RICE UNIVERSITY

The Aglucone of Sinigrin

by

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A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

MASTER OF ARTS

Thesis Director's signature:

Martin E. Ettinger

Houston, Texas

May 1965

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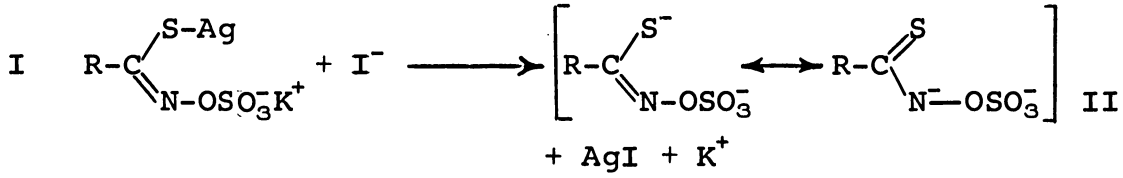
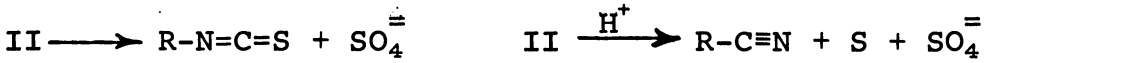
A B S T R A C T

The mechanism of the ascorbate-activated cleavage of sinigrin to mustard oil has been postulated as enzymatic hydrolysis of the thioglucoside to the aglucone, which undergoes spontaneous Lossen rearrangement to allyl isothiocyanate. Potassium silver sinigrate (I) served as a convenient source of the aglucone (II) for comparison. When the potassium

$R = H_2C{=}CH\text{-}CH_2\text{-}$



$$\text{I} \quad R\text{-}C(S\text{-}Ag)(N\text{-}OSO_3^-K^+) + I^- \longrightarrow \left[R\text{-}C(S^-)(N\text{-}OSO_3^-) \longleftrightarrow R\text{-}C(S)(N^-\text{—}OSO_3^-)\right] \text{II} + AgI + K^+$$



$$\text{II} \longrightarrow R\text{-}N{=}C{=}S + SO_4^= \qquad \text{II} \xrightarrow{H^+} R\text{-}C{\equiv}N + S + SO_4^=$$

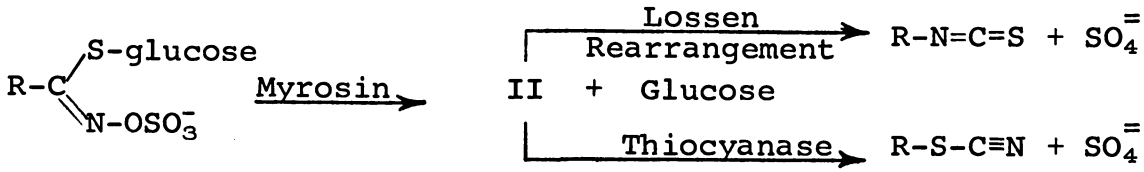
salt was decomposed with iodide at pH 2, 97% of the product was allyl cyanide; at pH 5, 97% was allyl isothiocyanate; and at pH 3.53, the two compounds were formed in equal amounts. The ratio of nitrile to isothiocyanate was proportional to hydrogen ion concentration. Enzymatic hydrolysis of sinigrin at pH 3.2-5, with or without ascorbate, gave essentially the same product distribution.

Treatment of potassium silver sinigrate with hydrogen cyanide at pH 6.6 allowed spectroscopic observation of the aglucone. It (II) has an ultraviolet absorption maximum at 245 mμ, ε 11,000, and a half-life of 37 seconds at 20° (estimated 4 minutes at 0°). These results agree with data for

the absorbing intermediate in the enzymatic hydrolysis of sinigrin and confirm the proposed mechanism.

Product formation from the silver salt (I) and iodide at pH 3.2 and 24° was half complete in 30 seconds. Ferrous ion (pH 5) decomposed the aglucone to nitrile. Iodine appeared to oxidize the aglucone to the more stable disulfide.

Maceration of Thlaspi arvense seed furnishes allyl isothiocyanate and allyl thiocyanate by an enzymatic process from sinigrin. The yield of thiocyanate is highest in a reducing environment at 0°. In the reaction sequence, the thioglucoside bond of sinigrin is cleaved by a myrosin-like enzyme,



and the resulting aglucone either undergoes Lossen rearrangement or is captured by another enzyme ("thiocyanase") and converted to allyl thiocyanate. Isothiocyanate once formed is not isomerized. That the thiocyanate is derived from the aglucone was demonstrated by reaction of potassium silver sinigrate with iodide and ground Thlaspi seed. The aglucone thus produced was converted efficiently to thiccyanate.

Allyl isothiocyanate and allyl thiocyanate reached an equilibrium in the undiluted liquid, with 5.1% allyl thiocyanate at 24° and 8.2% at 100°. Equilibrium is approached at 24° with a half-life of 17-19 days. In dilute aqueous and methanolic solutions, which decompose rapidly, the apparent equilibrium is 35-40% allyl thiocyanate.

TO MY PARENTS

A C K N O W L E D G M E N T

The author wishes to express his sincere gratitude to Dr. Martin G. Ettlinger for his direction of this problem. He also wishes to express thanks for support by a Public Health Service research grant (AM 06960) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

H. E. M.

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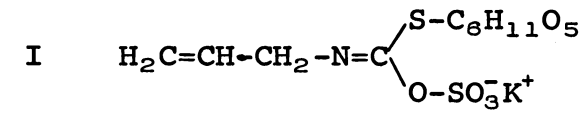
I N T R O D U C T I O N

The mustard oil glucosides are precursors of isothiocyanates, or mustard oils, and in certain cases of the isomeric thiocyanates and of nitriles. They are found in many plants such as horseradish, cabbage, turnips, and nasturtium, and are distributed throughout the entire plant. The known mustard oil glucosides are rapidly increasing in number and their distribution in nature has been summarized by Kjaer.[1]

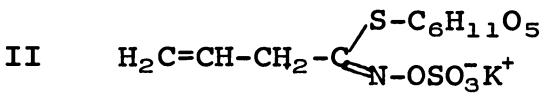
The existence of mustard oils appears to have been reported as early as three centuries ago.[2] Sinigrin, a mustard oil glucoside in the form of a crystalline potassium salt, was isolated in 1839 from the seeds of black mustard.[3] In 1863, the empirical formula was established by Will and Körner.[4] From their experiments and without distinction between isothiocyanates and thiocyanates, they suggested that sinigrin might not contain the mustard oil skeleton. However, Gadamer[5] in 1897 proposed structure (I) for sinigrin. The rapid enzymatic cleavage of sinigrin to allyl

isothiocyanate indicated to Gadamer that allyl isothiocyanate is present as



I

the same sequence of atoms in sinigrin. Although Gadamer was aware that (I) did not accommodate all the properties of sinigrin, it remained undisputed until 1956, when the correct structure (II) was established by Ettlinger and Lundeen.[6]



II

One of the most interesting properties of the mustard oil glucosides is their enzymatic hydrolysis. If the moist seeds or other material of glucoside-containing plants is ground, isothiocyanate, D-glucose, sulfate, and acid are liberated. In addition, thiocyanates or nitriles are produced from the macerated material of several species. The nature of these processes has been the subject of much debate.

The revised structure of sinigrin indicated the possibility that enzymatic hydrolysis of mustard oil glucosides

to yield isothiocyanates and nitriles could proceed through the action of a single enzyme.[6,7] The formation of isothiocyanate was proposed to occur concurrently with enzymatic removal of the glucosyl group, through a rearrangement analogous to the Lossen rearrangement. Production of nitriles in unbuffered enzymatic hydrolysis or by acid hydrolysis of mustard oil glucosides was explained by analogy to known fissions of thiohydroxamic acids to nitriles.[8,9]

It was pointed out by Lundeen[8] that since the Lossen rearrangement is of a concerted nature, cleavage of the glucosyl-sulfur bond in a mustard oil glucoside would be facilitated by simultaneous rearrangement and loss of sulfate. Consequently, anti configuration of the migrating and leaving groups about the carbon-nitrogen double bond in the glucoside would be expected. This configuration has since been proved by X-ray analysis.[10] Such a mechanism, however, would not allow even the transient existence of an intermediate.

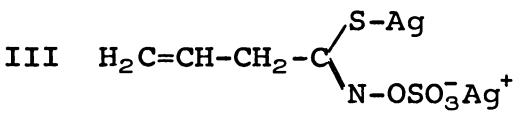
More than thirty years ago, von Euler[11] and Neuberg[12] suggested that the overall cleavage of sinigrin requires two enzymes. A single-enzyme system has been supported by

Reese, Clapp, and Mandels[13] and Nagashima and Uchiyama.[14] Recent work by Gaines and Goering [15,16] has been published in favor of a two-enzyme arrangement. They reported separation of the enzyme myrosin into two entities: one functions as a glucosidase, and the other as a sulfatase. They suggest that the sulfur-glucosyl bond is cleaved by the glucosidase and the fragment which results undergoes loss of sulfate and rearrangement while attached to the sulfatase factor. Such a system implies the existence of an intermediate.

Schwimmer[17] pointed out in a note that the course of sinigrin decomposition by myrosin and ascorbic acid to isothiocyanate or nitrile depends on pH. Schwimmer proposed that 3-butenylthiohydroximylsulfate forms at pH 3 and would accumulate in the absence of protein impurities or ascorbic acid. Further experiments were initiated by Schwimmer[18] in which sinigrin was hydrolyzed enzymatically and optical density changes were observed at 255 mμ, the isosbestic point between sinigrin and allyl isothiocyanate. A rapid rise in absorption was noticed, followed by a slower rise and then decay. These changes were attributed to side reactions, or the formation of relatively stable intermediates, or of an enzyme-substrate complex.

More recent work by Ettlinger and Thompson [19,20] on hydrolysis of sinigrin with ascorbate-activated enzyme preparations from yellow mustard has indicated that the ratio of nitrile to isothiocyanate produced is proportional to hydrogen ion concentration, which suggests that the product-forming step does not depend on an enzyme, but stems from an intermediate whose fate is determined by the pH. Further, at pH 5 or more a very definite, transient ultraviolet absorption was reported with a maximum between 245 and 250 mμ (log ε 4.0). Decomposition of the unstable, absorbing species was nonenzymatic. This intermediate, identified as the sinigrin aglucone, was reported to have a half-life of approximately 25-30 seconds at 20° and pH 5-9, and a pK_a below 3.5.

It is an established fact that treatment of sinigrin with silver nitrate yields the disilver salt, silver sinigrate (III). [4,5,14] This salt can be decomposed to allyl



III

isothiocyanate by neutral nucleophiles such as thiosulfate or chloride, and to allyl cyanide by acidic reagents such

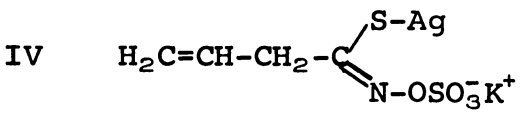
as hydrogen sulfide or hydrochloric acid. The analogy between the pH dependence of the products from enzymatic hydrolysis of sinigrin and the results from silver sinigrate is inescapable. If the sinigrate ion is the unstable product of enzymatic hydrolysis, formed through cleavage of the thioglucoside link, then the silver sinigrate decomposition should show the same pH dependence since the initial attack by a nucleophilic reagent must be on silver to displace the aglucone.

A careful examination of the reactions of sinigrate salts would be helpful in ascertaining the properties of the sinigrin aglucone, knowledge of which is essential for elucidating the pathways of enzymatic decomposition of mustard oil glucosides.

PART ONE

Kinetic and Spectral Studies with the Sinigrin Aglucone

Silver sinigrate, as was mentioned previously, is a well-known compound which can be decomposed to yield allyl isothiocyanate or allyl cyanide. However, silver sinigrate is not a convenient choice for quantitative studies because it is only slightly soluble in water (approximately 2 or 3 times more soluble than silver chloride). Potassium silver sinigrate (IV) had been prepared in more concentrated solutions[5, 14] and was reported to decompose to allyl isothiocyanate on treatment

IV 

with thiosulfate or chloride, but had not been isolated in solid form.

Work was initiated to prepare a water-soluble sinigrate salt that could be isolated and handled

satisfactorily.* Sinigrin was treated with silver nitrate to give disilver sinigrate. Potassium silver sinigrate was then prepared from a suspension of silver sinigrate in an equivalent of aqueous potassium chloride. Tetramethylammonium silver sinigrate was obtained in a similar manner and from it the ammonium, tetraethylammonium, tetra-n-propylammonium, and trimethylammonium salts were prepared with a cation exchange resin.

Tetra-n-propylammonium silver sinigrate and tetraethylammonium silver sinigrate were viscous oils, which would not crystallize. Tetramethylammonium silver sinigrate could be prepared readily and crystallized nicely from methanol or methanol-ethanol. Although tetramethylammonium silver sinigrate appeared to be the most stable of the salts and the easiest to crystallize, it was extremely hygroscopic and therefore impractical to use. The ammonium, potassium, and trimethylammonium salts were obtained as amorphous powders that crystallized with difficulty and decomposed slowly under ordinary conditions. There was little

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*Work on this project was begun by Mr. Robert Moon, who prepared samples of potassium silver sinigrate and ammonium silver sinigrate.

difference between these three compounds. The potassium salt, the only sinigrate used in further work, was prepared in quantity as a white powder, which was stored up to 9 months over silica gel at 5° with no sign of decomposition. It decomposed slowly in the course of one or two weeks out in the laboratory but was stable for experimental purposes.

A series of experiments were carried out at pH between 2 and 7 to determine the product dependence on pH for the reaction of potassium silver sinigrate with potassium iodide. The experiments were performed by decomposition of 60-80 mg. of potassium silver sinigrate with a small excess of potassium iodide in 20 ml. of buffer. After 20 minutes, the reaction mixture was extracted with ether and the ether solution analyzed with a vapor chromatograph and hydrogen flame ionization detector, which could measure allyl isothiocyanate and allyl cyanide in concentrations down to 8×10^{-4} M and 3×10^{-4} M, respectively. Figure 1 and Table II show the results of these experiments. The slope of the line in Figure 1 is unity: the ratio of nitrile to isothiocyanate is directly proportional to hydrogen ion concentration and the reaction proceeds equally to either compound at pH 3.53.

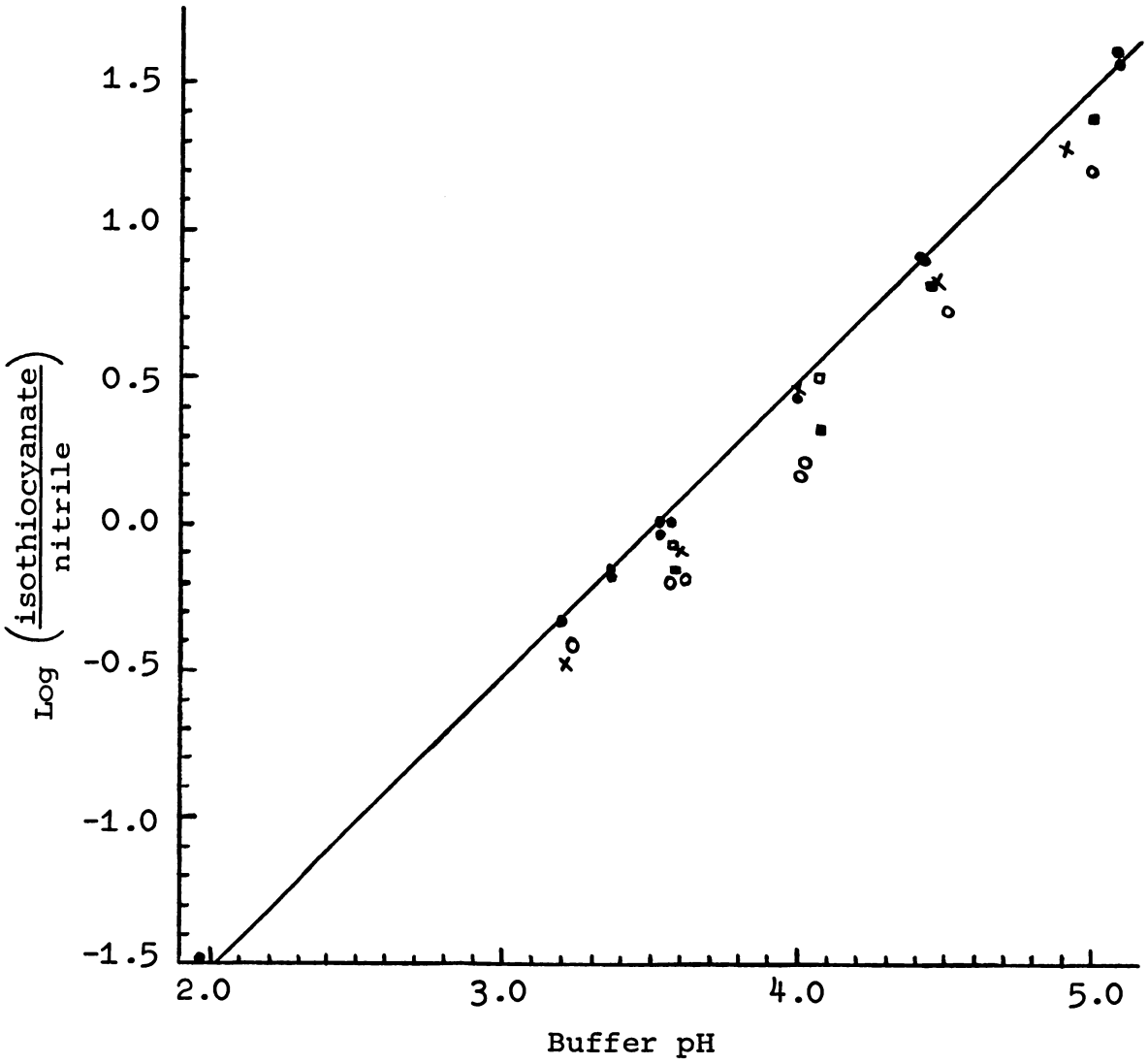


Fig. 1. Dependence of product composition on pH for reaction of silver sinigrate and iodide and for enzymatic hydrolysis of sinigrin. The experiments with potassium silver sinigrate are represented by ●, myrosin by x, ascorbate-activated enzyme by ○, ascorbate-activated enzyme using ½ as much sinigrin by ■, and ascorbate-activated enzyme using ¼ as much sinigrin by □.

The results were unchanged in a reaction with the buffer concentration doubled.

Sulfate, sulfur, and silver iodide were determined in representative experiments. Sulfate was determined with barium chloranilate.[21] Sulfur was extracted with carbon disulfide from the precipitate formed by decomposition of the silver salt and was identified by comparison with known material. The residue after extraction of the precipitate with carbon disulfide was silver iodide. Sulfate and silver iodide were found in near 100% yield. Sulfur was produced in small amounts (less than 3.5 mg.) and recovery was only roughly quantitative. The molar amounts of sulfur at pH 3.5 and 4.4 corresponded to those of allyl cyanide.

The reaction of iodide and potassium silver sinigrate was rapid. Precipitation of silver iodide began immediately in buffers of pH 4-7 and ended within 5 minutes. At lower pH, the precipitate formed slowly during 2 hours. This was not due to slow reaction, however, but to the colloidal nature of the precipitates. If disodium hydrogen phosphate was added in sufficient quantity to a reaction mixture of low pH, the pH was changed to a value above 5, at which allyl isothiocyanate is the only product. Any

allyl cyanide found after complete reaction must have been produced before the pH was raised. From experiments in which basic phosphate was added after various times at pH 3.18, it was determined that the decomposition with sinigrate and iodide initially 0.01 M at that pH and 24° was half complete in roughly 30 seconds.

The results of silver sinigrate decomposition demonstrated a simple relation between pH and the products. For direct comparison, the enzymatic hydrolysis of sinigrin was conducted under the same general conditions with two different enzyme preparations. One crude enzyme, the classical myrosin, was prepared from yellow mustard flour according to the method outlined by Schwimmer.[18] The second preparation, also from yellow mustard flour, was the ascorbate-activated enzyme (glucosinolase),[19,20] whose effect is increased as much as 400 times on addition of small amounts of L-ascorbic acid. The rate of hydrolysis by myrosin preparations is increased by a factor of 5 or 10 with the vitamin.

Two sets of experiments were conducted, one with myrosin and the other using L-ascorbic acid and the highly active enzyme. The only difference in experimental procedure was the addition of L-ascorbic acid. The enzyme activity

decreases at low pH, partly owing to denaturation.[20] However, reactions could be carried out at pH down to 3.5 with satisfactory results, which was adequate for comparison with the potassium silver sinigrate experiments. After mixing the sinigrin and enzyme (and L-ascorbic acid) in 20 ml. of buffer, they were set aside for ½-3 hours, as long as was necessary for the reaction to reach at least 90% completion. The results of these experiments also are plotted in Figure 1 and given in Table IV. The lines that would be determined by the experimental points again have unit slope, but are displaced to the right of the line for the silver sinigrate experiments. Some of the displacement was due to the L-ascorbic acid added and to the acid produced during hydrolysis. Thus the product composition represented an average pH below the buffer pH. When the amount of sinigrin used in the enzyme-catalyzed reactions was reduced to one fourth, the results approached more closely to those of the silver sinigrate decomposition.

Thus it was concluded that enzymatic hydrolysis of sinigrin by myrosin or ascorbate-activated glucosinolase yields the same ratio of allyl isothiocyanate to allyl

cyanide between pH 3.5 and 5.0 as the reaction of potassium iodide and potassium silver sinigrate.

Since the treatment of potassium silver sinigrate with iodide must generate the aglucone, its reported ultraviolet absorption was looked for. However, turbidity from silver iodide hid any absorption that existed. Hydrogen cyanide was found to be an effective agent for removal of silver. In phosphate buffer, pH 6.62 and 1.7×10^{-2} molar in hydrogen cyanide, the reaction was complete and nearly instantaneous. Under these standard conditions, the silver cyanide complex had a molecular extinction of 1,100 at 220 mμ, but only 150 at 230 mμ. The allyl isothiocyanate produced had a weak absorption (λ_{max} 240 mμ, ϵ 780), only 7% of that of the sinigrate at the same wave length. Thus, from 290 to 220 mμ there is only minor absorption due to reaction products other than the aglucone.

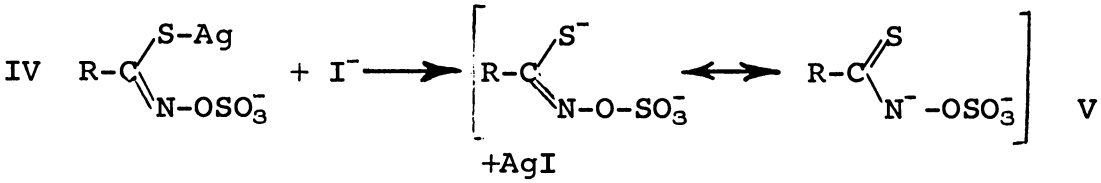
Experiments were conducted on a Cary automatic recording spectrophotometer. The cells, cell compartment, and all reagents were thermostatted. One ml. of sinigrate solution was added to the cell, which already contained 2 ml. of cyanide-phosphate mixture. After addition of the sinigrate, 3 seconds elapsed before recording could begin.

At 290 mμ, the molecular extinction of potassium silver sinigrate is 1,600, much greater than that of any product or intermediate. From the time of the first observation, the absorption never decreased by more than 1% of the amount calculated at this wave length for the potassium silver sinigrate in the mixture. However, from 270 through 220 mμ a very definite, transient absorption existed. This absorption could not be due to potassium silver sinigrate and therefore must belong to the intermediate, the sinigrin aglucone. Since it appeared that sinigrate lost its silver to cyanide at the instant it was added to the buffer, the sinigrate concentration became the aglucone concentration. Extrapolation of the decay curve from 3 seconds to zero time yielded the absorption for a known concentration of aglucone and silver cyanide. After subtraction of the small absorption of the silver cyanide, the extinction of the aglucone could be calculated. The results shown in Figure 2 were obtained at 20°.

The absorption of the aglucone (λ_{max} 245 mμ, log ϵ 4.05) is in good agreement with the maximum of a model compound, phenylacetothiohydroxamate in alkaline methanol at 247 mμ (log ϵ 3.8).[8]

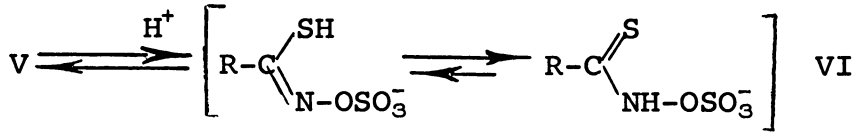
vinylacetothiohydroxamate-O-sulfonate (V), which rearranges in neutral media to allyl isothiocyanate.

R = $H_2C=CH-CH_2-$



$V \longrightarrow R-N=C=S + SO_4^{=}$

In acid, the thiohydroxamic acid-O-sulfonate (VI) decomposes by a first-order route to nitrile,[19,20] analogously to the known formation of nitriles from thiohydroxamic acids.[8,9]



$VI \longrightarrow R-C\equiv N + S + HSO_4^-$

It is equivalent to say that the transition state leading to nitrile includes the composition of (VI), since the acid-base equilibrium of the aglucone between (V) and (VI) must be extremely rapid and the conjugate acid (VI) is not an

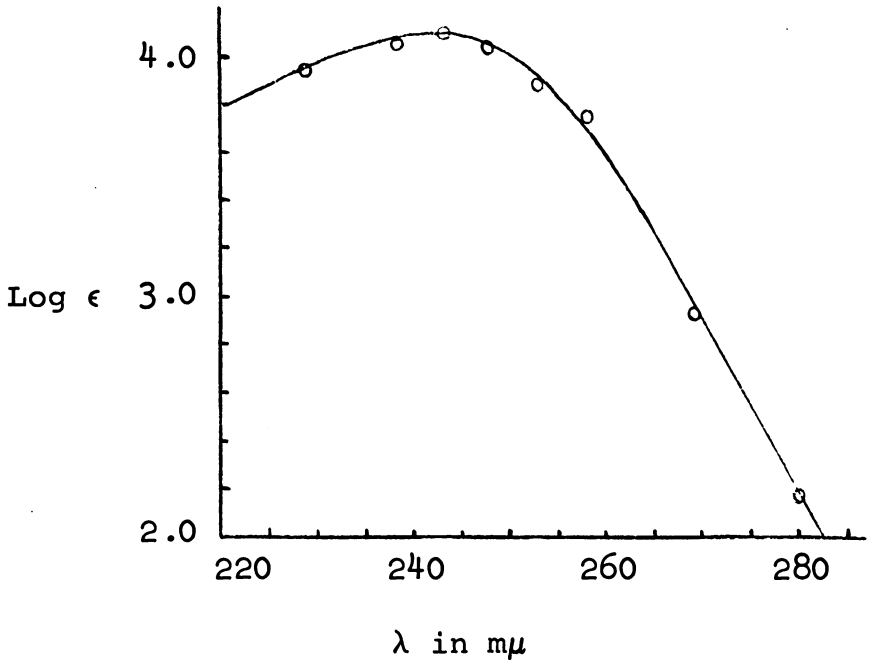


Fig. 2. Ultraviolet absorption spectrum of the sinigrin aglucone.

The half-life of the aglucone was determined from the fall of its absorption. At 20°, 25°, 30°, and 35°, half-lives were found of 37,23,16, and 10 seconds respectively. By extrapolation, the half-life of the aglucone at 0° was roughly estimated at 4 minutes. Decomposition of the aglucone follows first-order kinetics, in accord with a Lossen rearrangement.

Iodide, a nucleophilic species, must attack the covalently bonded silver of potassium silver sinigrate (IV) and displace the sinigrin aglucone,

independent kinetic intermediate. The quantitative relation between product ratio and pH corresponds simply to the existence of two competing first-order reactions of the aglucone (V), one uncatalyzed, the other unimolecular in hydrogen ion.

Since the product distributions from cleavage of potassium silver sinigrate and from enzymatic hydrolysis of sinigrin are virtually the same, it is natural to suppose a common intermediate. For the enzymatically formed intermediate, the half-life given in the literature[19] is 25-30 seconds at 20°. The report mentions that the transient absorption approached its maximal, steady-state value when the reaction was started, more rapidly than it disappeared when the attack on sinigrin was abruptly stopped, and the half-life selected was a compromise. In fact, the half-life observed for decay at 20° ranged from 32 to 41 seconds, averaging 36½ seconds, in excellent agreement with the value of 37 seconds now found for the aglucone from the silver salt. Possibly the error in the half-life derived from the growth of absorption at the beginning of the enzymatic hydrolysis lies in the assumption that the rate of sinigrin

hydrolysis is constant. The spectrum[22] of the enzymatic intermediate, recalculated on the basis of the longer half-life, is compared with that of the aglucone from silver sinigrate in Table I. The spectra agree well in proportion, and most of the absolute difference can be ascribed to uncertainties in aglucone concentration. The intermediate is confirmed as being the aglucone of sinigrin, and the subsequent product-forming steps must be the same in both reactions. Consequently, the formation of allyl isothiocyanate and allyl cyanide from sinigrin can result from one enzyme, a thioglucosidase that liberates the aglucone.

T A B L E I

ULTRAVIOLET SPECTRUM OF THE SINIGRIN AGLUCONE

	λ in mμ	245	250	255	260	265	270
Log ε	from enzymatic hydrolysis	3.91	3.90	3.80	3.57	3.26	2.96
	from silver sinigrate	4.05	4.02	3.90	3.70	3.40	3.01

Experimental

Vapor Phase Chromatography: Analytical Technique

Analyses of allyl isothiocyanate, allyl cyanide, and allyl thiocyanate were carried out on an Aerograph Hy-Fi (Wilkens Instrument and Research), Model A-600-B, vapor phase chromatograph with a hydrogen flame ionization detector. The ether extracts of reaction mixtures were dilute and it was difficult to find a column having adequate sensitivity in which the products were separated from the solvent peak. Packed columns (1/8 inch inner diameter) were prepared with Ucon 50-HB-660 glycol, DC-550 silicone oil, castor wax, Carbowax, N,N-dimethyloleamide*, m-xylylene dicyanide*, and oleonitrile* in differing concentrations on firebrick (30-50 mesh). The materials giving the best results were then tried on an 80-foot capillary column (0.03 inch inner diameter). The capillary column gave the same

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*These materials were suggested and generously supplied by Dr. A. Zlatkis, University of Houston.

separation of products as the packed column, but the peaks were considerably sharper. The sensitivity was further tripled by supplying oxygen to the hydrogen flame instead of air.

The most desirable properties were obtained on the 80-foot capillary coated with m-xylylene dicyanide. The column was coated by slowly forcing 50 ml. of a 5% methylene chloride solution through the column over a 2-hour period. One-μl. samples were injected with a Hamilton syringe of that capacity. The column gave the best separation and sensitivity near room temperature. The absence of heat was advantageous because allyl thiocyanate rearranged noticeably on the column above 50°.

Compositions of the extracts and yields were determined by comparison with standard solutions of allyl isothiocyanate, allyl cyanide, and allyl thiocyanate in ether. The standards were stored at 0° and were used for no longer than a week after preparation. Allyl cyanide was determined by peak height and either peak height or the area calculated from height times the width at half-maximal height was used for allyl isothiocyanate. Two or three chromatograms were run of each sample and the results averaged.

The ratio of cyanide and isothiocyanate generally differed only slightly among the analyses of a given extract. However, the total yield was not so easy to determine accurately, for the solvent tended to evaporate from the syringe needle before injection, and it was necessary to check the yield.

At 28°, allyl isothiocyanate and allyl cyanide had retention times of 12 and 4 minutes, and could be detected accurately at concentrations down to 8×10^{-4} M and 3×10^{-4} M respectively. At 40°, allyl isothiocyanate and allyl thiocyanate had retention times of 4 and 11 minutes and could be detected accurately at concentrations of 2×10^{-4} M and 5×10^{-4} M respectively. Allyl thiocyanate and allyl cyanide could not be measured at the same temperature because allyl thiocyanate had an extremely long retention time at 28° and allyl cyanide could not be separated from the solvent peak at higher temperatures.

Allyl isothiocyanate and allyl cyanide at 40° and 28° respectively were not entirely separated from the solvent, which tailed off gradually for nearly 10 minutes, but they gave sharp signals on the side of the solvent peak.

The base line was drawn as a straight line connecting the points on the solvent tail where the signal began and where it again merged with that of the solvent.

Experiments were conducted to determine the efficiency with which allyl cyanide and isothiocyanate could be extracted from aqueous solution with ethyl ether. Solutions 5-10 $\times 10^{-3}$ M were prepared and then 20-ml. portions were extracted successively with 20, 10, and 5 ml. of ether. Chromatography of the extracts showed that at least 98% of the allyl isothiocyanate was removed in the first extraction, while about 87%, 11%, and 1% of the allyl cyanide were removed in the respective extractions.

Chromatograms of the ether extracts of reactions at pH 4.0 and 4.5 contained a signal with almost the same retention time as allyl isothiocyanate. This made it difficult to establish the true height of the allyl isothiocyanate peak. When the ether phase was washed with 1 ml. of saturated sodium carbonate solution, the foreign signal was completely removed. The impurity was assumed to be acetic acid from the buffer.

An alternative method of product analysis was considered but did not prove to be as effective in general.

Treatment of allyl isothiocyanate with ammonia gave the corresponding thiourea, which can be determined spectrophotometrically[23] in very low concentrations. However, by this technique it was not possible to determine allyl cyanide directly. Also, gas chromatography was simpler and could be carried out in much less time.

Buffers

Buffer solutions were prepared at various times and their pH checked periodically. Variations were slight, and the following list is typical. Each formulation was dissolved in 100 ml. of distilled water.

1. Phosphate, pH 6.76
   0.804 g. of potassium dihydrogen phosphate
   1.60 g. of disodium hydrogen phosphate heptahydrate

2. Phosphate with hydrogen cyanide, pH 6.62
   1.0 g. of potassium dihydrogen phosphate
   0.2 g. of potassium cyanide

3. Acetate, pH 4.90
   0.2 ml. of glacial acetic acid
   0.60 g. of potassium acetate

4. Acetate, pH 4.46
   0.60 ml. of glacial acetic acid
   0.60 g. of potassium acetate

5. Phthalate, pH 4.01
   1.0 g. of potassium biphthalate

6. Acetate, pH 3.98
   1.0 ml. of glacial acetic acid
   0.30 g. of potassium acetate

7. Formate, pH 3.59
   0.20 ml. of 90% formic acid
   0.26 g. of sodium formate

8. Formate, pH 3.20
   0.45 ml. of 98-100% formic acid
   0.26 g. of sodium formate

9. Sulfate, pH 1.9
   0.32 g. of potassium pyrosulfate
   0.72 g. of potassium sulfate

Preparation of Potassium Silver Sinigrate

Two grams of sinigrin dissolved in 50 ml. of water was added to 1.8 g. of silver nitrate in 50 ml. of water and the mixture was stirred for thirty minutes at 24°. The white precipitate of silver sinigrate was filtered off, washed three times with water, twice with ethanol, and with ether, and dried in vacuum; yield 2 g. (98%). The silver sinigrate was suspended in 60 ml. of water at 24° by magnetic stirring and 0.34 g. of potassium chloride was added. After 2 hours, the reaction mixture was centrifuged at 20,000 X g to remove a fine suspension of silver chloride. The supernatant was decanted and evaporated to 35 ml. at room temperature on a rotary evaporator with an oil pump. About 30 ml.

of ethanol was added and the solution placed in a freezer. There an amorphous precipitate of potassium silver sinigrate formed, which was washed with acetone and dried under vacuum. The product weighed 1.49 g. (92% yield) and was stored at 0° over silica gel.

Potassium silver sinigrate prepared in the manner described with 10% excess of silver sinigrate contained traces of chloride, which was not removed from the sample even after repeated crystallization from aqueous ethanol. Chloride was identified as a white precipitate of silver chloride which appeared on addition of a little silver nitrate to a solution of the potassium silver sinigrate, or as the residue of silver chloride which remained after digestion of the sinigrate in boiling nitric acid.

About 600 mg. of tetra-n-propylammonium silver sinigrate was prepared on a cation exchange resin from chloride-contaminated tetramethylammonium silver sinigrate (preparation of these compounds is discussed later). The tetra-n-propylammonium salt was obtained as a thick oil, which was dissolved in 25 ml. of acetone, filtered, evaporated, taken up in water, and converted to potassium silver sinigrate on the resin. The material thus obtained

(542 mg.) was free of chloride. An analytical sample was obtained by dissolving 250 mg. of potassium silver sinigrate in 5 ml. of water and adding ethanol (about 5 ml.) until a flocculent precipitate began to form. This first precipitate was removed by centrifugation. When the supernatant was chilled very slowly, fine threads of crystals formed, which were washed with acetone and dried under vacuum. Repeated crystallization gave the analytical sample. Purification was hindered by the potassium silver sinigrate decomposing slowly in aqueous solution at room temperature.

The sample thus prepared decomposed at 120°. The n.m.r. spectrum (determined with a Varian A-60 spectrometer at 60 mc) contained a 1-proton multiplet at 350-392 cps, a 2-proton signal at 313-337 cps, and a 2-proton doublet centered at 201 cps below tetramethylsilane (J = 6 cps). The ultraviolet absorption, measured with a Beckman DU spectrophotometer, showed a molecular extinction (calculated for the dihydrate) of 10,500 at 220 and 230 mμ, 8,900 at 250 mμ, and 1,600 at 290 mμ.

Analyses for carbon, hydrogen, sulfur, and nitrogen were carried out by the Scandinavian Microanalytical Laboratories. The potassium and silver analyses were by Elek Microanalytical Laboratories.

Anal. Calcd. for $C_4H_5O_4S_2NKAg \cdot 2H_2O$:

C, 12.70; H, 2.40; S, 16.95;
N, 3.70; K, 10.22; Ag, 28.52.

Found:

C, 13.26; H, 2.61; S, 16.44;
N, 3.70; K, 9.66; Ag, 28.10.

The analyses indicated the presence of approximately two moles of water. The water of hydration was also determined by n.m.r. A standard solution was prepared of dioxane (ca. 0.046 M) in heavy water. The hydroxyl and dioxane peaks were integrated and the concentration of solvent protons calculated from the relative intensities. Then 100 mg. of sinigrate was added to 1 ml. of the dioxane solution, the hydroxyl concentration was again determined and the water of hydration was calculated from the difference. This method consistently gave 0.25-0.30 mole of water of hydration for samples that had been dried overnight under vacuum. A sample that was exposed in the laboratory overnight contained 0.6 mole of water. A possible explanation for the difference between the elemental analysis and n.m.r. results is that the analytical sample was crystalline, whereas the samples used for n.m.r. were amorphous powders.

Tetramethylammonium
silver sinigrate

Sinigrin (970 mg.) was added to 916 mg. of silver nitrate in 50 ml. of water. The silver sinigrate produced was washed three times with water and transferred to a flask which contained 1.937 mmoles of tetramethylammonium chloride in 180 ml. of water. The mixture was stirred for 1 hour at 4°, centrifuged free of silver chloride, and taken to dryness on a rotary evaporator. The residue was dissolved in 150 ml. of methanol, ethanol (30 ml.) was added, and the solution was evaporated to 100 ml. under reduced pressure. When the concentrate was chilled in a freezer, crystals formed to yield 664 mg. of tetramethylammonium silver sinigrate. This salt was readily crystallized from methanol and appeared to be more stable than the potassium salt, but was extremely hygroscopic and unsuited for use except in a dry atmosphere. The ultraviolet absorption closely paralleled the absorption of potassium silver sinigrate, but the extinction was about 10% lower, presumably because the sample absorbed moisture while being weighed.

Ammonium, Tetraethylammonium, Tetra-n-propylammonium, and Trimethylammonium Silver Sinigrates

These salts were prepared by use of ½ to 1 g. of Amberlite IR-120 cation exchange resin each. The ultraviolet absorption spectra of the crude ammonium and trimethylammonium salts were very similar to the spectrum of potassium silver sinigrate.

1. Tetra-n-propylammonium silver sinigrate: One gram of resin in the hydrogen form was converted to the tetra-n-propylammonium form by passing 10% tetra-n-propylammonium hydroxide through the column until the effluent was basic. After the resin was washed free of base, 38.6 mg. of tetramethylammonium silver sinigrate in 15 ml. of water was passed through the column at approximately 1 ml./min. The effluent was evaporated to a clear, yellow, acetone-soluble oil, which could not be induced to crystallize.

2. Tetraethylammonium silver sinigrate: Preparation was by the same method with 0.5 g. of resin and 43.9 mg. of tetramethylammonium silver sinigrate. The column was prepared with 10% tetraethylammonium hydroxide.

A clear, yellow oil was obtained, which was soluble in ethanol and could not be made to crystallize.

3. Trimethylammonium silver sinigrate: A 25% solution of trimethylamine in methanol was diluted tenfold with water and passed through a resin column until the effluent was basic. A 38.4-mg. sample of tetramethylammonium silver sinigrate was passed over the resin. The solution thus obtained was evaporated to dryness and the residue crystallized from 80-90% ethanol to yield 24 mg. of trimethylammonium silver sinigrate.

4. Ammonium silver sinigrate: One gram of resin was converted to the ammonium form with 0.75 M ammonium hydroxide, and then 68.5 mg. of tetramethylammonium silver sinigrate was converted to ammonium silver sinigrate, which crystallized from 50-60% aqueous ethanol; yield 36.6 mg.

Sinigrin Tetraacetate

Several attempts were made to synthesize sinigrin tetraacetate, a known compound,[8,24] from potassium silver sinigrate and acetobromoglucose. Reactions were carried out at room temperature and at 0°, in the dark or in ruby glassware. Reaction times ranged from 3 to 24 hours. The

solvents used were acetone, N,N-dimethylformamide, ethyl ether, and methanol. Excess acetobromoglucose[25] (m.p. 87-89°, $[\alpha]_D^{24}$ +196° in chloroform) was dissolved in the solvent and potassium silver sinigrate was suspended in this solution by magnetic stirring. After several hours, the mixture was centrifuged and the supernatant evaporated. No sinigrin tetraacetate was isolated in any of the experiments, nor could it be detected by paper chromatography.[24]

Decomposition of Potassium Silver Sinigrate

A 32-mg. sample of potassium iodide (1.92×10^{-4} mole) dissolved in a standard buffer was added to 60 mg. of potassium silver sinigrate (1.74×10^{-4} mole), also dissolved in buffer (20 ml. total volume) and contained in a 50-ml., round-bottom, screw-capped centrifuge tube. After 30 minutes, the reaction mixture was extracted with 20-, 10-, and 5-ml. portions of ethyl ether. Each extraction mixture was centrifuged and the ether layer pipetted off because the ether and water phases and the precipitates separated very slowly on standing in a separatory funnel. The combined extracts were then analyzed by vapor phase chromatography. The results are given in Table II.

T A B L E I I

RESULTS OF POTASSIUM SILVER SINIGRATE DECOMPOSITION

Buffer pH	Potassium silver sinigrate, moles x 10^{-5}	Allyl isothiocyanate (AITC), moles x 10^{-5}	Allyl cyanide (AC), moles x 10^{-5}	Log (AITC/AC)	pH - log (AITC/AC)
1.96	14.8	0.55	16.5	-1.482	3.44
3.19	15.8	5.72	11.9	-0.319	3.51
3.36	15.1	7.13	10.0	-0.149	3.51
3.36	15.8	7.18	10.2	-0.155	3.51
3.53[a]	5.34	2.36	2.25	0.021	3.51
3.53	5.38	2.46	2.62	-0.028	3.56
3.56	12.4	7.18	6.92	0.016	3.54
4.01[b]	5.7	3.77	1.21	0.496	3.51
4.07	5.9	3.68	1.07	0.539	3.53
4.08	14.5	11.3	3.2	0.544	3.54
4.08	15.8	13.9	4.00	0.540	3.54
4.41	18.8	15.9	1.90	0.919	3.49
4.43	15.1	11.6	1.41	0.914	3.52
4.44	14.6	12.7	1.53	0.918	3.52
5.09	13.1	10.5	0.29	1.570	3.52
5.09	18.1	17.2	0.40	1.633	3.46
6.80	17.4	16.8			

[a] Acetate buffer of double strength.
[b] Potassium biphthalate buffer; buffer of pH 4.07-4.08 was acetate.

After mixing the reagents in the acetate and phosphate buffers, the solution immediately became a clear yellow-orange. Then a light yellow precipitate of silver iodide rapidly appeared and settled out. In the formate buffers, the solution became a clear yellow-orange upon mixing, but the precipitate formed very slowly, over a period of 1 to 2 hours. The rate of product appearance was tested in the following way. Shortly after mixing the reagents in 20 ml. of formate buffer (pH 3.18), 1.2 g. of disodium hydrogen phosphate was added, resulting in a pH of 6.6. From the amount of allyl cyanide in the products and the known ratio at pH 3.18, the extent of the reaction that occurred at this pH could be calculated. It was found that the half-time at room temperature and pH 3.18 is roughly 25-30 seconds (see Table III).

Determination of Sulfur and Silver Iodide

The reaction of potassium iodide and potassium silver sinigrate was carried out as usual in acetate buffer, pH 4.5, or formate buffer, pH 3.4. After 30 minutes, the reaction mixtures were centrifuged and the supernatants

decanted. The formate mixture was heated before centrifugation to coagulate the colloidal sulfur. The dried precipitates were extracted with freshly distilled carbon disulfide. The extracts were evaporated to yellow residues which were weighed and recrystallized from ethanol; m.p. 115°. Known sulfur recrystallized from ethanol melted at 115°, as did a mixture with a specimen from the silver salt.

T A B L E I I I

RESULTS OF pH CHANGE, 3.18 TO 6.6

Time in seconds before change	Potassium silver sinigrate, moles x 10^{-5}	Allyl isothiocyanate, moles x 10^{-5}	Allyl cyanide, moles x 10^{-5}	Percent reaction completed
30	19.5	9.65	6.08	56
60	17.1	7.65	7.60	72
120	17.6	6.25	10.1	89

The reactions were run with 1.78×10^{-4} mole of potassium silver sinigrate. From the known ratio of

products, 55% or 9.8×10^{-5} mole and 10% or 1.78×10^{-5} mole of allyl cyanide would be expected in the formate and acetate respectively. From the formate buffer, 3.3 mg. or 1.03×10^{-4} mole of crude sulfur was recovered and from the acetate buffer, 0.7 mg. or 2.2×10^{-5} mole.

Reactions were conducted in phosphate, acetate, and formate buffers with 60 mg. of potassium silver sinigrate. After 30 minutes, the mixtures were centrifuged and the supernatants decanted. The precipitates were washed, dried, and extracted with carbon disulfide, and the insoluble residues, assumed to be silver iodide, were dried and weighed. The amounts from the phosphate, acetate, and formate buffers were 42.7 mg., 41.7 mg., and 38.9 mg., respectively. The theoretical yield of silver iodide from 60 mg. of potassium silver sinigrate is 40.6 mg., calculated on the basis of 0.25 mole of water of hydration.

Enzymatic Hydrolysis of Sinigrin

Two aqueous solutions of dialyzed enzymes were prepared, the classical myrosin[18] and the ascorbate-activated glucosinolase.[20] The solutions were analyzed for protein by the biuret method and their specific activity (in units of

μmoles of sinigrin hydrolyzed/mg. protein/min.) was assayed using the procedure described by Mabry[26] and by automatic titration.[20] The myrosin (2.6 mg. of protein /ml.) had specific activities of 0.81 without cofactor and 8.0 in 1.2×10^{-4} M L-ascorbate. The corresponding values for the ascorbate-activated glucosinolase (0.56 mg. of protein/ml.) were 0.73 and 277.

1. Hydrolysis by myrosin: Two ml. of myrosin was added to 75 mg. of sinigrin dissolved in 20 ml. of standard buffer in a 50-ml. centrifuge tube. After ½ to 3 hours, depending on the rate of hydrolysis, the reaction mixtures were extracted with 20-, 10-, and 5-ml. portions of ether. The longer reaction times were necessary at lower pH. Each extraction was centrifuged because otherwise the ether and water phases separated very slowly from the colloidal precipitates. The extracts were combined and analyzed by vapor phase chromatography.

2. Hydrolysis by ascorbate-activated enzyme: In these experiments, L-ascorbic acid was added to the buffer before the enzyme. At pH 5, 0.05 ml. of enzyme solution and 5 mg. of L-ascorbic acid, at pH 4.5, 0.10 ml. of enzyme and 10 mg. of L-ascorbic acid, at pH 4.0, 0.3 ml. of enzyme and 20 mg.

of L-ascorbic acid, and at pH 3.5 and 3.2, 20 mg. of L-ascorbic acid and 2 ml. of enzyme, were used. Table IV shows the results of the enzymatic hydrolyses.

Ultraviolet Spectrum and Half-life of the Aglucone

Measurements were carried out on a Cary Automatic Recording Spectrophotometer, Model 14, using 1-cm. cells. The reference cell contained distilled water. The reaction medium was a buffer, pH 6.6, nominally 0.05 M in potassium dihydrogen phosphate and 0.025 M in potassium cyanide.

The cell, cell compartment, solutions, and pipettes were thermostatted at the desired temperature. Two ml. of the buffer was pipetted into the cell, and 1 ml. of $2\text{-}3 \times 10^{-4}$ M aqueous potassium silver sinigrate was injected with a hypodermic syringe. The instrument, having been previously set at the desired wave length, could be turned on in three seconds. A syringe was used because the sinigrate could be added quickly and was thoroughly mixed with the buffer. The order of reagent addition was chosen because an extremely strong absorption, centered around 240 mμ, completely masked the aglucone absorption in experiments in which the buffer was added to the sinigrate with the syringe.

T A B L E I V

ENZYMATIC HYDROLYSIS OF SINIGRIN

Initial pH	Final pH	Sinigrin, moles x 10^{-5}	Allyl isothiocyanate (AITC), moles x 10^{-5}	Allyl cyanide (AC), moles x 10^{-5}	Log (AITC/AC)	pH (average) - log (AITC/AC)
A: Hydrolysis with Ascorbate-activated Enzyme						
3.22	3.19	14.6	0.882	2.12	-0.381	3.60
3.56	3.44	16.4	3.70	5.60	-0.176	3.68
3.61	3.49	13.8	3.93	5.84	-0.172	3.72
4.00	3.80	14.4	8.60	5.63	0.184	3.72
4.01	3.90	19.5	7.38	4.31	0.233	3.72
4.48	4.41	14.3	12.3	1.72	0.854	3.60
4.50	4.33	19.8	13.9	2.57	0.736	3.68
4.98	4.72	19.8	15.2	0.944	1.205	3.65
3.57	3.45	9.46	3.15	4.42	-0.147	3.66
4.06	3.96	8.90	5.00	2.10	0.348	3.66
4.44	4.36	9.14	6.40	0.94	0.833	3.57
4.96	4.86	8.90	6.4	0.26	1.40	3.51
3.56	3.51	4.66	1.74	1.98	-0.055	3.59
4.07	4.01	4.58	2.65	0.85	0.51	3.53

(continued)

T A B L E I V (continued)

Initial pH	Final pH	Sinigrin, moles x 10^{-5}	Allyl isothiocyanate (AITC), moles x 10^{-5}	Allyl cyanide (AC), moles x 10^{-5}	Log (AITC/AC)	pH (average) - log (AITC/AC)
		B: Hydrolysis with Myrosin				
3.20		18.4	4.58	13.6	-0.472	3.67
3.59		18.5	7.71	9.66	-0.097	3.69
3.98		18.9	12.2	6.50	0.273	3.71
4.46		18.3	13.7	2.15	0.84	3.62
4.90		18.1	16.2	0.85	1.28	3.62
3.56	3.52	4.75	1.74	1.98	-0.055	3.60
3.53[a]	3.50	4.75	1.94	1.87	0.016	3.50
3.51[b]	3.50	4.80	2.20	1.96	0.050	3.45

[a] Buffer double strength.

[b] Buffer quadruple strength.

This was apparently due to a complex formed when the cyanide passed through the metal syringe needle.

The removal of silver by cyanide was found to be rapid. The cyanide concentration in the reaction mixtures was 1.7×10^{-2} molar and potassium silver sinigrate was $7\text{-}9 \times 10^{-5}$ molar. Reactions were carried out in which the buffer was diluted to study its effectiveness in removing silver from the sinigrate. At 20° and cyanide concentration of 5.3×10^{-4} molar, the apparent half-life of the aglucone remained unchanged at 35 seconds, but when the cyanide was reduced to 2.7×10^{-4} molar, the apparent half-life increased to 45 seconds.

The decay of the aglucone was followed from 3 seconds to 4 minutes, until the reaction was practically complete. The half-life was calculated from the decay of absorption at several different wave lengths.

Since the reaction was assumed instantaneous, at the time of mixing the concentration of the aglucone would be one-third of the concentration of the silver sinigrate solution that had been added. By extrapolating the decay curve at a given wave length back to zero time, the total absorption was obtained. The molecular extinction of the aglucone was calculated from 220 to 290 mμ (see Table V).

T A B L E V

MOLECULAR EXTINCTIONS OF ULTRAVIOLET ABSORPTIONS

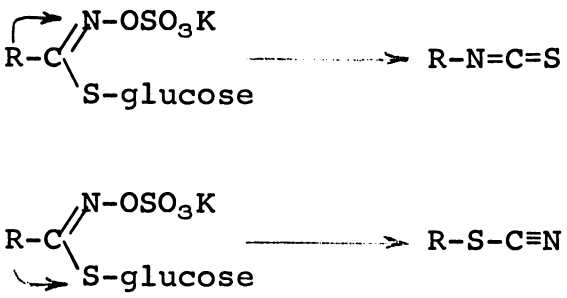
λ in mμ	Sinigrin aglucone	Potassium silver sinigrate	Silver cyanide	Allyl isothiocyanate
220	6,100	10,600	1,100	460
230	9,000	10,500	160	640
240	10,500	10,000	40	780
245	11,300	9,600		740
250	10,400	8,900		620
255	8,000			
260	5,000	6,500		230
265	2,500			
270	1,000	4,100		70
280	220	2,800		
290	50	1,600		

In these calculations, the removal of silver by cyanide was assumed to be instantaneous, occurring 3 seconds before the instrument was turned on. Since the reaction was shown to be complete in 3 seconds, the error in this assumption could not be more than that. From the rate of decay, error in time is estimated to correspond to 3-4% of the aglucone absorption per second; that is, the absorption given for the aglucone should be reduced by 3-4% if zero time was 2 seconds before the instrument was turned on instead of 3 as assumed.

P A R T T W O

The Formation of Allyl Thiocyanate in Thlaspi Arvense

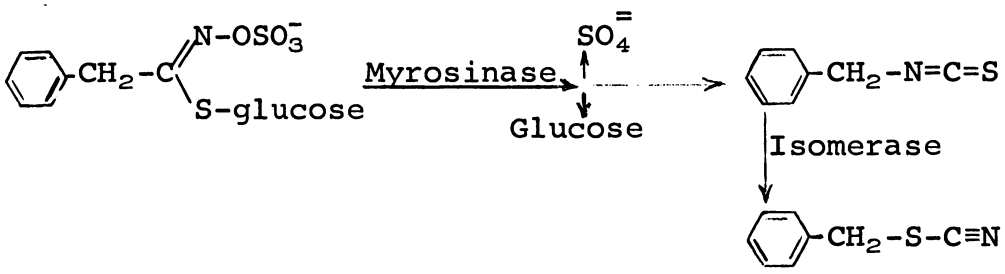
In 1959, Gmelin and Virtanen reported the formation of thiocyanates from certain plants of the mustard family, such as Thlaspi arvense, Lepidium sativum, and Lepidium ruderale.[27,28] By paper chromatography, sinigrin was the only sulfur-containing glucoside identified in Thlaspi arvense, which yielded allyl thiocyanate on aqueous maceration, while in Lepidium sativum and Lepidium ruderale, which yielded benzyl thiocyanate, the only glucoside found was glucotropaeolin. If the plant material was treated with boiling methanol, the glucoside was not degraded. The thiocyanate appeared to be derived from the parent glucoside by an enzymatic system. This unusual transformation was initially considered (scheme 1) to be possibly due to an enzyme that, after removal of glucose, directed the side chain of the aglucone to sulfur instead of nitrogen.[27,29,30]



1.

Further work [29,31,32] was performed with Lepidium sativum seed, which furnishes benzyl thiocyanate, isothiocyanate, and cyanide, on maceration. Virtanen and Saarivirta[29] found that the ratio of thiocyanate to isothiocyanate was much greater in water than in 0.5 M citrate buffer (pH 5), although the total yield remained constant. Thus, there is evidence that both products arise from the same glucoside. They also found that reactions conducted in water at 2° produced five times as much thiocyanate as isothiocyanate, but at higher temperatures the ratio diminished. When the reactions at 2° were stopped after short time intervals, it was reported that the product distribution changed considerably. After 10-15 seconds, the yields of thiocyanate and isothiocyanate were 2 and 6 mg./g. of seed, but after 60 seconds they had become respectively 6.7 and 2.5 and at 300 seconds 8.5 and 1.8 mg./g. The

reactions were carried out by adding water to ground seed in a microdistillation apparatus. After an appropriate time, the mixture was steam-distilled and the distillate analyzed.[32] In order to accommodate the new information, the following scheme was proposed.[29]



Thus, it was suggested that benzyl isothiocyanate was formed in the usual manner, but was then isomerized to thiocyanate. Virtanen[33] acknowledged that benzyl isothiocyanate added to Lepidium seed was not isomerized, but suggested that the oil did not react because it was insoluble. Attempts to isolate the thiocyanate-producing system from seed were unsuccessful. However, Virtanen has stated in a review[30] that an aqueous extract of the green parts of Lepidium ruderale can convert added glucotropaeolin to benzyl thiocyanate.

In the present work, initial experiments were carried out on ground, defatted Thlaspi arvense seed by addition to

neutral phosphate buffer at room temperature. After half an hour, the reaction was complete, and the mixtures were extracted with ether and analyzed by vapor phase chromatography. The products were allyl isothiocyanate and allyl thiocyanate. However, the product ratio was changeable. The ratio of thiocyanate to isothiocyanate was 2 for freshly ground seed, but only 0.9 for flour ten days old. The total yield remained nearly constant, but the amount of allyl thiocyanate produced had fallen to 70% of the original. Further, a sample of seed that had been ground and defatted 2 years previously produced only a third of the allyl thiocyanate of the last sample. This apparent decomposition of the "thiocyanate enzyme" was greatly retarded by storage of the seed meal at 0°. The enzyme was not reactivated by ordinary cofactors like adenosine triphosphate.

Further reactions were performed by addition of 20 mg. of ground seed, which could yield 2-2.5 μmoles of allyl compounds, to a buffer containing about 25 μmoles of sinigrin. In various experiments, allyl thiocyanate and isothiocyanate were obtained in considerable excess of 2.5 μmoles each. Thus, the added sinigrin must have been converted to thiocyanate and isothiocyanate.

A striking change was observed when D-glucoascorbic acid (usually ca. 0.002 M) was added to the reaction mixtures. In experiments without added sinigrin, the ratio of thiocyanate to isothiocyanate rose as high as 19 (Table VI, Expts. 15-19). At 24°, with sinigrin added and a reaction time of 30 minutes, the amount of thiocyanate produced was roughly doubled compared to an experiment with no D-glucoascorbic acid. The total rate of product formation was little altered (Table VII, Expts. 2 and 3). If 2-O-methyl-L-ascorbic acid was added instead, it caused no change (Table VI, Expt. 14). However, when L-ascorbic acid was included in the reaction mixture with Thlaspi meal, the ratio of thiocyanate to isothiocyanate was as high as 24 to 1 (Table VI, Expts. 9-13). With added sinigrin and reaction times of 30 minutes, the rate increased over that without ascorbic acid by at least a factor of six and about seven times as much thiocyanate was produced (Table VII, Expts. 2,5, and 6). The true rate increase by L-ascorbic acid was not determined.

In experiments with added sinigrin and D-glucoascorbic acid at 24°, it was found that if the buffer was swept with

a stream of nitrogen before the reaction, the thiocyanate produced over a 30-minute period increased by about 50%. Since the total yield was nearly the same, the effect must be directly on the system responsible for the thiocyanate-isothiocyanate distribution (Table VII, Expts. 3 and 4). If the reaction with sinigrin and D-glucoascorbic acid in air was carried out at 0° instead of room temperature, the amount of thiocyanate produced increased by 50% for a three-hour reaction period and the formation of isothiocyanate was almost entirely suppressed (Table VII, Expts. 7 and 9). In reactions at room temperature with added sinigrin, little allyl thiocyanate was produced after the first hour, but in systems containing D-glucoascorbic acid and chilled to 0°, substantial formation of thiocyanate appeared to continue after 3 hours (Table VII, Expts. 9, 11, and 12). None was produced after 12 hours and 25 μmoles was the maximum yield of allyl thiocyanate observed with excess sinigrin and 21 mg. of flour (Table VII, Expts. 12-13).

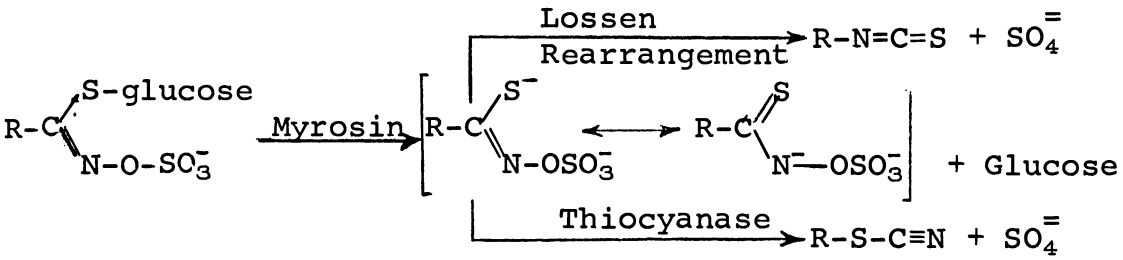
These experiments demonstrate that the enzyme leading to allyl thiocyanate in Thlaspi arvense is labile and

sensitive to oxygen. The ascorbic acids are reducing agents and protect the enzyme. Flushing the system with nitrogen removes dissolved oxygen, and lower temperature would be expected to stabilize any labile system. The rate at which isothiocyanate forms relative to thiocyanate is minor at the beginning of the reaction, but with time isothiocyanate increases until it surpasses thiocyanate at room temperature (Table VII, Expts. 3-4 and 7-8), which also indicates that the thiocyanate-producing enzyme is unstable. The difficulty encountered by Virtanen in trying to isolate this enzyme seems inherent to the system.

As was mentioned previously, the existence of an isomerase could not be studied satisfactorily with benzyl isothiocyanate because it would not dissolve. However, 0.02 M aqueous allyl isothiocyanate solutions can be prepared and hence there is no problem with solubility. Several experiments were performed by addition of Thlaspi meal to 5 ml. of neutral buffer in which 10-25 μmoles of allyl isothiocyanate was dissolved. The allyl isothiocyanate was either added to the buffer as a liquid or prepared in solution from sinigrin and ascorbate-activated glucosinolase. The results established that although endogenous

thiocyanate formation was uninhibited, the added isothiocyanate was not isomerized (Table VI, Expts. 20-22). Therefore, allyl thiocyanate cannot arise from allyl isothiocyanate in Thlaspi arvense.

Since an enzyme similar to myrosin has been extracted from thiocyanate-forming plants,[27,29] this enzyme might be thought to cleave sinigrin to the aglucone, which could form isothiocyanate by Lossen rearrangement, or could be captured by a second enzyme and converted to allyl thiocyanate. This mechanism, represented in the diagram below, was demonstrated from the products formed when the aglucone was generated independently in the presence of ground Thlaspi seed.



Twenty-seven μmoles of potassium silver sinigrate was dissolved in phosphate buffer, which contained D-glucoascorbic acid. The mixture was placed in an ice bath and flushed with nitrogen. Finely powdered potassium iodide

was added, followed promptly by a 20-mg. portion of Thlaspi flour, naturally containing 2.5 μmoles of sinigrin (Table VII, Expts. 14-16). The immediate formation of a light yellow precipitate indicated that silver iodide had formed and the aglucone was liberated. At 0°, the aglucone has a half-life of approximately four minutes and the enzyme in Thlaspi still functions at a high rate. The enzyme system was placed in immediate contact with more than ten times the amount of aglucone it normally encounters. The products recovered were 15 μmoles of allyl thiocyanate and 7 μmoles of allyl isothiocyanate. Clearly, the aglucone from potassium silver sinigrate, though it partially rearranged to allyl isothiocyanate, was rapidly converted to allyl thiocyanate.

Hence, allyl thiocyanate does not arise from allyl isothiocyanate in Thlaspi arvense, but arises directly from the sinigrin aglucone by enzymatic action. The "thiocyanase" is labile, but when preserved from decomposition can convert almost all the aglucone to thiocyanate, in competition with the spontaneous rearrangement to isothiocyanate. L-Ascorbic acid both protects the thiocyanase and accelerates the enzymatic hydrolysis of sinigrin to the aglucone.

The mechanistic conclusion is a return to Virtanen's first hypothesis from his second. The reason for his second proposal was, as already set forth, Saarivirta and Virtanen's observation[29] that when Lepidium sativum seed was treated with ice water, the yield of benzyl isothiocyanate varied with reaction time so as to show a very pronounced, rapidly transient maximum. Since the reaction was stopped and the products isolated by steam distillation,[32] which presumably would have decomposed the aglucone of glucotropaeolin to the isothiocyanate, the analytical method did not distinguish between the two molecules and the qualitative result is in fact compatible with direct genesis of the thiocyanate from either. A quantitative appraisal is clouded by the reported[31] simultaneous liberation of benzyl cyanide, in amount greater than the sum of thiocyanate and isothiocyanate. The colorimetric determination[32] of the nitrile may include other substances, however, and whether all the material detected had glucotropaeolin as precursor is unproved. More information is desirable, but the scheme now established for allyl thiocyanate formation in Thlaspi may well be general.

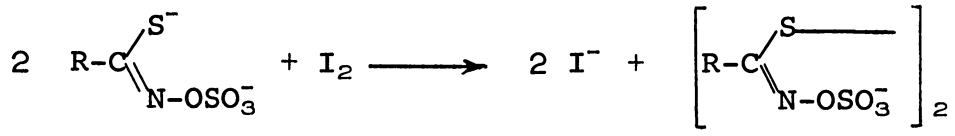
A number of experiments were carried out in attempts to prepare allyl thiocyanate non-enzymatically from potassium silver sinigrate. The sinigrate was added to an acetate buffer (pH 5) which had been chilled to 0° and contained potassium iodide and a metal salt (0.01 M). In this manner, the aglucone was generated in the presence of cations which, it was hoped, would complex with the aglucone and serve as a bridge for electron transfer that would lead to thiocyanate instead of the usual isothiocyanate.

About 50 μmoles of metal salt was used in each reaction, or about twice the amount of sinigrate. The reactions in the presence of manganese, cobalt, nickel, cadmium, or lead showed no effect of the metals and gave allyl isothiocyanate in good yield. Copper and mercuric ions formed stable salts with the aglucone, which were decomposed by potassium cyanide and yielded allyl isothiocyanate, with 42% and 16% respectively of allyl cyanide. With thallous and ferric salts, allyl isothiocyanate was formed, but only in about 60% yield. Ferrous ion converted the aglucone quantitatively to allyl cyanide. Conversion to the nitrile also occurred in enzymatic

experiments including the ferrous salt. With lower concentrations, it was seen that one equivalent of ferrous ion resulted in 2-4 equivalents of nitrile.

If potassium silver sinigrate was added to a solution of potassium iodide and iodine in phosphate buffer, allyl cyanide, allyl isothiocyanate, or allyl thiocyanate were not produced. A light yellow precipitate formed, which could be redissolved by potassium cyanide and presumably was silver iodide. The colorless solution obtained after removal of the silver iodide by filtration produced no ether-soluble material on standing overnight at 0°. If the aqueous solution was distilled, allyl cyanide and allyl isothiocyanate were obtained in roughly equal amounts.

Thus, iodine had presumably oxidized the aglucone, which dimerized to form the disulfide, a stable molecule.



The solution of the disulfide could be reduced with zinc dust, sodium borohydride, or sodium dithionite, to yield allyl isothiocyanate. Treatment with ferrous sulfate yielded allyl isothiocyanate and allyl cyanide, but no

allyl thiocyanate could be detected. Decomposition with 1 M sodium carbonate or 1 M hydrochloric acid at room temperature gave the same products.

EXPERIMENTAL

Thlaspi Arvense Seed Meal

Three grams of Thlaspi arvense seed* was crushed with a mortar and pestle and extracted three times (ten minutes each) with 45-ml. portions of petroleum ether (b.p. 30-60°). After drying in air overnight, 2.1 g. of seed meal was obtained. The meal was best stored at 0°.

Enzymatic Reactions

A weighed sample of Thlaspi arvense flour was added to 5 ml. of 0.05 M phosphate buffer (pH 6.76) in a screw-capped centrifuge tube. After standing 30 minutes at room temperature, the mixture was extracted with 10 ml. of ether and centrifuged. The ether phase was analyzed by vapor chromatography as described previously. This basic technique was used in all the experiments. If sinigrin, allyl isothiocyanate, or an ascorbic acid was used in the

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*The major sample of Thlaspi seed was generously provided by the late R. W. King, of the R. T. French Company, and had come originally from Ontario Seed Cleaners and Dealers Ltd., Toronto, Canada.

experiment, the material was added to the buffer before the solution was chilled or swept with nitrogen, and the seed meal was added at the last.

Reactions of Sinigrin Aglucone with Cadmium Sulfate, Cobalt Chloride, Copper Sulfate, Ferric Nitrate, Ferrous Sulfate, Lead Acetate, Manganese Chloride, Mercuric Chloride, Nickel Acetate, and Thallous Acetate

Ten mg. of potassium silver sinigrate (25 μmoles) was dissolved at 0° in 5 ml. of acetate buffer (pH 5) containing 50 μmoles of manganese, cobalt, nickel, ferrous, ferric, or cadmium salt. Then 6 mg. of potassium iodide was added and the solution was covered with 10 ml. of ether and allowed to stand overnight. The ether extract was analyzed by vapor phase chromatography. With the copper, mercury, thallium, or lead compounds, the salt was dissolved in half the buffer. Potassium silver sinigrate and potassium iodide were added to the rest of the buffer and the two solutions were immediately combined.

TABLE VI

EXPERIMENTS WITH THLASPI SEED, ALONE OR WITH ISOTHIOCYANATE, AS SOURCE OF ALLYL THIOCYANATE[a]

Number	Mg. of flour	Age of flour, days at room temp.	D-Gluco-ascorbic acid, mg.	L-Ascorbic acid, mg.	Allyl thiocyanate (ATC), μmoles	Allyl isothiocyanate (AITC), μmoles	ATC/AITC	Total yield, μmoles
1[b]	100	1/6			7.1	3.5	2.1	10.6
2	400	2			31.8	16.7	1.9	48.5
3[c]	200	4			16.8	7.6	2.2	24.4
4	200	5			10.8	7.0	1.6	17.8
5	100	7			6.8	5.9	1.2	12.7
6	100	7			5.7	5.0	1.1	10.7
7	100	10			5.5	6.3	0.87	11.8
8	100	ca.700			1.6	5.7	0.28	7.3
9	200	4		2.9	21.4	0.88	24.3	22.3
10	200	7		3.0	18	2.3	7.8	20.3
11	100	5		3.0	8.8	2.2	4.0	11.0
12	100	5		0.3	8.3	2.5	3.3	10.8
13	100	1/6		3.0	10.0	1.2	8.5	11.2

[a] Each experiment was performed in 5 ml. of phosphate buffer, pH 6.76. Except as noted, the reaction time was 30 min., and the temperature, 24°.

[b] Time, 26 hr.

[c] 90 min.

(continued)

T A B L E V I (continued)[a]

Number	Mg. of flour	Age of flour, days at room temp.	D-Gluco-ascorbic acid, mg.	L-Ascorbic acid, mg.	Allyl thiocyanate (ATC), μmoles	Allyl isothiocyanate (AITC), μmoles	ATC / AITC	Total yield, μmoles
14[d]	100	6			5.4	4.8	1.1	10.2
15	100	5	2.5		9.5	0.7	13.3	10.2
16	100	5	0.5		9.2	1.1	8.5	10.3
17	100	6	0.13		9.2	1.4	6.5	10.6
18	100	10	3.0		8.4	1.6	5.2	10.0
19	100[e]		3.0		9.5	0.5	19.0	10.0
20[f]	100	9		3.0	10.1	11.3		21.4
21[g]	100	9		5.0	7.8	22.0		29.8
22[f,h]	21[e]		2.0		2.6	10.0		12.6

[a] Each experiment was performed in 5 ml. of phosphate buffer, pH 6.76. Except as noted, the reaction time was 30 min., and the temperature, 24°.

[d] 2-O-Methyl-L-ascorbic acid (0.65 mg.) added.

[e] Stored at 0°.

[f] Ten μmoles of allyl isothiocyanate was dissolved in the buffer.

[g] Twenty-four μmoles of sinigrin was hydrolyzed with ascorbate-activated enzyme in the buffer before the *Thlaspi* flour was added.

[h] Reacted 3 hr. at 0°.

TABLE VII

EXPERIMENTS WITH THLASPI SEED AND SINIGRIN OR ITS AGLUCONE[a]

Number	Reaction time	Temperature	Sinigrin, μmoles	D-Gluco-ascorbic acid, mg.	L-Ascorbic acid, mg.	Allyl thiocyanate (ATC), μmoles	Allyl iso-thiocyanate (AITC) μmoles	ATC / AITC	Total yield, μmoles
1[b]	30 min.	24°	25.9	2.8		27.1	4.9	5.5	32
2	30 min.	24°	18			1.2	2.9	0.41	4.1
3	40 min.	24°	24	2.3		3.6	3.3	1.1	6.9
4[c]	30 min.	24°	25	3.0		4.5	0.53	8.4	5.0
5	30 min.	24°	26		2.2	8.0	16.0	0.5	24.0
6	30 min.	24°	26		2.4	9.2	16.0	0.57	25.2
7	3 hrs.	24°	24	2.2		4.3	9.0	0.48	13.3
8[c]	3 hrs.	24°	25	2.7		6.2	11.0	0.57	17.2
9	3 hrs.	0°	26	2.9		6.7	0.74	9	7.4
10[c]	3 hrs.	0°	18		2.5	14	3.3	4.2	17.3
11[c]	12 hrs.	0°	22	2.8		13	7.4	1.8	20.4
12[c]	12 hrs.	0°	53	2.9		28.0	7.0	4.0	35.0
13[c]	45 min.	0°	96	2.8		24.0	7.4	3.2	31.4
14[c,d]	12 hrs.	0°		2.8		17.0	4.8	3.5	21.8
15[c,d]	40 min.	0°		3.0		14.3	7.5	1.9	21.8
16[c,d]	30 min.	0'		2.7		15.0	7.2	2.1	22.2

[a] Each experiment was performed in 5 ml. of phosphate buffer, pH 6.76, using, except in No. 1, 21 mg. of Thlaspi meal that had been stored at 0°.

[b] With 100 mg. of meal, which had been stored at 0°.

[c] The buffer was swept with nitrogen beforehand.

[d] Twenty-seven μmoles (10 mg.) of potassium silver sinigrate was allowed to react with potassium iodide (5.6 mg.) in the buffer immediately before the Thlaspi meal was added.

Preparation of the Aglucone Disulfide

Nineteen mg. of iodine was dissolved with 60 mg. of potassium iodide in 5 ml. of phosphate buffer chilled to 0°. When 50 mg. of potassium silver sinigrate was added, the brown iodine color immediately disappeared and a colorless solution was obtained after filtration.

Reactions of the Disulfide

To 10 ml. of water was added 4.4 mg. of sodium borohydride or 10 mg. of sodium hydrosulfite, and 1 ml. was added to 0.5 ml. of the disulfide solution prepared above. After 1 hour at room temperature, the mixture was extracted with 2 ml. of ether and analyzed by vapor phase chromatography. In another reduction, 12 mg. of zinc dust was added to 0.5 ml. of disulfide solution and the mixture was stirred magnetically overnight while covered with 2 ml. of ether. Allyl isothiocyanate was the sole reduction product obtained.

To a 0.5-ml. aliquot of the disulfide solution was added 0.5 ml. of 2 M sodium carbonate or 0.05 ml. of concentrated hydrochloric acid. The mixture was allowed to sit 2½ hours at room temperature under 2 ml. of ether before the analysis was made.

PART THREE

Equilibration of Allyl Isothiocyanate and Allyl Thiocyanate

In order to determine if allyl isothiocyanate could be extracted with ether from aqueous solutions and analyzed quantitatively as the thiourea, several experiments of this type were performed with standard solutions. The results were consistently 8-12% low with ordinary, distilled allyl isothiocyanate. Analysis by vapor phase chromatography at 40° showed that the allyl isothiocyanate was contaminated with 7-8% of volatile material, including 5.5% of allyl thiocyanate. These calculations were made with the assumption that the mole to area ratio was the same for all peaks. Parallel analyses by nuclear magnetic resonance confirmed the proportions of allyl isothiocyanate and allyl thiocyanate. Samples of allyl isothiocyanate obtained from the Eastman Kodak Company and Matheson Coleman and Bell were found to contain 5.1% of allyl thiocyanate and 1% of allyl disulfide as the major impurities.

Allyl isothiocyanate 99.5% pure and 98% pure allyl thiocyanate were prepared. Samples were placed in ampules,

flushed with nitrogen, sealed, and heated 18 hours on a steam bath. Analysis by vapor phase chromatography and n.m.r. of the materials thus obtained showed that both contained 8.2% of allyl thiocyanate. The liquids became yellow after heating, but there was no other indication of decomposition. The heated samples, purified allyl isothiocyanate and allyl thiocyanate, and a control sample of allyl isothiocyanate that contained 5% of allyl thiocyanate, were placed in closed containers and stored in the dark at 24°. Analyses were made periodically by chromatography. The results listed in Table VIII show that for the undiluted liquids at 24°, the equilibrium composition is approximately 5% allyl thiocyanate. Also, the mixtures approach equilibrium with a half-life of 17-19 days. From the equilibrium ratios at 24° and 100°, the heat of isomerization can be calculated as 1.5 kcal./mole.

The liquids that had been heated showed no sign of decomposition during the entire storage time, but in the other three samples a yellow residue began to form after 1 or 2 weeks, and roughly half of the original sample had decomposed after 6 weeks. Chromatography showed only minor extraneous peaks.

The rearrangement was also studied in approximately 0.001 M aqueous and methanolic solutions. Within 1 or 2 days after preparation, the solutions became cloudy and a light yellow precipitate formed. Direct chromatographic analysis of these solutions disclosed that there was a steady decomposition of allyl isothiocyanate and allyl thiocyanate. The products were largely undetermined, but two peaks in the chromatograms did appear that became equal to those of allyl isothiocyanate and thiocyanate in total area after 6-8 weeks. The new peaks were identical with the pair (retention times 1 and 10 minutes at 27°) given by a commercial sample of allyl disulfide.

Solutions were prepared which contained different relative concentrations of allyl thiocyanate and isothiocyanate. Thus it was possible to estimate the equilibrium after a few days from the changes in composition. The results in Table VIII indicate that the apparent equilibrium mixture in aqueous and methanolic solutions is 35-40% allyl thiocyanate.

Aqueous solutions which contain allyl thiocyanate or allyl isothiocyanate must be analyzed without delay to

avoid rearrangement and decomposition. If such solutions are heated, the results will be altered.

A study of the equilibrium between thiocyanates and isothiocyanates by infrared and ultraviolet spectrophotometry has been reported.[34] Polar solvents were found to favor thiocyanate, but the presence of thiocyanate at equilibrium in undiluted allyl isothiocyanate was not recognized. Equilibrium at 100° for the pure liquid and in 0.001-0.1 M cyclohexane solution was given as less than 1% thiocyanate, and as 9-11% for acetonitrile solutions. In another investigation[35] of the rearrangement of allyl thiocyanate, including dependence of the rate on solvent and temperature, the reaction was assumed to go ultimately to completion. Recently, the equilibrium composition of allyl mustard oil heated at 100° has been determined by vapor chromatography at 115° to be 8-10% thiocyanate.[36] The high temperature of analysis may have caused some interconversion, as was acknowledged, for the extreme compositions observed were 55 and 96% isothiocyanate. Rates of isomerization were not measured.

T A B L E V I I I

SPONTANEOUS INTERCONVERSION OF ALLYL ISOTHIOCYANATE AND THIOCYANATE[a]

Allyl isothiocyanate, equilibrated	Time[b]	0	11	28	
	% Allyl thiocyanate[c]	5.1	5.0	5.2	
Allyl isothiocyanate	Time	0	18	37	
	% Allyl thiocyanate	0.5	2.9	3.9	
Allyl thiocyanate	Time	0	11	29	46
	% Allyl thiocyanate	98	68	41	25
Allyl isothiocyanate heated 18 hrs. on a steam bath under nitrogen	Time	0	11	28	46
	% Allyl thiocyanate	8.2	7.1	6.1	5.9
Allyl thiocyanate heated 18 hrs. on a steam bath under nitrogen	Time	0	11	28	46
	% Allyl thiocyanate	8.2	6.8	6.1	5.8

[a] Analyses were performed by vapor phase chromatography as previously described. One-μl. samples were injected of the dilute solutions.

[b] Days at 24°.

[c] Percent of isothiocyanate plus thiocyanate.

(continued)

T A B L E V I I I (continued)

Allyl isothiocyanate in dilute aqueous solution	Time[b]	0	4	21	44
	% Allyl thiocyanate[c]	5.2	11	21	35
	Total[d]	3.0	2.5	1.4	0.5
Allyl thiocyanate in dilute aqueous solution	Time	0	4	21	44
	% Allyl thiocyanate	98	81	53	46
Allyl isothiocyanate and thiocyanate in dilute aqueous solution	Time	0	4	21	
	% Allyl thiocyanate	52	51	44	
	Total	5.4	4.1	2.7	
Allyl isothiocyanate and thiocyanate in 40% methanol	Time	0	4	21	44
	% Allyl thiocyanate	22	24	30	33
	Total	8.8	7.9	4.9	1.4
Allyl isothiocyanate in 40% methanol	Time	0	4	21	44
	% Allyl thiocyanate	30	32	36	37
	Total	8.8	4.9	1.5	

[b] Days at 24°.

[c] Percent of isothiocyanate plus thiocyanate.

[d] Sum of isothiocyanate and thiocyanate areas on chromatogram, $mm.^2 \times 10^{-3}$.

(continued)

TABLE VIII (continued)

Allyl isothiocyanate and thiocyanate in 40% methanol	Time[b]		0	4	21	44	
	% Allyl thiocyanate[c]		41	40	39	39	
	Total[d]		10.3	9.6	5.3	1.8	
Allyl isothiocyanate in 40% methanol	Time	1 8	10	15	20	37	61
	% Allyl thiocyanate	1 10	10	14	17	29	34
	Total	7.7	7.7	6.2	6.4	2.8	0.9
Allyl isothiocyanate in 40% methanol	Time		0	6	10	27	51
	% Allyl thiocyanate		4.9	11	15	26	34
	Total		9.4	7.6	5.0	3.9	1.3
Allyl thiocyanate in 40 % methanol	Time	0	1	3	8	31	54
	% Allyl thiocyanate	98	93	87	73	55	44
	Total		9.1	9.5	5.4	1.6	1.1

[b] Days at 24°.

[c] Percent of isothiocyanate plus thiocyanate.

[d] Sum of isothiocyanate and thiocyanate areas on chromatogram, $mm.^2 \times 10^{-3}$.

PREPARATION OF MATERIALS

Allyl Isothiocyanate

A 50-ml. portion of allyl isothiocyanate, which contained 5% of allyl thiocyanate, was distilled through a center-rod column at 0.5 mm. A heart cut of 25 ml. that contained 2% of thiocyanate was redistilled and a 5-ml. sample of allyl isothiocyanate, b.p. 51° at 20 mm., 99.5% pure, was obtained.

Allyl Thiocyanate

Allyl thiocyanate 98% pure was prepared by the method of Gerlich.[37] Ten ml. of allyl bromide was added to 11 g. of potassium thiocyanate dissolved in 100 ml. of acetone at 0°. After 12 hours, the potassium bromide formed was filtered off and the acetone solution was concentrated with a rotary evaporator. Vacuum distillation yielded 8 ml. of clear, colorless liquid, b.p. 30° at 0.6 mm. The product was stored in a freezer.

The n.m.r. spectra of allyl isothiocyanate and allyl thiocyanate at 60 mc contained characteristic methylene signals, respectively a multiplet centered at 248 cps and a doublet (J = 7 cps) centered at 218 cps below tetramethylsilane.

B I B L I O G R A P H Y

1. A. Kjaer, Fortschr. Chem. Org. Naturstoffe, 18, 122 (1960).

2. F. Challenger, "Aspects of the Organic Chemistry of Sulphur," Butterworths Scientific Publications, London, 1959, Chapter 4.

3. A. Bussy, Compt. rend., 9, 815 (1839); J. pharm., 26, 39 (1840).

4. H. Will and W. Körner, Liebigs Ann., 125, 257 (1863).

5. J. Gadamer, Arch. Pharm., 235, 44 (1897).

6. M. G. Ettlinger and A. J. Lundeen, J. Am. Chem. Soc., 78, 4172 (1956).

7. Idem , ibid., 79, 1764 (1957).

8. A. J. Lundeen, "The Structure of the Mustard Oil Glucosides and Synthesis of the Glucotropaeolate Ion," Ph. D. thesis, The Rice Institute, 1957.

9. L. Cambi, Atti reale accad. Lincei, Rend. classe sci. fis., mat. e nat., 18, I, 687 (1909).

10. J. Waser and W. H. Watson, Nature, 190, 1297 (1963).

11. H. von Euler and S. E. Eriksson, Fermentforschung, 8, 518 (1926).

12. C. Neuberg and O. von Schoenebeck, Biochem. Z., 265, 223 (1933).

13. E. T. Reese, R. C. Clapp, and M. Mandels, Arch. Biochem. Biophys., 75, 228 (1958).

14. Z. Nagashima and M. Uchiyama, Bull. Agr. Chem. Soc. Japan, 23, 555 (1959); Nippon Nôgei-Kagaku Kaishi, 33, 1144 (1959).

15. R. D. Gaines and K. J. Goering, Biochem. Biophys. Research Communs., 2, 207 (1960).

16. R. D. Gaines and K. J. Goering, Arch. Biochem. Biophys., 96, 13 (1962).

17. S. Schwimmer, Acta Chem. Scand., 14, 1439 (1960).

18. Idem , ibid., 15, 535 (1961).

19. M. G. Ettlinger, G. P. Dateo, Jr., Barbara W. Harrison, T. J. Mabry, and Charlyne P. Thompson, Proc. Natl. Acad. Sci. U.S., 47, 1875 (1961).

20. M. G. Ettlinger and Charlyne P. Thompson, "Studies of Mustard Oil Glucosides (II)," Final Report Contract DA 19-129-QM-1689, Proj. 7-99-01-001, Simplified Food Logistics; AD-290,747, Office of Technical Services, U.S. Department of Commerce.

21. R. F. Bertolacini and J. E. Barney, Anal. Chem., 29, 281 (1957).

22. M. G. Ettlinger, personal communication.

23. Cf. A. Kjaer, J. Conti, and I. Larsen, Acta Chem. Scand., 7, 1276 (1953).

24. O.-E. Schultz and W. Wagner, Arch. Pharm., 288, 525 (1955).

25. R. L. Whistler and M. L. Wolfrom, eds., "Methods in Carbohydrate Chemistry," vol. II, Academic Press, New York, 1962.

26. T. J. Mabry, "Syntheses and Coenzymatic Activities of Analogs of Vitamin C," Ph. D. thesis, The Rice Institute, 1960.

27. R. Gmelin and A. I. Virtanen, Acta Chem. Scand., 13, 1474 (1959).

28. Idem , Suomen Kemistilehti, B 32, 236 (1959).

29. A. I. Virtanen and M. Saarivirta, ibid., B 35, 102 (1962).

30. A. I. Virtanen, Arch. Biochem. Biophys., Suppl. 1, 200 (1962).

31. A. I. Virtanen and M. Saarivirta, Suomen Kemistilehti, B 35, 248 (1962).

32. M. Saarivirta and A. I. Virtanen, Acta Chem. Scand., 17, Suppl. 1, S74 (1963).

33. A. I. Virtanen, lecture, May 1964, personal communication.*

34. A. Iliceto, A. Fava, and U. Mazzucato, Tetrahedron Letters, No. 11, 27 (1960); idem , and P. Radici, Gazz. chim. ital., 90, 919 (1960).

35. P. A. S. Smith and D. W. Emerson, J. Am. Chem. Soc., 82, 3076 (1960).

36. L. D. Kirk, L. T. Black, and G. C. Mustakas, J. Am. Oil Chemists Soc., 41, 599 (1964).

37. G. Gerlich, Liebigs Ann., 178, 89 (1875).

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*Phytochemistry, 4, 207 (1965).