Chisholm, 1964; Benn, 1962), L-valine (Meakin, 1965) and L-tryptophane (Schraudolf and Bergmann, 1965) respectively, and that 2-phenylethylglucosinolate may be formed from L-phenylbutyrine which in turn is derived from L-phenylalanine and acetate (Underhill, 1965). Only with p-hydroxybenzylglucosinolate has doubt been raised as to whether the corresponding amino acid tyrosine was a direct precursor. It was shown by Kindl (1965) that p-coumaric acid was more readily incorporated into this glucosinolate than was L-tyrosine in white mustard plants. This may have been due to differences in the transport of the two precursors to the site of synthesis. Extensive metabolism of the L-tyrosine occurred.

There is no analogous amino acid known in nature for allylglucosinolate. However this glucosinolate frequently occurs in plants along with 3-methylthiopropylglucosinolate (glucoibervirin) and the sulphoxide (glucoiberin) and sulphone (glucocheirolin) derivatives (Ettlinger and Thompson, 1962). Evidence has accumulated to show that all of these may be derived from L-homomethionine which in turn may be formed from methionine and acetate (Chisholm and Wetter, 1966; Matsuo and Yamazaki, 1966). It was proposed that 3-methylthiopropylglucosinolate may be an intermediate in the formation of allylglucosinolate. D,L-Allylglycine was not efficiently incorporated. Higher homologues of these glucosinolates may be formed similarly from higher homologues of methionine.

Experiments with ^{14}C and ^{15}N labelled L-phenylalanine have shown that benzylglucosinolate retains the amino group but the carboxyl group is lost,

presumably as carbon dioxide during the biosynthesis (Underhill and Chisholm, 1964; Meakin, 1965). As with cyanogenic glycosides some non-nitrogenous compounds were studied as possible precursors of glucosinolates before it was shown that the C-N bond of L-phenylalanine remained intact during the biosynthesis of benzylglucosinolate.

Some attempts have been made to find intermediates between amino acids and glucosinolates. Phenylacetamide, 2-phenylethylamine, phenylacetonitrile, phenylacethydroxamic acid and 2-oximino-3-phenylpropionic acid (phenylpyruvic acid oxime) were not efficiently incorporated into benzylglucosinolate (Underhill and Chisholm, 1964; Meakin, 1965).

Recent experiments, some of which form part of this thesis, have shown that isobutyraldoxime (Tapper and Butler, 1967), phenylacetaldoxime (Tapper and Butler, 1967; Underhill, 1967) and 3-phenylpropionaldoxime (Underhill, 1967) may be incorporated into isopropyl-, benzyl-, or 2-phenylethylglucosinolates respectively at rates greater than that obtained with corresponding amino acids under similar conditions. Further, phenylacetaldoxime formation has been detected in the nasturtium (Tropaeolum majus L.) shoots used to study benzylglucosinolate biosynthesis (Underhill, 1967) and a cell free preparation can convert N-hydroxyphenylalanine to phenylacetaldoxime (Kindl and Underhill, 1968).

The efficiency of various sulphur compounds as precursors of both the sulphate and isothiocyanate sulphur has been investigated. Sulphur dioxide (Kutacek et al., 1966) sulphate, sulphide, thiosulphate, methionine (Wetter, 1964) and cysteine (Kindl, 1965) may all serve as precursors, with varying efficiencies, of the two glucosinolate sulphur atoms. The O-sulphonate group may be directly derived from adenosine-5'-phosphosulphate or 3'-phosphoadenosine-5'-phosphosulphate (Wetter, 1964). Recently, Meakin (1967) has claimed that phenylacetothiohydroxamic acid does not contribute the isothiocyanate sulphur atom of glucosinolates but it may come from β-D-1-thioglucose. This

implies that glucosylation may occur by mechanisms quite different from those usually considered.

1.9 Methods for biosynthesis studies

Adelberg (1953) has suggested that three general approaches may be employed to obtain evidence of biosynthesis pathways. These are the use of isotopic tracers, the *in vitro* study of enzyme systems, and the use of organisms with biosynthetic pathways which can be blocked either by mutations or by use of specific inhibitors. Considerable insight into the possible pathways may come from using non-radioactive precursors including compounds not of natural origin, the study of sequential synthesis of apparently related substances during the course of the growing season, and chemical speculation as to the possible relationship of similar structures. All of these procedures may be criticised depending on particular circumstances. The first three criteria are most commonly used but considerable caution is required when interpreting experiments relying on only one type of evidence.

It may be necessary to distinguish between a normal pathway with normal intermediates and other possible pathways which may contribute to a particular biosynthesis. Further, what may be considered to be the end-product of one biosynthetic pathway may be further metabolised and therefore considered to be an intermediate on a broader examination.

Swain (1965) in a review of methods used for biosynthesis studies on higher plants, has discussed some of the limitations of experiments using isotopic tracers. If an administered compound is to be demonstrated as a precursor the plant tissue must be able to absorb it, generally from dilute solution; transport it to the site of biosynthesis; convert a substantial proportion of it to the desired end-product; and then accumulate the end-product. These conditions must be taken into account when plant material is selected. Several factors may affect the interpretation of experiments of this type. Firstly a

compound is administered, frequently to a detached organ, under far from normal physiological conditions. Secondly the compound may be toxic when administered under these conditions or may be excluded from the actual site of synthesis. Thirdly the administered compound may not be a natural intermediate but be converted to the desired product by way of reactions unrelated to the normal biosynthesis or by natural pathways of limited significance.

A further difficulty arises in the expression of the results of experiments. The effectiveness of a compound as a precursor may be expressed as the percentage incorporated into the product after a certain time interval. This is best when a relatively small amount of labelled compound can be introduced into the effective natural pool with minimum disturbance. The possibility of the compound being metabolised in other ways should be assessed and measured if possible and this taken into account when interpreting the results. For example, an amino acid might be incorporated substantially into protein and give only a small percentage incorporation into the desired product yet still be an effective, indeed obligatory, natural precursor. In contrast a compound considered to be an intermediate and convertible only to the desired product should have a high percentage conversion.

If a relatively large amount of precursor is administered and if the rate of biosynthesis is limited the percentage converted may be low. An indication of precursor effectiveness may then be given by a comparatively low dilution of label. Underhill et al., (1957) used the dilution figures as the principal criterion for assessing precursors of quercetin biosynthesis. However Watkin and Neish (1960), also studying quercetin, showed that the percentage of L-phenylalanine converted was a more reliable guide to precursor effectiveness. Their results are notable for the effect of the amount of administered L-phenylalanine on the extent of incorporation. In particular a five-fold increase in percentage conversion occurred with a ten-fold increase

in the amount administered and the corresponding dilution was 600 times less. Thus the interpretation may become dependent on the amount and concentration of the administered compound.

CHAPTER II

MATERIALS AND METHODS

2.1 Plant Material

Flax cell culture. Biosynthetic pathways have frequently been studied in microorganisms with greater ease than in higher plants. Cyanogenic glycosides, however, are known in only a few microorganisms and have been poorly described until very recently. Further, no glucosinolates have been reported in microorganisms. Some of the advantages of working with microorganisms (i.e. uniformity of tissue type, reproducibility, sterility, and ease of handling) can be obtained with suspended cell cultures of higher plant tissues. An unsuccessful attempt was made to establish linen flax (Linum usitatissimum L.) in suspended cell culture using a comprehensive liquid medium with supplements of coconut milk, kinetin, indole-3-acetic acid, 2,4-dichlorophenoxyacetic acid and glutamine (Murashige and Skoog, 1962). For this attempt, callus tissue grown from seedling hypocotyl was obtained from Dr E. G. Bollard, D.S.I.R., Auckland and maintained on a similar agar medium.

Flax seedling shoots. Linen flax seedlings, variety "Redwood", were used for the study of linamarin biosynthesis. The conditions for optimal incorporation of L-valine-^{14}C into linamarin have been discussed by Butler and Conn (1964a). Seeds were obtained from Mr P. Palmer, Crop Research Division, D.S.I.R., Lincoln, and germinated on a layer of moist cotton wool in plastic trays. The seeds were held at 25° in darkness and a saturated atmosphere for four days, by which stage the etiolated shoots were about 3 to 4 cm tall. They were then covered with a sheet of clear "perspex" and placed in a growth chamber where they received 18 hours exposure to artificial light of approxi-

mately 2000 foot candles intensity. Rapid greening of the cotyledons occurred.

The top 2.5 to 3 cm portion of the linen flax shoots were excised under water to restrict damage to the vascular system and used in experiments, usually in lots of twenty. The compounds to be administered were dissolved in 200 ul or less of water or tris(hydroxymethyl)aminomethane buffer in 1 ml beakers and the shoots allowed to take up all the solution, which was followed by water as required. In certain cases where volatile compounds were administered, the seedling shoots were enclosed in suitable transparent vessels. The shoots were usually allowed to assimilate and metabolise the administered compounds for 7 hours of continuous artificial light.

Cherry laurel and peach shoots. Cherry laurel shoots (Prunus laurocerasus L.) were used by Mentzer et al., (1963) to show that phenylalanine could be converted to prunasin. Although cherry laurel was originally described as containing prulaurasin, Plouvier (1935) has shown that this arises by isomerisation during the extraction procedure originally used and that cherry laurel contains prunasin. This was confirmed in this investigation by the isolation of prunasin as white crystalline needles from oven dried cherry laurel shoots according to the method of Plouvier. Paper chromatography failed to show evidence for any other cyanogenic glycoside in ethanolic extracts of fresh cherry laurel shoots. In the experiments described here, young and actively growing shoot tips bearing three or four expanded leaves were selected for isotope experiments.

In similar experiments to those of Mentzer et al., (1963), Ben-Yehoshua and Conn (1964) showed that prunasin in peach seedlings was efficiently synthesised from L-phenylalanine. However, sterile peach seedlings (Prunus persica, Batsch) are not particularly easily obtained, so the possibility of using young growing shoots of mature trees was investigated. Again, extraction of oven dried shoots yielded crystalline prunasin and paper chromatography failed

to give evidence of any other cyanogenic glycosides. Batches of shoots of both cherry laurel and peach selected at different times of the year from trees growing under natural conditions may have varied in their metabolic responses. Both species produce shoots abundantly in the early summer but later in the summer, only after pruning. A supply of shoots was possible for about half of the year. Because of the possible seasonal variations most experiments included control treatments where L-phenyalanine-U-^{14}C was administered. Peach shoots were selected from two trees at different times of the year.

Non-volatile compounds were assimilated by peach and cherry laurel shoots from solutions in 2 ml beakers and were followed by water as required. Volatile compounds were administered with the shoots enclosed in glass vials. Two or more shoots were used for each treatment and the fresh weight recorded.

Scurvy grass. Plant systems for studies on glucosinolate biosynthesis were chosen bearing in mind the availability of isotopically labelled compounds, all of which were also used in the studies on cyanogenic glucoside biosynthesis. Scurvy grass (Cochlearia officinalis L.) was chosen for its reported content of isopropylglucosinolate in seed (Kjaer and Conti, 1953) and a preliminary investigation indicated that this glucoside along with others was present in the leaf tissue. Seeds were obtained from Professor A. Kjaer, Copenhagen, and after germinating on wet filter paper, the seedlings were grown by water culture in a growth chamber under long day conditions until they were about 3 cm high. Labelled compounds were assimilated from solution through part of the roots - about half of the root system was removed prior to treatments. Excised leaves were not suitable for experiments as they tended to wilt under even mild conditions.

Garden cress. Seedling shoots of garden cress (Lepidium sativum L) were used for the study of benzylglucosinolate which is the predominant glucosinolate in this plant. Seeds were obtained from Arthur Yates and Co. Ltd.,

Auckland. The seeds were germinated on moist filter paper for five days in darkness at 25°, and then given 18 hours of exposure to light in a growth chamber prior to treatment with labelled compounds. The seedling shoots were used in batches of 30 in similar procedures to those used for linen flax shoots. Assimilation was for 48 hours under continuous artificial light of approximately 2000 foot candles intensity.

2.2 Chemicals

2-Oximinoisovaleric acid. The method of synthesis was adapted from Ahmad and Spenser (1961). A solution of 5 mmoles of hydroxylamine hydrochloride and 5 mmoles of sodium bicarbonate at 5° in 1.5 ml of water was added with cooling to 5 mmoles of 2-ketoisovaleric acid dissolved in 3 ml of water. The mixture was left standing for 18 hours at about 20° prior to being cooled and acidified to pH 1.5 with 6% w/v hydrochloric acid. It was then extracted three times with diethyl ether, the combined extracts being dried over anhydrous sodium sulphate and the solvent removed under reduced pressure. The oily residue crystallised slowly and 2-oximinoisovaleric acid was obtained by recrystallisation from a mixture of ether and hexane, in 76% yield. It was identified as the anti(HC=NOH—COOH)isomer by comparing its infrared spectrum with the data recorded by Ahmad and Spenser (1961).

Isobutyraldoxime. The method of synthesis was adapted from Vogel (1948). A mixture of 0.1 mole of freshly distilled isobutyraldehyde and 0.11 of hydroxylamine hydrochloride was placed in a flask fitted with a dropping funnel, thermometer and stirrer together with 15 ml of water. A small excess of 0.06 mole of sodium carbonate dissolved in approximately 20 ml of water was added slowly with stirring, keeping the temperature below 45°. The mixture was stirred for a further hour before the crude oxime was separated off. The aqueous phase was extracted twice with a small volume of diethyl ether and this was added to

the crude oxime and dried over anhydrous sodium sulphate prior to distillation. The principal fraction boiling at 140-142° (755 mm) was isobutyraldoxime in 73% yield. (Lit. b.p. 140° Heilbron, et al., 1965). According to Phillips (1958) isobutyraldoxime exists in syn and anti forms in the equilibrium ratio of 2.1:1. The presence of two isomers in this preparation was confirmed by gas chromatography. The isomers were partially separated using a preparative scale Carbowax 400 column and examined by infrared spectrometry. The two fractions gave slightly different spectra initially but after standing overnight at 20° both fractions gave spectra identical to the initial mixture.

Phenylacetaldoxime. Sodium hydroxide solution (10% w/v) was added to 50 mmole of hydroxylamine hydrochloride and ice in a small flask fitted with a stirrer, until the solution was just alkaline. To this, 25 mmole of phenylacetaldehyde was slowly added with vigorous stirring for an hour. An oily paste which separated was recrystallised twice from diethyl ether and hexane mixtures to give a 30% yield of phenylacetaldoxime, m.p., 96.5°, (lit. m.p., 98.5°, Heilbron, et al., 1965).

2-Oximino-3-phenylpropionic acid (phenylpyruvic acid oxime). The method of synthesis was adapted from Ahmad and Spenser (1961). A solution of 10 mmoles of hydroxylamine hydrochloride, and 12 mmoles of sodium bicarbonate in 5 ml of water was added slowly to a cool filtered solution of 10 mmoles of sodium phenylpyruvate. The mixture, after standing at 30° for 30 minutes, was again cooled, acidified with concentrated hydrochloric acid, and extracted three times with diethyl ether. The solvent was removed under reduced pressure from the combined extracts and the residue recrystallised twice from a mixture of diethyl ether and hexane. More 2-oximino-3-phenylpropionic acid was recovered from the mother liquors giving a yield of 45%. It was identified as the anti(HO--COOH)isomer by comparing its infrared spectrum with data record-

ed by Ahmad and Spenser (1961). M.p. 173° (decomp.), (lit. m.p. 166-167°, decomp. Ahmad and Spenser, 1961).

Isopropyl isothiocyanate. The method of synthesis was adapted from Moore and Crossley (1955). A mixture of 0.18 mole of carbon disulphide and 0.18 mole of sodium hydroxide was placed in a flask fitted with stirrer, reflux condenser and dropping funnel together with 25 ml of water. This mixture was cooled to 10° and 0.18 mole of isopropylamine added over a period of 30 minutes. It was then stirred with heating to about 40° for one hour before adding 0.18 mole of ethylchloroformate and a little more water over a period of an hour. The isothiocyanate separated off at the top and the aqueous portion was extracted with a small portion of diethyl ether. This was combined with the isothiocyanate for drying over anhydrous sodium sulphate. Isopropyl isothiocyanate was isolated by fractional distillation under vacuum and boiled at about 80° at 25 mm.

Isopropyl thiourea. About 0.2 ml of isopropyl isothiocyanate was treated with 5 ml of concentrated ammonia solution overnight at room temperature. Some crystalline material dissolved on boiling this mixture to drive off ammonia and recrystallised on cooling. The thiourea was filtered off and recrystallised with hot water. M.p. 172° (lit. m.p. 169-170°, Kjaer and Conti, 1953).

D,L-O-Methylthreonine. This compound was prepared by Mr I. Manning in these laboratories according to the method of West and Carter (1937).

D,L-2-Methoxypropionaldoxime. A mixture of 0.5g D,L-O-methylthreonine and 2g of ninhydrin in 20 ml of pH 2.5, 1M citrate buffer was heated to 100° for 10 minutes and then steam distilled. The distillate was collected in a vessel containing 1g of hydroxylamine hydrochloride and 1.5g of sodium bicarbonate dissolved in 5 ml of water and allowed to stand at room temperature for 24 hours. The oxime was then extracted into diethyl ether and the extract,

after concentration under reduced pressure, purified by gas chromatography. The extract contained only one volatile compound which was trapped in cooled glass U tubes fitted to the outlet of a thermal conductivity detector. This compound was examined by high resolution mass spectrometry using an A.E.I. M59 mass spectrometer. The base peak was at mass 59 with other peaks in decreasing magnitude at masses 73, 71, 88 and 86. The latter two peaks gave accurate mass measurements consistent with ions of $C_4H_8NO^+$ and $C_5H_6NO_2^+$. As both of these contain one nitrogen atom and an even number of hydrogen atoms, both must be fragments from a hypothetical parent ion which was not observed. The fragments may correspond to the loss of CH and CH_3 from the expected parent ion of mass 103.

D,L-2-Methoxypropionitrile. The procedure followed was that of Adams et al., (1959). A mixture of 1 mole of paraldehyde and 3 moles of methanol was cooled to below 0° and treated with an excess of dry hydrogen chloride. The mixture separated into two phases and dry calcium chloride was added to the lower phase to assist further separation. The top phase was cooled and bubbled with nitrogen to remove excess hydrogen chloride prior to distillation. The fraction boiling at 70-74° (755 mm) contained 1-chloroethylmethyl ether at approximately 70% yield.

A slurry of 0.9 mole of cuprous cyanide ($Cu_2(CN)_2$) in diethyl ether, was added slowly to 1.8 mole of 1-chloroethylmethyl ether in a further 500 ml of ether and the suspension stirred and gently refluxed for 3 hours. The ethereal solution was decanted, the residue washed with further portions of diethyl ether, and the combined material was distilled to give D,L-2-methoxypropionitrile. Redistillation gave a fraction boiling at 115-117° (746 mm) (lit. b.p. 118°, Heibron et al., 1965).

D,L-3-Methoxyvaline (D,L-2-amino-3-methoxyisovaleric acid). The method of West et al., (1937) was used. A solution of 0.01 mole of 3,3-dimethyl-

acrylic acid in 20 ml of methanol was added to 0.01 mole of finely ground silver nitrate suspended in 80 ml of methanol and followed by the slow addition with stirring of 0.01 mole of bromine. After a further hour of stirring the silver bromide was filtered off and the filtrate neutralised with 2N sodium hydroxide. Excess solvent was evaporated off and the residue extracted once with diethyl ether which was discarded. It was then acidified with 2N sulphuric acid and extracted three times with equal volumes of diethyl ether. This combined ether extract was dried over anhydrous sodium sulphate, filtered through activated charcoal (Darco G60) and the solvent evaporated. 2-Bromo-3-methoxyisovaleric acid was recrystallised from a mixture of benzene and hexane with a yield of 60%.

The amino acid was prepared by heating 0.4g of 2-bromo-3-methoxyisovaleric acid to 90° for 16 hours with 3 ml of concentrated ammonia solution in a sealed tube. The excess ammonia was evaporated off and the residue, dissolved in water, was absorbed on a Zeocarb 225 column (50-100 mesh, 8 cm x 1 cm, in the $H^{(+)}$ form). After washing the column with water, the amino acid was eluted with 10% w/v ammonia solution and taken to dryness. D,L-3-Methoxyvaline was recrystallised from a water and acetone mixture. Paper chromatography, followed by a ninhydrin detecting reagent, showed only one amino acid to be present with an appropriate R_f in the system used. A total yield of 0.23g was obtained.

Methacrylonitrile. A sample of 17g of methacrylamide was heated with 20g of phosphorous pentoxide for 40 minutes and the nitrile distilled off. It was redistilled to give a fraction with b.p. 88-89° (745 mm) at 69% yield. (Lit. b.p. 90-92° Heilbron et al., 1965).

Methacrolein oxime. This was prepared by adding slowly, with stirring, 0.1 mole of sodium carbonate dissolved in 40 ml of water to a mixture of 0.2 mole hydroxylamine hydrochloride and 0.18 mole of methacrolein. After 30

minutes at room temperature, the top layer was collected and the aqueous phase extracted with diethyl ether. The combined organic phases were dried over anhydrous potassium carbonate and distilled with a short fractionating column at a reduced pressure of 45 mm. A fraction boiling at 76-78° gave a 20% yield while other fractions also contained less pure methacrolein oxime.

t-Butylisobutyramide. The method was adapted from Plaut and Ritter (1951). Approximately 11 mmoles of isobutyronitrile was mixed with 20 mmoles of t-butanol in 5 ml of glacial acetic acid. The mixture was cooled prior to adding 20 mmoles of concentrated sulphuric acid and then held at 40°C for 50 minutes. t-Butylisobutyramide was precipitated by adding 20g of ice, filtered off and recrystallised from ethanol and water. It sublimed at 110°.

2,4-Dinitrophenylhydrazine derivatives. DNPH derivatives were prepared from acetone, methylethyl ketone, isobutyraldehyde, phenylacetaldehyde and benzaldehyde. The general procedure was to add a small excess of the carbonyl compound to a warm saturated ethanolic solution of 2,4-dinitrophenylhydrazine hydrochloride containing also a small portion of dilute hydrochloric acid. The crystalline derivatives which separated on cooling were filtered off and recrystallised from ethanol or benzene and hexane.

2.3 Source of labelled compounds.

L-valine-U-^{14}C (107 mC/mmole), L-phenylalanine-U-^{14}C (7.0 mC/mmole), potassium cyanide-^{14}C (0.5 mC/mg) and sodium isobutyrate-1-^{14}C (180 μC/mg) were all obtained from the Radiochemical Centre, Amersham. Phenylacethydroxamic acid-1-^{14}C (0.90 μC/mg) and 2-oximino-3-phenylpropionic acid-2-^{14}C (1.52 μC/mg) were generous gifts from Dr E. W. Underhill, Prairie Regional Laboratory, Saskatoon. Hydroxylamine hydrochloride-^{15}N of 95 atoms per cent excess (APXS) was obtained from Dr E. E. Conn, University of California, Davis. L-valine-^{15}N of 45 APXS was obtained from Schwarz Bioresearch Inc., New York. All other

labelled compounds were prepared as described below. In most cases methods of synthesis were tried with unlabelled or low specific activity compounds before committing the high specific activity compound. The compounds from these trial preparations were identified by comparing their infrared spectra with spectra of well characterised standards.

2-Oximinoisovaleric acid-U-^{14}C. 2-Ketoisovaleric acid-U-^{14}C was obtained by the action of 4 mg of L-amino acid oxidase (dried Crotalus adamanteus venom) on 10 μmoles of L-valine-U-^{14}C (50 μC) in the presence of 25 units of catalase in 2.5ml of water. The mixture was incubated with toluene as preservative for 12 hours at 27° and acidified with 0.5 ml 2M hydrochloric acid before extracting with diethyl ether (Meister, 1952). The combined ether extract was added to a solution of 4.55 mg of non-radioactive sodium 2-ketoisovalerate and 15 mg of sodium bicarbonate in 100 μl of water. To this 40 μl of 4M hydroxylamine hydrochloride was added and the mixture left standing at room temperature for 18 hours. The 2-oximinoisovaleric acid was extracted into diethyl ether after acidification with 0.3 ml of 1M hydrochloric acid and the solid residue obtained upon evaporating the ether was recrystallised from a mixture of ether and hexane. 2-Oximinoisovaleric acid-U-^{14}C was identified as the anti(HO-COOH) isomer by comparison of its infrared spectrum with the spectral data recorded by Ahmad and Spenser (1961).

Isobutyraldoxime-U-^{14}C. Isobutyraldehyde-U-^{14}C was prepared by heating 0.1 mmole of L-valine-U-^{14}C with 100 mg of ninhydrin in 2 ml of pH 2.5, 1M citrate buffer for 5 minutes at 100°. It was then steam distilled directly into 0.2 mmole of hydroxylamine hydrochloride and 0.2 mmole of sodium bicarbonate dissolved in 1 ml of water. The isobutyraldoxime-U-^{14}C that was formed on standing 18 hours was partitioned into diethyl ether and purified by gas chromatography. The oxime was trapped in a glass U tube inserted into a thermal conductivity detector outlet and cooled with solid carbon dioxide. It was

presumed to be a mixture of syn and anti isomers.

Isobutyraldehyde-U-^{14}C. This was prepared by heating 50 μmoles of L-valine-U-^{14}C with 5 mg of ninhydrin in 0.1 ml of pH 2.5 1M citrate buffer on a boiling water bath in an evacuated system. The isobutyraldehyde-U-^{14}C was collected by condensing in a portion of the closed system cooled with liquid nitrogen. The aqueous solution obtained on thawing was administered directly to the seedlings.

Isobutyramide-1-^{14}C. Approximately 30 μC of sodium isobutyrate-1-^{14}C and a small excess of sodium bicarbonate was refluxed with 0.3 ml of thionyl chloride in 2 ml of diisopropyl ether for 15 minutes. It was then cooled and 100 μl of isobutyric acid added before refluxing for a further 15 minutes. The mixture was cooled over dry ice and 2 ml of concentrated ammonia solution added by syringe through a rubber seal. It was left standing for two hours at room temperature before evaporating to dryness under reduced pressure. The solid residue was extracted with ethyl acetate from which the isobutyramide-1-^{14}C crystallised in low overall yield when the volume was reduced by evaporation. The product had a specific activity of 46.5 μC/mmole.

Isobutyronitrile-1-^{14}C. This was prepared by heating 0.11 mmole of isobutyramide-1-^{14}C (23.3 μC/mmole) with approximately 80 mg of phosphorous pentoxide to 200° for one hour. The nitrile was flushed out with a gentle stream of nitrogen and trapped in a U tube cooled with liquid air. In a repeat preparation 0.35 mmole isobutyramide-1-^{14}C (6.5 μC/mmole) was similarly heated with 250 mg of phosphorous pentoxide.

Phenylacetaldoxime-U-^{14}C. A solution of 1 mg of phenylalanine-U-^{14}C in 0.6 ml of pH 6.7, 0.05 M phosphate buffer was treated with 2 mg of N-bromo-succinimide and 50 mg succinimide. Reaction was for 30 minutes at 5° with continuous shaking with a total of 1 ml of diethyl ether in portions. The ether extract containing phenylacetaldehyde-U-^{14}C was then added to 25 mg of hydroxy-

lamine hydrochloride and 30 mg of sodium bicarbonate dissolved in 200 µl of water. The ether was removed with a gentle stream of nitrogen and approximately 20 mg of carrier phenylacetaldehyde added. The mixture was shaken for 40 minutes at 5° to precipitate phenylacetaldoxime-U-^{14}C, the aqueous phase was removed, and the residue dried and recrystallised twice from diethyl ether and hexane. A low overall yield was obtained.

Phenylacetonitrile-1-^{14}C. A solution of 11 mg potassium cyanide-^{14}C in 140 µl of 70% v/v aqueous ethanol was sealed in a glass tube with an excess of 50 µl of benzylchloride. The tube was heated at 75° for 150 minutes. The nitrile was isolated by gas chromatography in a similar way to isobutyraldoxime-U-^{14}C.

Mandelonitrile-1-^{14}C and 2-hydroxyisobutyronitrile-1-^{14}C. Both these were prepared from potassium cyanide-^{14}C immediately before administration and were used without prior purification.

Isobutyraldoxime-^{15}N. A solution of 0.2 mmole of hydroxylamine hydrochloride-^{15}N (47.5 APES) and 0.2 mmole of sodium bicarbonate in 0.4 ml of ice cold water was treated with about 0.6 mmole of isobutyraldehyde. After 18 hours at 18° the mixture was extracted three times with a total of 6 ml of diethyl ether. The excess ether was evaporated off and the isobutyraldoxime-^{15}N purified by gas chromatography as for the ^{14}C labelled compound.

2-Oximinoisovaleric acid-^{15}N. A solution of 0.2 mmole of hydroxylamine hydrochloride-^{15}N (47.5 APES) and a small excess of sodium bicarbonate in 1 ml of water was treated with 0.2 mmole of sodium 2-ketoisovalerate. The mixture was held at 18° for 28 hours before acidifying to pH 1.5 with dilute hydrochloric acid and extracting three times with a total of 12 ml of diethyl ether. The combined ether extract was dried over anhydrous sodium sulphate and the solvent evaporated. 2-Oximinoisovaleric acid-^{15}N was recrystallised twice from ether and hexane.

2.4 Enzymes

L-amino acid oxidase. Dried *Crotalus adamanteus* venom was used as a source of L-amino acid oxidase and was obtained from CalBiochem, Los Angeles. The enzyme was dialysed against distilled water before use.

Catalase. Crystalline catalase was obtained from CalBiochem, Los Angeles, and was also dialysed against distilled water before use.

β-Glucosidase. "Emulsin" obtained from B.D.H. Ltd, England, or "β-glucosidase, (Sweet Almonds)" obtained from Seravac Laboratories, Cape Town, were used for the hydrolysis of prunasin. They were dissolved in phosphate buffers prior to use.

Linamarase. This β-glucosidase with specificity for linamarin and lotaustralin was prepared in this laboratory by Mr W. D. Bennett according to the method of Coop (1940). A total of 100g of finely ground defatted linseed meal (variety "Redwood") was shaken in portions with 2 litres of water and immediately centrifuged. The extract was buffered by adding 100 ml of 0.2M sodium acetate and 100 ml of 0.2M acetic acid. A white precipitate formed on standing but contained only a fraction of the enzymic activity and was centrifuged off and discarded. The supernatant was cooled to below 10° and one half its volume of cold ethanol added slowly to produce a further precipitate which was also discarded. More alcohol was added to bring the final concentration to 60% v/v. The precipitate which then formed contained the linamarase and was centrifuged off before dissolving in approximately 250 ml of distilled water. Dilute sodium hydroxide was added to bring the solution to pH 7 and it was stored under a few drops of toluene at 5°. The enzyme, which was purified approximately twenty-fold by this procedure, was diluted with phosphate buffers before use.

Myrosinase. This enzyme was prepared from commercial mustard flour (Reckitt, Coleman, Nugget (N.Z.) Ltd.) by ammonium sulphate precipitation. A

portion of 113g of the flour was defatted with light petroleum and air dried powder suspended in 340 ml of water for 4 hours. The solids were centrifuged off and washed with a further 100 ml of water which was then added to the first water extract. Solid ammonium sulphate was added in portions and the precipitate forming between 50% and 70% saturation at 18° centrifuged off. This precipitate which contained myrosinase was dissolved in 25 ml of water and dialysed in water for two days. It was stored in a deep freeze.

2.5 Extraction

Ethanol extracts. In general, linen flax shoots and scurvy grass seedlings were extracted by boiling without prior blending, in 80% v/v ethanol for 15 or 20 minutes. About 25 ml of solvent was used for 20 flax shoots or each scurvy grass seedling. After standing several hours at room temperature the extract was decanted and the tissue residue extracted again with a similar volume of solvent. The combined extracts were taken to dryness under vacuum at below 50° on a rotary evaporator before taking up in 2 ml of water or v/v 20% aqueous n-propanol. They were stored at -10°. Suitable portions of these extracts were used for chromatography.

Peach, cherry laurel and garden cress shoots were similarly extracted twice with 80% v/v ethanol in appropriately larger volumes of solvent after blending. The solvent was again removed with a rotary evaporator and the concentrated extracts dissolved in water to make 5 ml in the case of peach and cherry laurel or 2 ml for garden cress. A small amount of peach and cherry laurel extracts not soluble in water was removed by centrifugation. Again suitable portions of these concentrated extracts were used for analysis by enzymic treatment or chromatography.

Diethyl ether extracts. In experiments P and Q, procedures different from those above were used. In these the linen flax shoots were first treated with diethyl ether to obtain extracts suitable for analysis by gas chromatography.

For experiment Q the batches of 20 shoots were placed in brass cylinders of about 7 ml volume together with 1.5 ml of ether, 1.5g of 4mm glass beads and 0.5g of anhydrous sodium sulphate. The cylinders, sealed with brass caps and polythene washers were shaken, to disintegrate the plant tissue, for 30 seconds at 5° in a shaker built according to the design of Nossal (1953). The brass caps were then replaced by porous polythene filters and brass filter holders, and the ether extracts filtered by centrifugation into small vials. About 1 ml of the extracts was recovered and samples were taken for gas chromatography. The solid residues were transferred to beakers for extracting with cold ethanol.

2.6 Chromatography.

Cyanogenic glycosides. Whatman 3 MM paper was used for both preparative and analytical paper chromatography. Prunasin was separated using n-butanol saturated with water (BW) while separations of linamarin were made using this solvent as well as methylethyl ketone:acetone:water, 15:5:3 v/v (MAW) and propanol:water, 7:3 v/v (PW). Thin layer chromatography was also used with supports including MN-300 cellulose (Macherey, Nagel and Co., Germany), mixed layer of MN-300 and silica gel H (Merck, Germany) (Bieleski and Turner, 1966), Avicel (FMC Corp., U.S.A.) and Eastman Chromogram sheet 6064. Two dimensional separations on paper or thin layers used MAW in the first dimension followed by either BW or PW. With thin layers the sample was applied as a short band near an edge, and after developing in the first dimension, the partially resolved bands were eluted into compact spots near the margin of the plate by immersing most of the plate up to the bands in water for a few minutes (Bieleski and Turner, 1966). The plates were then dried and developed with the second solvent.

Cyanogenic glucosides could be detected by spraying with an appropriate β-glucosidase solution and, after standing some hours in a closed humid

vessel, the glucose produced by hydrolysis gave spots with typical reducing sugar spray reagents such as aniline phosphate in n-butanol (Bryson and Mitchell, 1951).

A more sensitive and specific method was developed in this laboratory (Bennett and Tapper, 1968, see appendix 4) which involved detecting cyanide released by enzymic action. The cyanide, held on the paper or thin layer, catalysed the formation of a blue dye from p-nitrobenzaldehyde and o-dinitrobenzene under slightly alkaline conditions (Guilbault and Kramer, 1966).

Glucosinolates. These were separated on Whatman 3MM paper using n-butanol:ethanol:water, 4:1:4 v/v, or on thin layers using a combination of electrophoresis and chromatography. The procedure was a modification of the methods used by Bieleski and Turner (1966) for amino acid separations. The sample was applied as a short band near the middle of, and at a right angle to, one edge of a 20 cm square cellulose thin layer plate. After electrophoresis in pH 2 buffer (17 ml 90% formic acid plus 57 ml acetic acid per litre) the partially resolved bands were eluted into spots, and developed with the chromatography solvent of n-propanol:water:n-propylacetate:acetic acid:pyridine, 120:60:20:4:1 v/v. With suitably chosen voltages and times for electrophoresis (e.g. 1000 volts for 15 minutes) a partial amino acid separation could be obtained on the same plates as the glucosinolate separation. The glucosinolates were detected by spraying with 0.02M silver nitrate, drying in an oven for a few minutes and then overspraying with 0.02M potassium dichromate. Glucosinolates appeared as pale yellow spots on a light brown background (Challenger, 1959).

Thioureas, prepared from glucosinolates, were separated by ascending paper chromatography with chloroform saturated with water as solvent (Kjaer and Rubinstein, 1953) and they were detected by spraying with an iodine-azide reagent (3g sodium azide, 1.27g iodine and 0.83g potassium iodide in 100 ml

of water) which gave white spots on a brown background (Feigl, 1956). Identification was by R_f measurements relative to known standards.

2,4-Dinitrophenylhydrazones. These derivatives of simple aldehydes and ketones were separated on silica gel G (Merck, Germany) thin layers, or Eastman Chromagram sheet K 301 R, using benzene, toluene, or n-butylacetate:light petroleum (40-60°), 1:9, as solvents. Standard markers were used to identify spots or bands.

Amino acids. Amino acids were separated and estimated by the method of Bieleski and Turner (1966). The amino acids were separated by two-dimensional thin layer chromatography, ninhydrin complexes were formed under controlled conditions, and the eluted complexes determined colorimetrically.

Volatile compounds. Gas chromatography was used both in the preparation of some compounds and in the analysis of plant tissue extracts. Instruments used were a modified Aerograph 664 (Varian Aerograph, California) fitted with both thermal conductivity and flame ionisation detectors, and an Aerograph 1520. This instrument was fitted with a stream splitter such that a small proportion of the gas from the column passed into a flame ionisation detector. The remainder was conducted to a proportional gas flow detector (Nuclear-Chicago, Illinois) for measuring the radioactivity of volatile compounds as they were eluted from the gas chromatography column.

The following columns were found most suitable:

(i) 15% w/w Carbowax 400 (polyethylene glycol) on Chromosorb W (60/80 mesh, DMCS treated) was packed in a 3m x 7.6 mm ID aluminium column. This column was temperature programmed between 35° and 140° in the Aerograph 664 by simple control of the oven heater power input, and was used for separating a range of compounds with boiling points up to that of isobutyraldoxime. A partial separation of isobutyraldehyde from acetone and between the stereoisomers of isobutyraldoxime was possible.

(ii) A smaller aluminium column, 1.5m x 4.5mm ID, packed with 11% w/w Carbowax 400 on Chromosorb P (60/80 mesh, HMDS treated) was also used in the Aerograph 664, for some preparative separations as well as to examine linen flax shoots for the presence of isobutyronitrile. The thermal conductivity detector was used with this column.

(iii) A stainless steel column, 2m x 2.5mm ID packed with 10% w/w Carbowax 1500 on Chromosorb P (60/80 mesh, HMDS treated) was used with the flame ionisation detector in the Aerograph 664. The column could clearly resolve the two isomers of isobutyraldoxime and was used to examine linen flax shoots for the presence of isobutyronitrile and isobutyraldoxime.

(iv) A stainless steel column, 2.5m x 4.5mm ID, containing 21% w/w Hi-Eff 1B (diethylene glycol succinate from Applied Science Laboratories, Inc., U.S.A.) on Chromosorb W (60/80 mesh, re-sieved, acid washed and treated with 3% DCS in toluene in this laboratory) was used for separating a range of compounds with the Aerograph 1520. Helium carrier gas was used at about 80 ml per minute with linear temperature programmes up to 190°.

2.7 Analysis of glucosides.

Linamarin. The analysis of glucosides involved measuring radioactivity, identifying by enzymic hydrolysis, and measuring the amount of compound present to obtain the specific activity values for the intact glucosides or some of the hydrolysis products.

Linamarin was purified by one or two dimensional chromatography before analysis. Its presence was usually indicated by zones of radioactivity in appropriate areas detected by radioautography or by geiger counter. Enzymic hydrolysis was carried out in 50 ml or smaller flasks fitted with either centre wells or cups suspended from glass stoppers. The linamarin, dissolved in 0.2 to 1.5 ml of water was hydrolysed with 0.5 to 1 ml of linamarase solution and 0.5 to 1 ml of pH 5.9, 0.1M phosphate (sodium salts) buffer. The flasks were

generally shaken for at least 24 hours at 30°. The hydrogen cyanide, produced by hydrolysis, was absorbed in 0.5 ml of 1M sodium hydroxide in the centre wells or cups and suitable portions diluted and quantitatively analysed for cyanide by the method of Aldridge (1944). The specific activity of linamarin was then calculated on the basis of the total activity of linamarin and the yield of hydrogen cyanide upon hydrolysis.

Prunasin. As paper chromatography gave no evidence for cyanogenic glycosides other than prunasin in the peach and cherry laurel extracts, no preliminary separation was required. Portions (0.5, 1 or 2 ml) of the extract dissolved in water were treated with 1 mg of β-glucosidase (Seravac) and, to reduce any possibility of bacterial metabolism during the hydrolysis, 125 µg chloramphenicol dissolved in 0.5 ml 0.1M phosphate buffer, pH 5.9 was added. The hydrolyses were conducted in 50 ml flasks with centre wells containing 0.5 ml of 15% w/v semicarbazide hydrochloride and cups suspended from the stoppers containing 0.5 ml of 1M sodium hydroxide. The flasks were held at 30° for 48 hours and given occasional or continuous gentle shaking. The benzaldehyde liberated from prunasin formed crystals of benzaldehyde semi-carbazone in the centre wells which, after removing the excess reagent, were recrystallised two or three times from ethanol. The purity and quantity of the recrystallised benzaldehyde semicarbazone was measured by recording the U.V. absorption spectrum in ethanol with a Unicam SP800 recording spectrophotometer. No impurities were detected. The quantity was determined by the optical density at the peak absorption of 283 nm. The radioactivity of known quantities was determined by liquid scintillation counting and a specific activity measurement calculated. Both the quantity and the radioactivity of the liberated cyanide was measured. Two checks with recrystallised prunasin showed 95 and 98% recovery of hydrogen cyanide by this method.

In those cases where prunasin was labelled predominantly in the nitrile moiety and where paper chromatography showed that little else in the extract was labelled, then contamination of the cyanide with other volatile radioactive compounds capable of being absorbed in alkali was considered unlikely or insignificant. In other cases where a range of compounds were strongly labelled and where benzaldehyde was also labelled, some risk of contamination of the cyanide was considered possible. In particular, benzaldehyde might react in the 1M sodium hydroxide giving non-volatile products and also some radioactivity in the sample may have been released as carbon dioxide.

To check on these possibilities, portions of the cyanide solutions in 1M sodium hydroxide were treated with excess acetic acid in the presence or absence of silver acetate in stoppered flasks with centre wells containing 0.5% "Hyamine 10x" hydroxide (Rohm and Haas, U.S.A.) in methanol. After allowing diffusion of the acid-liberated volatile compounds to take place for at least 18 hours with gentle shaking, the centre well contents were analysed by liquid scintillation counting. In the presence of excess silver ions, cyanide formed a precipitate which was not decomposed by the acetic acid, and it was possible to estimate the extent of the errors mentioned above, and make corrections accordingly. Even so, the possible errors in determination of the specific activity of the nitrile moiety are estimated to be greater than the errors involved in benzaldehyde specific activity measurements.

In some of the earlier experiments with prunasin, a procedure based on that of Ben-Yehoshua and Conn (1964) was also used and gave essentially the same results. A phenylhydrazine reagent (5 ml of phenylhydrazine and 5 ml of acetic acid, made to 100 ml with water and filtered through activated charcoal) was used.

Glucosinolates. After electrophoretic and chromatographic purification, the glucosinolates were eluted and the radioactivity measured directly in the

liquid scintillation counter. No evidence for glucosinolates other than benzylglucosinolate was obtained with the garden cress. Portions of 200 µl of the extract dissolved in water were treated with 20 µl of myrosinase solution and 50 µl of pH 5.9, 0.1M phosphate buffer at room temperature. This mixture was extracted periodically (from 15 minutes to 8 hours after mixing) with diethyl ether. The combined ether extracts of approximately 3 ml were treated with 2 ml of concentrated ammonia solution and 3 ml of ethanol to form N-benzylthiourea. After standing 18 hours at room temperature the excess ammonia and solvents were removed by a stream of nitrogen and the residue applied to chromatography paper for thiourea separation. Radioactive bands on the developed papers were detected by a geiger counter, eluted with aqueous ethanol and the U.V. absorption spectrum and radioactivity measured to determine the specific activity of the benzyl isothiocyanate. The absorption peak at 244 nm was used for quantitative measurements. A trial using tetramethylammonium benzylglucosinolate (Calbiochem) showed an overall recovery by this procedure of about 40%. Isothiocyanates are known to undergo reactions with amino and sulphydryl groups (Edman, 1950; Goksoyr, 1964) which would tend to reduce the yields obtained when treating glucosinolates with myrosinase. Some formation of phenylacetonitrile may also occur at pH 5.9 and reduce the yield.

Radioactive isopropylglucosinolate was identified by a similar procedure after electrophoresis and chromatography. The purified radioactive glucoside in 0.4 ml of water was mixed with 50 µl of a scurvy grass leaf extract which acted as a supply of carrier glucoside, and treated with 20 µl of myrosinase at room temperature to liberate isopropyl isothiocyanate. This was extracted with diethyl ether after 15, 45 and 120 minutes and the combined ether extracts treated with ammonia solution and ethanol. The thioureas were chromatographed as described for the benzyl derivative. The addition of carrier glucosinolates

was necessary to avoid excessive losses in yield of radioactive isopropylthiourea. This prevented estimation of the specific activity of the aglycone.

2.8 Measurement of Radioactivity

Radioautography (with Kodak medical X-ray film) was usually used for the detection of radioactive compounds after two dimensional electrophoretic and chromatographic separations and, to a lesser extent, after one dimensional separations when geiger counting was also used. Paper strips or 5 cm wide thin layer chromatograms were scanned with either a Nuclear-Chicago Actigraph II, 4π thin window counter, or a Packard 7200, 4π windowless radiochromatogram scanner.

Most quantitative measurements of radioactivity were made by liquid scintillation counting. Instruments used were a Beckman LS100 and Packard models 4312, 3375 and 2211. All instruments were capable of routinely counting ^{14}C at 60 to 85% efficiency depending on instrumental settings and scintillation mixture. A range of mixtures, similar or identical to published formulae (Schram, 1963) were used and the efficiency of counting determined using standard [illegible] ^{14}C (Radiochemical Centre, Amersham).

On several occasions "quench curves" were constructed by either the internal channels ratio or the external standard channels ratio methods. A set of vials including a blank and standards with increasing degree of quenching from either water or chloroform was usually included in each lot of samples for automatic counting to check the instrument's performance. All samples were counted at least twice and where possible to a standard error of less than 1%.

A variety of sample preparation procedures were used. The zones of radioactivity on thin layer chromatograms were scraped off the plates and transferred to scintillation vials. This transfer was facilitated by the use

of short pieces of glass tubing with a small wad of cotton wool held at a central constriction and which were connected to a vacuum pump by rubber hose. After sucking the thin layer material on to the cotton wool, the glass tube was detached from the hose, the thin layer material and cotton wool pushed into a vial and the tube rinsed with 1 ml of 50% v/v aqueous methanol. In most cases the compounds to be counted were limamarin, an unidentified compound (IV) (of similar polarity to limamarin), or valine. Tests showed that, after standing a few hours in the 50% methanol, these compounds remained dissolved when a dioxane based scintillation mixture was added.

Radioactive compounds on chromatography paper were counted by placing the piece of paper in a vial together with 1 or 2 ml of a toluene based scintillator mixture - sufficient to saturate the paper. This method tended to give high backgrounds and therefore an alternative procedure was frequently used where the compounds were first eluted with water. Rectangular pieces of paper were stood in a small volume of water in a shallow dish and the radioactive compounds washed to the upper end. The piece of wet paper was then inverted, wrapped in "Parafilm" (Gallenkamp, London) and aluminium foil, and gently rolled so that it could be placed in a centrifuge tube. The top most portion of the "Parafilm" and foil was folded over the lip of the centrifuge tube to hold the roll up from the bottom of the tube. Only light centrifugation was required to collect most of the water and virtually all the radioactivity from the paper. Where all the sample was to be counted, a scintillation vial with a short glass tube supported from the top of the vial was used in place of the centrifuge tube. In this way the eluted sample was collected directly in a standard vial and required only addition of a dioxane based scintillation mixture prior to counting.

Volatile radioactive compounds from experiment P separated using the Aerograph 664, were trapped in a wad of cotton wool saturated with xylene and

held in the barrel of a 5 ml syringe fitted into the thermal conductivity detector. The syringes were changed to collect fractions and the xylene with the radioactivity was rinsed into vials with scintillation mixture prior to counting.

2.9 Analysis of ^{15}N

Small samples of the 2-oximinoisovaleric acid-^{15}N and isobutyraldoxime-^{15}N used for administration to linen flax seedlings were digested by the Kjeldahl method in the presence of 10 mg glucose as an additional reducing compound and 3 ml of digestion mixture. The ammonia, liberated by sodium hydroxide, was steam distilled into 2% w/v boric acid and titrated with dilute hydrochloric acid. The aldoxime gave a quantitative recovery but some of the oximino acid was lost, presumably as isobutyronitrile.

L-Valine-^{15}N was analysed by an adaption of the method of Sobel et al., (1945). The amino acid was heated with 100 mg of ninhydrin in 7 ml of pH 2.5, 0.3M citrate buffer to 100° for 15 minutes. The excess ninhydrin was destroyed by the addition of 10 drops of 30% w/v hydrogen peroxide followed by 5 minutes further heating. The ammonia, again liberated by sodium hydroxide, was steam distilled and titrated as above. Approximately 98% recovery was obtained.

Linamarin, isolated by paper chromatography, was treated with 1.5M sodium hydroxide for 30 minutes at 100° to hydrolyse the nitrile moiety to ammonia. The ammonia was then steam distilled from the same vessel for 15 minutes into 2% w/v boric acid and titrated. Recovery of ammonia was 95-100%.

All samples for ^{15}N analysis were again steam distilled from alkali and the distillate acidified with dilute sulphuric acid prior to concentration by boiling.

The ^{15}N analysis was performed by mass spectrometry according to the method of Sprinson and Rittenberg (1949) on an instrument designed and constructed at the Institute of Nuclear Science, Wellington.

CHAPTER III

EXPERIMENTAL RESULTS

3.1 Introduction

The experiments and results fall into three groups for purposes of discussion although this was not in all cases the order in which they were conceived. In the first section experiments are discussed where various isotopically labelled compounds were administered to plant tissue and the incorporation into cyanogenic glucosides measured. In some cases attention is drawn to other compounds which also became labelled. The second section comprises attempts to show the presence of intermediates. All the experiments in this section were performed with linen flax seedlings. In the third section three experiments are described which, as in the first section, are attempts to demonstrate incorporation of labelled compounds into glucosides. In this case the glucosides are glucosinolates.

3.2 Incorporation of labelled compounds into cyanogenic glucosides.

Oximes as precursors of linamarin. It has been shown conclusively that L-valine is an efficient precursor of linamarin in several plants and suggested that corresponding 2-oximino acids might be intermediates between amino acids and cyanogenic glycosides (Butler and Conn, 1964a). Table II gives the results of experiment A where this suggestion was tested by comparing the incorporation of L-valine-U-^{14}C and 2-oximinoisovaleric acid-U-^{14}C into linamarin in linen flax seedlings. The conditions were essentially similar to those used by Butler and Conn (1964a). The labelled precursors were dissolved in 100 µl of pH 7.4, 0.2M tris(hydroxymethyl)-aminomethane buffer and administered to 20 seedling shoots in open 1 ml beakers. Additional water was supplied as required and the labelled compounds were assimilated and metabolised for 7 hours under artificial

TABLE II

OXIMES AS PRECURSORS OF LINAMARIN

Expt.	Compound Administered			Linamarin				Recovered Valine	
		Amount (μmole)	S.A.[a]	Amount (μmole)	S.A.[a]	% precursor converted	Dilution of ^{14}C	Amount (μmole)	S.A.[a]
A	L-valine-U-^{14}C	1.2	910	12	17.1	23[b]	43[b]	0.67	390
	2-Oximinoisovaleric acid-U-^{14}C	1.0	1150	11	7.6	9[b]	121[b]	0.25	15
B	L-valine-U-^{14}C	3.3	300	15	13.5	25[b]	18[b]	-	-
	Isobutyraldoxime-U-^{14}C	3.4	390	15	18.5	21	21	-	-
	Isobutyraldehyde-U-^{14}C	1.3	1510	13	1.0	0.7	1510	-	-

a Specific activity (including the carboxyl carbon atom) in μC/mmole

b Corrected for an assumed loss of carboxyl carbon atom - see text

light. Less than 10% of the administered radioactivity remained in the beakers.

In experiment B isobutyraldoxime was similarly tested as a precursor. L-Valine-U-^{14}C and isobutyraldoxime-U-^{14}C were dissolved in water and administered from 1 ml beakers. Isobutyraldehyde-U-^{14}C was also tested as a precursor to determine whether isobutyraldoxime could be incorporated after hydrolysis of the oxime group. The aldehyde was administered from solution in a closed 25 ml glass vial. These results are included in Table II.

Butler and Butler (1960) showed that the administration of D,L-valine-1-^{14}C gives insignificant label in linamarin as would be expected if the carbon skeleton of L-valine forms the basis of the linamarin aglycone. Others have shown a similar loss of amino acid carboxyl groups in the biosynthesis of dhurrin (Gander, 1962) and prunasin (Mentzer et al., 1963). For this reason the values for radioactivity of linamarin derived from L-valine-U-^{14}C have been adjusted by the factor 5/4 to give better indications of the percentage of this precursor incorporated and the dilution of specific activity. If 2-oximinoisovaleric-U-^{14}C was an intermediate between L-valine and linamarin then a similar adjustment for loss of the carboxyl group is justified.

The results, expressed as percentage precursor converted to linamarin, show that 2-oximinoisovaleric acid was a little less than half as effective and isobutyraldoxime was about equally effective as L-valine as precursors of linamarin. The incorporation from L-valine observed in these experiments was consistent with that found by Butler and Conn (1964a). Figure 5 illustrates the usual pattern obtained by radioautographs of two dimensional chromatograms.

Linamarin was the major labelled product from L-valine-U-^{14}C. There was a low level of incorporation into leucine and a few other unidentified compounds.

Although the incorporation from 2-oximinoisovaleric acid-U-^{14}C was less than from L-valine-U-^{14}C, it was still considered significant and indicated that

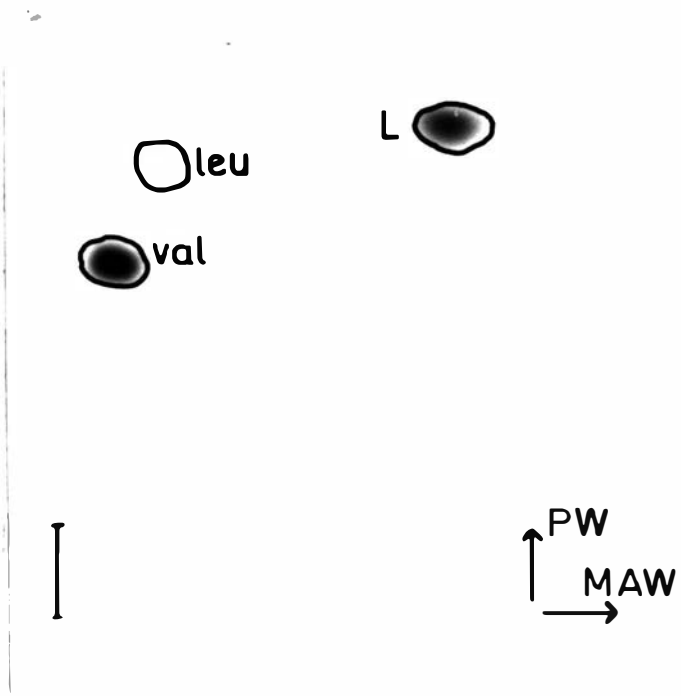


FIGURE 5 INCORPORATION OF RADIOACTIVITY FROM L-VALINE-U-^{14}C IN LINEN FLAX. A RADIOAUTOGRAPH FOLLOWING THIN LAYER CHROMATOGRAPHY OF AN ETHANOLIC EXTRACT. L IS LINAMARIN.

the 2-oximinoisovaleric acid could have been an intermediate in linamarin biosynthesis. A lower incorporation could have been due to a toxic effect of this compound when administered at this concentration or difficulty in transport to the site of biosynthesis. Alternatively 2-oximinoisovaleric acid might have been converted to L-valine in the plant tissue either by hydrolysis to 2-ketoisovaleric acid and hydroxylamine followed by transamination or by direct reduction of the oximino group to an amine. These possibilities were examined by isolating valine at the end of the experiment and comparing its specific activity with that of the linamarin. The results are also in Table II. Where L-valine-U-^{14}C was administered, the recovered valine had a specific activity many times greater than the linamarin whereas with 2-oximinoisovaleric acid-U-^{14}C the specific activity was relatively little more than that of the linamarin. Further, the amount of valine was small compared with the amount of linamarin. An indication of the small amount of radioactivity in valine when 2-oximinoisovaleric acid-U-^{14}C was administered is given by Figure 6, which also shows that other unidentified compounds were present in small amounts. These results indicated it was unlikely, but not impossible, that 2-oximinoisovaleric acid was incorporated into linamarin by way of a preliminary conversion to L-valine.

The incorporation of isobutyraldoxime-U-^{14}C into linamarin to about the same extent as from L-valine-U-^{14}C was good evidence for the aldoxime being a natural intermediate. It was considered that the observed conversion of 21% was a minimal figure because of an expected loss of a portion of the compound by evaporation during administration.

Figure 7 indicates that linamarin was the principal labelled ethanol soluble product from isobutyraldoxime-U-^{14}C although some other unidentified labelled compounds were also noted in small amounts.

The possibility of a conversion of isobutyraldoxime into linamarin by way

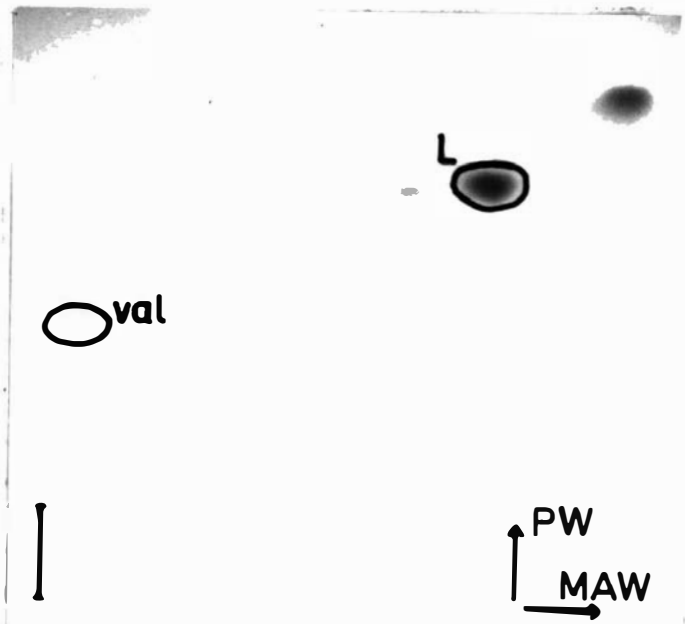


FIGURE 6 INCORPORATION OF RADIOACTIVITY FROM 2-OXOISOVALERIC ACID-U-^{14}C IN LINEN FLAX. A RADIOAUTOGRAPH FOLLOWING THIN LAYER CHROMATOGRAPHY OF AN ETHANOLIC EXTRACT. L IS LINAMARIN.

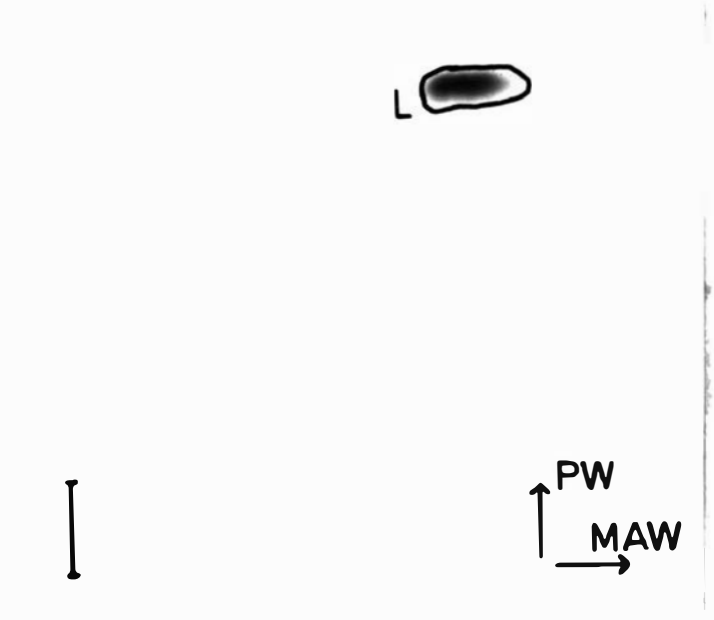


FIGURE 7 INCORPORATION OF RADIOACTIVITY FROM ISOBUTYRALDOXIME-U-^{14}C IN LINEN FLAX. A RADIOAUTOGRAPH FOLLOWING THIN LAYER CHROMATOGRAPHY OF AN ETHANOLIC EXTRACT. L IS LINAMARIN.

of isobutyraldehyde can be excluded on the basis of the low incorporation from that compound - despite the fact that it was taken up readily by the seedlings and extensively metabolised as indicated in Figure 8.

Administration of ^{15}N labelled oximes to flax. The results of experiments A and B indicated that 2-oximinoisovaleric acid and isobutyraldoxime may have been intermediates in linamarin biosynthesis. On the basis of the finding by Butler and Conn (1964a) of the retention of ^{15}N from L-valine, it was considered that the two oximes should also be incorporated with the C-N bond intact. In experiment C, 20 umoles of the double labelled oximes or L-valine, dissolved in water, were administered to 160 flax seedling shoots and allowed to metabolise for 9 hours under artificial light. For the first 2 hours of exposure, all the shoots were enclosed in clear plastic containers to reduce evaporative losses of isobutyraldoxime. Each batch of shoots yielded about 100 μmoles of linamarin which was purified by paper chromatography. The extent of dilution of the two isotopic labels was determined and compared for each treatment.

Table III contains the results which show that the ^{15}N label was diluted to approximately the same extent as the ^{14}C of the four aglycone carbon atoms in each treatment. If the C-N bond remained intact then a ratio of one would be expected for the dilutions of ^{15}N and ^{14}C. The relative dilutions obtained with 2-oximinoisovaleric acid and isobutyraldoxime are considered to not deviate significantly from one when the cumulative experimental errors are taken into account. The C-N bond therefore remained essentially intact.

The relative dilution from double labelled L-valine was just significantly greater than one. This indicates that some transamination of the L-valine may have occurred prior to the biosynthesis. These results confirm the possibility of the two oximes being intermediates in linamarin biosynthesis. They also exclude any significant incorporation of 2-oximinoisovaleric acid by a pathway via valine, involving transamination, as in that case a considerable relative dilution of the ^{15}N would have been expected.

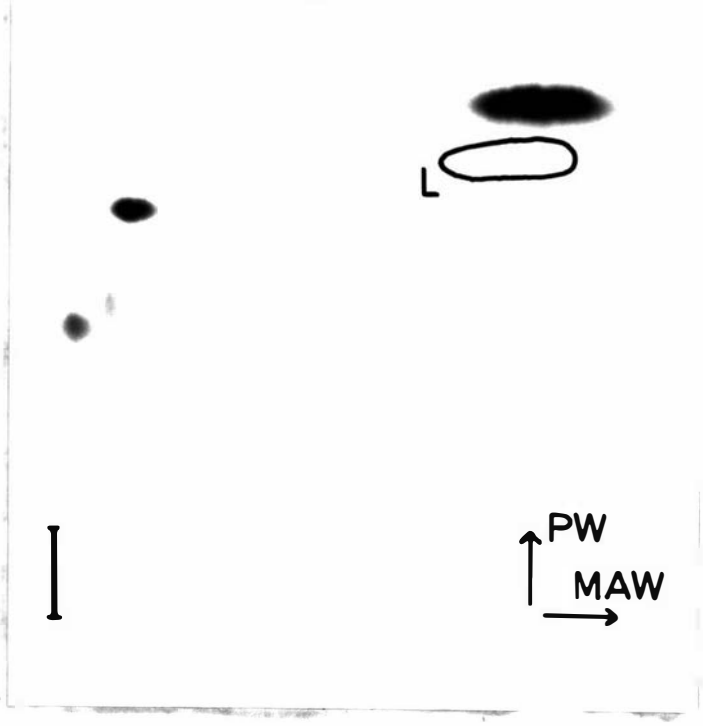


FIGURE 8 INCORPORATION OF RADIOACTIVITY FROM ISOBUTYRALDEHYDE-U-^{14}C IN LINEN FLAX. A RADIOAUTOGRAPH FOLLOWING THIN LAYER CHROMATOGRAPHY OF AN ETHANOLIC EXTRACT. L IS LINAMARIN.

TABLE III

INCORPORATION OF ^{15}N-OXIMES INTO LINAMARIN

Compound Administered			Linamarin					
	S.A.[a]	APES ^{15}N	S.A.[a]	APES ^{15}N	Dilution of ^{14}C	Dilution of ^{15}N	Relative Dilution of $^{15}N/^{14}C$	% precursor converted [c]
L-valine-U-^{14}C, ^{15}N	152	22.7	3.27	0.49	37.2[b]	46	1.24[b]	13[b]
2-Oximinoisovaleric acid-U-^{14}C, ^{15}N	30.1	22.1	0.625	0.53	38.5[b]	42	1.09[b]	13[b]
Isobutyraldoxime-U-^{14}C, ^{15}N	187	22.1	9.10	1.22	20.5	18	0.88	24

a Specific activity in μC/mmole

b Corrected for an assumed loss of carboxyl carbon atom

c Calculated from the incorporation of ^{14}C into linamarin

Isobutyramide and isobutyronitrile as precursors of linamarin. Although the previous experiments indicated that 2-oximinoisovaleric acid and isobutyraldoxime might have been intermediates in linamarin biosynthesis, the possibility remained that both, or either, were incorporated by way of steps not occurring naturally. In particular, 2-oximinoisovaleric acid could have been incorporated by prior non-enzymic conversion to isobutyronitrile (Ahmad and Spenser, 1961) if this latter compound was also incorporated. Isobutyronitrile could be a natural intermediate in linamarin biosynthesis.

In experiments D and E isobutyronitrile-1-^{14}C was administered to twenty flax seedling shoots in closed 12 ml glass vessels together with 100 µl of water, and L-valine-U-^{14}C was administered to similar shoots in open 1 ml beakers as controls. Isobutyramide-1-^{14}C, which had been prepared as an intermediate in isobutyronitrile-1-^{14}C synthesis, was similarly administered in open beakers.

The results in Table IV show that isobutyronitrile was incorporated into linamarin and, although the percentage converted to linamarin was not as great as from L-valine, the dilution was less. The dilution by a factor of ten for isobutyronitrile upon incorporation into linamarin in experiment E was also less than the dilution of the 2-oximinoisovaleric acid in experiments A and C, (Tables II and III respectively), where approximately the same percentage of conversion to linamarin was observed. These results were consistent with the possibility of a nonenzymic conversion of 2-oximinoisovaleric acid to the nitrile which was then incorporated into linamarin. They also indicate that nitriles may be intermediates in cyanogenic glycoside biosynthesis.

The low incorporation of label from isobutyramide-1-^{14}C into linamarin indicates that this compound was not an intermediate. The figure of 0.2% converted into linamarin is a maximum figure as the small amount of radioactivity eluted from the linamarin zone of chromatograms was not sufficient to positively identify as linamarin.

TABLE IV

INCORPORATION OF ISOBUTYRONITRILE INTO LINAMARIN

Expt.	Compound Administered	Amount (μmole)	S.A.[a]	Linamarin: % precursor converted	Linamarin: Amount (μmoles)	Linamarin: S.A.[a]	Linamarin: Dilution of ^{14}C
D	L-valine-U-^{14}C	1	1210	23[b]	-	-	-
	Isobutyronitrile-1-^{14}C	10	23.3	9	-	-	-
	Isobutyramide-1-^{14}C	7.3	46.5	< 0.2	-	-	-
E	L-valine-U-^{14}C	1	1210	23[b]	13	17.6	55[b]
	Isobutyronitrile-1-^{14}C	10.1	6.5	11	11	0.65	10

a Specific activity in μC/mmole

b Corrected for an assumed loss of -$^{14}COOH$ from L-valine-U-^{14}C

The pattern of radioactive zones obtained upon paper chromatography of some of the extracts of experiments D and E are illustrated in Figure 9. The count rate is plotted on a logarithmic scale to facilitate comparisons between chromatograms with differing total activity and between minor and major components in the same extract. It may be seen that the major radioactive zones were associated with valine and linamarin when L-valine-U-^{14}C was administered. With the isobutyronitrile-1-^{14}C treatment the linamarin zone contained radioactivity while no other portion of the chromatogram was sufficiently radioactive to be detected. The pattern from administration of isobutyramide-1-^{14}C confirms the observation that linamarin was not labelled although some other labelled compounds were present.

2-Hydroxyisobutyronitrile-1-^{14}C as a precursor of linamarin. The results of experiments D and E indicated that isobutyronitrile might be an intermediate in linamarin biosynthesis in which case the introduction of a 2-hydroxyl group and a subsequent glucosylation could be the final two steps of the biosynthesis. The likely intermediate would be 2-hydroxyisobutyronitrile (acetone cyanohydrin).

In experiment F, this compound was prepared and administered to 20 flax seedling shoots. Because of the difficulties of purifying and handling small amounts of labelled 2-hydroxyisobutyronitrile, a procedure was adopted whereby the compound was prepared from acetone and hydrogen cyanide-^{14}C immediately before use without a purification step. A solution of 2.5 μmoles of potassium cyanide-^{14}C in 5 μl of water, was added to 5 μmoles of potassium dihydrogen phosphate dissolved in 5 μl of water and 5 μl of acetone. The potassium dihydrogen phosphate was used to liberate hydrogen cyanide under conditions where synthesis of 2-hydroxyisobutyronitrile-1-^{14}C would have been rapid in the presence of an excess of acetone. The mixture was held at room temperature for one hour prior to diluting to approximately 100 μl with water and administering to the shoots in a closed 12 ml vessel.

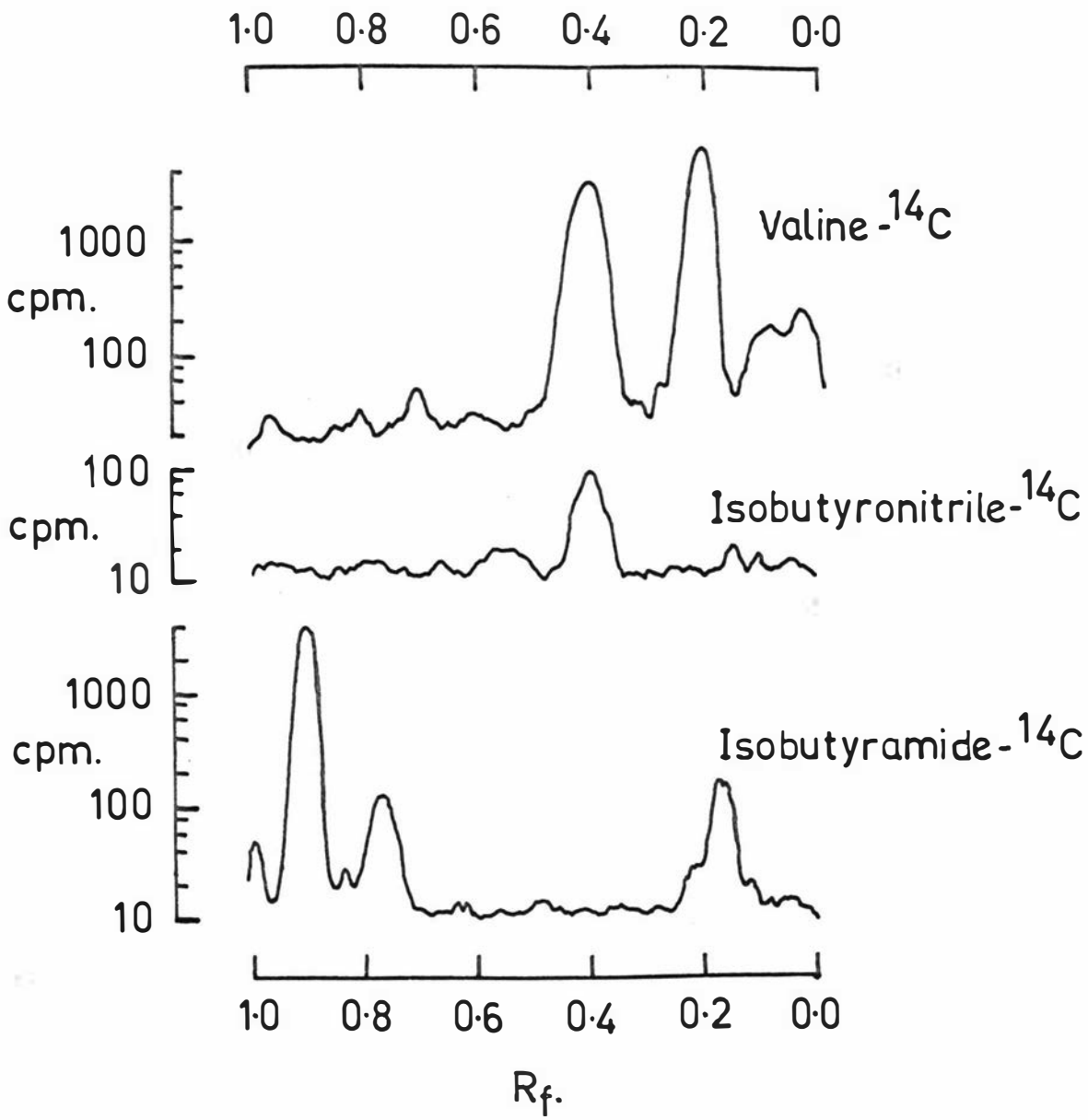


FIGURE 9 INCORPORATION OF RADIOACTIVITY FROM L-VALINE-U-^{14}C, ISOBUTYRONITRILE-1-^{14}C AND ISOBUTYRAMIDE-1-^{14}C IN LINEN FLAX, SEMI-LOGRITHMIC SCANS OF RADIOACTIVITY ON PAPER CHROMATOGRAM STRIPS DEVELOPED WITH BW; LINAMARIN HAS AN R_f OF 0.40 AND VALINE AN R_f OF 0.20.

It was recognised that the formation of 2-hydroxyisobutyronitrile-1-^{14}C would not be complete even if equilibrium was reached and some ^{14}C would be present as hydrogen cyanide. A similar batch of seedling shoots were therefore exposed to hydrogen cyanide-^{14}C as a control. A solution of 2.5 µmoles of potassium cyanide-^{14}C and 5 µmoles of potassium dihydrogen phosphate in 100 µl of water was used.

The results are given in Table V and show that 2-hydroxyisobutyronitrile was incorporated into linamarin at a substantial rate. The figure of 28% precursor converted to linamarin was the highest such figure observed in the series of experiments with flax seedling shoots and indicates this compound may be a natural intermediate. In contrast hydrogen cyanide was not significantly incorporated into linamarin, confirming a result obtained by Butler and Conn (1964a).

The extracts of the treated seedlings were examined by paper and thin layer chromatography and electrophoresis. Figure 10 illustrates the patterns of radioactivity observed upon one dimensional separation. In the 2-hydroxyisobutyronitrile-1-^{14}C treatment, a considerable zone of radioactivity was associated with linamarin, but was not detectable with the hydrogen cyanide-^{14}C treatment. Both chromatograms showed that a range of other compounds were labelled; notably compounds of low R_f in this solvent system. The amino acids of both extracts were separated on thin layers as shown by radioautographs in Figures 11 and 12. Asparagine, identified by the typical brown colour produced with a ninhydrin spray reagent (1% ninhydrin and 1% collidine in ethanol), was the major labelled amino acid. Other amino acids were tentatively identified by their colour reaction with ninhydrin and relative position on the chromatograms. Glutamine had considerably less radioactivity than asparagine while some other amino acids contained only traces of radioactivity. Several compounds, neutral at pH 2, were observed to be labelled.

From these separations and radioactivity measurements, it is apparent

TABLE V

INCORPORATION OF 2-HYDROXYISOBUTYRONITRILE INTO LINAMARIN

Compound Administered		Linamarin			
	S.A.[a]	Amount (μmoles)	S.A.[a]	% precursor converted	Dilution of ^{14}C
2-Hydroxyisobutyronitrile-1-^{14}C	1830	12	105	28	17.5
Hydrogen cyanide-^{14}C	1830	11	< 2.5	< 0.6	> 700

a Specific activity in μC/mmole

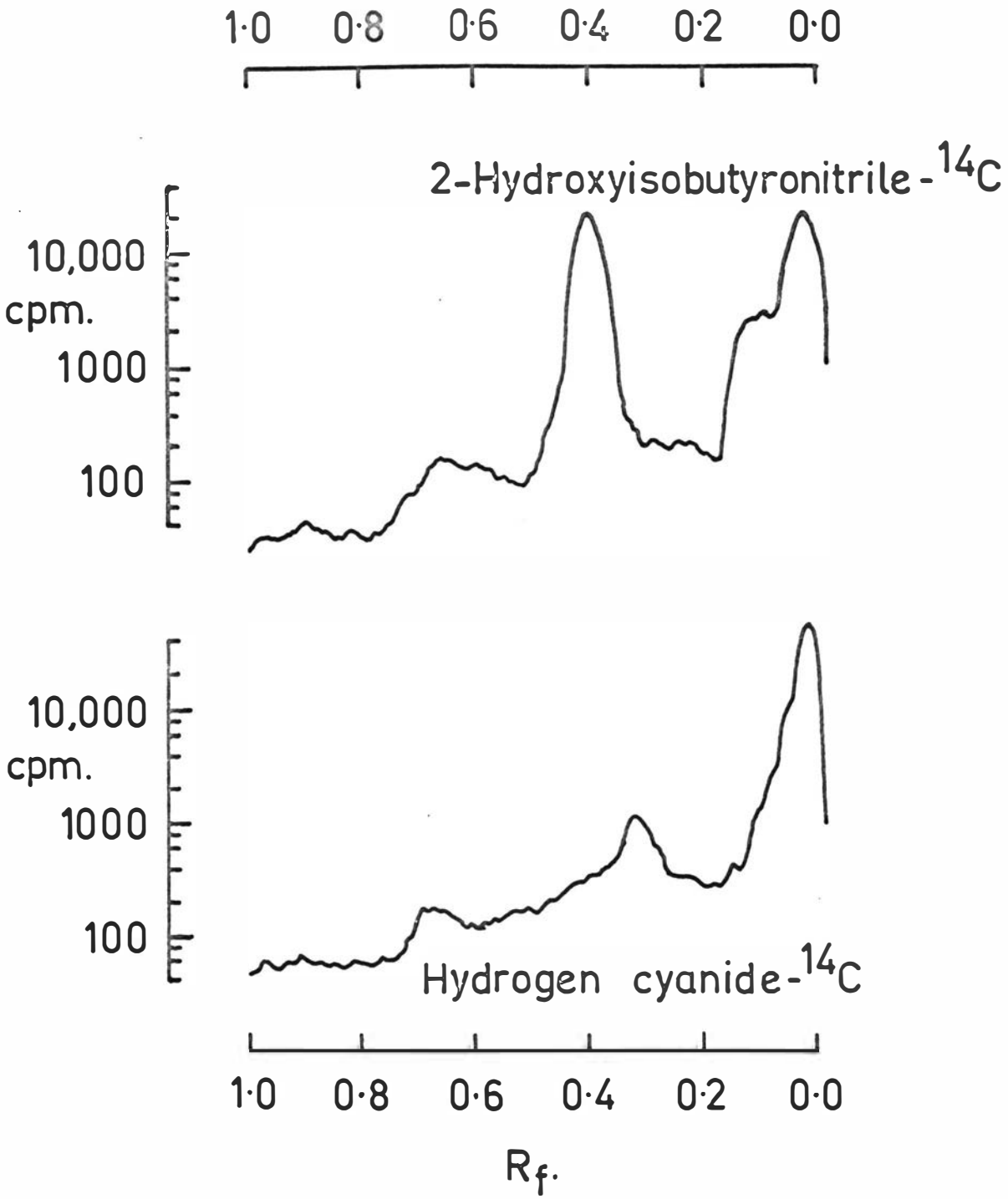


FIGURE 10 INCORPORATION OF RADIOACTIVITY FROM 2-HYDROXYISOBUTYRONITRILE-1-^{14}C AND HYDROGEN CYANIDE-^{14}C IN LINEN FLAX. SEMI-LOGRITHMIC SCANS OF RADIOACTIVITY ON PAPER CHROMATOGRAM STRIPS DEVELOPED WITH BW; LINAMARIN HAS AN R_f OF 0.40.

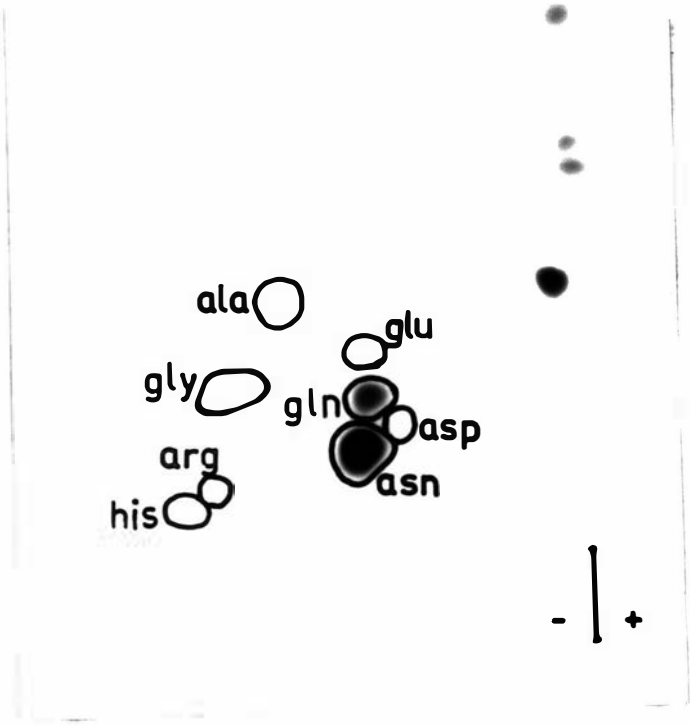


FIGURE 11 INCORPORATION OF RADIOACTIVITY FROM HYDROGEN CYANIDE-^{14}C INTO AMINO ACIDS IN LINEN FLAX. A RADIOAUTOGRAPH OF AN AMINO ACID SEPARATION AFTER ELECTROPHORESIS AT pH 2 AND CHROMATOGRAPHY.

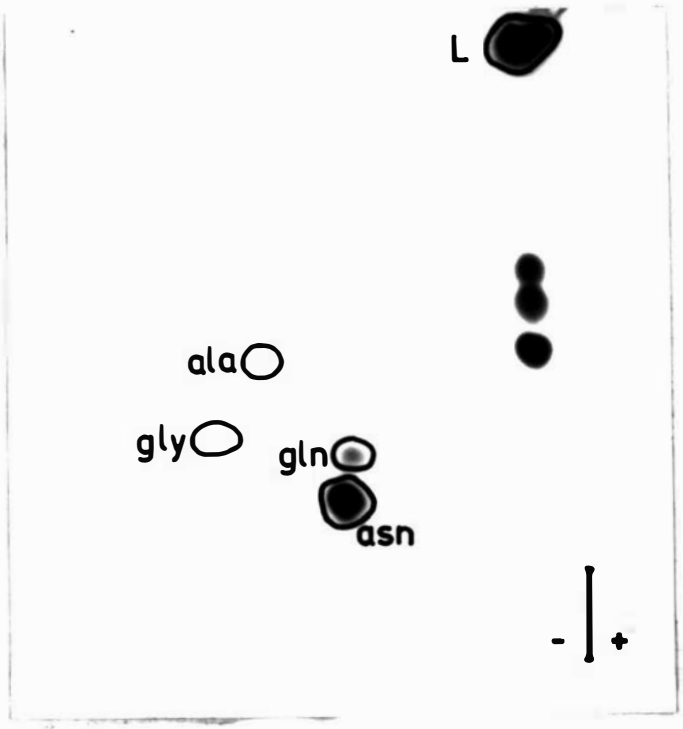


FIGURE 12 INCORPORATION OF RADIOACTIVITY FROM 2-HYDROXYISOBUTYRONITRILE-1-^{14}C INTO AMINO ACIDS IN LINEN FLAX. A RADIOAUTOGRAPH OF AN AMINO ACID SEPARATION AFTER ELECTROPHORESIS AT pH 2 AND CHROMATOGRAPHY. L IS LINAMARIN.

that asparagine was a major labelled product in both cases, although more was formed in the hydrogen cyanide-^{14}C treatment. This confirms the results of others on the metabolism of hydrogen cyanide. Most of the labelled compounds observed in the 2-hydroxyisobutyronitrile-1-^{14}C treatment, excepting linamarin and two other compounds, can be ascribed to incorporation of labelled hydrogen cyanide.

These results would be consistent with 2-hydroxyisobutyronitrile being an intermediate in a small natural pool which was not formed from acetone and hydrogen cyanide and which had a rapid turnover. Taken together with the results of earlier experiments, part of the pathway of biosynthesis to linamarin may be postulated as involving at least some of the reactions in Figure 13.

Oximes as precursors of prunasin. In the following few experiments, an attempt has been made to substantiate the type of pathway illustrated in Figure 13 in different plant systems producing a different glucoside. It has been shown that both peach (Ben-Yehoshua and Conn, 1964) and cherry laurel (Mentzer et al., 1963), can synthesize the aglycone of prunasin from L-phenylalanine. Both of these plants have been used in experiments F to I.

In experiment F, four actively growing autumn shoots of cherry laurel with a total fresh weight of about 3.4g were used in each treatment. For experiment H, four autumn shoots of peach with a total fresh weight of about 3.6g were selected for each treatment. L-Phenylalanine-U-^{14}C and phenylacetaldoxime-U-^{14}C were each administered from beakers in 250 µl of water, although the oxime was first dissolved in 5 µl of ethanol. Additional water was added as required and the assimilation and metabolism was for 44 hours in a controlled environment with two dark periods of 6 hours each.

Four autumn shoots of cherry laurel with a total fresh weight of about 3.8g were used in each treatment of experiment G while three autumn shoots of peach were used in each treatment of experiment I. These peach shoots, with

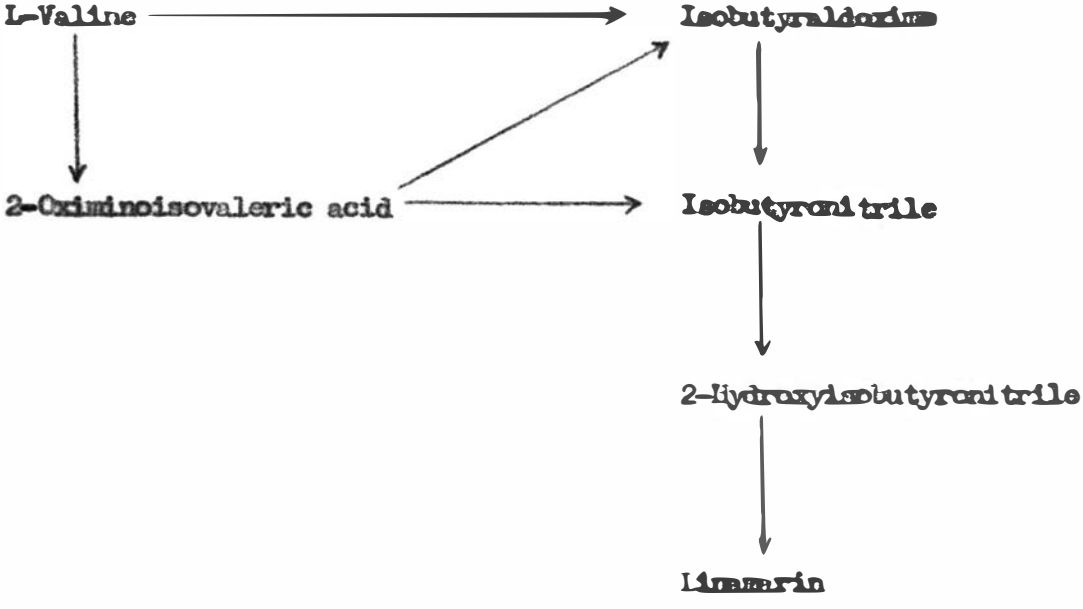


FIGURE 13 TENTATIVE PATHWAY OF LINAMARIN BIOSYNTHESIS

combined fresh weight of about 3.5g, were of a different variety from those used in experiment H and were a little more mature. The labelled compounds were administered as 0.6 ml of solution and additional water was added as required. Where 6.1 μmoles of 2-oximino-3-phenylpropionic acid-2-^{14}C was administered, it was first dissolved in 10 μl of ethanol as was the phenylacetaldoxime-U-^{14}C in experiment I. In the other treatment with 2-oximino-3-phenylpropionic acid-2-^{14}C in experiment I, 2.58 μmoles was administered as the sodium salt in approximately 0.04M, pH 7.4, tris(hydroxymethyl)aminomethane buffer. In experiments G and I, assimilation and metabolism was for 28 hours of continuous light.

The results presented in Tables VI and VII show that phenylacetaldoxime and 2-oximino-3-phenylpropionic acid were both better precursors of prunasin than L-phenylalanine as demonstrated by the higher percentage converted to prunasin and the lower dilution of ^{14}C.

Where uniformly labelled precursors were administered, the percentage converted to prunasin was calculated on the basis of the specific activity of benzaldehyde, determined as the semicarbazone, and the yield of hydrogen cyanide obtained upon treatment with β-glucosidase. The carboxyl carbon atom of L-phenylalanine-U-^{14}C was assumed lost as was the case with L-valine-U-^{14}C in linamarin biosynthesis. Paper chromatography of both peach and cherry laurel extracts showed only one cyanogenic glycoside to be present and this had an R_f identical to prunasin.

If the L-phenylalanine-U-^{14}C and phenylacetaldoxime-U-^{14}C were converted to prunasin without any cleavage of the phenyl ring from the side chain then the ratio of specific activities of the recovered benzaldehyde and hydrogen cyanide should have been 7:1. The appropriate ratio figures in Tables VI and VII were considered to not deviate significantly from 7, taking into account the experimental errors, and therefore retention of the side chain was confirmed.

TABLE VI

OXIMES AS PRECURSORS OF PRUNASIN IN CHERRY LAUREL

Expt.	Compound Administered	Amount (μmoles)	S.A.[a]	Products from prunasin: HCN (μmoles)	S.A.[a] HCN	S.A.[a] Bens.[b]	% precursor converted	Ratio[c] ^{14}C	Dilution of ^{14}C
F	L-Phenylalanine-U-^{14}C	1.07	439	326	0.026	0.176	16[d]	6.7	1940[d]
	Phenylacetaldoxime-U-^{14}C	1.2	23	314	0.0040	0.033	43[d]	8.3	610[d]
G	L-Phenylalanine-U-^{14}C	2.14	439	278	0.032	0.22	8.3[d]	6.9	1550[d]
	2-Oximino-3-phenyl-propionic acid-2-^{14}C	6.1	271	303	2.23	<0.002	41[e]	<0.001	120[e]

a Specific activity in μc/μmole

b Benzaldehyde determined as the semicarbazone

c Ratio of radioactivity of benzaldehyde and hydrogen cyanide

d Based on conversion of C_6-C_1 moiety of precursor to benzaldehyde

e Based on incorporation of radioactivity in hydrogen cyanide

TABLE VII

OXIMES AS PRECURSORS OF PRUNASIN IN PEACH

Expt.	Compound Administered	Amount (μmoles)	S.A.[a]	Products from prunasin: HCN (μmoles)	S.A.[a] HCN	S.A.[a] Benz.[b]	% precursor converted	Ratio[c] ^{14}C	Dilution of ^{14}C
H	L-Phenylalanine-U-^{14}C	1.07	439	177	0.034	0.234	11[d]	6.9	1460[d]
	Phenylacetaldoxime-U-^{14}C	1.2	23	157	0.0082	0.060	39[d]	7.3	340[d]
I	L-Phenylalanine-U-^{14}C	2.14	439	257	0.089	0.66	25[d]	7.4	520[d]
	2-Oximino-3-phenyl-propionic acid-2-^{14}C	6.1	271	231	3.36	<0.002	47[e]	<0.001	80[e]
	2-Oximino-3-phenyl-propionic acid-2-^{14}C	2.58	271	251	1.73	<0.001	62[e]	<0.001	160[e]
	Phenylacetaldoxime-U-^{14}C	2.08	23	230	0.0077	0.053	29[d]	6.9	380[d]

a Specific activity in μC/mmole

b Benzaldehyde determined as the semicarbazone

c Ratio of radioactivity of benzaldehyde and hydrogen cyanide

d Based on conversion of C_6-C_1 moiety of precursor to benzaldehyde

e Based on incorporation of radioactivity into hydrogen cyanide

The Tables also show that 2-oximino-3-phenylpropionic acid-2-^{14}C was incorporated with negligible randomisation of label into the benzaldehyde moiety, further indicating its effectiveness as a precursor.

These results show that oximes were good precursors of prunasin and were perhaps natural intermediates in peach and cherry laurel. It is notable that in both peach and cherry laurel the 2-oximino-3-phenylpropionic acid was a more effective precursor of prunasin than 2-oximinoisovaleric acid was a precursor of linamarin in linen flax.

Administration of phenylacetonitrile-1-^{14}C and phenylacethydroxamic acid-1-^{14}C to peach and cherry laurel. Similar criticisms apply to the incorporation of oximes into prunasin as was the case with oximes incorporated into linamarin. In particular, 2-oximino-3-phenylpropionic acid might have been incorporated into prunasin by prior non-enzymic conversion to phenylacetonitrile. In experiments J and K, phenylacetonitrile-1-^{14}C was administered to spring shoots of cherry laurel and peach respectively. Controls with L-phenylalanine-U-^{14}C were used. Phenylacethydroxamic acid-1-^{14}C was administered to cherry laurel shoots only to test the hypothesis that hydroxamic acids may rearrange to give 2-hydroxyl compounds which could be converted to cyanogenic glycosides.

Four cherry laurel shoots with total fresh weight about 2.9g and three peach shoots with total fresh weight about 1.7g were used for each treatment. Both L-phenylalanine-U-^{14}C and phenylacethydroxamic acid-1-^{14}C were dissolved in 0.5 ml of water and administered in open beakers with additional water added as required. Phenylacetonitrile-1-^{14}C was administered with 0.2 ml of water in a 33 ml glass container. It did not completely dissolve at the beginning of the experiment but the droplets gradually disappeared within an hour, presumably being taken up into the shoots in solution or as vapour.

The results given in Table VIII show that phenylacethydroxamic acid was

TABLE VIII

INCORPORATION OF PHENYLACETONITRILE-1-^{14}C AND PHENYLACETHYDROXAMIC ACID-1-^{14}C INTO PRUNASIN

Expt.	Compound Administered			Products from prunasin				
		Amount (μmoles)	S.A.[a]	HCN (μmoles)	S.A.[a] HCN	S.A.[a] Benz.[b]	% precursor converted	Dilution of ^{14}C
Cherry laurel								
J	L-Phenylalanine-U-^{14}C	2.14	439	260	0.071	0.39	14[c]	880[c]
	Phenylacetonitrile-^{14}C	3.14	2150	250	17	<0.002	63[d]	127[d]
	Phenylacethydroxamic acid-1-^{14}C	1.81	136	300	<0.003	<0.001	<0.3[d]	-
Peach								
K	L-Phenylalanine-U-^{14}C	2.14	439	38	0.26	1.44	7.6[c]	240[c]
	Phenylacetonitrile-^{14}C	3.32	2150	41	101	<0.01	57[d]	21[d]

a. Specific activity in μC/mmole

b Benzaldehyde determined as the semicarbazone

c Based on conversion of C_6-C_1 moiety of precursor to benzaldehyde

d Based on incorporation of radioactivity into hydrogen cyanide

not effective as a precursor of prunasin, thereby indicating that the formation of cyanogenic glycosides by rearrangement and glycosylation of hydroxamic acids was most unlikely. The large incorporation of phenylacetonitrile-1-^{14}C into prunasin in both peach and cherry laurel strongly suggested that it was a natural intermediate. The level of incorporation may have been sufficient to account for the considerable incorporation from 2-oximino-3-phenylpropionic acid observed in experiments G and I if conversion of the 2-oximino acid to nitrile occurred freely.

Figure 14 shows the distribution of radioactivity obtained upon paper chromatography for each of the cherry laurel treatments. L-phenylalanine-U-^{14}C was extensively metabolised with only a small portion remaining. Prunasin was by far the major non-volatile product from phenylacetonitrile-1-^{14}C. In the phenylacetohydroxamic acid-1-^{14}C treatment, no significant radioactivity occurred in the prunasin zone but other unidentified radioactive compounds were observed. Peach shoot extracts gave chromatograms essentially similar to respective cherry laurel extracts.

Mandelonitrile-1-^{14}C as a precursor of prunasin. In experiment L an attempt was made to test mandelonitrile as a precursor of prunasin in cherry laurel shoots. The basic procedure was similar to that used in experiment E to show that 2-hydroxyisobutyronitrile-1-^{14}C may be incorporated into linamarin by flax seedling shoots. A solution of 5 µmoles of potassium dihydrogen phosphate and 2 µmoles of potassium cyanide-^{14}C in 10 µl of water, was allowed to stand with 2 µl of freshly distilled benzaldehyde for 40 minutes at 5°C. D,L-Mandelonitrile-1-^{14}C was expected as a reaction product. The reaction mixture was transferred with 190 µl of water to a 33 ml glass vessel enclosing three cherry laurel shoots of about 2.4g total fresh weight. A further three shoots of similar fresh weight were treated with 2 µmoles of potassium cyanide-^{14}C and 5 µmoles of potassium dihydrogen phosphate in 200 µl of water in a similar

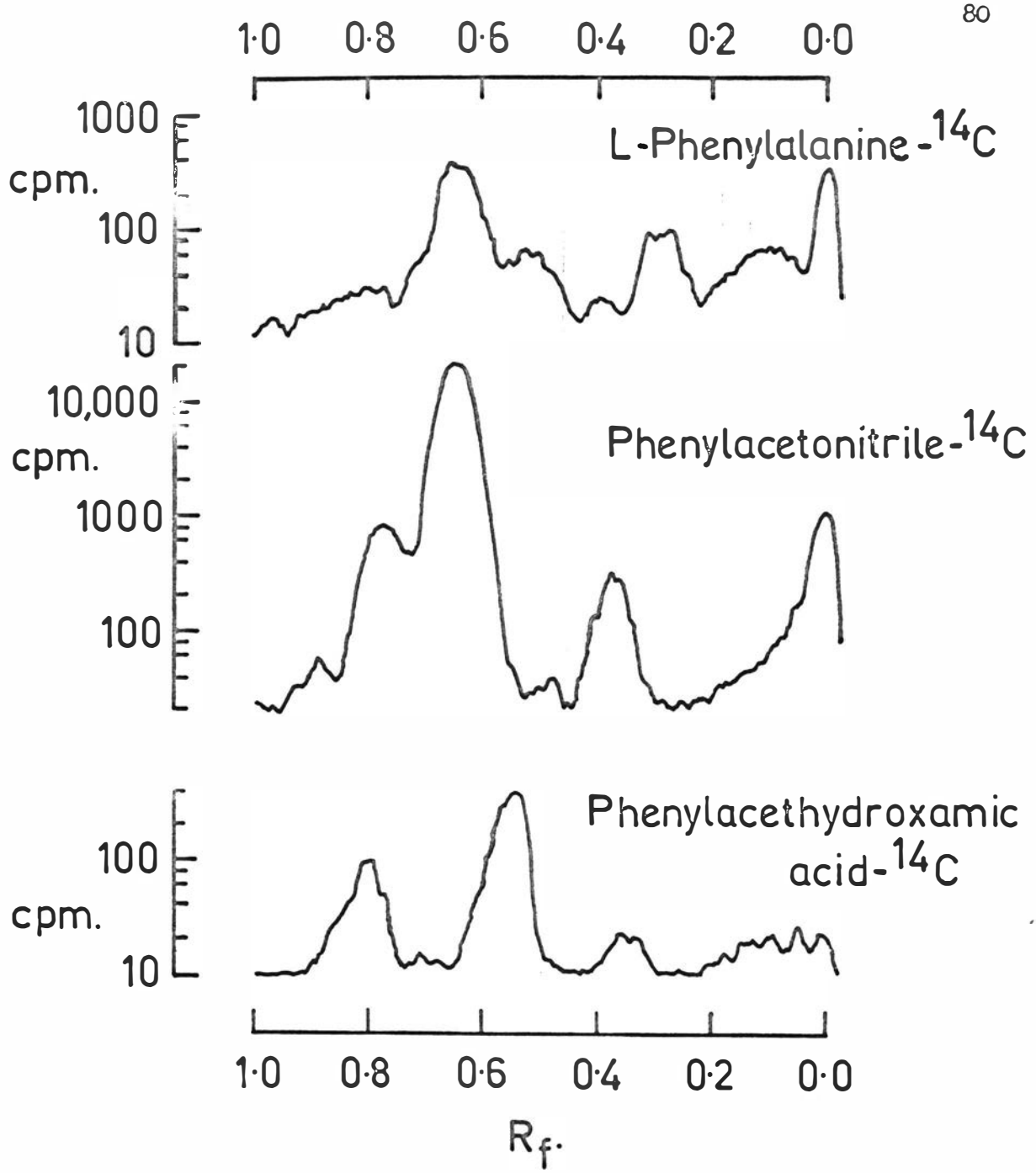


FIGURE 14 INCORPORATION OF RADIOACTIVITY FROM L-PHENYLALANINE-U-^{14}C, PHENYLACETONITRILE-1-^{14}C AND PHENYLACETHYDROXAMIC ACID-1-^{14}C IN CHERRY LAUREL SHOOTS. SEMI-LOGRITHMIC SCANS OF RADIOACTIVITY ON PAPER CHROMATOGRAM STRIPS DEVELOPED WITH BW; PRUNASIN HAS AN R_f OF 0.65 AND PHENYLALANINE AN R_f OF 0.30.

closed glass vessel as a control treatment. Assimilation and metabolism was for 28 hours in continuous light.

The results of this experiment given in Table IX show that hydrogen cyanide was incorporated into the nitrile moiety of prunasin to the small but significant extent of 4.0% of the amount administered. Prior treatment with excess benzaldehyde increased the incorporation of radioactivity but not to a great degree. The low specific activity of the benzaldehyde from prunasin indicates that little randomisation of label occurred.

These results may be consistent with the possibility that L-mandelonitrile is a natural intermediate if certain conditions applied during the experiment. The incorporation of hydrogen cyanide-^{14}C may have arisen from reaction with benzaldehyde released from the shoots, possibly due to damage during the administration of the label. This reaction may have been catalysed by a specific oxynitrile lyase which would be expected in this tissue and which would give only L-mandelonitrile. The observed incorporation of hydrogen cyanide-^{14}C was inconsistent with a pathway of biosynthesis from an amino acid, aldoxime, or nitrile.

The relatively low level of incorperation from the D,L-mandelonitrile-1-^{14}C treatment may have been due to a number of factors. In particular, mandelonitrile may not have been formed efficiently under the conditions used but rather some of the benzaldehyde may have been converted to unlabelled benzoin. Further, the mandelonitrile, excess benzaldehyde, or any other compounds formed in the reaction may have been toxic. It was observed that the bottom 3 mm of the shoots, which were immersed in the solution, showed "browning" after about 30 minutes exposure and appeared dead at the end of the experiment. This effect cannot be attributed to only a toxic effect of hydrogen cyanide as the shoots treated with cyanide showed only modest "browning" of the cut surface at the end of the experiment. A toxic effect of the D,L-mandelonitrile-1-^{14}C treatment

TABLE IX

MANDELONITRILE AS A PRECURSOR OF PRUNASIN

Compound Administered			Products from prunasin				
	Amount (μmoles)	S.A.[a]	HCN (μmoles)	S.A.[a] HCN	S.A.[a] Benz.[b]	% precursor converted[c]	Ratio[d] ^{14}C
D,L-Mandelonitrile-1-^{14}C	2.0	690	265	0.33	0.0024	6.5	0.007
Hydrogen cyanide	2.0	690	280	0.19	0.015	4.0	0.08

a Specific activity in μC/mmole

b Benzaldehyde determined as the semicarbazone

c Based on incorporation of radioactivity in the nitrile moiety of prunasin

d Ratio of radioactivity of benzaldehyde and nitrile moiety

may therefore have prevented significant uptake from solution through the stem. Some of the incorporation observed may be attributed to incorporation of hydrogen cyanide-^{14}C similar to that already mentioned. If D,L-mandelonitrile-1-^{14}C were formed initially, then during the experiment it may have dissociated to give hydrogen cyanide-1-^{14}C which could be assimilated from the vapour phase.

Figure 15 shows that some other unidentified compounds, neutral at pH 2, were formed when hydrogen cyanide-^{14}C was administered. A notable feature of this treatment was the low level of radioactivity recovered in amino acids. The amino acid separation of the D,L-mandelonitrile-1-^{14}C treatment gave an essentially similar radioautograph.

3.3 Intermediates in linamarin biosynthesis

Introduction. Among the criteria used to establish a pathway, the demonstration of the natural existence of possible intermediates plays an important central role. If conventional methods of chemical analysis are not sufficiently sensitive to detect a small concentration of an intermediate then a limited number of other techniques are available. The use of radioactive precursors of high specific activity may increase the sensitivity of an isolation procedure several-fold. If a sample of the compound suspected as an intermediate is administered along with labelled precursor then the labelled intermediate may be diluted and accumulated in the tissue. The use of this type of procedure has been described as "trapping". Alternatively, accumulation of an intermediate may be induced by administering a specific inhibitor of the biosynthesis. Each of these techniques has been used to investigate intermediates in cyanogenic glycoside biosynthesis.

In a series of experiments discussed briefly at the Tenth International Botanical Congress, Butler and Conn (1964b) administered a range of general metabolic inhibitors and specific structural analogues of valine and isoleucine

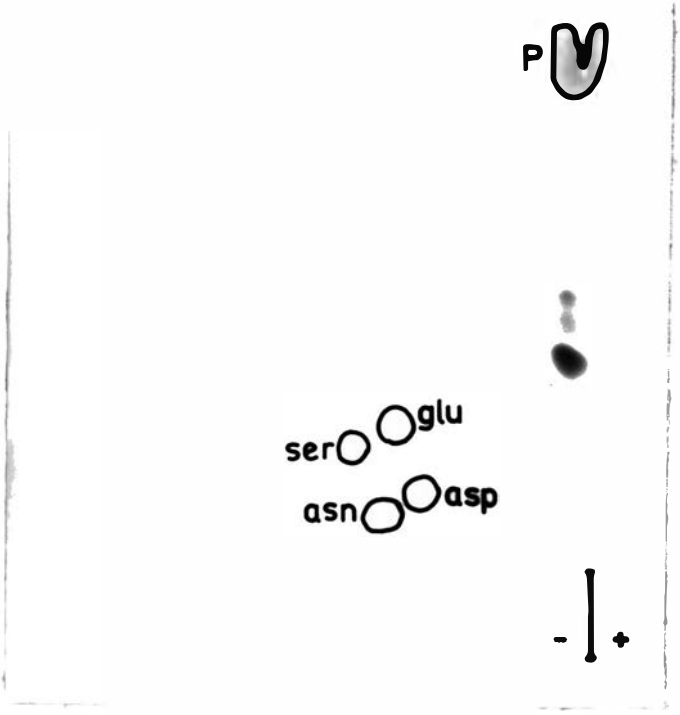


FIGURE 15 INCORPORATION OF RADIOACTIVITY FROM HYDROGEN CYANIDE-^{14}C IN CHERRY LAUREL. A RADIOAUTOGRAPH OF AN AMINO ACID SEPARATION AFTER ELECTROPHORESIS AT pH 2 AND CHROMATOGRAPHY. P IS PRUNASIN.

to linen flax shoots together with L-valine-U-^{14}C. In some treatments partial inhibition of incorporation of label into linamarin was observed and in a few cases two dimensional paper chromatography revealed a new unidentified labelled compound (IV). Table X lists the compounds administered and gives a qualitative indication of the effect. More unpublished work by Butler showed that when D-glucose-^{14}C was administered with D,L-O-methylthreonine some label accumulated in the same area of chromatograms as for IV. Treatment with emulsin released glucose-^{14}C from this compound. When isoleucine-U-^{14}C was administered with D,L-O-methylthreonine another unidentified compound was observed. This was presumably homologous with IV. It was concluded that IV was a glucoside and possibly an intermediate, or was derived from an intermediate of linamarin biosynthesis. An attempt to observe incorporation of ^{14}C-labelled IV into linamarin by administering back to flax shoots was unsuccessful. In some of the experiments which follow, the effect of O-methylthreonine and related compounds and the nature of IV was further investigated.

Effect of D,L-O-methylthreonine and D,L-3-methoxyvaline. In experiment M, 10 μmoles of D,L-O-methylthreonine or D,L-3-methoxyvaline were administered with 1.2 μmoles of L-valine-U-^{14}C or 1.0 μmole of 2-aminoisovaleric acid-U-^{14}C to linen flax seedlings. The conditions were identical to those used in experiment A which therefore represents control treatments.

Table XI combines the results of experiments A and M. D,L-O-Methylthreonine gave significant inhibition of linamarin biosynthesis and accumulation of IV when L-valine-U-^{14}C was administered. While D,L-3-methoxyvaline had a significant inhibitor effect, it did not induce accumulation of IV and therefore had a different mode of action from D,L-O-methylthreonine. It is possible that, D,L-3-methoxyvaline could exhibit an inhibitor effect as an analogue of a natural amino acid. A small conversion of L-valine-U-^{14}C to leucine which

TABLE X

RESULTS OF INHIBITOR EXPERIMENTS BY BUTLER AND CONN

Compound	Inhibition	Compound IV
Streptomycin	Nil	Nil
Chloramphenicol	Nil	Nil
L-Penicillamine	Nil	Nil
L-allo-Isoleucine	Nil	Nil
2-Hydrazinoisovaleric acid	Nil	Nil
D,L-*erythro*-2-Amino-3-chlorobutyric acid	Nil	Nil
D,L-*threo*-2-Amino-3-chlorobutyric acid	Nil	Nil
2-Amino-3-ethyl-valeric acid	Strong	Nil
D,L-2-Amino-3-methiobutyric acid	Strong	Nil
Isopropylmalate	Nil	Nil
D,L-O-Methylthreonine	Strong	Considerable
D,L-O-Methyl-allo-threonine	Strong	Considerable
Isobutylamine hydrochloride	Weak	Trace
2-Methoxypropionamine hydrochloride	Weak	Moderate

TABLE XI

EFFECT OF TWO AMINO ACIDS AS INHIBITORS

Expt.	^{14}C compound	Inhibitor	Incorporation[a] from ^{14}C compound	
			% converted to linamarin	% converted to IV
A	L-Valine-U-^{14}C	-	23	Nil[b]
M	L-Valine-U-^{14}C	D,L-O-Methylthreonine	9	3
M	L-Valine-U-^{14}C	D,L-3-Methoxyvaline	10	Nil[b]
A	2-Oximinoisovaleric acid-U-^{14}C	-	9	Nil[b]
M	2-Oximinoisovaleric acid-U-^{14}C	D,L-O-methylthreonine	5	Nil[b]

a Assuming a loss of carboxyl-^{14}C from precursor

b Estimated as less than 0.5% which was the minimum detectable level

occurred in the control treatment was absent in the presence of D,L-3-methoxyvaline; other effects on general metabolism were not studied.

D,L-O-Methylthreonine inhibited the conversion of 2-aminoisovaleric acid-U-^{14}C to linamarin although the effect was relatively less than when L-valine-U-^{14}C was administered. Compound IV did not accumulate. This observation indicates that D,L-O-methylthreonine may act in two ways to reduce the synthesis of linamarin. It may be a general inhibitor of cell metabolism causing a depression in the level of linamarin biosynthesis by affecting other functions of the plant tissue as well as a specific inhibitor of linamarin biosynthesis leading to the accumulation of IV.

Effect of 2-methoxypropionaldoxime and 2-methoxypropionitrile. The finding by Butler and Conn (Table X) of a weak inhibitory action of isobutylamine and 2-methoxypropionamine, together with the formation of traces of IV, suggested that other related compounds without a carboxyl group might also be active as this type of inhibitor. On the basis of the hypothesis that aldoximes and nitriles are intermediates in cyanogenic glycoside biosynthesis, the corresponding methoxyaldoxime and methoxynitrile were prepared and tried as inhibitors.

In experiment N, D,L-2-methoxypropionaldoxime and D,L-O-methylthreonine (as an inhibitor control treatment) were administered with 1 µmole of L-valine-U-^{14}C as in experiment D. For experiment O, 10 µmoles of D,L-2-methoxypropionitrile were administered to the usual batch of 20 linen flax seedlings, together with 1 µmole of L-valine-U-^{14}C dissolved in 70 µl of water and enclosed in a 12 ml glass container. Assimilation and metabolism was for 7 hours in continuous light.

The results of these experiments listed in Table XII show that D,L-2-methoxypropionaldoxime was an even more effective inhibitor of linamarin biosynthesis than D,L-O-methylthreonine. It is possible that D,L-O-methylthreonine may obtain its specific inhibitor action by prior conversion to the aldoxime,

TABLE XII

EFFECT OF D,L-2-METHOXYPROPIONALDOXIME AND D,L-2-METHOXYPROPIONITRILE

Expt.	Inhibitor	Amount (μmoles)	Incorporation[a] from L-valine-U-^{14}C % converted to linamarin	% converted to IV
D		-	23	Nil[b]
N	D,L-O-Methylthreonine	10	7	3
N	D,L-2-Methoxypropionaldoxime	9	5	4
O	D,L-2-Methoxypropionitrile	10	2.5	Nil[b]

a Assuming a loss of carboxyl-^{14}C from L-valine-U-^{14}C

b Estimated as less than 0.5% which was the minimum detectable level

perhaps by the same enzymes which may convert L-valine to isobutyraldoxime.

The low conversion of L-valine-U-^{14}C to linamarin with the D,L-2-methoxypropionitrile treatment may have been due to an inhibition effect by this compound, or may have been due to slow uptake and transfer of L-valine-U-^{14}C to the site of synthesis of linamarin when the seedling shoots were enclosed in a small vessel which inhibits transpiration. Compound IV was not detected in this treatment.

Volatile intermediates. In the preceeding experiments including those in Table X, no attempt was made to examine the linen flax seedlings for volatile intermediates. In this section attempts are described to obtain direct evidence for isobutyronitrile and isobutyraldoxime in the linen flax seedlings.

A batch of 100g of seedling shoots, grown as usual, were blended with 200 ml of water and steam distilled until 500 ml of distillate had accumulated. The distillate was extracted with 20 ml of light petroleum (b.p. 30-40°) and samples taken for gas chromatography using a Carbowax 400 column (1.5m x 4.5mm ID) as described in section 2.6. No isobutyronitrile was detected. The minimum detectable level was estimated as 0.2 mg/g fresh weight.

In a subsequent attempt to show volatile intermediates, 100 seedling shoots were allowed to assimilate and metabolise 10 µmoles of L-valine for 24 hours from solution, with and without the addition of 50 µmoles of D,L-O-methylthreonine. The two batches of shoots were then steam distilled and the 20 ml of distillate extracted with 5 ml of diethyl ether. The extract was reduced in volume to 0.5 ml and portions taken for gas chromatography on a Carbowax 1500 column. Neither isobutyronitrile nor isobutyraldoxime were observed although other unidentified volatile compounds were detected in both treatments.

Labelled volatile intermediates, I. In experiment P, three batches of 20 seedling shoots were treated with 2 µmoles of L-valine-U-^{14}C containing 1 µC of radioactivity dissolved in 200 µl of water. In one treatment 10 µmoles of D,L-O-methylthreonine was added and in another 60 µmoles of isobutyraldoxime

was added to the solution and 0.6 mmole placed in an adjacent 1 ml beaker. All treatments were enclosed in 200 ml transparent containers for two hours and exposed to light for a total of eight hours after which the shoots were blended with 50 ml of cold diethyl ether for one minute. The ether extract was decanted and the residue extracted twice with boiling 80% ethanol. Portions of 5 ml of the ether extracts were concentrated in the presence of added carrier acetone, isobutyraldehyde, isobutyronitrile, and isobutyraldoxime. These compounds were separated on a large Carbowax 400 column (3m x 7.6mm ID, section 2.6) and the volatile radioactive compounds trapped and counted as described in section 2.8.

Figure 16 shows histograms of the average counting rate of the fractions for the three treatments as well as an indication of the temperature programme and a typical separation obtained. The notable feature of the results was the accumulation of radioactivity in the fractions containing isobutyraldoxime when it or D,L-O-methylthreonine was administered.

Further portions of the ether extracts were treated with 2,4-dinitrophenylhydrazine dissolved in dilute ethanolic hydrochloric acid prior to thin layer chromatography. Bands of radioactivity corresponding to isobutyraldehyde-2,4-dinitrophenylhydrazone were observed for the treatments with isobutyraldoxime and D,L-O-methylthreonine.

These results were consistent with isobutyraldoxime being an intermediate and also indicated that the specific inhibition of linamarin biosynthesis may occur at a step between isobutyraldoxime and linamarin such that the oxime may accumulate. Radioactivity occurred in other volatile compounds especially in the absence of isobutyraldoxime or D,L-O-methylthreonine.

The ethanolic extracts of the plant tissue residues were reduced in volume under vacuum and chromatographed. Table XIII gives results which indicated the usual inhibitory effect of D,L-O-methylthreonine accompanied by synthesis of IV.

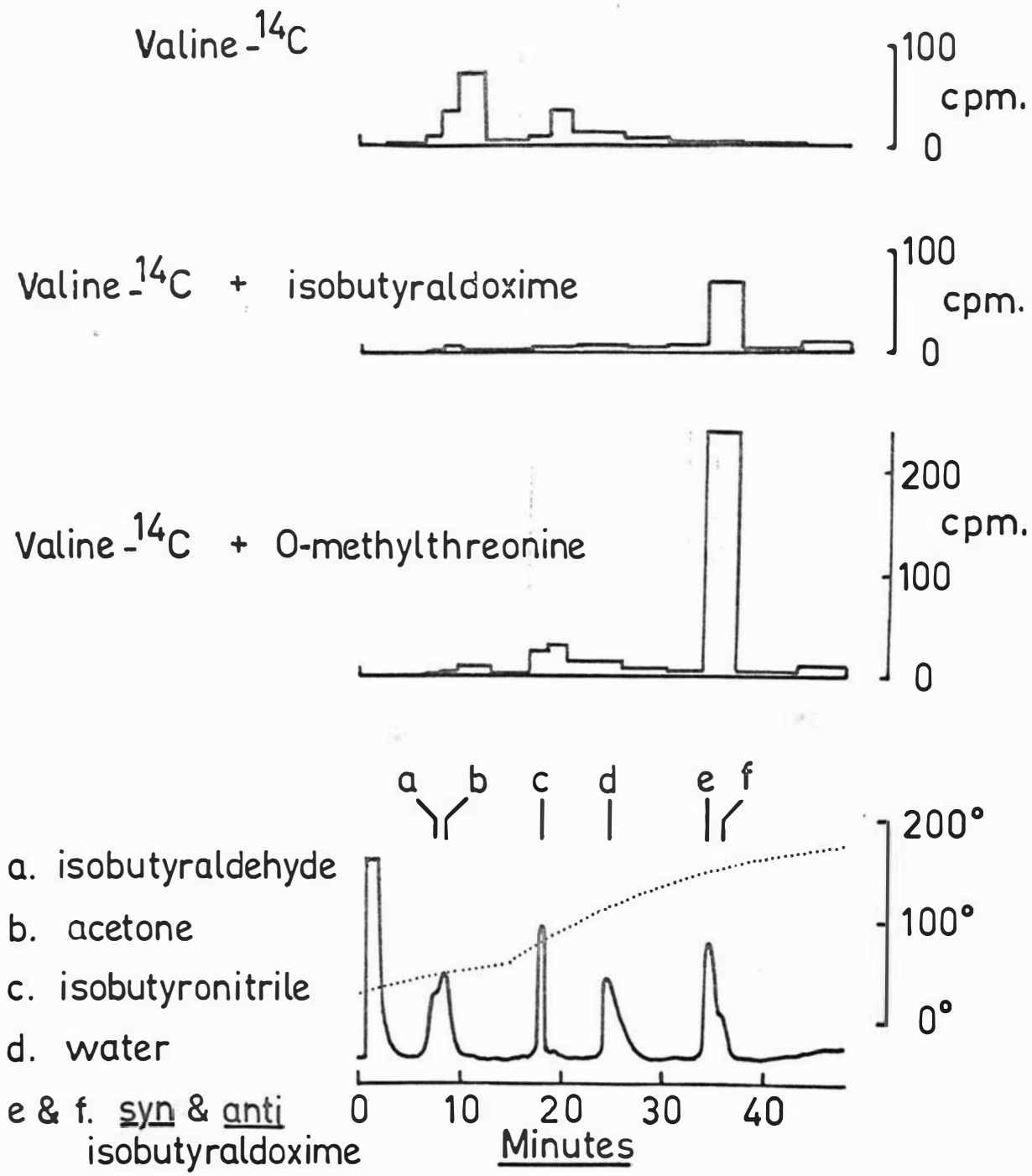


FIGURE 16 GAS CHROMATOGRAPHIC SEPARATION OF VOLATILE RADIOACTIVE COMPOUNDS FROM LINEN FLAX FOR THREE TREATMENTS.

TABLE XIII

EFFECT OF D,L-O-METHYLTHREONINE AND ISOBUTYRALDOXIME ON LINAMARIN BIOSYNTHESIS

Treatment	Incorporation[a] from L-valine-U-^{14}C	
	% converted to linamarin	% converted to IV
Control	20	Nil[b]
D,L-O-methylthreonine	6	2
Isobutyraldoxime	Nil[b]	Nil[b]

a Assuming a loss of carboxyl-^{14}C from L-valine-U-^{14}C

b Estimated as less than 0.5% which was the minimum detectable level

The treatment with isobutyraldoxime prevented incorporation of radioactivity into linamarin. This may be attributed to either a toxic effect or to competitive dilution of the label.

Labelled volatile intermediates, II. Experiment 4 was designed to examine the effects of some other possible inhibitors or intermediates as well as confirm the finding of isobutyraldoxime-^{14}C as an accumulated intermediate. L-valine-U-^{14}C containing 2 μC in 1 μmole was administered to batches of twenty seedling shoots with treatments of unlabelled isobutyraldoxime, isobutyronitrile, methacrolein oxime, methacrylonitrile, 2-hydroxyisobutyronitrile, 2-oxoisovaleric acid, 2-methoxypropionaldoxime, and D,L-O-methylthreonine. The last three compounds, being considered non-volatile, were administered with the L-valine-U-^{14}C in 142 μl of water. Two portions of 50 μl of water were added during the first 2.5 hours after which the shoots were enclosed in 12 ml glass vessels. The other treatments were similar except that the unlabelled compounds were administered after the first 2.5 hours which allowed prior uptake of virtually all of the L-valine-U-^{14}C solution. Volatile compounds were probably assimilated from the vapour phase. A further 7.5 hours was allowed for metabolism of the L-valine-U-^{14}C in the presence of the added unlabelled compounds. Results may be compared with a control where no unlabelled compound was used.

The shoots were extracted first with approximately 1.5 ml of diethyl ether. The procedure, which was designed to eliminate the necessity of concentration of the ether extract prior to gas chromatography, is described in detail in section 2.5. The solid residue was extracted three times by standing for several hours in 20 ml of cold 95% ethanol. The combined ethanol extracts were concentrated and portions separated by paper chromatography.

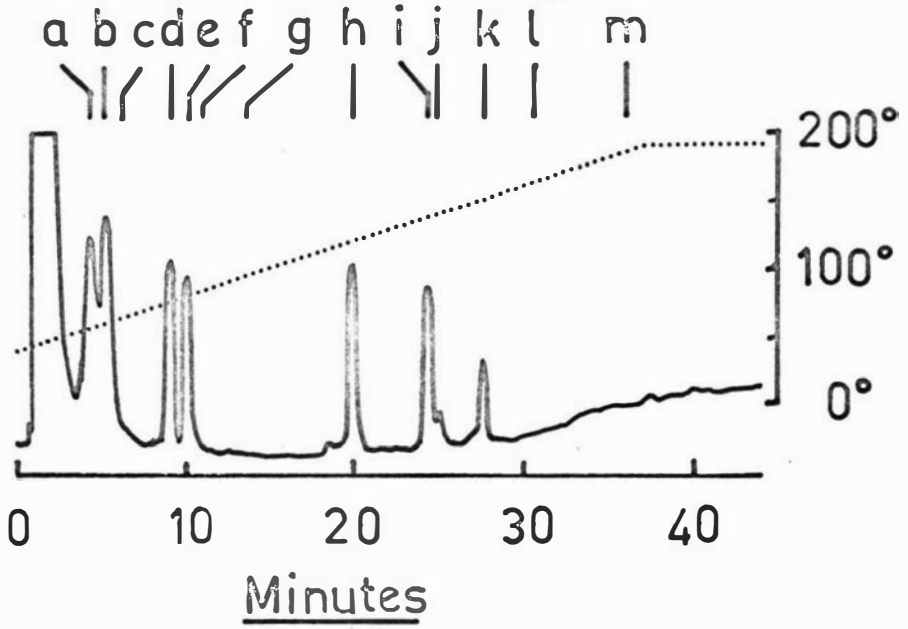
Details of the gas chromatography and determination of radioactivity of the ether extracts using a diethylene glycol succinate column are described in section 2.6. A flame ionisation detector was used to detect mass peaks of all

compounds. Figure 17 gives a typical example of the separations and the temperature programme used. The letters refer to areas of the chromatograms which were of interest - some were the positions of known compounds. The small peak at j is believed to be an impurity formed from isobutyraldoxime.

Figures 18 and 19 are semi-logarithmic histograms of the count-rate obtained with the proportional counter on the same time scale and under identical conditions to the separation shown in Figure 17, using approximately 150 µl portions of the ether extracts. The units of radioactivity are hundreds of counts recorded in the 0.4 minute intervals of the histograms. The shaded areas are those which exceed twice the standard error of the background in 0.4 minute counting periods and represent significant peaks of radioactivity.

Figure 18 compares the patterns of radioactivity obtained with the control treatment and the treatments with 60 µmoles of isobutyronitrile and 20 µmoles of isobutyraldoxime. Certain peaks of radioactivity appeared in all or most treatments to varying extents and may be due to labelled compounds not related to, nor involved in, linamarin biosynthesis. They included peaks at positions b, f, g, j, k, l and m. These peaks remained unidentified although it was likely that the peak at b was due to acetone derived from labelled linamarin during the extraction or from 2-hydroxyisobutyronitrile which was a possible intermediate.

The most notable feature of the isobutyraldoxime treatment was the considerable radioactivity associated with isobutyraldoxime at h which also appeared as a large mass peak in the flame ionisation detector. This peak was trapped in a U tube cooled with solid carbon dioxide, and 2,4-dinitrophenylhydrazine derivatives were prepared which gave a single peak of radioactivity with an R_f identical to that of isobutyraldehyde-2,4-dinitrophenylhydrazone upon chromatography with toluene on silica gel (Eastman Chromagram sheet, type K301 R). This confirmed the identification of labelled isobutyraldoxime. The considerable peak of radioactivity at j in the isobutyraldoxime treatment had an



a. isobutyraldehyde
b. acetone
d. methacrylonitrile
e. isobutyronitrile
h. isobutyraldoxime
i. methacrolein oxime
k. 2-methoxypropionaldoxime

FIGURE 17 TYPICAL GAS CHROMATOGRAPHIC SEPARATION USING A DIETHYLENE GLYCOL SUCCINATE COLUMN WITH THE TEMPERATURE PROGRAMME INDICATED.

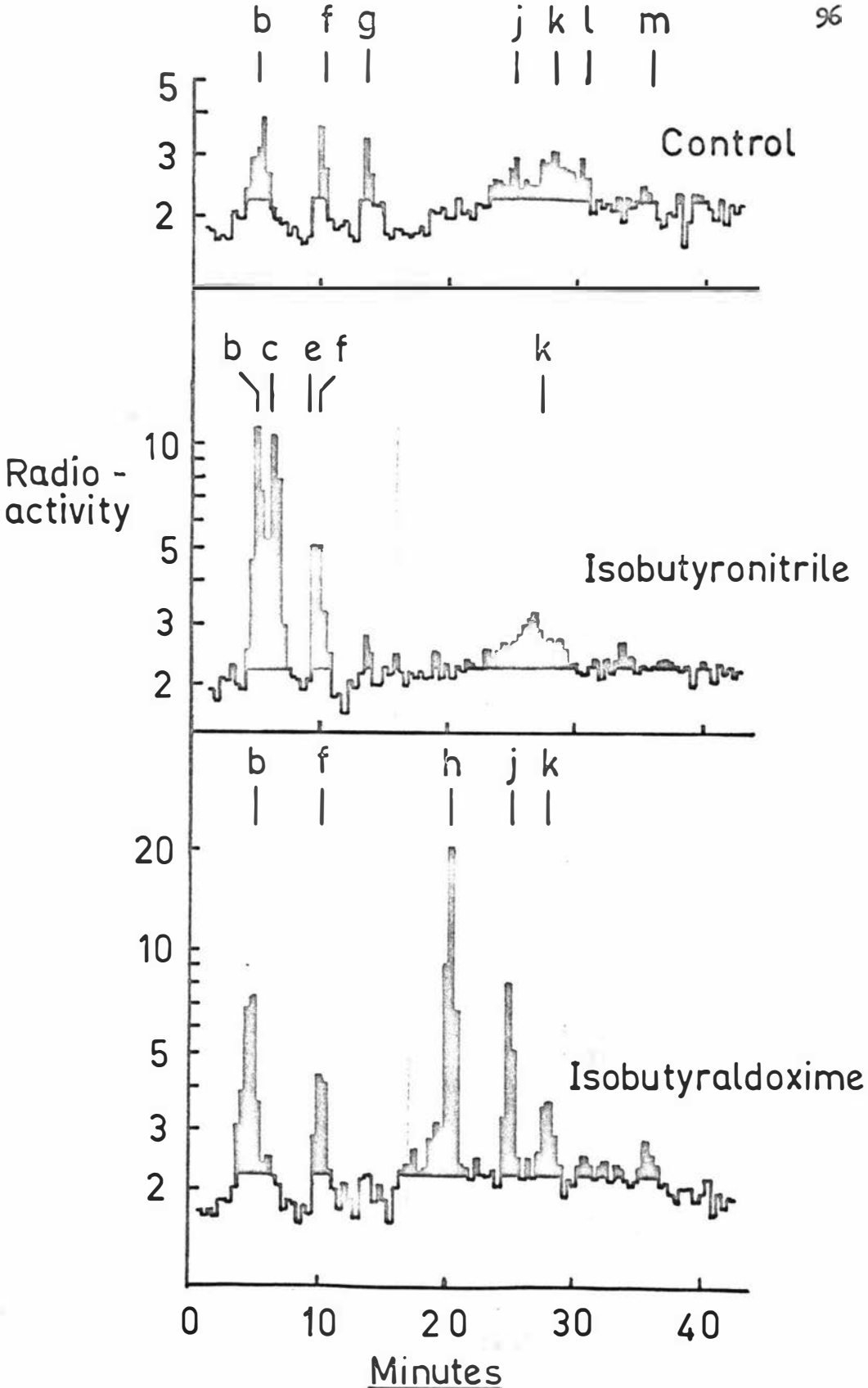


FIGURE 18 RADIOACTIVITY IN SEPARATED VOLATILE COMPOUNDS FROM LINEN FLAX TREATED WITH L-VALINE-U-^{14}C AND OTHER UNLABELLED COMPOUNDS. UNITS OF RADIOACTIVITY ARE HUNDREDS OF COUNTS RECORDED IN THE 0.4 MINUTE INTERVALS OF THE HISTOGRAMS.

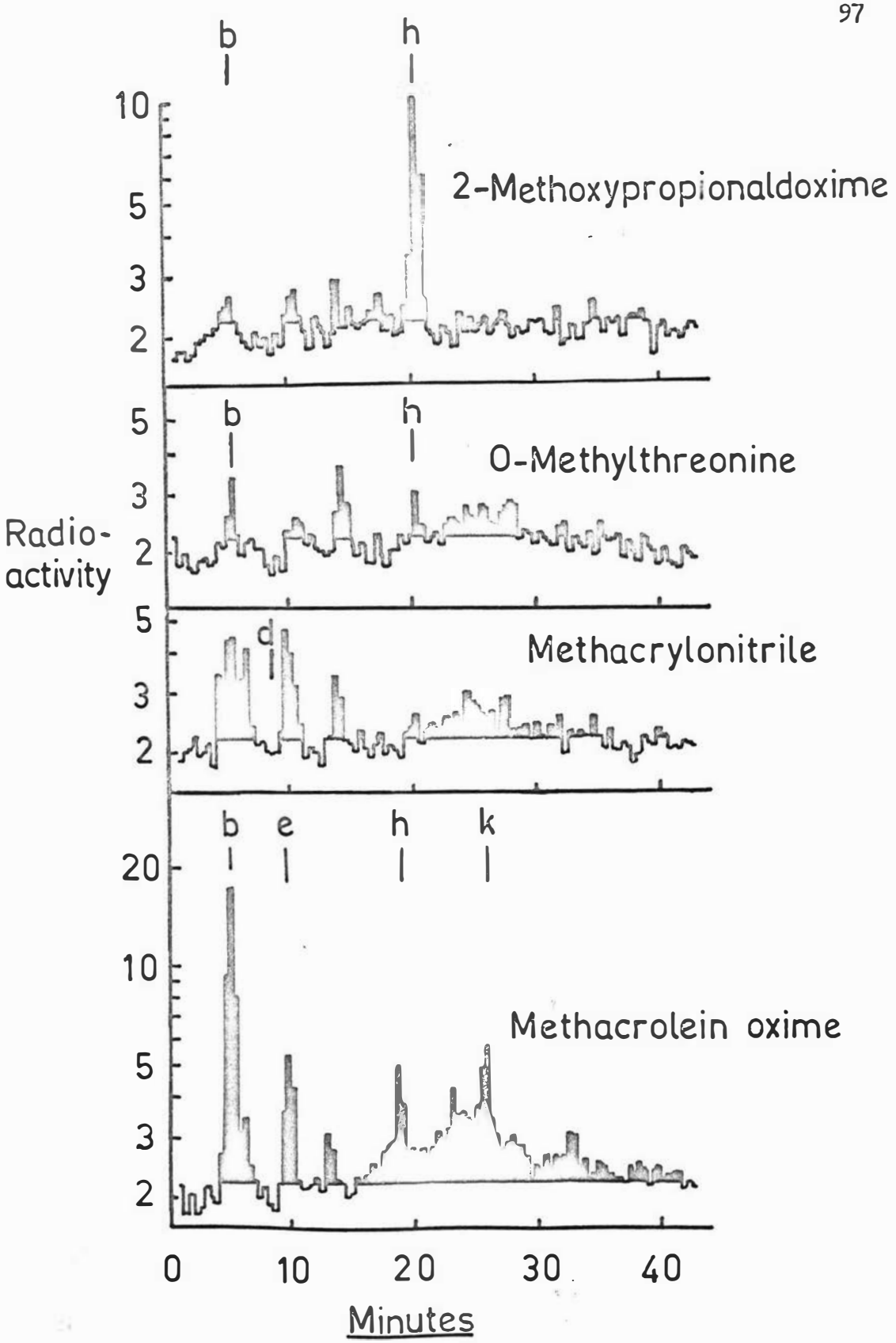


FIGURE 19 RADIOACTIVITY IN SEPARATED VOLATILE COMPOUNDS FROM LINEN FLAX TREATED WITH L-VALINE-U-^{14}C AND OTHER UNLABELLED COMPOUNDS. UNITS OF RADIOACTIVITY ARE HUNDREDS OF COUNTS RECORDED IN THE 0.4 MINUTE INTERVALS OF THE HISTOGRAMS.

associated peak with the flame ionisation detector and appeared to have approximately the same specific activity as the recovered isobutyraldoxime. It may have been formed by the plant tissue or may have arisen as an artifact during or after extraction. A small mass peak also occurred at e which may have been isobutyronitrile with a small amount of associated radioactivity not resolved from radioactivity at f. Another small mass peak with an elution time of about 16 minutes was not radioactive. None of these mass peaks were observed with the control.

The treatment with 60 μmoles of isobutyronitrile gave a chromatogram with considerable radioactivity corresponding to acetone at b and an unidentified compound at g, traces of which occurred in other treatments. A significant peak of radioactivity was associated with a large mass peak for isobutyronitrile at e. This peak was trapped and diluted with pure isobutyronitrile. t-Butylisobutyramide was prepared from it and recrystallised to a constant specific activity which indicated that approximately 70% of the radioactivity trapped in this peak was isobutyronitrile. This result supported the hypothesis that isobutyronitrile was an intermediate in linamarin biosynthesis.

Treatment with a smaller amount of 12 μmole of isobutyronitrile produced a pattern of radioactivity very similar to that of the control. The only significant difference was a broader peak at e and f indicating some "trapping" of radioactivity in isobutyronitrile.

The chromatograms of treatments with 10 μmoles of D,L-2-methoxypropionaldoxime and 10 μmoles of D,L-O-methylthreonine are illustrated in Figure 19. Radioactivity attributable to isobutyraldoxime occurred with both treatments at h although to a lesser extent in the presence of D,L-O-methylthreonine. A very small mass peak corresponding to isobutyraldoxime was also detected along with a larger peak of D,L-2-methoxypropionaldoxime when this latter compound was administered. These results confirm the findings of experiment P that iso-

butyraldoxime accumulation can be induced by selected inhibitors which also induce the accumulation of compound IV.

The effect of administration of 24 μmoles of methacrylonitrile or 20 μmoles of methacrolein oxime was examined as both of these compounds could in theory be intermediates in linamarin biosynthesis, in which case an accumulation of radioactivity in them would have been expected. Methacrylonitrile, in particular, could be considered as an intermediate between isobutyronitrile and 2-hydroxyisobutyronitrile. The results of gas chromatography shown in Figure 19 did not give any evidence for radioactivity in either of the methacryl compounds and therefore these compounds are probably not intermediates. Methacrylonitrile was recovered as a single large mass peak at d without significant associated radioactivity. The treatment with the nitrile did, however, have the effect of increasing radioactivity at c and e relative to the control. The radioactive peak at e could be isobutyronitrile.

Methacrolein oxime had the effect of increasing the radioactivity in a number of volatile compounds, most notably at b which could be acetone. A peak at h may be attributed to some accumulation of isobutyraldoxime in the presence of methacrolein oxime.

Treatment with 10 μmoles of 2-oximinoisovaleric acid or 22 μmoles of 2-hydroxyisobutyronitrile had little effect on the pattern of volatile radioactive compounds which was, in each case, similar to that of the control. A moderate sized mass peak of isobutyronitrile was observed from the 2-oximinoisovaleric acid treatment but the associated radioactivity was only just significant.

With the 2-hydroxyisobutyronitrile treatment, a moderate sized mass peak of acetone appeared but the radioactivity was similar to that of the control. A small and barely significant peak of radioactivity could be attributed to isobutyraldoxime.

Table XIV gives a qualitative indication of the effects of each treatment on the formation of linamarin and IV. Reduced incorporation of label into linamarin occurred in every treatment relative to the control. The notable reductions were with isobutyraldoxime and with 60 μmoles of isobutyronitrile which were consistent with a competition effect. The effect of D,L-2-methoxy-propionaldoxime was to again give an even greater synthesis of compound IV and inhibition of linamarin biosynthesis than did D,L-O-methylthreonine. No other unlabelled compounds induced significant accumulation of compound IV. Except in the case of isobutyronitrile no attempt was made to show the effect of varying concentration of the unlabelled competitor or inhibitor.

Properties and structure of compound IV. In this section, experiments are described which attempted to extend information about IV. The finding by Butler that this compound was a glucoside which accumulated only in the presence of a specific inhibitor of linamarin biosynthesis was taken as the basis for studies. In the first instance IV was purified from part of the extract of the treatment with D,L-O-methylthreonine in experiment L. It was passed through a small column (3cm x 0.3cm) of Biodemineralit and purified by paper chromatography in two solvent systems when it appeared to be radiochemically homogenous although not necessarily chemically pure. Its free passage through the deionising resin demonstrated an absence of acidic or basic groups.

In experiment R small portions of IV were treated with 1M hydrochloric acid with and without the addition of 0.1% 2,4-dinitrophenylhydrazine for 2 hours at 0° or for 24 hours at 30°. The solutions were then neutralised with excess sodium bicarbonate and extracted with 16 volumes of a mixture of equal parts of ethanol and n-butanol. The supernatant was drawn off and evaporated under reduced pressure prior to applying to Whatman 3 MM chromatography paper. After developing with BW the paper strips were scanned for radioactivity.

The results indicated that the prolonged treatment with acid destroyed

TABLE XIV

EFFECT OF COMPETITOR OR INHIBITOR ON LINAMARIN BIOSYNTHESIS FROM L-VALINE-U-^{14}C

Unlabelled Compound		Radioactivity[a]	
	(Amount µmoles)	Linamarin	IV
Control	-	++++	-
Isobutyraldoxime	20	++	-
Isobutyronitrile	60	++	-
Isobutyronitrile	12	+++	-
D,L-2-Methoxypropionaldoxime	10	+	++
D,L-O-Methylthreonine	10	++	+
Methacrylonitrile	24	+++	-
Methacrolein oxime	20	++	-
2-Oximinoisovaleric acid	10	+++	-
2-Hydroxyisobutyronitrile	22	++	-

a Compounds separated by chromatography on Whatman 3MM paper with BW solvent and scanned for radioactivity

all of IV and, as no radioactivity was recovered on the paper chromatograms, that portion of the molecule derived from L-valine was rendered volatile. Prolonged treatment with 2,4-dinitrophenylhydrazine yielded a non-volatile radioactive compound with a high R_f value presumed to be a hydrazone derivative. After treatment with acid for only two hours at 0° some, but not all, of IV was recovered. Treatment with 2,4-dinitrophenylhydrazine under similar conditions again yielded the hydrazone.

The radioactive hydrazone spots were eluted in ethanol and transferred to silica gel G thin layer chromatography strips. The radioactivity had the same R_f as isobutyraldehyde-2,4-dinitrophenylhydrazone in three solvent systems of benzene, toluene, and n-butylacetate:light petroleum (40° - 60°); 1:9. It was thereby distinguished from the corresponding hydrazones of acetaldehyde, acetone and methylethyl ketone. Subsequently a sample of the radioactive hydrazone was diluted with 200 mg of crystalline isobutyraldehyde-2,4-dinitrophenylhydrazone and recrystallised three times from a mixture of benzene and ethanol. After each crystallisation a sample of about 85 mg was counted at virtually infinite thickness with a thin window Geiger counter. The specific activity remained constant, confirming the identification of the radioactive hydrazone.

Further experiments with IV were carried out with a specially prepared sample. One batch of 40 flax seedlings was treated with 2 µmoles of L-valine-U-^{14}C containing 10 µC of radioactivity together with 20 µmoles of D,L-O-methylthreonine for 10 hours with continuous light. Additional water was added as required. The shoots were extracted twice with boiling 80% aqueous ethanol and the combined extracts reduced in volume prior to passing through a small column (4 cm x 0.3 cm) of Biodeminrolit. The solution together with washings was transferred to chromatography paper and purified by developing with BW and MAW.

In experiment S, compound IV was treated with 0.6M hydrochloric acid at 30°.

Portions were taken at intervals and added to an excess of sodium bicarbonate prior to drying in a vacuum to remove the volatile products of hydrolysis. Figure 20 records the non-volatile residue of radioactivity. After 18 hours a portion was chromatographed on paper to confirm that the small amount of non-volatile radioactivity remaining was IV. Under similar conditions there was negligible hydrolysis of linamarin indicating the relatively high sensitivity of IV to acid hydrolysis.

Both IV and linamarin with ^{14}C label were treated with hydrolytic enzymes in experiment T. Each compound was treated with 1.2 mg of β-glucosidase from sweet almonds or 50 µl of linamarase in a total of 250 µl pH 5.9, 0.04M phosphate buffer at 40°. Samples of each were taken at the beginning and after four hours and spotted on paper for chromatography with BW. The zones containing the remaining IV or linamarin were cut out and transferred to vials for scintillation counting in the presence of a toluene based scintillation fluid. After four hours only 26% of the IV and 70% of the linamarin remained in the β-glucosidase treatment. In contrast 95% of IV remained after treatment with linamarase while less than 1% of the linamarin was left. These results show that IV is strongly resistant to hydrolysis by linamarase but relatively susceptible to the action of the β-glucosidase from almonds. This confirms unpublished findings by Butler.

Further information on the structure of IV was obtained in experiment U by examining the volatile products of treatments of IV with the gas chromatograph and an attached proportional counter. Conditions for chromatography and subsequent counting were identical to those used in experiment S and illustrated in Figure 17.

In the first treatment illustrated in Figure 21, IV was held at 40° in 0.5M hydrochloric acid for 20 hours prior to extracting with a small volume of diethyl ether. The single peak of radioactivity obtained with a portion of this

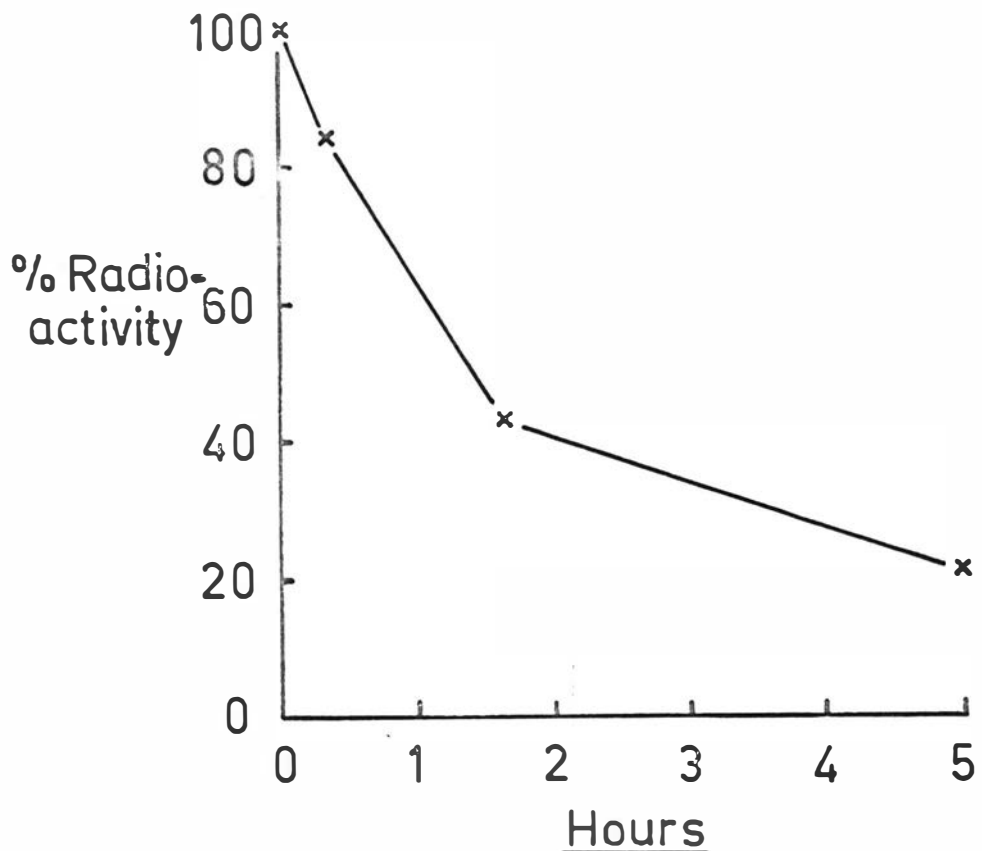


FIGURE 20 NON VOLATILE RADIOACTIVITY FROM COMPOUND IV AFTER TREATMENT WITH 0.6M HYDROCHLORIC ACID AT 30°.

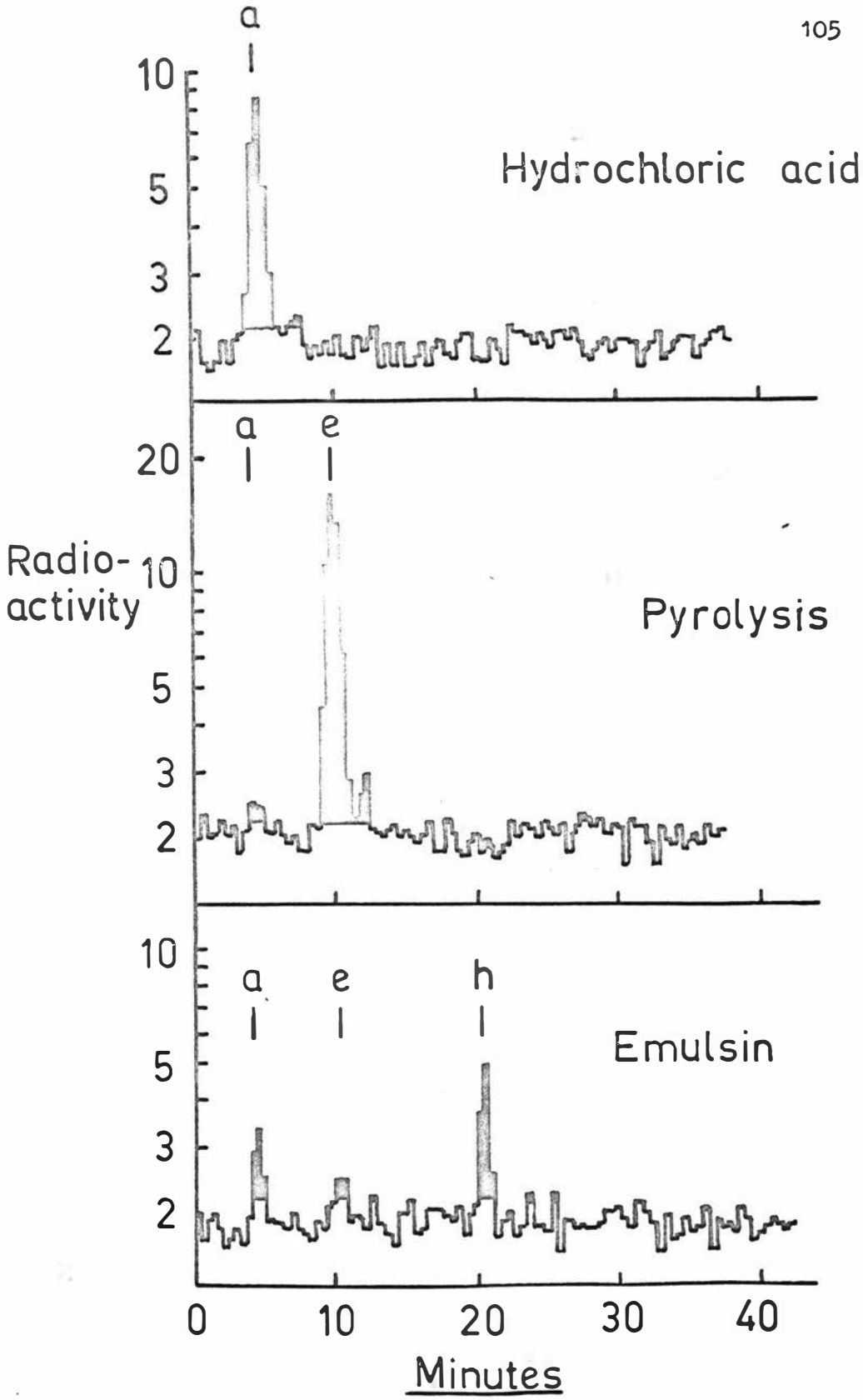


FIGURE 21 RADIOACTIVITY IN VOLATILE COMPOUNDS OBTAINED BY TREATMENTS OF COMPOUND IV.

ether extract had an elution time consistent with it being isobutyraldehyde, as was expected on the basis of experiment R.

The second treatment was an attempt to pyrolyse IV and find volatile decomposition products. A small volume of IV was dried on a Nichrome wire coil which was then inserted into the inlet of the gas chromatograph and heated by an electric current to a dull red temperature. The major peak of radioactivity had an elution time the same as isobutyronitrile. It was trapped and diluted with pure isobutyronitrile. From this, t-butylisobutyramide was prepared and recrystallised to a constant specific activity which indicated all the radioactivity in this peak could be attributed to isobutyronitrile. Another sample of IV was shown to be partially decomposed to isobutyronitrile at 160° on the Nichrome wire without electrical heating.

In the third treatment, IV was dissolved in 100 µl of pH 5.1, 0.05M phosphate buffer together with 2.5 mg of emulsin and held at 40° for 20 hours. The mixture was then extracted with a small volume of diethyl ether and this used for gas chromatography. The major radioactive peak had an elution time the same as for isobutyraldoxime and was trapped and treated with acidic 2,4-dinitrophenylhydrazine. The hydrazone preparation gave a peak of radioactivity with the R_f of isobutyraldehyde-2,4-dinitrophenylhydrazone upon thin layer chromatography with toluene as solvent. A smaller peak of volatile radioactivity had the elution time of isobutyraldehyde and could be derived from isobutyraldoxime.

These results indicate that isobutyraldoxime was at least part of the aglycone of IV. This was consistent with the finding of isobutyraldehyde upon acid hydrolysis as isobutyraldoxime is itself hydrolysed by acid. Further it is known that C-derivatives of oximes may be readily converted to nitriles. The simplest structure for IV in accord with these findings and those of Butler is isobutyraldoxime-C-glucoside as illustrated in Figure 22. Further studies are required to confirm this structure.

FIGURE 22 ISOBUTYRALDOXIME-O-GLUCOSIDE; A POSSIBLE STRUCTURE FOR COMPOUND IV.

3.4 Glucosinolate biosynthesis

In the preceding experiments it was shown that 2-oximino acids and aldoximes may be precursors of cyanogenic glycosides. In the following experiments these compounds were tested as precursors of glucosinolates.

Precursors of isopropylglucosinolate. In experiments V and W, L-valine-U-^{14}C, 2-oximinoisovaleric acid-U-^{14}C, and isobutyraldoxime-U-^{14}C were administered to scurvy grass seedlings. For experiment V the amino acid and the 2-oximino acid were dissolved in 200 µl of pH 7.4, 0.05M tris(hydroxymethyl)aminomethane buffer and administered to single seedlings which were then exposed to continuous light for 21 hours. In experiment W isobutyraldoxime-U-^{14}C or L-valine-U-^{14}C, each dissolved in 450 µl of water, were administered to groups of three plants which were enclosed in transparent 220 ml containers to reduce evaporative losses of the aldoxime. The plants were harvested after 48 hours including two 6 hour dark periods. In both cases water was added to the seedling roots as required.

Table XV gives the results of experiments V and W. The incorporation of L-valine-U-^{14}C into isopropylglucosinolate, while not particularly high, was significant. The radioautograph of the L-valine-U-^{14}C treatment chromatogram of experiment W reproduced in Figure 23, showed that most radioactivity remained in the valine pool with only small conversion to other compounds including leucine. No significant radioactivity was observed in other glucosinolate zones. Isopropylglucosinolate was identified by the formation of radioactive isopropylthiourea. This indicates that L-valine was a specific precursor of the isopropylglucosinolate aglycone.

Isobutyraldoxime-U-^{14}C was efficiently converted to isopropylglucosinolate which was the most strongly labelled compound as shown by a radioautograph in Figure 24. This result suggests isobutyraldoxime was an intermediate in isopropyglucosinolate biosynthesis. The incorporation from 2-oximinoisovaleric acid-U-^{14}C was low although radioautographs of thin layer separations showed

TABLE XV

COMPARISON OF PRECURSORS OF ISOPROPYLGLUCOSINOLATE

Expt.	Compound Administered	Amount (μmole)	S.A.[a]	% converted to Isopropylglucosinolate
V	L-Valine-U-^{14}C	1.0	2490	0.87[b]
	2-Oximinoisovaleric acid-U-^{14}C	1.2	1150	0.25[b]
W	L-Valine-U-^{14}C	4.0	622	0.70[b]
	L-Valine-U-^{14}C	1.0	2490	1.74[b]
	Isobutyraldoxime-U-^{14}C	3.8	390	15.9

a Specific activity in μC/mmole

b Corrected for an assumed loss of -^{14}COOH

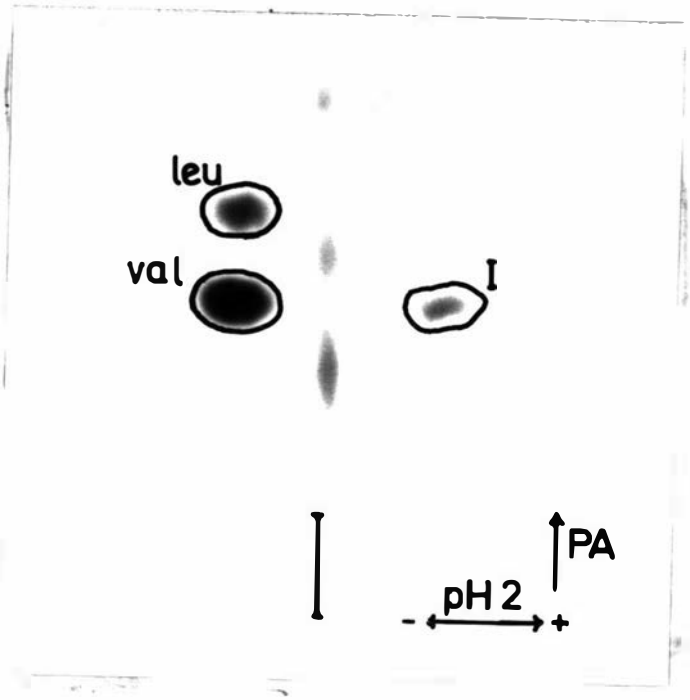


FIGURE 23 INCORPORATION OF RADIOACTIVITY FROM L-VALINE-U-^{14}C IN SCURVY GRASS. A RADIOAUTOGRAPH FOLLOWING THIN LAYER ELECTROPHORESIS AND CHROMATOGRAPHY. I IS ISOPROPYLGLUCOSINOLATE.

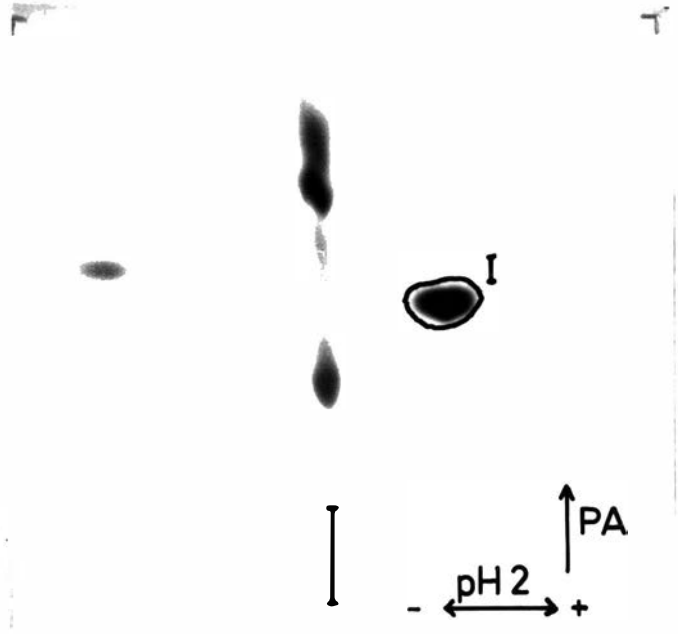


FIGURE 24. INCORPORATION OF RADIOACTIVITY FROM ISOBUTYRALDOXIME-U-^{14}C IN SCURVY GRASS. A RADIOAUTOGRAPH FOLLOWING THIN LAYER ELECTROPHORESIS AND CHROMATOGRAPHY. I IS ISOPROPYLGLUCOSINOLATE.

other compounds were labelled. Figure 25 is one such radioautograph which excludes the valine zone. Valine was the only cationic compound observed to be labelled in this treatment and it is possible that the small percentage conversion of the 2-oximinoisovaleric acid to isopropylglucosinolate may have involved valine as an intermediate. Further, the incorporation rate given in Table XV must be treated as a maximum value as the glucosinolate was not positively identified as containing the radioactivity in this treatment. Figure 25 shows the isopropylglucosinolate zone could not be clearly separated from a streak of other radioactive compounds. The pattern of spots on this radioautograph indicates decomposition of one or more labelled compounds may have occurred during electrophoresis and chromatography. It may be concluded that 2-oximinoisovaleric acid is probably not an intermediate in isopropylglucosinolate biosynthesis.

Precursors of benzylglucosinolate. In experiment X, L-phenylalanine-U-^{14}C and phenylacetaldoxime-U-^{14}C were administered in 0.25 ml of water to batches of 30 excised seedling shoots of garden cress. The aldoxime was first dissolved in 5 µl of ethanol. Assimilation and metabolism was for 48 hours of continuous light. The results are given in Table XVI.

The percentage of L-phenylalanine incorporated into benzylglucosinolate was significant and indicated this amino acid was a precursor of benzylglucosinolate. Radioautographs of the extract separated on thin layers by electrophoresis and chromatography showed benzylglucosinolate as a major labelled soluble product from L-phenylalanine-U-^{14}C. A few radioactive compounds, neutral at pH 2, were also observed although some radioactivity remained in the phenylalanine zone.

L-Phenylalanine had previously been shown to be a precursor of benzylglucosinolate in nasturtium (Tropaeolum majus L.)(Benn, 1962; Underhill and Chisholm, 1964).

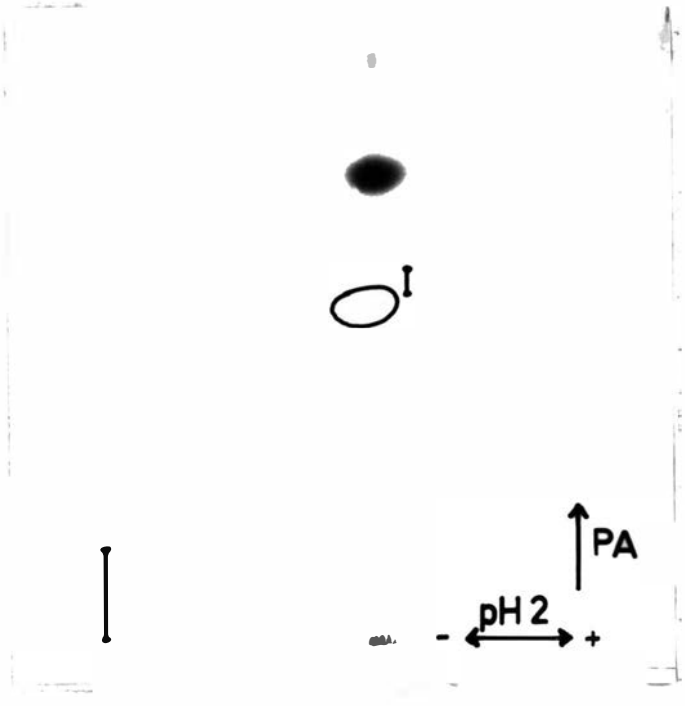


FIGURE 25 INCORPORATION OF RADIOACTIVITY FROM 2-OXIMINOISOVALERIC ACID-U-^{14}C IN SCURVY GRASS. A RADIOAUTOGRAPH FOLLOWING THIN LAYER ELECTROPHORESIS AND CHROMATOGRAPHY. I IS THE ISOPROPYLGLUCOSINOLATE ZONE.

TABLE XVI

PRECURSORS OF BENZYLGLUCOSINOLATE

Compound Administered			Products		
	Amount (μmoles)	S.A.[a]	% converted to glucosinolate	S.A.[a] of thiourea	Dilution of ^{14}C
L-Phenylalanine-U-^{14}C	1.1	44.0	4.3[b]	3.56	110[b]
Phenylacetaldoxime-U-^{14}C	1.2	23.1	26	1.22	19

a Specific activity as μC/mmole

b Corrected for an assumed loss of -$^{14}COOH$ from L-phenylalanine-U-^{14}C

The high incorporation of phenylacetaldoxime-U-^{14}C into benzylglucosinolate with comparatively low dilution showed this compound was an effective precursor. Again, radioautographs showed benzylglucosinolate to be a major labelled product. These results together with the results of experiments V and W indicate aldoximes are probably intermediates in the biosynthesis of structurally related glucosinolates.

CHAPTER IV

DISCUSSION

4.1 Glucosinolate biosynthesis

The results obtained from three experiments with two species of cruciferous plants showed that suitable aldoximes may be converted to glucosinolates to a considerably greater extent than corresponding amino acids. In view of the previous unsuccessful attempts to show similar incorporation for a range of other possible intermediates these results with aldoximes may be taken as good evidence for an intermediate role.

2-Oximinoisovaleric acid was not notably incorporated into isopropylglucosinolate. Underhill and Chisholm (1964) reported a similar low incorporation of an oximino acid into benzylglucosinolate with nasturtium shoots and suggested that the anti (HO—COOH) isomer which they administered was the incorrect isomer while the syn (HO—COOH) isomer might be an intermediate. The syn isomers have been reported for only a few 2-oximino acids (Ahmad and Spenser, 1964). Taking into account the facile isomerisation of syn (HO—COOH)-2-oximinophenylacetic acid (glyoxylic acid oxime) to the anti form, the greater ease of isomerisation of alkyl aldoximes compared with the benzaldoximes, and the effects of steric hindrance, it is possible that equilibration between the two isomers of many 2-oximino acids is rapid and favours the anti (HO—COOH) configuration. If this is so, then a search for syn (HO—COOH)-2-oximino acids suitable for administering to plants, may be futile.

More recent work by Underhill (1967) has confirmed that corresponding aldoximes are incorporated into benzylglucosinolate and phenylethylglucosinolate and it was found that accumulation of labelled phenylacetaldoxime could be induced by a "trapping" experiment with nasturtium. Kindl and Underhill (1968)

have shown that N-hydroxyphenylalanine is incorporated into benzylglucosinolate and they have described an enzyme system capable of forming the aldoxime from the N-hydroxyamino acid. Thus a sequence of two steps for the formation of aldoxime has been suggested which does not include 2-oximino acids.

The small incorporation of 2-oximino acids observed with isopropylglucosinolate in experiment V and with benzylglucosinolate by Underhill and Chisholm (1964) and Meakin (1965) may be attributed to a pathway involving prior conversion to the amino acid, either by way of a hypothetical hydrolysis of the oximino group to give a keto acid or by direct reduction of the oximino group to amine. An enzyme capable of this reduction has been reported by Omura et al., (1967) in higher plants. During the analysis of the 2-oximinoisovaleric acid treatment, a radioactive spot attributed to valine was noted which would be consistent with this suggestion.

Subsequent steps of glucosinolate biosynthesis have been studied in less detail. Meakin (1967) has claimed that 1-thioglucose may donate the isothiocyanate sulphur and that phenylacetothichydroxamic acid is not incorporated into benzylglucosinolate. More recently, Matsuo (1968) has cited unpublished results which showed that thioglucose (as sodium thioglucoside) was not a precursor of allylglucosinolate. Further studies may be required to find the immediate source of the isothiocyanate sulphur atom and to clarify this discrepancy.

The biosynthesis of the sulphate moiety may involve transfer of sulphate from adenosine-5'-phosphosulphate or 3'-phosphoadenosine-5'-phosphosulphate to an acceptor such as an aldoxime or thichydroximic acid glucoside by a sulphotransferase.

4.2 Cyanogenic glycoside biosynthesis

The experiments with linen flax shoots and peach and cherry laurel shoots have demonstrated that suitable aldoximes - isobutyraldoxime and phenylacetaldoxime respectively - may be converted to cyanogenic glucosides. Isobutyraldoxime

which was converted to linamarin with the C-N bond intact is probably normally formed in the linen flax in minute concentrations but accumulation of it can be induced by specific inhibitors of linamarin biosynthesis. It may be accepted on these results that aldoximes are intermediates in the biosynthesis of linamarin and prunasin and also, by analogy, of structurally related cyanogenic glycosides including lotaustralin, sambunigrin, amygdalin, vicianin, dhurrin and taxiphyllin.

Similarly, isobutyronitrile and phenylacetonitrile were incorporated into linamarin and prunasin respectively, and evidence demonstrating the ability of linen flax shoots to form isobutyronitrile was obtained from "trapping" experiments. However, the accumulation of isobutyronitrile could not be induced by known specific inhibitors of linamarin biosynthesis. These results indicate nitriles are also intermediates at a subsequent stage.

While neither isobutyronitrile nor isobutyraldoxime were incorporated to a significantly greater extent than L-valine during the experiments, both were incorporated to an extent sufficient to implicate them as intermediates. Factors which could work towards reduced incorporation of them were their volatility and possible toxic effect when administered in abnormally large doses. Both compounds are lipophilic and could well have a disruptive effect on cell membranes and organelles at these concentrations.

The relatively high and significant incorporation of 2-oximinoisovaleric acid and 2-oximino-3-phenylpropionic acid into linamarin and prunasin respectively is interesting. These compounds are not considered intermediates in the biosynthesis of structurally related glucosinolates although aldoximes, which are glucosinolate intermediates, could in theory be derived from them by decarboxylation. It is reasonable to suppose that the observed incorporation of 2-oximino acids was via the nitriles. The non-enzymic conversion of a range of 2-oximino acids to nitriles has been reported by Ahmad and Spenser (1961). In

experiment C, evidence was obtained for occurrence of this conversion in the flax seedlings but unpublished results obtained by Conn and Tapper failed to demonstrate an enzyme system in linen flax capable of accelerating the reaction. The status of 2-oximino acids as intermediates in cyanogenic glycoside biosynthesis must remain uncertain until evidence for a conversion to aldoximes is obtained or until the presence of enzymes for their formation and utilisation is demonstrated.

In the case of linen flax, good evidence was obtained to suggest that 2-hydroxyisobutyronitrile was also an intermediate although the pattern for incorporation from hydrogen cyanide was also obtained. Confirmatory evidence has come from experiments by Hahlbrock and Conn and forms part of a joint publication (Hahlbrock et al., 1968 - See Appendix 3).

Results for incorporation of D,L-mandelonitrile into prunasin with cherry laurel shoots were less definitive. A notable feature was the significant incorporation of radioactivity from hydrogen cyanide into prunasin. This was in apparent contradiction to the evidence for the nitrile moiety formation from phenylacetaldoxime and phenylacetonitrile. It may be reconciled by postulating that a reaction between benzaldehyde from the plant tissue and the administered hydrogen cyanide occurred under the particular experimental conditions. The mandelonitrile so formed could be converted to prunasin. A further notable feature of the treatment of cherry laurel with labelled hydrogen cyanide was the apparently low incorporation into asparagine and relatively high incorporation into a range of neutral or weakly acidic compounds. This may have arisen as a result of further metabolism of asparagine if it was the first major labelled compound. The problem deserves further attention.

It should be noted that the effect of varying the concentration of the administered compounds on the pattern of metabolism was not investigated. While significant concentration effects may occur it was considered that they would

probably be of a lower order of magnitude than the differences of incorporation rate accepted as evidence for or against a possible intermediate role.

The results obtained from the experiments with linen flax seedlings, peach shoots and cherry laurel shoots, together with the recent findings of Kindl and Underhill (1968), support a general pathway of cyanogenic glycoside biosynthesis involving a sequence of reactions from amino acids through aldoximes, nitriles and 2-hydroxynitriles as illustrated in Figure 26.

Some of the reactions postulated deserve further discussion. Experimental precedent for the first reaction (1) has come from studies by Stevens and Emery (1966) on the biosynthesis of the antibiotic, hadacidin. It was shown that this hydroxamic acid derivative was formed by N-hydroxyglycine and a formyl donor and that N-hydroxyglycine could be obtained from glycine.

Mention has already been made of the conversion of N-hydroxyphenylalanine to phenylacetaldoxime demonstrated by Kindl and Underhill (1968). This corresponds to reaction (2) of Figure 26. Molecular oxygen is probably required. An intermediate may intervene in reaction (3). There is no direct evidence to show that aldoximes can be converted to nitriles in dilute aqueous solution. However, under dehydrating conditions which permit the formation of intermediate O-acyl derivatives, many aldoximes are converted to nitriles. The biological equivalent of this type of mechanism could involve the formation of an intermediate with a suitable leaving group. One possibility would be the phosphorylation of the aldoxime by the action of a suitable kinase and a nucleoside-5'-triphosphate. The phosphorylated aldoxime could then be converted to nitrile as in Figure 27.

An alternative intermediate in the case of linamarin biosynthesis could be compound IV which may be isobutyraldoxime O-glucoside. Its formation from a nucloside-5'-diphospho-D-glucose would follow the usual glycosylation pattern and it could be enzymically converted to isobutyronitrile as illustrated also

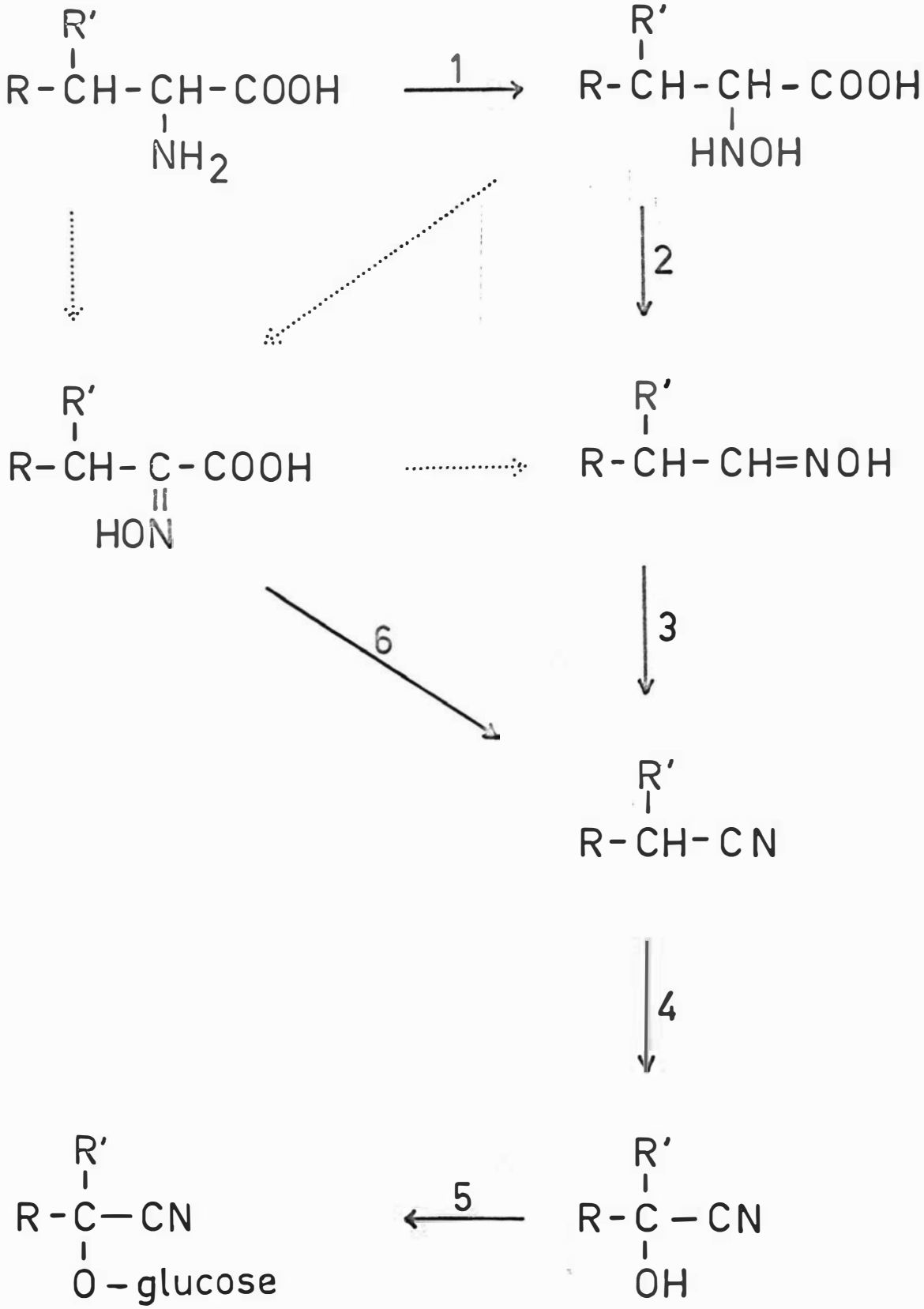
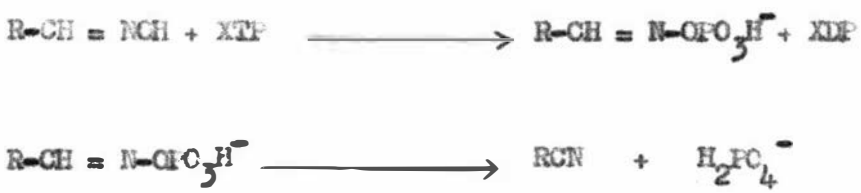


FIGURE 26 PROPOSED PATHWAY OF CYANOGENIC GLUCOSIDE BIOSYNTHESIS.



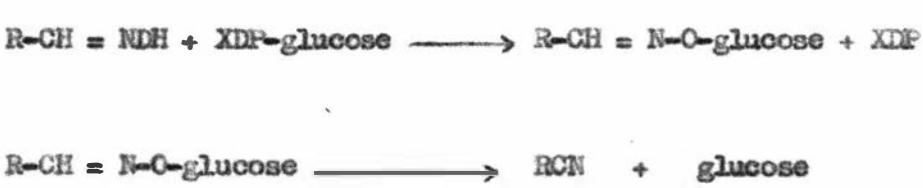


FIGURE 27 POSSIBLE STEPS BETWEEN ALDOXIMES AND NITRILES

in Figure 27.

It should be noted that Butler unsuccessfully attempted to show that IV could be converted to linamarin. It was possible that the administered IV was not transported to the normal site of synthesis of linamarin and therefore the status of IV as an intermediate must remain uncertain. The conditions required to induce accumulation of IV are of interest. 2-Methoxypropionaldoxime was the most effective inhibitor of linamarin biosynthesis and also induced the most of IV. This oxime or a derivative from it could well be a competitive inhibitor of the conversion of isobutyraldoxime to isobutyronitrile. D,L-O-Methylthreonine may have a specific inhibitory effect because of a prior conversion to D,L-2-methoxypropionaldoxime by some of the enzymes normally active in linamarin biosynthesis. The other compounds which Butler showed could induce a small accumulation of IV may also be competitive inhibitors. If compound IV is not an intermediate in linamarin biosynthesis, then it is presumably formed from unusually large pools of isobutyraldoxime which may accumulate in the presence of these specific inhibitors.

In the case of linamarin biosynthesis a conversion of isobutyronitrile to 2-hydroxyisobutyronitrile could have methacrylonitrile, formed by the action of a dehydrogenase, as an intermediate. Water could then be added across the double bond. The results from a single experiment where methacrylonitrile was administered along with labelled L-valine do not support this hypothesis but are not sufficiently rigorous to exclude all possibility of methacrylonitrile being an intermediate.

An alternative mechanism could involve direct oxidation with molecular oxygen. A simple unsaturated intermediate is not possible with the aromatic cyanogenic glycoside biosynthesis and the 2-hydroxyl group may be introduced in a reaction analogous to the mixed function oxidation of dopamine to noradrenaline.

The final step of biosynthesis of linamarin or prunasin and several

closely related cyanogenic glucosides is probably the transfer of glucose to a 2-hydroxynitrile from a nucleoside-5'-diphospho-D-glucose by a mechanism of the general type for formation of glycosidic bonds. Hahlbrook and Conn (1968) have purified an enzyme capable of forming linamarin from UDP-D-glucose and 2-hydroxyisobutyronitrile. The biosynthesis of the glycosides amygdalin and vicianin probably involves the transfer of a further sugar group to the corresponding mono-glucoside.

Although the evidence presented in this study supports the main pathway of cyanogenic glucoside biosynthesis shown in Figure 26, some other possible pathways have not been excluded. Mention has already been made of the possibility of 2-oximino acids being intermediates. While the results tend to exclude simple amides and hydroxamic acids as intermediates, 2-hydroxyaldoximes and corresponding glycosides cannot be excluded as an alternative pathway to simple nitriles and 2-hydroxynitriles. It was possible that the unidentified phenolic glucoside reported by Gander (1966), but about which doubt has since been expressed (Gander, personal communication), was a 2-(p-hydroxyphenyl)-2-(glucosyl)oxyacetaldoxime. This compound, depending on its stereo-chemistry, would yield dhurrin or taxiphyllin upon dehydration of the oxime group. Another structure, possibly consistent with the report by Gander, is p-glucosyloxyphenylacetaldoxime which could be an intermediate in dhurrin, taxiphyllin and p-glucosyloxymandelonitrile biosynthesis.

4.3 General Comment

Most of the studies, including this one, of cyanogenic glycoside or glucosinolate biosynthesis have centred on those compounds with a direct biogenic relationship to known amino acids. There are however a number of glucosinolates and a few cyanogenic glycosides for which there are no analogous amino acids known. The unusual structure of some of these compounds suggests that the plants in which they occur have novel pathways of metabolism and uncommon amino acids

on which there have not yet been any significant studies.

Also of interest is the relationship of the present investigation to the biosynthesis and metabolism of other natural products where nitrogen is not in its fully reduced state. A reference has already been made to hadacidin which is one of a class of substituted hydroxamic acids where the hydroxamyl moiety is probably derived from an N-hydroxyamine which in turn is derived by oxidation of an amine. N-Hydroxyamino acids may also be formed *in vitro* from hydroxylamine (Emery, 1963).

A restricted number of nitro compounds are known from higher plants. Some of these such as karakin and hiptagin are derivatives of 3-nitropropionic acid (Robinson, 1963). Hiptagin is reported to yield hydrogen cyanide following treatment with alkali (Carter, 1921). The nitro group appears to be derived from the amino group of aspartic acid (Shaw and McCloskey, 1967) and an aldoxime could be an intermediate. Similarly phenylacetaldoxime could be an intermediate in the biosynthesis of 1-nitro-2-phenylethane which is found in the bark oil of *Aniba canellila* (Robinson, 1963). Oxidation of an amine to a nitro group is also implied in the biosynthesis by microorganisms of chloramphenicol (McGrath *et al.*, 1968) and 2-nitroimidazole (Lancini *et al.*, 1966) although in these cases nitroso compounds could be intermediates in place of aldoximes.

It is possible that aldoximes are also intermediates in the biosynthesis of the interesting, but little studied, azoxy compounds. These include the glycosides macrozamin (Langley *et al.*, 1951) and cycasin (Nishida *et al.*, 1956) which give some hydrogen cyanide following alkaline degradation.

In contrast to cyanogenic glycosides, ricinine, which is a pyridine alkaloid, contains a nitrile moiety apparently derived from an amide by dehydration (Waller *et al.*, 1966). The formation of 3-cyanoalanine, 2-aminopropionitrile and a small group of related compounds from hydrogen cyanide has already been discussed, as has the formation of nitriles as products of glucosinolate decomposition.

There is a possibility for the biosynthesis of hydrogen cyanide by pathways other than decomposition of a cyanogenic glycoside in some microorganisms. Michaels et al., (1965) has shown hydrogen cyanide biosynthesis from glycine and suggested cyanoformic acid as an intermediate. In view of the comparative ease by which oximes may be converted to nitriles, formaldoxime and oximinoacetic acid (glyoxylic acid oxime) should also be investigated as intermediates.

It is notable that in all studies of these organic nitrogen compounds there has been no evidence to show free hydroxylamine or other similar oxidised forms of nitrogen as intermediates and therefore there appears to be no direct relationship with nitrogen assimilation, fixation or dissimilation.

BIBLIOGRAPHY

ABROL, Y. P. "Occurrence of Linamarin and Lotaustralin in Iceland Poppy (Papaver nudicaule)." Indian J. Chem. 4: 251-252, 1966.

ABROL, Y. P. and CONN, E. E. "Non-occurrence of Lotusin in Lotus arabicus L." Nature 206: 399, 1965.

ABROL, Y. P. and CONN, E. E. "Studies on Cyanide Metabolism in Lotus arabicus L. and Lotus tenuis L." Phytochemistry 5: 237-242, 1966.

ABROL, Y. P., CONN, E. E. and STOCKER, J. R. "Studies on the Identification, Biosynthesis and Metabolism of a Cyanogenic Glucoside in Nandina domestica Thunb." Phytochemistry 5: 1021-1027, 1966.

ADAMS, R. et al. "Synthesis of Heliotramide." Aust. J. Chem. 12: 706, 1959.

ADELBERG, E. A. "The Use of Metabolically Blocked Organisms for the Analysis of Biosynthetic Pathways." Bact. Reviews 17: 253-267, 1953.

AHMAD, A. and SPENSER, I. D. "The Conversion of α-Keto Acids and of α-Keto Acid Oximes to Nitriles in Aqueous Solution." Can. J. Chem. 39: 1340-1359, 1961.

ALDRIDGE, W. N. "A New Method for the Estimation of Micro Quantities of Cyanide and Thiocyanate." Analyst 69: 262-265, 1944.

ANDERSON, A. S. and MUIR, R. M. "Auxin Activity of Glucobrassicin." Physiol. Plant. 19: 1038-1048, 1966.

BACH, E. Dansk Botan. Arkiv. 16: 1-200, 1956. Cited by Ward, E.W.B. Can. J. Botany 42: 319-327, 1964.

BARBER, G. A. "Enzymic Glycosylation of Quercetin to Rutin." Biochemistry 1: 463-468, 1962.

BECKER, W. et al. Biochem. Zeit. 337: 156-166, 1963.

BENN, M. H. "Biosynthesis of Mustard Oils." Chem. Ind. p. 1907, 1962.

BENNETT, T. D. and TAPPER, B. A. "A Sensitive Method for Detecting Cyanoglycosides on Paper and Cellulose Thin Layers." J. Chromatog. 34: 428-429, 1968.

BEN-YEHOSHUA, S. and CONN, E. E. "Biosynthesis of Prunasin, the Cyanogenic Glucoside of Peach." Plant Physiol. 39: 331-333, 1964.

BIELESKI, R. L. and TURNER, N. A. "Separation and Estimation of Amino Acids in Crude Plant Extracts by Thin-layer Electrophoresis and Chromatography." Anal. Biochem. 17: 278, 1966.

BLEICHERT, E. F., NEISH, A. C. and TOWERS, G. H. N. "Biosynthesis of Taxiphyllin in Taxus." Biosynthesis of Aromatic Compounds. Proc. of the 2nd Meeting of the Fed. of European Biochemical Societies, edited by G. Billek. Oxford: Pergamon Press, 1966. pp 119-127.

BLUM, M. S. and WOODRING, J. P. "Secretion of Benzaldehyde and Hydrogen Cyanide by the Millipede Pachydesmus crassicutis (Wood)." Science 138: 512, 1962.

BLUMENTHAL-GOLDSCHMIDT, S., BUTLER, G. W. and CONN, E. E. "Incorporation of Hydrocyanic Acid Labelled with Carbon-14 into Asparagine in Seedlings." Nature 197: 718-719, 1963.

BRESON, J. L. and MITCHELL, T. J. "Improved Spraying Reagent for Detection of Sugars on Paper Chromatograms." Nature 167: 864, 1951.

BUTLER, G. W. "The Distribution of the Cyanoglucosides Linamarin and Lotaustralin in higher plants." Phytochemistry 4: 127-131, 1965.

BUTLER, G. W., BAILEY, R. W. and KENNEDY, L. D. "Studies on the Glucosidase Linamarase." Phytochemistry 4: 369-381, 1965.

BUTLER, G. W. and BUTLER, B. G. "Biosynthesis of Linamarin and Lotaustralin in White Clover." Nature 187: 780, 1960.

BUTLER, G. W. and CONN, E. E. "Biosynthesis of the Cyanogenic Glucosides Linamarin and Lotaustralin." J. Biol.Chem. 239: 1674-1679, 1964a.

BUTLER, G. W. and CONN, E. E. "The Metabolism of Cyanogenic Glycosides in Higher Plants." A paper presented to the Tenth International Botanical Congress, Edinburgh, 1964b.

CALDERON, P., PEDERSON, C. S. and MATTICK, L. R. "Nature of Myrosinase Enzyme." J. Agr. Food Chem. 14: 665, 1966.

CARTER, C. E. "Metabolism of purines and pyrimidines." Annual Review of Biochemistry 25: 123-146, 1956.

CHAKRAVARTI, R. N. Bull. Calcutta School Trop. Med. 3: 162, 1955. Cited by Fowden, L. in "Aspects of Amino Acid Metabolism in Plants." Annual Review of Plant Physiology 18: 85-106, 1967.

CHALLENGER, F. Aspects of the Organic Chemistry of Sulphur. London: Butterworths Publications Ltd., 1959.

CHISHOLM, M. D. and WETTER, L. R. "Biosynthesis of Mustard Oil Glucosides VII. Formation of Sinigrin in Horseradish from Homomethionine-2-^{14}C and Homoserine-2-^{14}C." Can. J. Biochem. 44: 1625-1632, 1966.

COBURN, R. A. and LONG, L. "Gynocardin". J. Org. Chem. 31: 4312-4315, 1966.

CONN, E. E. and AKAZAWA, T. "Biosynthesis of p-Hydroxybenzaldehyde." A paper presented at the Annual Meeting of the Fed. of American Societies for Experimental Biology, Philadelphia, 1958. Abstract in Fed. Proc. 17: 205, 1958.

COOP, I. E. "Cyanogenesis in White Clover (Trifolium repens L.)". New Zealand J. Sci. Tech. 22: 71B-83B, 1940.

CORI, G. T. and CORI, C. F. "The Kinetics of the Enzymatic Synthesis of Glycogen from Glucose-1-phosphate." J. Biol. Chem. 135: 733-756, 1940.

CORKILL, L. "Cyanogenesis in White Clover (Trifolium repens L.)." New Zealand J. Sci. Tech. 23: 178B-193B, 1942.

DILLEMAN, G. Encyclopedia of Plant Physiology. Edited by W. Ruhland. Vol. 8: 1050-1075. Berlin: Springer-Verlag, 1958.

DUNHILL, P. M. and FOWDEN, L. "Enzymatic Formation of β-Cyanoalanine from Cyanide by Escherichia coli Extracts." Nature 208: 1206-1207, 1965.

DUNSTAN, and HENRY. Phil. Trans. 12B: 515, 1901. Cited by Robinson, M. E. "Cyanogenesis in Plants." Biological Reviews 5: 126-141, 1930.

DUTTON, G. J. and STOREY, I.D.E. "Glucuronide Synthesis in Liver Homogenates." Biochem. J. 48: XXIX, 1951.

EDMAN, P. Acta Chem. Scand. 4: 283, 1950.

EMERY, T. F. "Aspartase-Catalyzed Synthesis of N-Hydroxyaspartic Acid." Biochem. 2: 1041-1045, 1963.

ETTLINGER, M. G. et al. "Vitamin C as a Coenzyme: The Hydrolysis of Mustard Oil Glucosides." Proc. Nat. Acad. Sci. 47: 1875-1880, 1961.

ETTLINGER, M. G. and DuTEC, G. P. Studies of Mustard Oil Glucosides (I). Contract DA 19-129-QM-1059, Project 7-84-06-032, William M. Rice University, Houston, Texas, 1961.

ETTLINGER, M. G. and LUNDEEN, A. J. "The structures of Sinigrin and Sinalbin; An Enzymatic Rearrangement." J. Am. Chem. Soc. 78: 4172-4173, 1956.

ETTLINGER, M. G. and LUNDEEN, A. J. "First Synthesis of a Mustard Oil Glucoside; The Enzymatic Lossen Rearrangement." J. Am. Chem. Soc. 79: 1764, 1957.

ETTLINGER, M. G. and THOMPSON, C. P. Studies of Mustard Oil Glucosides (II). Contract DA 19-129-QM-1689, Project 7-99-01-001, William M. Rice University, Houston, Texas, 1962.

FEIGL, F. Spot Tests in Organic Analysis. Fifth edition. Amsterdam: Elsevier Publishing Co., 1956. p. 88.

FLOSS, H. G., HADWIGER, L. and CONN, E.E. "Enzymatic Formation of β-Cyanoalanine from Cyanide." Nature 208: 1207-1208, 1965.

FOWDEN, L. "Amino Acid Biosynthesis." Biosynthetic Pathways in Higher Plants. Edited by J. B. Pridham and T. Swain. London: Academic Press Inc., 1965. Pp 73-100.

GADAMER, J. Ber. dtsch. chem. Ges. 30: 2322-2330, 1897. Cited by Challenger, F. Aspects of the Organic Chemistry of Sulphur. London: Butterworths Publications Ltd., 1959.

GAINES, R. D. and GOERING, K. J. "Myrosinase II. The Specificity of the Myrosinase System." Arch. Biochem. Biophys. 96: 13-19, 1962.

GANDER, J. E. "In Vivo Biosynthesis of Glycosidic Cyanide in Sorghum." Fed. Proc. 17: 226, 1958.

GANDER, J. E. "On the Biosynthesis of p-Hydroxymandelonitrile-β-glucoside in Sorghum." Fed. Proc. 18: 232, 1959.

GANDER, J. E. "Incorporation of ^{14}C into p-Hydroxymandelonitrile-β-glucoside and other Phenolic Substances in Sorghum Seedlings." J. Biol. Chem. 237: 3229-3232, 1962.

GANDER, J. E. "Evidence for Two Phenolic Glucosides Derived from L-Tyrosine in Sorghum Seedlings." Phytochemistry 5: 125-131, 1966.

GESSNER, T. and ACARA, M. "Metabolism of Thiols S-Glucosylation." J. Biol. Chem. 243: 3142-3147, 1968.

GMELIN, R. and VIRTANEN, A. I. "Glucobrassicin, the Precursor of 3-Indolylacetonitrile, Ascorbigen, and SCN^- in Brassica oleracea species." S. Kemistilehti B 34: 15, 1961.

GOKSOYR, J. "Chemical and Fungicidal Reactions of 3,5-Dimethyltetrahydro-1,3,5-thiadiazin-2-thione (3,5-D). A comparison with Sodium N-methyl Dithiocarbamate and Methyl Isothiocyanate." Acta Chem. Scand. 18: 1341-1352, 1964.

GOODMAN, I. et al. "A Mammalian Thioglycosidase." Science 130: 450-451, 1959.

GORTER, K. Chem. Abstr. 15: 1299, 1921.

GUIGNARD, L. J. Botanique 4: 385, 1890. Cited by Kjaer, A. "Naturally Derived Isothiocyanates (Mustard Oils) and their Parent Glucosides." Fort. Chem. Organ. Naturstoff. 18: 122-169, 1960.

GUILBAULT, G. G. and KRAMER, D. N. "Ultra Sensitive Specific Method for Cyanide using p-Nitrobenzaldehyde and o-Dinitrobenzene." Anal. Chem. 38: 834, 1966.

HAHLBROCK, K. et al. "The Conversion of Nitriles and ∝-Hydroxynitriles to Cyanogenic Glucosides in Flax Seedlings and Cherry Laurel Leaves." Fed. Proc. 27: 593, 1968. and Arch. Biochem. Biophys. 125: 1013, 1968.

HAHLBROCK, K. and CONN, E. E. "Biosynthesis of Cyanogenic Glucosides: Partial Purification and Properties of UDP-Glucose:Cyanohydrin β-Glucosyltransferase." A Paper presented to the American Society of Plant Physiologists, Logan, Utah, 1968.

HAISMAN, D. R. and KNIGHT, D. J. "The Enzymic Hydrolysis of Amygdalin." Biochem. J. 103: 528-534, 1967.

HASSID, W. Z. "Transformation of Sugars in Plants." Annual Review of Plant Physiology 18: 253-280, 1967.

HEGNAUER, R. Pharmaceutisch Weekblad 94: 248-262, 1959.

HEILBRON, I. et al. Dictionary of Organic Compounds. 4th Edition. London: Eyre and Spottiswoode (Publishers) Ltd., 1965.

HELMRICH, VON B. and KLEINSCHMIDT, T. Hoppe-Seyler's Z. Physiol. Chem. 349: 25-28, 1968.

HENDRICKSON, H. R. "The β-Cyanoalanine Synthase of Blue Lupine." Fed. Proc. 27: 593, 1968.

HERISSEY. Compt. rend. 141: 959, 1905. Cited by Caldwell, R. J. and Courtauld, S. L. "Mandelonitrile Glucosides. Prulaurasin." J. Chem. Soc. 91: 671-676, 1907.

HOVANITZ, W. and CHANG, V.C.S. "Comparison of the Selective Effect of Two Mustard Oils and their Glucosides to Pieris larvae." J. Res. Lepidoptera 2: 281-288, 1963.

HOWARD, G. A. and GAINES, R. D. "Glucosyl transfer by β-thioglucosidase." Phytochemistry 7: 585-588, 1968.

JONES, D. A. "On the Polymorphism of Cyanogenesis in Lotus corniculatus." Can. J. Genet. Cytol. 8: 556-567, 1966.

JONES, D. A., PARSONS, J. and ROTHSCHILD, M. "Release of Hydrocyanic Acid from the Crushed Tissues of all Stages in the Life-cycle of Species in the Zygaeninae (Lepidoptera)." Nature 193: 52, 1962.

JOSEFSSON, E. "Distribution of Thioglucosides in Different Parts of Brassica Plants." Phytochemistry 6: 1617-1627, 1967.

KAWAKISHI, S. et al. "Studies on the Decomposition of Sinalbin." Agr. Biol. Chem. 31: 830, 1967.

KINDL, H. Monatshefte fur Chemie 96: 527-532, 1965.

KINDL, H. and UNDERHILL, E. W. "Biosynthesis of Mustard Oil Glucosides: N-Hydroxyphenylalanine, a Precursor of Glucotropaeolin and a substrate for the Enzymatic and Nonenzymatic Formation of Phenylacetaldehyde Oxime." Phytochemistry 7: 745-756, 1968.

KJAER, A. "Naturally Derived Isothiocyanates (Mustard Oils) and their Parent Glucosides." Fort. Chem. Organ. Naturstoff. 18: 122-169, 1960.

KJAER, A. "Isothiocyanates of Natural Derivation." Pure and Applied Chemistry 7: 229-245, 1963.

KJAER, A. "The Distribution of Sulphur Compounds." Comparative Phytochemistry. Edited by T. Swain. London: Academic Press Inc., 1966. Pp 187-194.

KJAER, A. and CONTI, J. "Isothiocyanates V: The Occurrence of Isopropyl Isothiocyanate in Seeds and Fresh Plants of Various Cruciferae." Acta Chem. Scand. 7: 1011-1012, 1953.

KJAER, A. and RUBINSTEIN, K. "Paper Chromatography of Thioureas." Acta Chem. Scand. 7: 528-536, 1953.

KOJIMA, M. and TAMIYA, K. "The Effect of L-Ascorbic Acid on Myrosinase Activity." J. Vitaminol. 10: 44-54, 1964.

KOUKOL, J., MILJANICH, P. and CONN, E. E. "The Metabolism of Aromatic Compounds in Higher Plants VI: Studies on the Biosynthesis of Dhurrin, the Cyanogenic Glucoside of Sorghum vulgare." J. Biol. Chem. 237: 3223-3228, 1962.

KUTACEK, L., BULGAKOV, R. and CHLISTILOVA, K. "On the Auxin Activity of Glucobrassicin in Biological Tests." Biologia Plantarum 8: 252-255, 1966.

KUTACEK, L., SMALCNY, J. and CHLISTILOVA, K. Experientia 22: 24-25, 1966. "The Biosynthetic Incorporation of External $^{35}SO_2$ in Glucobrassicin." Biological Abstracts 48: No. 19627, 1967.

LANCINI, G. C. et al. "Origin of the Nitro Group of Azomycin." Biochem. Biophys. Acta 130: 37, 1966.

LANGLEY, B. W., LYTHGOE, B. and RIGGS, N. V. "Macrozamin, Part III: The Aliphatic Azoxy Structure of the Aglycone Part." J. Chem. Soc. p.2309, 1951.

LARSEN, P. O. "Occurrence of p-Hydroxybenzylamine in White Mustard (Sinapis alba L.)." Biochem. Biophys. Acta 107: 134-136, 1965.

LEBEAU, J. B. and DICKSON, J. G. "Physiology and Nature of Disease Development in Winter Crown Rot of Alfalfa. Phytopathology 45: 667-673, 1955.

LIBBY, P. Unpublished data, 1962. Cited by Uribe, E. G. "A study on the Biosynthesis of Dhurrin in Sorghum vulgare." Ph.D. Thesis, University of California, Davis, 1965.

LICHTENSTEIN, E. P. "Insecticides Occurring Naturally in Crops." Natural Pest Control Agents. Edited by D. G. Crosby, Washington: American Chemical Society, 1966. p. 34.

McGRATH, R. et al. "Biosynthesis of Chloramphenicol III. Phenylpropanoid Intermediates." Can. J. Biochem. 46: 587, 1968.

McILROY, R. J. The Plant Glycosides. London: Edward Arnold and Co., 1951.

MATSUO, M. "Biosynthesis of Sinigrin VII. Incorporation of 4-Methylthiobutyraldoxime-1-^{14}C,^{15}N into Sinigrin." Tetrahedron Letters Pp 4101-4104, 1968.

MATSUO, M. and YAMAZAKI, M. "Biosynthesis of Sinigrin, III." Biochem. Biophys. Res. Comm. 25: 269, 1966.

MAZELIS, M. and INGRAHAM, L. L. "The Pyridoxal Phosphate-Dependent Oxidative Decarboxylation of Methionine by Peroxidase." J. Biol. Chem. 237: 109-112, 1962.

MEAKIN, D. "Studies of Mustard Oil Glucosides." Ph.D. Thesis, University of Alberta at Calgary, 1965.

MEAKIN, D. "The Biosynthesis of the Thioglucoside Moiety of Benzyl Glucosinolate." Experientia 23: 174-175, 1967.

MEISTER, A. "Enzymatic Preparation of α-Keto Acids." J. Biol. Chem. 197: 309-317, 1952.

MENTZER, C., FAVRE-BONVIN, J. and MASSIAS, M. Bull. Soc. Chimie Biol. 45: 745-760, 1963.

MICHAELS, R., HANKE, L. V. and CORPE, W. A. "Cyanide Formation from Glycine by Nonproliferating Cells of Chromobacterium violaceum." Arch. Biochem. Biophys. 111: 121-125, 1965.

MILLER, L. P. "Induced Formation of a β-glucoside in the Radish." Contr. Boyce Thompson Inst. 12: 359, 1942.

MOORE, M. L. and CROSSLEY, F. S. "Methyl isothiocyanate." Organic Syntheses. Coll. Vol. 3. Edited by E. C. Horning, New York: John Wiley & Sons, Inc. 1955.

MURASHIGE, T. and SKOOG, F. "A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures." Physiol. Plant. 15: 473, 1962.

NAGASHIMA, Z. and UCHIYAMA, M. "Possibility that Myrosinase is a Single Enzyme and Mechanism of Decomposition of Mustard Oil Glucoside by Myrosinase." Bull. Agric. Chem. Soc. 23: 555, 1959.

NARTEY, F. "Studies on Cassava, Manihot utilissima Pohl." Phytochemistry 7: 1307-1312, 1968.

NEISH, A. C. "Coumarins, Phenylpropanes and Lignin." Plant Biochemistry. Edited by J. Bonner and J. E. Varner, New York: Academic Press Inc., 1965 p. 601.

NISHIDA, K., KOBAYASHI, A. and NAGAHAMA, T. "Cycasin, a Toxic Glycoside of Cycas revoluta." Chem. Abstr. 50: 13756, 1956.

NOSSAL, P. M. "A Mechanical Cell Disintegrator." Aust. J. Exp. Biology 31: 583-589, 1953.

OGINSKY, E. L., STEIN, A. E. and GREER, M. A. "Myrosinase Activity in Bacteria as Demonstrated by the Conversion of Progoitrin to Goitrin." Proc. Soc. Exp. Biol. Med. 119: 360-364, 1965.

OKURA, H., OSAJIMA, Y. and TSUTSUMI, M. "Proof of Existence of Oxidase Activity in Leaf of Higher Plants." Enzymologia 32: 135, 1967.

PALLARES, E. S. "Note on the Poison Produced by the Polydesmus (Fontaria) Vicinus, Lin." Arch. Biochem. 9: 105, 1946.

PHILLIPS. Annals New York Acad. Sciences 70: 825, 1958.

PLAUT, H. and RITTER, J. J. "A New Reaction of Nitriles VI: Unsaturated Amides." J. Am. Chem. Soc. 73: 4076-4077, 1951.

PLOUVIER, V. Compt. rend. 200: 1985, 1935. Cited by Trim, A. R. "Glycosides as a General Group." In Modern Methods of Plant Analysis. Edited by K. Pasch and M. V. Tracey, Vol. 2, p. 305. Berlin: Springer-Verlag, 1955.

REESE, E. T., CLARK, R. C. and MANDELS, M. "A Thioglucosidase in Fungi." Arch. Biochem. Biophys. 75: 228-242, 1958.

RESSLER, C., GIZA, Y. and NIGAM, S. N. "Biosynthesis and Metabolism in Species of Vetch and Lathyrus of γ-Glutamyl-β-cyanoalanine: Relation to the Biosynthesis of Asparagine. J. Am. Chem. Soc. 85: 2874-2875, 1963.

ROBINSON, M. E. "Cyanogenesis in Plants." Biological Reviews 5: 126-141, 1930.

ROBINSON, T. The Organic Constituents of Higher Plants. Minneapolis: Burgess Publishing Co., 1963.

SANDBERG, M. and HOLLY, O. M. "Note on Myrosin." J. Biol. Chem. 96: 443, 1932.

SCHNEIDER, W., FISCHER, H. and SPECHT, W. Ber. deutsch. Chem. Ges. 63: 2787, 1930. Cited by Waser, J. and Watson, W. H. "Crystal Structure of Sinigrin." Nature 198: 1297-1298, 1963.

SCHRAM, E. Organic Scintillation Detectors. Elsevier Publishing Co., 1963.

SCHRAUDOLF, H. and BERGMANN, F. "Metabolism of Indole derivatives in Sinapis alba L." Planta 67: 75-79, 1965.

SEDLAK, J. et al. Biologia (Bratislava) 18: 210-220, 1963.

SEELY, M. K., CRIDDLE, R. S. and CONN, E. E. "The Metabolism of Aromatic Compounds in Higher Plants: VIII On the Requirement of Hydroxynitrile Lyase for Flavin." J. Biol. Chem. 241: 4457-4462, 1966.

SHAW, P. D. and McCLOSKEY, J. A. "Biosynthesis of Nitro Compounds II: Studies on Potential Precursors for the Nitro Group of β-Nitropropionic Acid." Biochem. 6: 2247-2253, 1967.

SIDGWICK, N. V. The Organic Chemistry of Nitrogen. Third edition revised by Millar, I. T. and Springhall, H. D. Oxford: Clarendon Press, 1966. p. 314.

SOBEL, A. E., HIRSCHMAN, A. and BERMAN, L. "A Convenient Microtitration Method for the Estimation of Amino Acids." J. Biol. Chem. 161: 99-103, 1945.

SPRINSON, D. B. and RITTENBERG, D. "The Rate of Utilization of Ammonia for Protein Synthesis." J. Biol. Chem. 180: 707-714, 1949.

STEVENS, R. L. and EMERY, T. F. "The Biosynthesis of Hadacidin." Biochem. 5: 74-81, 1966.

STEVENS, D. L. and STROBEL, G. A. "Origin of Cyanide in Cultures of a Psychrophilic Basidiomycete." J. Bact. 95: 1094-1102, 1968.

STROBEL, G. A. "The Fixation of Hydrocyanic acid by a Psychrophilic Basidiomycete." J. Biol. Chem. 241: 2618-2621, 1966.

STROBEL, G. A. "4-Amino-4-cyanobutyric acid as an Intermediate in Glutamate Biosynthesis." J. Biol. Chem. 242: 3265-3269, 1967.

SWAIN, T. "Methods used in the Study of Biosynthesis." Biosynthetic Pathways in Higher Plants. Edited by J. B. Pridham and T. Swain. London: Academic Press Inc., 1965. Pp 9-36.

TAPPER, B. A. and BUTLER, G. W. "Conversion of Oximes to Mustard Oil Glucosides (Glucosinolates)." Arch. Biochem. Biophys. 120: 719-721, 1967.

THOMPSON, K. N. Abstract of Thesis, Diss. Abstr. B27: 1791, 1966.

TRAYNIER, R.M.M. "Chemostimulation of Oviposition by the Cabbage Root Fly Erioischia brassicae (Bouche)." Nature 207: 218-219, 1965.

TOWERS, G.H.N., McINNES, A. G. and NEISH, A. C. "The Absolute Configurations of the Phenolic Cyanogenic Glucosides Taxiphyllin and Dhurrin." Tetrahedron 20: 71-77, 1964.

TSCHIERSCH, B. "Metabolism of Hydrocyanic Acid." Phytochemistry 3: 365-367, 1964.

TSUKUC, I. and HATA, T. "Studies on the Myrosinase in Mustard Seed." Agr. Biol. Chem. 31: 27, 1967.

UNDERHILL, E. W. "Biosynthesis of Mustard Oil Glucosides V: Formation of Gluconasturtiin from L-γ-Phenylbutyrine-^{14}C-^{15}N in Water Cress." Can. J. Biochem. 43: 179-187, 1965.

UNDERHILL, E. W. "Biosynthesis of Mustard Oil Glucosides: Conversion of Phenylacetaldehyde Oxime and 3-Phenylpropionaldehyde Oxime to Glucotropaeolin and Gluconasturtiin." European J. Biochem. 2: 61-63, 1967.

UNDERHILL, E. W. and CHISHOLM, M. D. "Biosynthesis of Mustard Oil Glycosides III: Formation of Glucotropaeolin from L-Phenylalanine-^{14}C-^{15}N." Biochem. Biophys. Res. Comm. 14: 425, 1964.

UNDERHILL, E. W., WATKIN, J. E. and NEISH, A. C. "Biosynthesis of Quercetin in Buckwheat, I." Can. J. Biochem. Physiol. 35: 219, 1957.

URIBE, E. G. "A Study on the Biosynthesis of Dhurrin in Sorghum vulgare." Ph.D. Thesis, University of California, Davis, 1965.

URIBE, E. G. and CONN, E. E. "The metabolism of Aromatic Compounds in Higher Plants VII: The Origin of the Nitrile Nitrogen Atom of Dhurrin (β-D-Glucopyranosyloxy-L-p-hydroxymandelonitrile)." J. Biol. Chem. 241: 92-94, 1966.

VIRTANEN, A. I. "Some Organic Sulphur Compounds in Vegetables and Fodder Plants and their Significance in Human Nutrition." Angew Chemie (Int. Ed.) 1: 299, 1962.

VIRTANEN, A. I. and SAARIVIRTA, M. "The Formation of Benzyl Nitrile, Benzyl Isothiocyanate and Benzyl Thiocyanate in the Crushed Seeds of Lepidium sativum." S. Kemistilehti B 35: 248, 1962.

VOGEL, A. I. Practical Organic Chemistry. London: Longmans, Green and Co., 1948.

WAGNER, H. "Flavonoid C-Glycosides." Comparative Phytochemistry. Edited by T. Swain. London: Academic Press, Inc., 1966. Pp 309-320.

WALLER, G. R. et al. "The Pyridine Nucleotide Cycle and its Role in the Biosynthesis of Ricinine by Ricinus communis L." J. Biol. Chem. 241: 4411-4418, 1966.

WASER, J. and WATSON, W. H. "Crystal Structure of Sinigrin." Nature 198: 1297, 1963.

WATKIN, J. E. and NEISH, A. C. "Biosynthesis of Quercetin in Buckwheat, III." Can. J. Biochem. Physiol. 38: 559, 1960.

WENSLER, R.J.D. "Mode of Host Selection by an Aphid." Nature 195: 830-831, 1962.

WEST, H. D. and CARTER, H. E. "Synthesis of α-Amino-β-hydroxy-n-butyric acids." J. Biol. Chem. 119: 109-119, 1937.

WEST, H. D., KRUMEL, G. T. and CARTER, H. E. "Synthesis of α-amino-β-hydroxy-butyric acids." J. Biol.Chem. 122: 605-609, 1937.

WETTER, L. "Biosynthesis of Mustard Oil Glucosides II: The Administration of Sulphur-35 Compounds to Horseradish Leaves." Phytochemistry 3: 57, 1964.

YAMAHA, T. and CARDINI, C. E. "The Biosynthesis of Plant Glycosides. II Gentiobiosides." Arch. Biochem. Biophys. 86: 133-137, 1960.