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GLUCOSINOLATES

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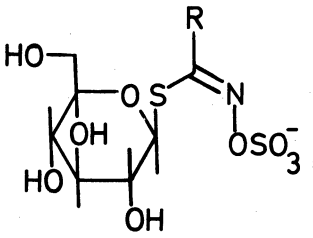
Abstract - The catabolism of glucosinolates is reviewed, with particular attention to the formation of "abnormal" products (thiocyanates, and cyano-epithioalkanes). The biological properties of the glucosinolates and catabolites are briefly surveyed.

INTRODUCTION

Some twenty years have elapsed since Ettlinger and Lundeen proposed 1 as the correct structure for glucosinolate anions.



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Since passing that milestone there have been major achievements in identifying glucosinolates (the list of which now exceeds 70), and in mapping the biosynthetic pathways at the substrate level. There are recent and authoritative reviews of these advances (1) and I propose to say no more about them here. There has also been a fresh discussion of the value of glucosinolates in plant systematics (2), and that too I shall leave aside. Instead I have chosen to concentrate upon the catabolism of glucosinolates, and their biological properties.

GLUCOSINOLATE CATABOLISM

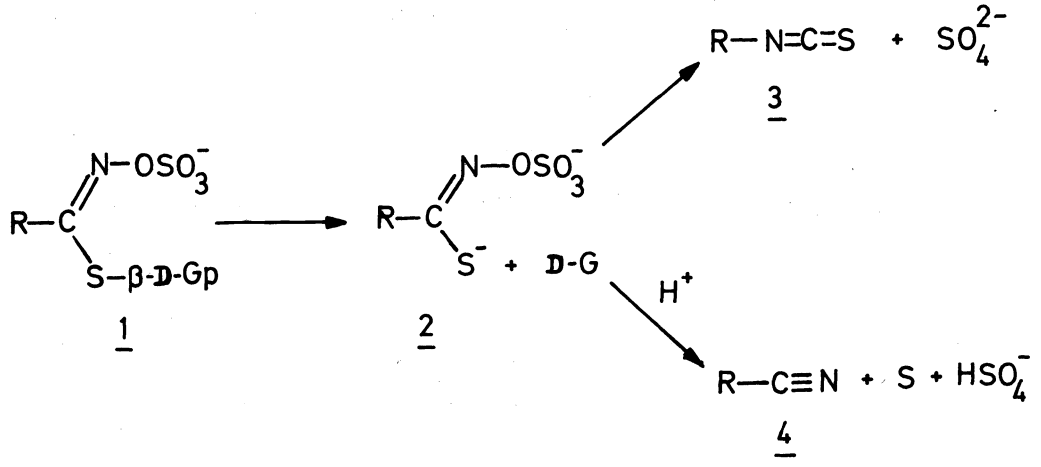
"Normal Catabolism".

When plant tissues containing glucosinolates are crushed there is usually formation of isothiocyanates ("mustard oils") (3) corresponding to the aglucone (2). (It was the early recognition of this "normal" catabolism which resulted in the old name "mustard oil glucosides" for the glucosinolates).

The isothiocyanate-forming enzymes are known as myrosinases and, after some controversy, it has been established that their role is to function as thioglucoside glucohydrolases (E. C. 3. 2. 3. 1) remarkably catholic in their acceptance of 1 with a wide variety of R sub-

stituents. Björkman (3) has provided a recent review of the properties of plant myrosinases (fungal-, bacterial-, mamalian- and aphid-myrosinases are also known (3)). Various isozymes have been isolated, all are glycoproteins. Some show a remarkable enhancement in activity upon the addition of ascorbate (apparently allosteric activation).

The corresponding nitriles 4 are often also formed in greater or lesser amount, at the expense of the isothiocyanate. Low pH favours nitrile production.



Scheme I

"Normal Catabolism" (after Miller, 1965)

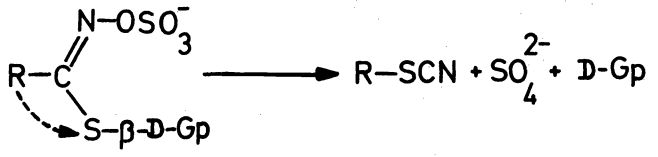
Using allylglucosinolate, Ettlinger's student Miller has demonstrated that the aglucone (2, R = allyl), generated chemically or by myrosinase action, decomposes by two competing non-enzymatic pathways: a proton independent isothiocyanate-forming one; and a proton-dependent nitrile forming one (Scheme I, R = allyl) (4).

Nitrile formation could also be promoted by some metal cations, ferrous ion being particularly effective (4). This observation may account for some of the high nitrile yields obtained from plant and seed preparations under pH conditions expected to favour isothiocyanate formation; frequent claims that nitrile formation can be under enzymatic control have also been made, although the particular systems involved are almost invariably ones in which "abnormal" catabolism is also occurring (see below).

"Abnormal" Catabolism. In contrast with the normal pathway, there are two other, "abnormal", catabolic transformations of glucosinolates, about which much less is known.

Catabolism to Organic Thiocyanates. In 1846 Pless observed that seeds of Thlaspi arvense L. gave a "garlic oil" as well as a "mustard oil" when crushed, but he misidentified the former as diallyl sulfide and over a century elapsed before Gmelin and Virtanen showed that it was in fact allyl thiocyanate (5). They also discovered that benzylglucosinolate was converted into benzyl thiocyanate (and benzyl cyanide, and isothiocyanate) by crushed seeds or fresh plants of Lepidium ruderale L. and seed, but not plant extracts (6), of L. sativum L. A third example was provided quite recently by Schlüter and Gmelin (7) who found that extracts of fresh Eruca sativa L. plants produced 4-methylthiobutyl thiocyanate from 4-methylthiobutylglucosinolate, while the seeds gave the "normal" isothiocyanate, Most recently Cole (8) has extended the list of plant species which yield benzyl, and 4-methylthiobutyl thiocyanates, but no new thiocyanates were reported.

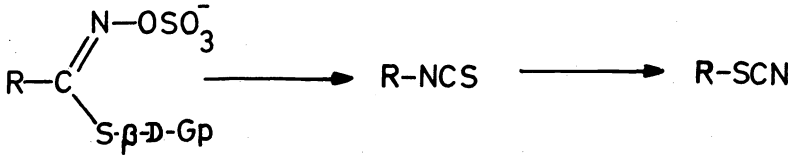
Gmelin and Virtanen first suggested that the thiocyanate-forming reaction involved an enzyme mediated rearrangement (Scheme II) (5).



Scheme II

Thiocyanate Formation (Gmelin and Virtanen, 1959)

Subsequently Virtanen and Saarivirta proposed that an isomerase acted on a first-formed isothiocyanate, i. e. the thiocyanates were visualized as being further (enzymatic) transformation products of the "normal" isothiocyanate catabolites (Scheme III) (9).



Scheme III

Thiocyanate Formation (Virtanen and Saarivirta, 1962)

This latter proposal was based in large part upon a product analysis as a function of time, which revealed a transitory early maximum in isothiocyanate production which was thought to correlate with a slower building-up of thiocyanate. Although benzyl isothiocyanate was not isomerised by these thiocyanate-forming systems the failure was ascribed to the low solubility of the isothiocyanate.

More recently Miller has demonstrated that allyl isothiocyanate dissolved in water is not isomerised by T. arvense seed flour (4), and Saarivirta has decided that the benzyl isothiocyanate maximum in the Lepidium is due to destruction of the compound in some non-thiocyanate-forming reaction (6). Thus the second hypothesis is now unsupported, while the first remains as a generalised concept, without any chemical analogy.

Significantly, Miller observed that the allylglucosinolate aglucone (2, R = allyl) was converted to allyl thiocyanate by T. arvense seed meal. Apparently the production of thiocyanates from glucosinolates needs two enzymes (at least): a myrosinase; and an aglucone-rearranging enzyme. Since the aglucone 2 normally rearranges spontaneously to isothiocyanate we must conclude that the rearranging enzyme is very efficient, or that the spontaneous isothiocyanate-forming reaction is somehow inhibited, or both.

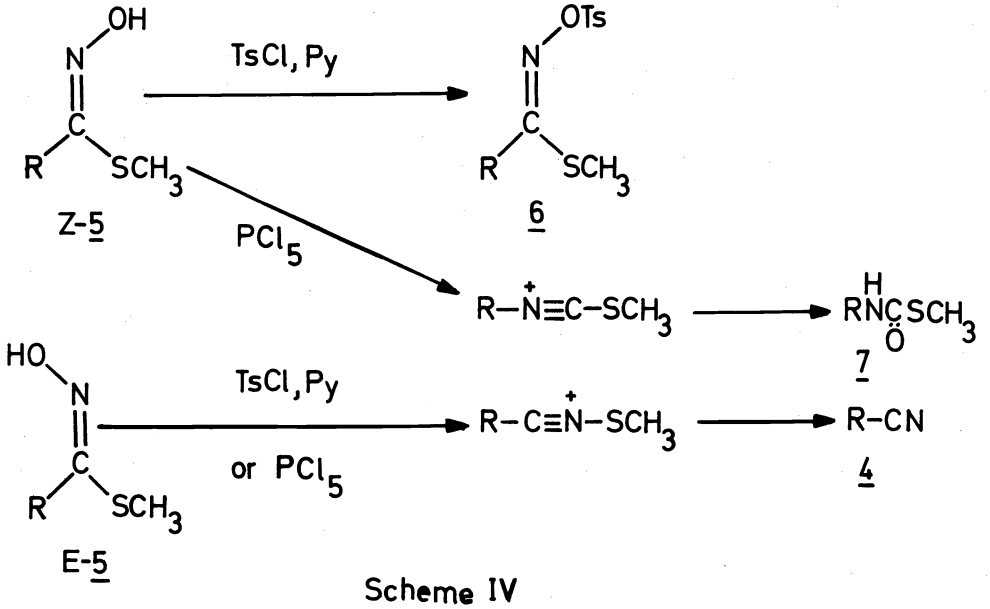
To date all attempts to isolate the thiocyanate-forming enzymes have failed, they are ap-

parently much more prone to denaturation than the relatively robust myrosinases. This is a field which merits continued efforts, meanwhile studies continue to employ seed meal or plant extracts. Noteworthily, Saarivirta has reported that in the Lepidium seed powder system ascorbate activates the thiocyanate forming enzymes, while hydrogen sulfide suppresses isothiocyanate formation (6). It would be interesting to know if the effect of hydrogen sulfide is to inactive the "normal" myrosinase, or to accelerate the conversion of aglucone to thiocyanate.

Inhibition of the non-enzymatic rearrangement of aglucone to isothiocyanate might be achieved by absorbing the aglucone onto some protein site such that steric interactions prevent the migration of R (i.e. convert the decomposition of the aglucone from non-enzymatic to enzymatic). However, the most obvious possibility, with ample chemical precedent, would be to destroy the Z-geometry about the C = N of 2 known to be a requirement for the concerted loss of sulfate and migration of R in the normal rearrangement to 3. Both of these possibilities have been recognized by Schlüter and Gmelin (7).

If indeed Z-2 was isomerized to E-2 there are some intriguing possibilities.

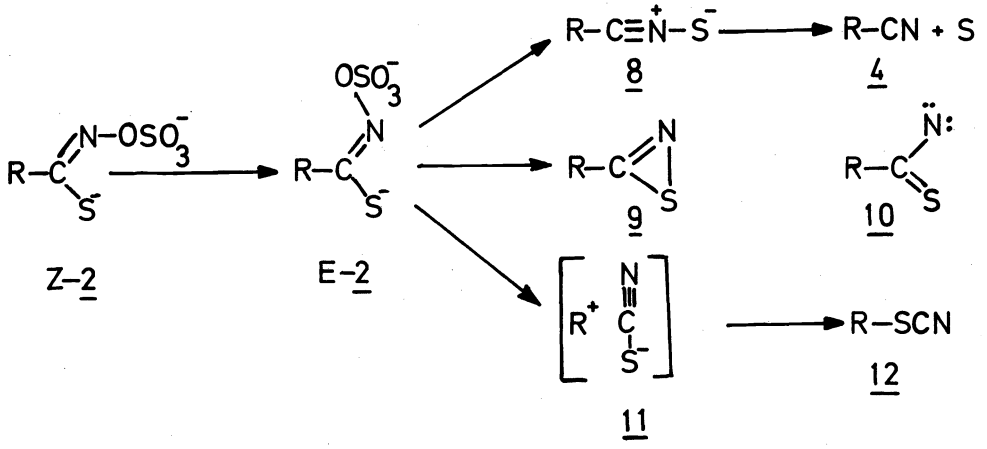
Davies et al. (10) prepared the Z- and E- isomer pair 5 (R = ethyl), and found that upon tosylation in pyridine at room temperature the Z-isomer gave a stable tosylate, while the E-isomer underwent spontaneous decomposition to yield nitrile 4 (R = ethyl) in 70 % yield. Similarly, treatment with phosphorus pentachloride, followed by water, converted Z-5 (R = methyl) to the thiocarbamate (7) while once again the E-isomer gave the nitrile (4, R = methyl), in high yield. These reactions were interpreted in terms of the Grob fragmentation for the Beckmann rearrangement (See Scheme IV).



Scheme IV

Z-, and E-Thiohydroximate Fragmentations (Davies et al., 1968)

In the case of the glucosinolate aglucone (E-2) the thiohydroximate sulfur is not alkylated and if sulfur migration occurred the product would be a nitrile sulfide (8) (Scheme V).



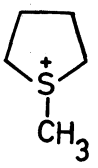
Scheme V

Aglucone Isomerisation and Fragmentation

Very little is known about this class of compound: they appear to be highly unstable, and decompose to nitriles (4) (11, 12). There is no report of a rearrangement analogous to that of nitrile oxides to isothiocyanates (13) i. e. no isothiocyanate (or thiocyanate) formation was observed in reactions of 8.

Another hypothetical product from E-2 is the thiazirine 9 (cf. the azirine intermediate in the Neber reaction). This is an apparently as yet unknown heterocycle. (The dihydro, thiaziridine, system appears to be very unstable with respect to sulfur loss and imine formation (14)). Similarly, we also lack knowledge of the properties of thiocarbonyl nitrenes (10), which could conceivably arise from Z- or E-2 via prototropic rearrangement and α-elimination, or from elimination of sulfate from E-2. Carbonyl nitrenes apparently do not rearrange to isothiocyanates and, if they do not react with solvent, they seem to fragment to alkyl and NCO radicals (15).

Considering fragmentation possibilities brings us to an ion-pair (11), formed directly from E-2 or some other intermediates (16). Collapse of 11 would certainly be expected to produce extensively, if not exclusively, the thiocyanate consequent upon the relative nucleophilicities of the S and N termini of the ambident anion. Another attractive feature of 11 is that the glucosinolates which yield thiocyanates are precisely those which would favour ion-pair formation by virtue of yielding relatively good cations R^+ : allyl, benzyl, and 13 (from 4-methylthiobutyl).



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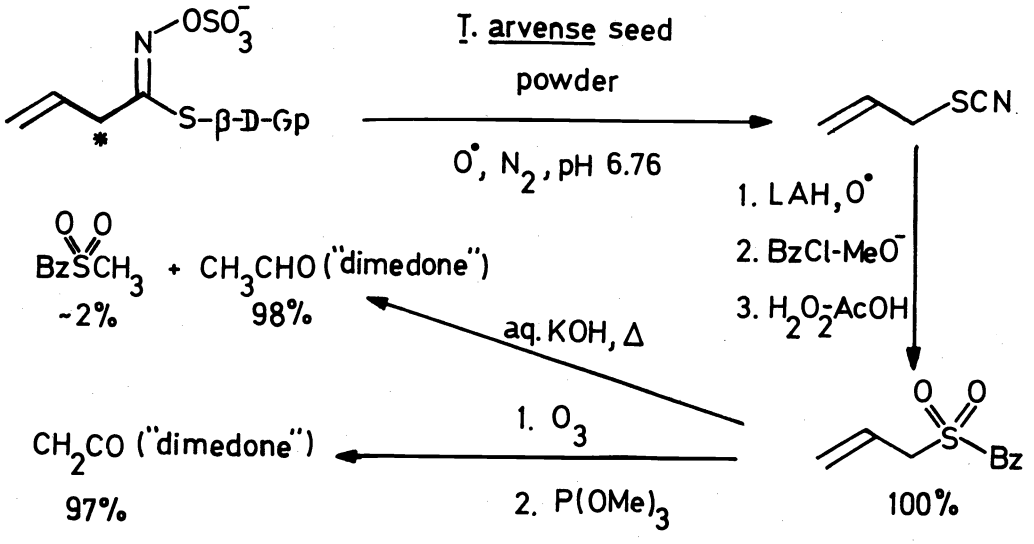
The most direct test of the hypotheses involved in Scheme V would be to prepare a set of E-2 aglucones (crucially R = allyl, benzyl, and 4-methylthiobutyl) and to examine their non-enzymatic decompositions, e. g. a set of Miller-type experiments involving E-2 (R = allyl). Unfortunately to date our attempts to prepare E-1 (R = allyl, or benzyl), from which we had hoped to prepare the corresponding aglucones, have been fruitless. Accordingly, we turned to other experiments designed to probe the possible intermediacy of 11.

We tried heating 13 (as the trifluorosulfonate or iodide salt) with potassium thiocyanate in heavy water and monitored the reaction by p. m. r., and g. c. analyses. There was no evidence of ring opening, only some demethylation occurred. In a sense this result is consistent with the known lability of 4-methylthiobutyl thiocyanate (which presumably cyclises to 13 thiocyanate).

We reasoned that propylglucosinolate might be accepted by the T. arvense enzyme system as an alternative to the natural allyl substrate, but would not be convertable to an ion pair on account of the difficulty in generating the highly energetic propyl cation i. e. that propylglucosinolate would be converted to butyronitrile by T. arvense seed powder. Propylglucosinolate is not known as a natural product, and we prepared it (as the potassium salt) by standard procedures from butyraldoxime. When treated with the T. arvense seed powder in phosphate buffer at pH 6.7 it gave butyronitrile and propyl isothiocyanate in ca. 4:3 ratio. There was no propyl thiocyanate.

This result, although at least in accord with expectation, is at best negative evidence and is clouded by uncertainty as to whether or not the propyl aglucone was accepted by the allyl thiocyanate-forming enzyme.

More to the point, we biosynthesised 1-^{14}C-allylglucosinolate from methionine-2-^{14}C (17), converted it to allyl thiocyanate with T. arvense seed powder and examined the distribution of label in the product. Scheme VI shows the degradations, and the results.

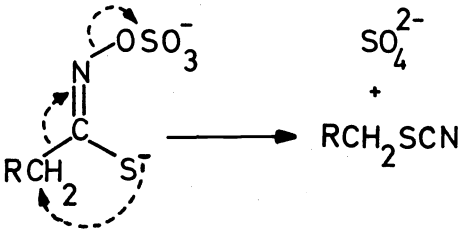


Scheme VI

Allyl Thiocyanate Biosynthesis (Lüthy and Benn, 1975)

Rearrangement is accompanied by label migration. This finding would most easily be rationalized in terms of a [3,3]-sigmatropic rearrangement, as in the thermal isomerisation of allyl thiocyanate and isothiocyanate. However, under the conditions of the experiment uncatalyzed rearrangement does not occur. We are therefore forced to conclude that if an ion-pair (11) is involved in thiocyanate formation it must be a tight one in which the termini of the allyl cation are not equivalent or, more likely, an ion-pair is not formed.

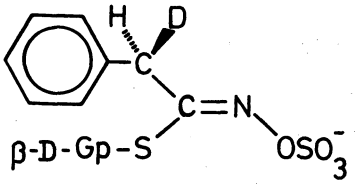
The result is also not compatible with a generalized rearrangement such as that in Scheme VII, though in this respect it is consistent with the observation that the thiocyanate-forming enzymes are substrate specific.



Scheme VII

Generalised Thiocyanate Formation ?

Further experiments which come to mind include an examination of the thiocyanate produced from 14 and 15 to see whether the rearrangements are stereospecific. Such information would help in clarifying whether the final, post-aglucone, stages of the thiocyanate rearrangement are enzymatic.



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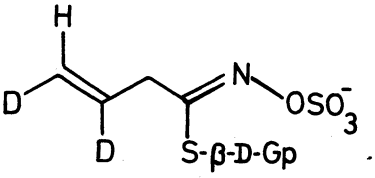

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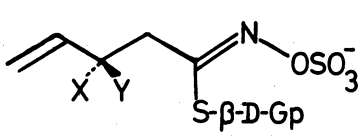
Catabolism to Cyano-epithioalkanes

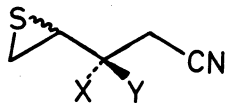
Another "abnormal" catabolism involves the conversion of alkenylglucosinolates to cyano-epithioalkanes. This transformation was first observed in autolysing seed meal preparations (18, 19, 20), but apparently it can also occur in fresh-plant extracts (8, 21). Scheme VIII summarises these reports.

As with thiocyanate production, the formation of the cyano-epithioalkanes is under the control of a labile enzyme system, and, when this is inactivated, the products are nitriles, and isothiocyanates (and derivatives thereof).

There has however been some success in studying its components. Tookey (22) has stabilised extracts of C. abyssinica seed meal by removing phenolics, adding ferrous ion and

Seed meals



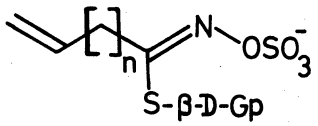


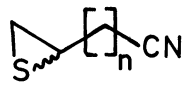
16a X=OH, Y=H —Crambe abyssinica; Hochst ex R.E.Fries (18)→ 17a X=OH, Y=H

b X=H, Y=OH —Brassica napus L. (19)→ b X=H, Y=OH

c X=Y=H —Brassica campestris L. (some cultivars) (20)→ c X=Y=H

Fresh plants





18 n=1-3 —numerous cases (8,21)→ 19 n=1-3

Scheme VIII

Cyano-epithioalkanes from Alkenylglucosinolates

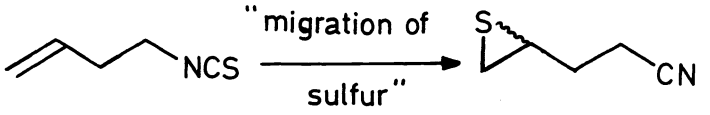
small amounts of dithiothreitol, and keeping the solution at 0° under nitrogen. Precipitation with ammonium sulfate then gave several fractions among which were one (A) (at 60-70 % saturation) which converted 16a to the nitrile 17a and was weakly activated by ascorbate; another (B) (at 50-60 % saturation) which gave some conversion of 16a to 17a and was strongly activated by ascorbate; and (C) a small protein or glycoprotein (precipitating at 40-60 % saturation, and present in B) which could be purified by Sephadex G-100 chromatography. Purified C was itself incapable of transforming 16a to any products, but when added to A gave a preparation which efficiently converted 16a to 17a (it also has the effect of reducing the thioglucosidase activity of A). It has been suggested that the role of C might be to combine with an intermediate released from A and divert it from alkenyl cyanide to cyano-epithioalkane, or to interact with A in an allosteric manner. The latter seems much more likely.

Tookey points out the resemblance of this two-protein enzyme system to lactose synthetase,

where also a small specific protein determines the nature of the products (23). As well, he notes that the liability of the epithio-nitrile forming enzyme system is reminiscent of the T. arvense thiocyanate-forming one and that this too may prove to involve a specifier protein.

An interesting feature of the formation of the episulfide group is that it is non-stereospecific. As the original investigators (18, 19, 20) noted this suggests some non-enzymatic process, and leads us to consider possibilities.

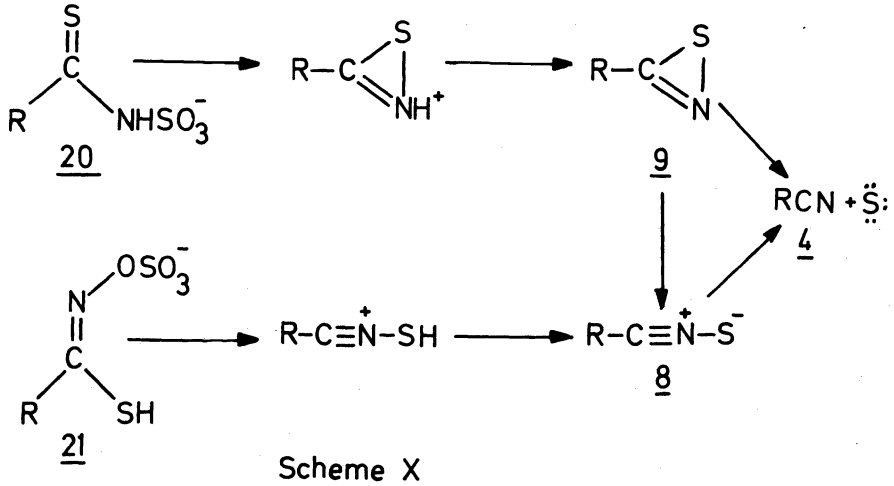
Cole (8) postulates a rearrangement of initially formed alkenyl isothiocyanate (Scheme IX) but this seems highly unlikely.



Scheme IX

Cyano-epithioalkane Formation (Cole, 1976)

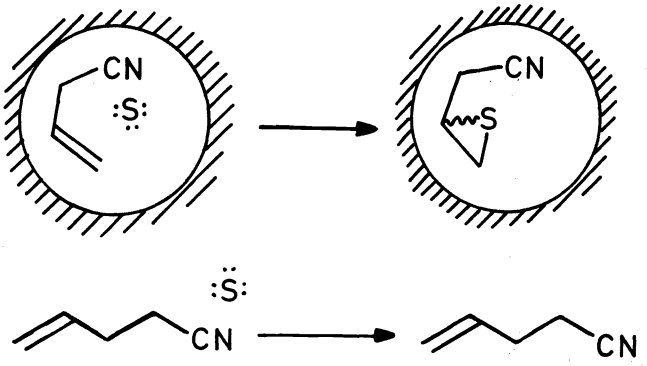
Much more plausible would be some modification of the aglucone desulfurisation, the nitrile-forming reaction, which resulted in capture of the sulfur by the olefinic chain. This brings us back to the matter of the mechanism for the "normal" aglucone (2) to nitrile (4) conversion (Scheme I). The proton dependency of this reaction indicates that a conjugate acid is involved, and this invites the thoughts that 20 may be yielding 9, or that acid catalysed isomerisation to the E-2 conjugate acid (21) leads to 8 (cf. Scheme V). The formation of 8 from 9 has also been postulated (11). Decomposition with the formation of nitrile and sulfur then follows (Scheme X).



Scheme X

Nitrile Formation?

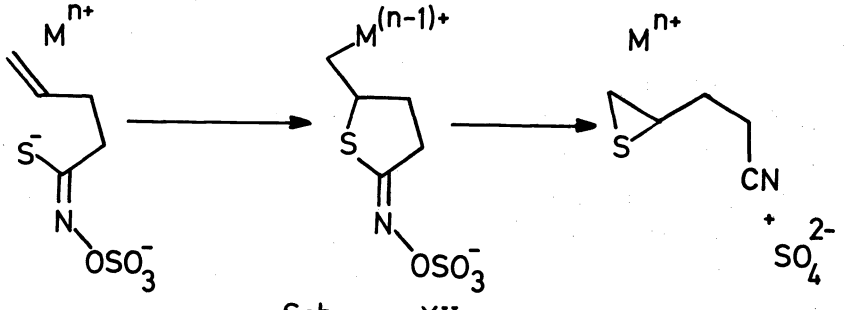
As written the sulfur would be generated as singlet excited atoms which could decay to the triplet ground state. Both species are known to react with olefins to yield epithioalkanes (the singlet atom also giving thiol products arising from insertions into C-H bonds) (24). Efficient intra-molecular capture of the sulfur atoms by the alkenyl side-chain of a glucosinolate would require folded conformations, while reaction in extended conformations would be expected to result in the escape of the sulfur atoms (and their subsequent polymerisation). It can be postulated that the former occurs in an enzyme cleft, and the latter after relief of the constraint to fold (Scheme XI).



Scheme XI

Sulfur Atom Capture or Escape

Conventional epithioalkane synthesis via epoxides, or 1,2-disubstituted alkanes (e.g. Scheme XII), have also been considered, but appear less attractive. The same is true of the intramolecular cyclization of a nitrile sulfide to a bicyclic isothiazoline, which might then fragment to epithio-nitrile.



Scheme XII

An Alternative Route to Epithio-nitriles

At present we are pursuing attempts to achieve the non-enzymatic transfer of sulfor from the allyl aglucone (2, R = allyl) to alkene acceptors, and other trapping experiments.

Biological Effects of Glucosinolates

The impetus for studying glucosinolates has always been provided by biological effects associated with their catabolism, and it seems appropriate to make some brief comments about this aspect.

The famous goitrogenic effects of the oxazolidine-2-thiones derived by intramolecular cyclization of 2-hydroxyalkyl isothiocyanates, as well as of thiocyanate ion, can be understood in the light of Hager's work with chloroperoxidase (25). Soluble thioureas resulting from the reaction of basic amino acids and small peptides with isothiocyanates may similarly be responsible for the goitrogenic properties of the latter.

The expected reaction of glucosinolate derived isothiocyanates with proteins has been observed (26), and the acylating properties of the isothiocyanates probably account for much of their toxicity towards microorganisms and insects. (Myrosinases may escape suicide as a consequence of the aglucone decomposition being non-enzymatic!).

The toxicity of the cyano-epithioalkanes has not been thoroughly explored, but they are potential biological alkylating agents, and are accordingly suspect (20, 27).

Probably the most important biological effects of glucosinolate catabolites are associated with their role as flavour components of food stuffs. The function of isothiocyanates, thiocyanates and nitriles in this respect has been discussed as part of a recent general review of the flavour and palatability of Cruciferous vegetables (28). For discerning humans the glucosinolate metabolites are luxuries, for insects they are, at least in some cases, matters of life and death. Verschaffelt's classic paper on the selection of food by phytophageous insects demonstrated that potassium allylglucosinolate rendered acceptable the leaves of plants usually rejected by larvae of *Pieris brassica* and *Pieris rapae*, and constitutes the first recognition of the role of a secondary metabolite in directing the relationship of insect and host-plant (29). The lode he uncovered has been mined extensively, and much of present day thinking on the function of plant secondary metabolites has derived from it (30). Although potassium allylglucosinolate has been reported to be a feeding stimulant for a number of insect pests on Crucifers it can act as a deterrent: an effect revealed with aphid species (31). It is lethal for *Papilio polyxenes* (32).

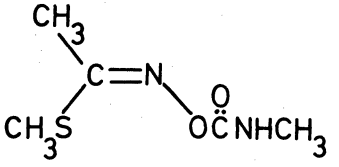
It is possible to suspect that some or all of these effects might de due to the glucosinolate catabolites, and indeed allyl isothiocyanate has been shown to attract many of the insects whose host-plants contain glucosinolates, and stimulate feeding, and ovipositing (33). (A Braconoid which in turn parasitises Crucifer feeding aphids is also attracted (34)). Given the relative volatility of the isothiocyanates *vis a vis* their parent glucosinolate salts, it seems reasonable that this should be the case if olfactory cues are involved in plant-finding. And although a recent study of the volatiles emitted a number of crucifers failed to detect any isothiocyanates (35) some insects are well known to be remarkably sensitive to sex-pheromones, and a similar situation may pertain to plant odours, *i. e.* that the insect can detect the plant-scent chemicals at exceedingly low concentration levels.

The most direct evidence that intact glucosinolates can stimulate feeding comes from electrophysiological studies by Schoonhoven (36), who found maxillary chemoreceptor cells in *P.brassicae* which responded to glucosinolates (at ca. 10^{-5} M) but were insentive to allyl isothio-

cyanate (at 10^{-3} M). (The only shadow on these experiments seems to be Miller's finding (4) that dilute aqueous solutions of allyl isothiocyanates are unstable).

It would be interesting to know how insects which habitually ingest glucosinolate handle their metabolism. Reference has already been made to aphid myrosinase, but if isothiocyanates are produced the problem of their toxicity remains. Perhaps in the insects the normal metabolism is directed towards nitriles. The finding that larvae and pupae of P. brassica accumulate glucosinolates and isothiocyanates (37) seems worthy of further scrutiny.

The catabolism of glucosinolates may have some bearing on that of the insecticide "Methomyl" ("Lanate"), O-[N-methylcarbonyl] S-methyl acetothiohydroximate (22), and vice versa. It is known that in vivo degradation of 22 gives acetonitrile (38).



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It should be noted that in this discussion of the biological properties of glucosinolate catabolites, as in the earlier discussion of the chemistry of their generation, we have not pursued them beyond the initial stages. Presumably nitriles will yield carboxylic acids; isothiocyanates the corresponding amines (there is some circumstantial evidence that indeed they do (39)) and thence aldehydes etc.; and thiocyanates thiols and their derivatives (28).

Conclusion

In the diversity of glucosinolate catabolism there is also a unity. The transformations to isothiocyanates, nitriles, thiocyanates, and cyano-epithioalkanes appear to involve alternative modes of decomposition of an initially generated aglucone (2). An understanding of the processes involved, and their control, promises valuable insight into mechanistic organo-sulfur chemistry, and enzymology, while much economic value attaches to the biological properties associated with the catabolites.

To conclude, the importance of the glucosinolates resides in their disapperance.

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