Degradation of Natural Thioglucosides with Ferrous Salts

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epi-Progoitrin, the predominant thioglucoside of crambe seed meal, is degraded nonenzymically by ferrous salts to form (*S*)-1-cyano-2-hydroxy-3-butene and (*S*)-3-hydroxy-pent-4-enethionamide. The influence on yields of reactant concentrations, as well as of pH and temperature, has been investigated. Rate studies imply that second-order kinetics are involved in thionamide formation. Nitriles normally result from the action of ferrous salts on thioglucosides. Thionamide production occurs only with those thioglucosides that possess a hydroxyl group in the 2-position of an alkyl glucosinolate. This structural feature is the same which permits oxazolidinethione formation on enzymic conversion of thioglucosides.

For several years the U. S. Department of Agriculture has been carrying out research and development work on the Crucifer plant, *Crambe abyssinica*, to promote its introduction as a new crop. The industrial potential of the seed oil as an erucic acid source has been recognized and is being exploited (*Chem. Eng. News*, 1966).

The composition of crambe seed meal has been scrutinized in considerable detail with the objective of producing an acceptable feed supplement. It has much in common with rapeseed meal and, hence, shares similar problems (Associate Committee on Animal Nutrition, 1965).

Our compositional studies on crambe meal have been primarily directed to the thioglucosides, which account for 8 to 10% of the oil-free seed. Of the total thioglucosides, by far the greater amount (up to 90%), is represented by a single chemical compound, *epi*-progoitrin (I), named to show its relation to the progoitrin of rapeseed. The two differ only in configuration at the asymmetric carbon atom in the thioglucoside aglucon.

Hydrolytic breakdown of thioglucosides takes place spontaneously by the action of native enzymes when the seed meal is merely brought into contact with water. The breakdown is remarkably complex and the conditions, as well as detailed chemistry of the products, have been the subject of several reports from our laboratory (Daxenbichler *et al.*, 1965, 1966a, 1966b; VanEtten *et al.*, 1966). Two of these products, episulfide nitriles, possess a chemical structure not previously recognized among the enzymic cleavage products of thioglucosides.

Alternatives to enzymic degradation of thioglucosides have also been studied, and recently we showed that ferrous salts will attack I to form a nitrile (II) and a thionamide (III) (Austin and Gent, 1967). These reaction products are shown in Figure 1. The nitrile is the same as that produced by enzymic cleavage, whereas the thionamide has not previously been obtained from I. These authors also showed that ferrous salts gave the same products from defatted and enzyme-inactivated crambe meal.

Further studies of the conditions for this unusual reaction are reported here. The scope of thioglucoside degradation by ferrous ion and the structural features necessary to produce thionamides are also described.

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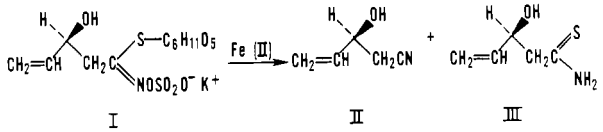


I II III

Figure 1. Degradation of *epi*-progoitrin by ferrous salts

EXPERIMENTAL

Extracts for unsaturated nitrile were assayed by the procedure of Daxenbichler *et al.* (1966a). The thionamide from *epi*-progoitrin was determined with a Beckman DK-2A recording ultraviolet spectrophotometer by measurement of the absorption at 267 mμ in ethanol solution, on the basis of the extinction value (log ϵ = 4.05) determined from a purified sample.

Reactions with Purified *epi*-Progoitrin (I). I (estimated purity 90%) was obtained from defatted crambe meal by the procedure of Daxenbichler *et al.* (1965). For the experiments described, a weighed amount of I was dissolved in water or buffer mixture and ferrous ammonium sulfate added. The solution was covered with nitrogen and allowed to stand for the requisite time period. The aqueous reaction mixture was then saturated with sodium chloride and extracted five times with 50-ml. portions of ether. After the extract had been dried over sodium sulfate, solvent was removed and the residue assayed for nitrile and thionamide. The sum of these two products normally constituted 90 to 100% of the ether-extractable substances.

Rate Study. A rate study was carried out at 25° C. Reagents were mixed and aliquots withdrawn at periodic intervals. These aqueous solutions were examined in the ultraviolet after appropriate dilution, and concentrations of thionamide were estimated from the absorption at 267 mμ.

Reactions with Defatted Seed Meals. The seed meals were covered with boiling water (700 ml. per 100 grams of meal) and heated to inactivate enzyme(s) by the procedure of VanEtten *et al.* (1965). Ferrous ammonium sulfate (50 grams per 100 grams of meal) was added to the cooled meal suspension. The mixture was covered with nitrogen and allowed to stand 16 hours at room temperature. Meal solids then were removed by centrifugation and washed with water; the combined aqueous extracts were saturated with sodium chloride. Products were obtained by thorough extraction (five 600-ml. portions) with ether. The extracts were dried, solvent was removed, and

the residue was assayed for nitrile and thionamide. If individual products were desired, they were separated on a column of aluminum oxide as previously described (Austin and Gent, 1967).

(*S*)-3-Hydroxy-3-phenylpropanthionamide. Defatted seed meal of *Barbarea vulgaris* (46 grams) was covered with boiling water (350 ml.) and heated to inactivate enzymes. Ferrous ammonium sulfate (25 grams) was added to the cooled mixture. Reaction was allowed to proceed, and separation of meal solids and ether extraction were carried out as described above. Spectral examination of the residue from the ether extraction showed the presence of both a nitrile and a thionamide (3-hydroxy-3-phenylpropionitrile and 3-hydroxy-3-phenylpropanthionamide). A portion of the residue (317 mg.) was dissolved in chloroform (5 ml.) and transferred to a column (2.5 × 11 cm.) consisting of aluminum oxide, Camag acidic (28 grams). All nitrile was removed from the column in the first eight 50-ml. fractions of chloroform eluate. Eluting solvent was then changed to chloroform-methanol (95 to 5). After an intermediate fraction (50 ml.), most of the thionamide was removed in three subsequent 25-ml. fractions. After solvent was evaporated from these fractions, the residue was extracted repeatedly with benzene at room temperature. Concentration of the benzene solution to 0.5 ml. resulted in the separation of colorless crystals, which were recrystallized from ether-benzene. Recovery was 50 mg. of product, which melted at 106–107°C. λ_{max} (EtOH): 328 mμ (log ϵ = 1.83); 267 mμ (log ϵ = 4.05).

Analysis. Calculated for $C_9H_{11}NOS$; C, 59.7; H, 6.1; N, 7.7; S, 17.7. Found: C, 60.0; H, 6.3; N, 7.7; S, 17.1.

(*S*)-3-Acetoxy-3-phenylpropionitrile. Solvent was removed from a nitrile-containing fraction from the column separation of products from *Barbarea vulgaris*. To the residue (60 mg.) was added pyridine (1 ml.) and acetic anhydride (0.5 ml.). The reaction mixture was kept in a bath at 42° C. for 1 hour, after which ice was added. The precipitate which separated was collected by filtration and washed with water. The nitrile acetate was recrystallized from 95% ethanol (1.5 ml.) to yield colorless crystals (50 mg., 65%) which melted at 124.5–125° C.

Analysis. Calculated for $C_{11}H_{11}NO_2$; C, 69.8; H, 5.8; N, 7.4. Found: C, 69.7; H, 6.0; N, 7.4.

The acetate (29 mg.) was hydrolyzed by refluxing for 1 hour with 4*N* sodium hydroxide (5 ml.). The hydrolysis solution was cooled, acidified, and extracted with ether to give cinnamic acid (20 mg., 87%), identified by mixed melting point and ultraviolet absorption spectrum.

RESULTS AND DISCUSSION

Ferrous Salt Requirements at Room Temperature. As originally carried out, a ferrous salt (usually ferrous ammonium sulfate, but ferrous sulfate also served) was added to an aqueous solution of I. The reaction medium was covered with nitrogen to prevent premature oxidation of ferrous ion and allowed to stand overnight. Not only is the reaction slow, but also an excess of ferrous salt is necessary for significant yields. After an arbitrary reaction time (16 hours), the solution was saturated with sodium chloride and products were recovered by ether extraction. The weight ratio of III to II was approximately 4 to 1. The effect of varying the amount of added ferrous ammonium sulfate is shown in Figure 2. Yields are reported as per cent of theoretical based on added I.

Yields of II and III approached limiting values as the amount of ferrous salt was increased. From six to eight molar equivalents of ferrous ammonium sulfate per mole of I was generally chosen as an acceptable compromise. Ferrous ion was specific for the reaction. Cobaltous, nickel, stannous, and ferric ions had no effect on I. Hydroquinone also failed to attack this thioglucoside.

Concentration Effects at Room Temperature. The concentration of reactants also influenced the course of the reaction. For a fixed molar ratio of ferrous ammonium sulfate to I (7 to 1), the yield of III decreased considerably in more concentrated solutions. At the same time, the yield of II remained constant or increased moderately. This effect is shown in Figure 3. In concentrated solutions, the thioglucoside suffered complex degradation and decomposition. Elemental sulfur was released and deep colors developed. These changes have not yet been explored in detail.

Duration of Reaction at Room Temperature. For purposes of yield comparisons, all the reactions so far described were extracted and assayed at the end of 16 hours. In actual fact, however, thionamide production continued, reaching a maximum value at about 25 hours, after which a slow loss of product occurred. Figure 4 shows the effect of reaction time on formation of III. Spectral examination of the reaction medium after ex-

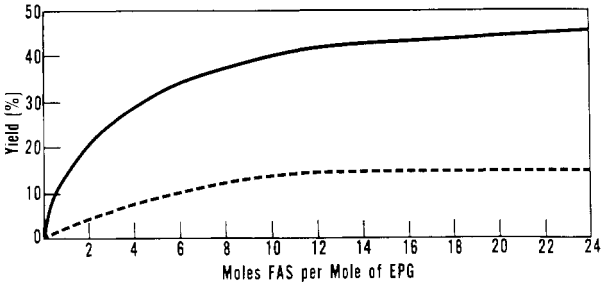


Figure 2. Yields of thionamide and of nitrile from *epi*-progoitrin (EPG) *vs.* amount of ferrous ammonium sulfate (FAS)

Yield calculated as per cent of theory based on initial EPG concentration (8.4 m*M*). Reaction conditions, 16 hours at room temperature

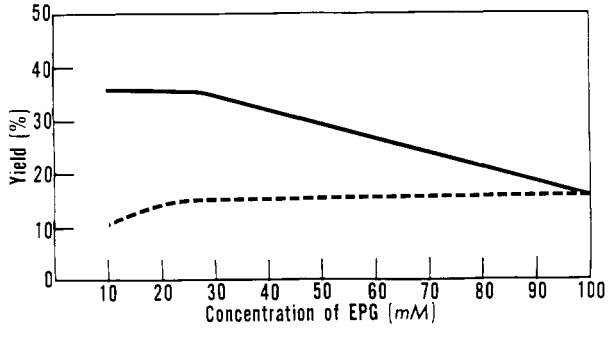


Figure 3. Yields of thionamide and nitrile from EPG as function of concentration

Yields calculated as per cent of theory based on amount of EPG used. Fixed initial molar ratio of FAS to EPG (7 to 1). Reaction conditions, 16 hours at room temperature

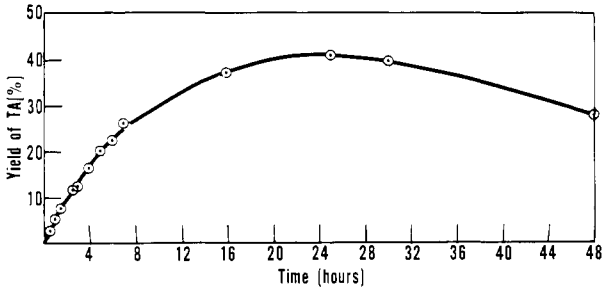


Figure 4. Yield of thionamide (TA) *vs.* duration of reaction

Initial EPG concentration (8.4 m*M*). Molar ratio FAS to EPG (7 to 1). Room temperature

traction of III and II gave qualitative evidence that unchanged I (λ_{max} = 227 mμ) was still present at 16 and 19 hours. It was undetectable after 23½ hours. The later stages of the reaction were complicated by competition between thionamide formation and its attrition.

Effect of pH. The reaction of ferrous salts with I at room temperature occurred over a range of pH values. Yields from solutions buffered at pH values from 1.4 to 5.4 were nearly the same, except for a reduction in thionamide content in the most acidic solution, probably the result of secondary decomposition. These data are summarized in Table I.

Rate Study. The first 7 hours of reaction time at 25° C. were selected to permit the establishment of an initial rate before side reactions became a complicating influence. During these 7 hours, the *epi*-progoitrin concentration was an inverse function of time (Figure 5), suggesting that second-order kinetics were involved. No spectral evidence was observed for the presence of an intermediate involving ferrous ion, although the transient existence of such a species cannot be excluded. The second-order rate constant at 25° C. was 5.6 per millimole per hour.

Temperature Effects. Initial investigations of the effect of temperature showed that the reaction of I with ferrous salts was practically inhibited at 5° C. At higher temperatures a slightly greater proportion of II was produced, but III remained the major product. At the extreme temperature investigated, 95° C., maximum yield of III was attained in 14 minutes, at which time the reaction mixture was cooled and extracted, and the products were assayed. The increased rate at the higher temperature was, of course, expected, but additionally 1 mole of ferrous salt per mole of I was sufficient. Table II shows that an excess of the metal salt did not improve thionamide yields, in contrast to the results at room temperature. Similar degradation of

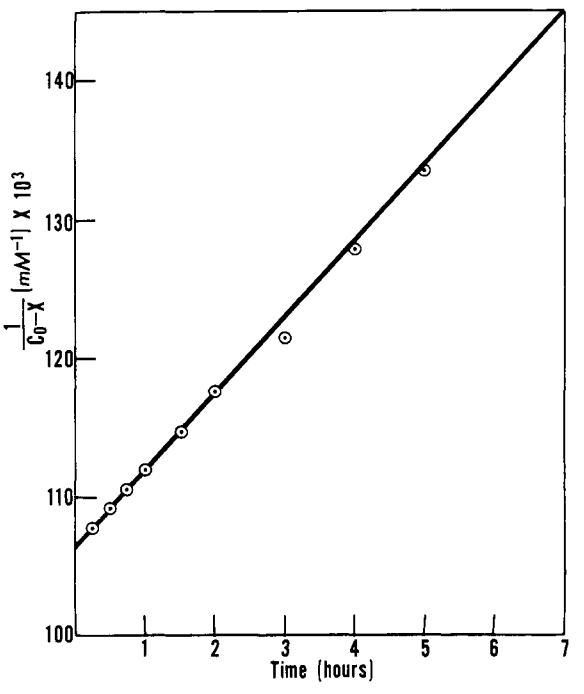


Figure 5. Reciprocal of EPG concentration as a function of time at 25° C.

C_o = initial EPG concentration (9.40 m*M*). X = thionamide concentration (m*M*) at time, t

sinigrin by ferrous sulfate at 95° C. was recently reported (Youngs and Perlin, 1967). In this instance, allyl cyanide was the sole product extracted by organic sovlents. The degradation of I, like that of sinigrin, was ferrous ion catalyzed at 95° C. We conducted the reaction with 0.5 and 0.1 molar equivalents of ferrous ammonium sulfate with results similar to those reported in Table II. The sole difference was a slower reaction time to attain maximum yields—namely, 30 and 90 minutes, respectively.

No unchanged I remained after reaction with ferrous ammonium sulfate at 95° C. This conclusion was evidenced by absence of an absorption band at 227 mμ, by paper chromatography, and by the lack of further reaction when either additional ferrous salt or mustard myrosinase was introduced. From 90 to 100% of the total ether-extractables was accounted for by the nitrile and thionamide contents. Accordingly, all other products of the nonenzymic breakdown of thioglucosides were water-soluble.

The most significant observation from all the variations investigated, whether ferrous salt concentration, temperature, time, or pH, is that yields reach a limiting value.

Table I. Reaction of *epi*-Progoitrin and Ferrous Ammonium Sulfate[a] in Buffered Solutions at Room Temperature

Solution pH	Yield[b] Nitrile, %	Yield[b] Thionamide, %
5.4	16	39
2.8	15	35
1.4	14	13

[a] Molar ratio ferrous ammonium sulfate to *epi*-progoitrin, 7 to 1. [b] Yield as per cent of theory based on initial *epi*-progoitrin concentration (8.4 m*M*).

Table II. Effect of Added Ferrous Ammonium Sulfate (FAS) on Product Yields at 95° C.[a]

Moles FAS Added per Mole of EPG	Yield[b] Nitrile, %	Yield[b] Thionamide, %
1	23	32
3	24	35
6	25	36
12	24	36

[a] Reaction time 14 minutes. [b] Yield reported as per cent of theory, based on initial *epi*-progoitrin (EPG) concentration (21 m*M*).

Under optimum conditions this value ranges from 50 to 60% for the sum of nitrile and thionamide.

Attention was next directed to ascertaining whether thioglucosides other than I also were attacked by ferrous salts under mild conditions. Both isolated thioglucosides and defatted seed meals were investigated under conditions which had been developed for I and crambe. The findings with isolated thioglucosides are summarized in Table III.

Glucotropaeolin was selected, since it should have given rise to the known phenyl thioacetamide if a reaction took place in the same way as with *epi*-progoitrin. However, only benzyl cyanide was detected.

Corresponding data for seed meals are given in Table IV.

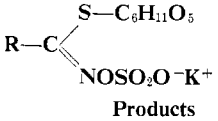
The information in Tables III and IV suggests that thioglucosides can be placed in two categories with respect to their degradation by ferrous salts. In one, the ether-soluble products consist of nitriles only; in the other, of both nitriles and thionamides. The distinguishing structural characteristic of this second category is the presence of a hydroxyl group in the 2-position of the alkyl glucosinolate. This structural feature is precisely the same one that permits cyclization to oxazolidinethiones during enzymic cleavage (Kjaer, 1963).

Whenever thionamides were produced, they were isolated and characterized. The (*S*)-3-hydroxypent-4-enethionamide from crambe has been described (Austin and Gent, 1967). Its optical isomer from *Brassica napus* (rapeseed) has the same melting point but opposite optical rotation (Austin *et al.*, 1968). A new compound is that from *Barbarea vulgaris* (Figure 6).

This product, (*S*)-3-hydroxy-3-phenylpropanthionamide, was isolated in the same manner as the corresponding compound from crambe. Stereochemistry was assigned by analogy with the related oxazolidinethione, for which absolute configuration has been established (Kjaer and Gmelin, 1957, 1958).

The cyano compound that accompanied the thionamide from *Barbarea vulgaris* was confirmed as 3-hydroxy-3-phenyl propionitrile by formation of the acetate and hydrolysis (with simultaneous dehydration) to cinnamic acid.

Table III. Reaction of Purified Thioglucosides and Ferrous Ammonium Sulfate

Thioglucoside formula: 

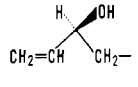
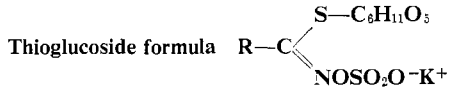
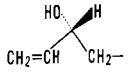
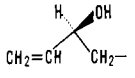
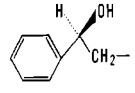
Thioglucoside	R	Products: Nitrile	Products: Thionamide
Glucotropaeolin		+	–
Sinigrin	$CH_2=CHCH_2-$	+	–
epi-Progoitrin		+	+

Table IV. Reaction of Thioglucosides in Seed Meals with Ferrous Ammonium Sulfate

Thioglucoside formula: 

Plant Species	Major Thioglucoside	R	Products: Nitrile	Products: Thionamide
Brassica nigra	Sinigrin	$CH_2=CHCH_2-$	+	–
Brassica napus	Progoitrin		+	+
Crambe abyssinica	*epi*-Progoitrin		+	+
Barbarea vulgaris	Gluco-barbarin		+	+

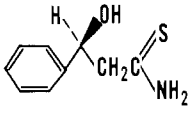


Figure 6. Thionamide from *Barbarea vulgaris*

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