Preparation of Crystalline Isouramil from Convicine by Acid Hydrolysis and Isolation of Two Decomposition Products of Isouramil

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A procedure was developed for the preparation of crystalline isouramil [IU, 6-amino-5-hydroxy-2,4(1H, 3H)-pyrimidinedione] from the glycoside convicine (CV) by acid hydrolysis. IU was not contaminated with CV, glucose, or any of the decomposition products. It was characterized by its ultraviolet absorbency pattern, HPLC elution profiles, factors affecting its stability and solubility, and its biological activity. In addition, two decomposition products of IU were isolated. One compound, referred to as compound X, was isolated in crystalline form and was shown to undergo reversible oxidation–reduction reactions in the presence of oxygen and a reducing reagent. The other crystalline compound (Y) was the final product of the oxidative decomposition of IU. A proposed sequence of intermediates in the conversion of IU of Y is given. The procedure for the isolation of IU and two of its decomposition products should facilitate further structural analysis and biological studies on these compounds.

IU is the aglycon of the glycoside convicine (CV) and has an empirical formula of $C_4H_5N_3O_3$ and a molecular weight of 143.10 (Mager et al., 1980). CV and its analogous compound vicine are found in faba beans (*Vicia faba*) and have been shown to be responsible for the human metabolic disease favism (Mager et al., 1980; Chevion et al., 1982; Arese et al., 1981). The parent compound CV is hydrolyzed by the intestinal anaerobic microflora to produce IU (Frohlich and Marquardt, 1983; Hegazy and Marquardt, 1984), which is absorbed by the blood. In the presence of an abundant supply of oxygen it autocatalytically forms the superoxide free radical (Albano et al., 1984), which if not neutralized by the free-radical scavenging system will cause irreversible cell damage (Chevion et al., 1982; Yannai and Marquardt, 1985; Winterbourn et al., 1986; Arbid and Marquardt, 1988). In addition to causing favism in humans, IU has been shown to affect egg size and fertility and hatchability in the laying hen (Muduuli et al., 1982), to cause diabetic like signs in rats (Rocic et al., 1985), and to inhibit the growth of the malarial parasite (*Plasmodium falciparum*) (Golenser et al., 1983; Clark et al., 1986).

Davidson and Bogert (1932) first synthesized IU using isobarbituric acid as the starting material. It was subjected to nitrosation or coupled with the (p-chlorophenyl)diazonium salt, and the reaction products were reduced with $(NH_4)S$ to yield IU. McOmie and Chesterfield (1956) modified the procedure by replacing the reducing reagent ammonium sulfide with sodium dithionite and thereby greatly increased the yield, as well as the final purity of the product. This synthetic route was further facilitated by the synthesis of isobarbituric acid from 5-bromouracil (Wang, 1959a,b). The procedure of Davoll and Laney (1956) for the synthesis of divicine can also be used for the syntheses of IU. The method is the same as that for the synthesis of divicine except guanidine was replaced by urea. IU may also be synthesized from CV by enzymatic hydrolysis with β-glycosidase (Herissey and Cheymol, 1931; Mager et al., 1965). This later procedure yields only small amounts of IU as CV is extremely insoluble at the pH of hydrolysis (Marquardt et al., 1983) and, therefore, is useful

for production of only small amounts of IU for in vitro studies. Finally, IU may be prepared from CV by acid hydrolyses. Ritthausen (1896) prepared crude IU from CV by acid hydrolysis, but factors affecting yield and purity of the preparation, particularly with regard to presence of decomposition products, were not established and certain crucial steps in the procedure were not given. A simple procedure is needed to prepare IU in view of the low yields and quantity of IU obtained by enzymatic hydrolysis of CV and the rather involved procedure for IU preparation by the synthetic routes. This compound is not commercially available, yet it has many interesting biological properties that warrant further studies. The development of a procedure for the preparation of IU by acid hydrolysis will be facilitated by the ready availability of CV easily isolated in crystalline form from faba beans (Marquardt et al., 1983; Arbid and Marquardt, 1985).

The objectives of this study were to develop an acid hydrolysis procedure for preparing IU from CV in relatively high yields and purity, to establish the purity, biological activity, and some solubility and stability characteristics of the purified IU, and to identify and isolate some of its intermediate decomposition products.

MATERIALS AND METHODS

Materials and Preparation of Solutions. All chemicals were from Sigma Chemical Co. (St. Louis, MO), from Fisher Scientific Co. Ltd. (Winnipeg, MB), or as given in Marquardt and Frohlich (1981). CV was prepared as described by Marquardt et al. (1983). Dithiothreitol (DTT) was prepared fresh daily as a 600 mM solution in water. A stock solution of IU (200 mg/100 mL) in degassed 0.1 N HCl at 2 °C was prepared twice per day and was generally used within 10 min of preparation in those studies where oxidative decomposition was to be minimized. Other solutions were prepared as subsequently described. Degassed solutions (treated with oxygen-free nitrogen followed by sonication) were used for all aqueous preparations.

Standard Procedure for Preparing IU. The following procedure was selected on the basis of several preliminary experiments optimizing conditions for yield and purity of IU. CV (4 g) was added to 10 mL of hot (98 °C) 12 N H_2SO_4 in a glass tube (Pyrex, 16 × 125 mm). The suspension was heated until it became clear (approximately 1.5 min) plus an additional 1 min, the total incubation time being approximately 2.5 min. It was immediately cooled in an ice bath with gentle mixing so as to facilitate a rapid

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decrease in temperature. The preparation was diluted with five volumes of cold degassed water (50 mL) and transferred to a 250-mL centrifuge tube. Aliquots of the water were used to rinse the glass tube. A copious precipitate formed within a few minutes, and this was harvested after 1 h by centrifugation at 13000g for 20 min at 2 °C. The precipitate was washed twice with six volumes of degassed water (2 × 60 mL) by resuspending the precipitate and recentrifuging the sample. An ethanol wash may be included if the sample is to be dried under nitrogen. The supernatant fractions were discarded. The entire procedure can be scaled up by increasing the number of hydrolysis tubes. The volume of H_2SO_4 (10 mL) and amount of CV (4 g) per tube should not be changed as this may alter rate of hydrolysis and therefore affect yield and purity. The precipitates, however, can be pooled after dilution of the hydrolysate with water. The washed precipitate was frozen (−70 °C) and then dried overnight in a freeze-drier. IU was pulverized with the aid of a mortar and pestle, and the product was stored in a darkened and sealed container at −20 °C. The weight of the sample was recorded, and the molar absorbance coefficient in 0.1 N HCl at 281 mM was determined. Two isolations were prepared, with a total of 24 g of CV (six tubes × 4 g/tube) being hydrolyzed each time.

Crystalline IU was prepared by allowing a near-saturated solution of IU in water (final pH ≈5) containing 6 mM DTT at 80 °C to crystallize at 2 °C. Undissolved IU can be removed from the 80 °C solution by filtration as described in the solubility studies. After the mixture was allowed to stand for 2 h, a 4% volume of ether was placed on the top of the solution after which crystals formed at the interphase. These crystals were prepared for photography after 4 h. The aqueous suspension was removed with a Pasteur-type pipet, the crystals were washed twice with acetone, transferred to the aluminum stubs, and photographed within 2 h of preparation.

For comparative purposes IU was also prepared by enzymatic hydrolysis of CV in a manner identical with that for vicine (Marquardt et al., submitted) except the amount of CV hydrolyzed was 15 mg/100 mL of pH 5.5 buffer.

Isolation of a Yellow Decomposition Product (X) and Preparation of the Final Decomposition Product (Y). A yellow decomposition product of IU (referred to as compound X) was prepared by adding CV (300 mg/mL) to hot (98 °C) 5 N H_2SO_4 as described in the standard procedure for preparing IU except a 15-min clarification period was required and the sample was left in the hot acid (98 °C) for an additional 6 min (21-min total incubation period). This procedure yielded a heat stable compound. After removal of floating debris in the hot hydrolysate by filtration through glass wool, the filtrate was cooled in an ice bath and allowed to stand for 1 h during which time a yellow crystalline precipitate formed. The precipitate was harvested by centrifugation at 40000g for 15 min at 2 °C, was washed twice with 10 volumes of water relative to amount of original CV suspension, and then freeze-dried, ground, and stored in a sealed darkened container at 20 °C until use. Compound X was prepared for electron microscopy by using a procedure similar to that described above except the acid was removed from the crystalline material by decantation and careful draining followed by washing the precipitate with water and gently stirring for approximately 3 min. After the solution was decanted and drained, the suspension was rinsed three times with acetone using the same procedure.

The yellow product isolated in a reduced state can be converted to the oxidized form by treatment of X (200 mg

of X/100 mL of 0.1 N HCl) with air for 2.5 h at 25 °C.

The final decomposition product of IU (referred to as compound Y) was prepared by bubbling air through a solution of IU (250 mg/100 mL of pH 7.0, 0.05 M sodium phosphate buffer) for 2 h. HPLC analysis was carried out to ensure that the conversion went to completion. These solutions were used directly for most analyses. To prepare crystalline Y, IU (200 mg) was added as a suspension to 5 mL of water, the pH was adjusted to 7.0 with 2 N NaOH, and air was passed through the sample at 25 °C. The preparation was treated with air (36 h) until only Y, as shown by HPLC analysis, was present. The IU suspension solubilized over a 20-h period, which reflected its conversion to Y. The sample was concentrated to a volume of 3 mL and then left at 2 °C for 4 days during which time crystals of Y formed. Water was removed by suction; the crystals were washed with acetone and were photographed.

HPLC Analysis. HPLC analysis using reversed-phase chromatography at pH 2.0 (0.05 M ammonium phosphate buffer) was as described previously (Marquardt and Frohlich, 1981). HPLC analysis was also carried out on a sulfonic acid cation-exchange column. The system of analysis was identical with that described by Marquardt and Frohlich (1981) except the HPLC stainless steel column (250 × 4.6 mm i.d.) was prepacked with Ultracil-CX (Beckman, Fullerton, CA). The elution buffer for this column was 10 mM hexanesulfonic acid and 25 mM ammonium phosphate buffer (final pH 2.0). Compounds elating from the cation-exchange column were monitored at either 220 or 281 nm by an ISCO Model 1840 absorbance monitor (Lincoln, NB) while those eluting from the reversed-phase column were detected with an ISCO Model UA-5 multiwavelength absorbance monitor. An absorbency filter was used with the UA-5 monitor so that the maximum absorbency was in the region of 280 nm.

All compounds analyzed in Figure 2 were prepared in 0.1 M HCl (200 mg/100 mL) and diluted 20-fold with the pH 2.0 eluting buffer except for CV. The initial concentration of CV was 15 mg/100 mL, and it was diluted 6-fold with eluting buffer prior to injection. The attenuation and threshold on the recorder were changed so that the peak height of the CV sample was enhanced 3.7-fold relative to the other compounds. Final amounts of sample injected were 2.0 μg/20 μL for all compound except CV (0.5 μg/20 μL). Temperature was ambient and flow rate 1.8 mL/min.

IU (200 mg/100 mL) for compounds analyzed in Figure 4 was prepared in 0.05 M sodium phosphate buffer (pH 7.0) and treated with air for different periods of time. DTT was added to an aliquot of the IU solution to a final concentration of 80 mM after 20-min incubation with air and allowed to stand 10 min at 25 °C. All samples were then diluted 20-fold with eluting buffer and immediately injected into the column. The flow rate was 1.8 mL/min. The amount of IU or its equivalent injected was 2 μg/20 μL for all treatments except that in frame E where the amount was 1 μg/20 μL.

Ultraviolet Spectra. The absorption spectra of IU and related compounds were determined with a Model DU8 spectrophotometer (Beckman). Stock solutions of IU, X-red, and Y used in Figure 3A were prepared in 0.1 N HCl (200 mg/100 mL) and diluted 150-, 100-, and 100-fold, respectively, with 0.1 N HCl. For Figure 3B, X-red was prepared in degassed 0.1 N HCl (200 mg/100 mL) and X-ox was prepared from X-red by passing air through the sample for 2.5 h at 25 °C. Both samples were diluted 100-fold with 0.1 N HCl and scanned. DTT (6 mM) was also added to aliquots of the diluted samples, and after 10 min they were rescanned. Blanks contained DTT (5 mM).

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Table I. Properties of IU and Its Decomposition Compounds

compd	HPLC elution time,[a] min	abs max, nm	abs ratio in 0.1 N HCl (281/220 nm)	molar abs, M^{-1} cm	effect of oxygen	effect of reducing reagent (DTT)
convicine	1.85	271[b]	3.0	17400[b]	nil	nil
IU-red	1.96	281 (280)[c]	3.8	13000 (13600)[c]	decompn to Y or X-ox[d]	protective[e]
IU-ox	1.82	279 (255)[f]			decompn to Y or X-ox[d]	conversion to IU-red
X-red	1.82	271, <200	0.4	2600[g]	decompn to Y or X-ox[d]	protective[e]
X-ox	1.80	<200	0.02		decompn to Y[d]	protective, conversion to X-red[e]
Y	1.52	<200	<0.01		nil	nil

[a] Elution time on a cation-exchange column. [b] Bein et al. (1968). Value determined at 271 nm. [c] Determined at 281 nm (pH 1.0). Bracketed value determined at 280 nm, pH 1.0, by Davoll and Laney (1956). [d] At pH 7.0, IU, IU-ox, X-red, and X-ox appear to be converted to Y. At low pH values IU-red yields X-red. [e] Protective against oxidative decomposition. [f] Value at pH 7.0. Bracket value from Chevion et al. (1982), pH 7.0. [g] Estimated at 271 mn assuming a molecular weight equal to that of the parent compound IU (143 Da).

For Figure 3C, stock solution of IU was diluted 150-fold with 0.05 M sodium phosphate buffer (pH 7.0) and air was bubbled through the diluted sample for a period of up to 25 min. Scans were run at 0, 5, 10, and 25 min after treatment with air.

Stability of IU. IU (7.4 mg/mL) was dissolved in water (final pH with IU was 3.7), 6% perchloric acid (pH 0.4), 0.05 M ammonium phosphate buffer used for HPLC analysis (pH 2.0), and 0.05 M sodium phosphate buffer (pH 7.2). The solutions were incubated for different time periods at 25 °C, and IU was analyzed for percent decomposition by monitoring for decreases in optical density at 281 nm after appropriate dilutions (40-fold) with 0.1 N HCl. The stability of IU (7.4 mg/mL) was also established at 0, 25, 40, and 60 °C after incubation in water (final pH 3.7) for periods up to 2 h. All studies were replicated twice.

Solubility of IU. A severalfold excess of IU was added to water (final pH after IU solubilization was 3.7) or 0.05 M sodium phosphate buffer (final pH 7.2) that had been preequilibrated to 1 and 25 °C. The suspensions were mixed for periods of 5, 15, and 30 min. One minute prior to removal of an aliquot, mixing was stopped to allow excess IU to settle and an aliquot was passed through a 13-mm cellulose acetate filter having a pore size of 0.45 μM (Millipore, Bedford, MA). Appropriate dilutions (100- or 200-fold) were made with 0.1 N HCl, and the absorbency at 281 nm was determined. The concentration of IU was calculated assuming a molar absorbance coefficient of 13600 M^{-1} cm. The study was replicated twice.

Other Analyses. IU was tested for its reaction with blood glutathione, free glutathione, and blood hemoglobin (Arbid and Marquardt, 1986, 1988) and for its reaction with ammoniacal FeCl$_3$ (Bendich and Clements, 1953). Toxicity studies were as described by Arbid and Marquardt (1986). Glucose analysis was carried out with the glucose oxidase method following the instructions of the manufacturer (Bulletin 510, Sigma). The crystals of IU, X, and Y were photographed at magnifications of 60-, 100-, and 2000-fold, respectively, on a Cambridge Stereo Scan Mark 2 electron microscope. The procedure was as described previously (Marquardt et al., 1989).

RESULTS AND DISCUSSION

Preliminary Studies on the Preparation of IU and Its Decomposition Products. These studies demonstrated that CV was initially insoluble in either hot or cold H$_2$SO$_4$ but that a suspension of CV in hot H$_2$SO$_4$ (98 °C) became clear with time. The time required for clarification of the suspension was dependent on the concentration of both H$_2$SO$_4$ and CV. For example, 30, 10, 3, 0.9, and 0.5 min were required, respectively, to convert a suspension of 200 mg/mL of CV to a clear solution in 2, 5, 8, 12, and 16 N H$_2$SO$_4$ at 98 °C. Clarification time at a given H$_2$SO$_4$ concentration also tended to change in proportion to the concentration of CV.

Reversed-phase chromatography following acid hydrolysis yielded four peaks tentatively identified as being unhydrolyzed CV, the reduced form of IU, the oxidized form of IU, and compound X and compound Y. These studies demonstrated that IU was rapidly converted to compound X in hot acid and that compound X was relatively stable under these conditions. Therefore, short hydrolysis times in concentrated H$_2$SO$_4$ at 98 °C and at high concentrations of CV yielded predominantly IU whereas longer incubation times yielded compound X. For example, incubation of 3-4 g/mL CV in 12 or 16 N H$_2$SO$_4$ at 98 °C for 2.5 min yielded predominantly IU whereas incubation of 2-3 g/mL CV in 5 or 8 N H$_2$SO$_4$ at 98 °C for more than 20 min resulted in the production of predominantly compound X and essentially no IU.

An interesting observation was that IU but not the other contaminants precipitated from solution when the 12 N H$_2$SO$_4$ hydrolysate containing 400 or more mg of CV/mL was diluted with 2.5-10 volumes of degassed water. This property was utilized in the preparation of pure IU. These initial studies demonstrated that the highest purity and yield of IU are obtained when high concentrations of both acid and CV are hydrolyzed at 98 °C for short periods of time, the prerequisite being that the hydrolysate be rapidly cooled 1 or 2 min after the hydrolysate becomes clear and the hydrolysate be diluted with 5 volumes of cold deaerated water to permit precipitation of IU from solution.

Other studies demonstrated that compound Y was formed by incubation of either IU or compound X in the presence of air (oxygen) in an aqueous solution at a neutral but not an acid pH.

Crystalline Structure, HPLC Elution Patterns, and Ultraviolet Spectra of IU, Compound X, and Compound Y. IU, compound X (reduced form), and compound Y were prepared in crystalline form (Figure 1) by procedures outlined in Materials and Methods. Each compound exhibited a unique crystalline structure.

HPLC analyses using both the reversed-phase column (chromatograms not shown) and the cation-exchange column (Figure 2) of freshly prepared solutions of the purified crystalline forms of CV, IU-red, X (reduced form), and Y revealed only a single symmetrical peak at both 281 and 220 nm for each compound. Elution times for each compound from the cation-exchange column are given in Table I.

CV and glucose, one of the hydrolytic products of CV, were not detected in any of the preparations. Also, HPLC analysis indicated that there was no or very little cross-contamination of IU, compound X, and compound Y in crystalline preparations of each of these compounds.

The ultraviolet absorption spectra of IU, X (reduced form), and Y in 0.1 N HCl (Figure 3A) demonstrate that they have different absorbency maxima (281, 271, and <200 nm, respectively) with the ratio of absorbency at
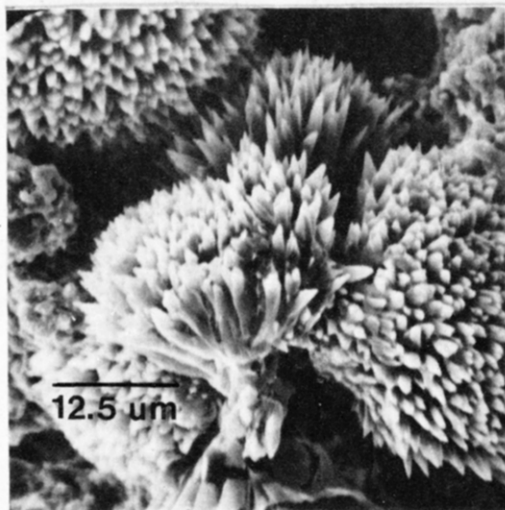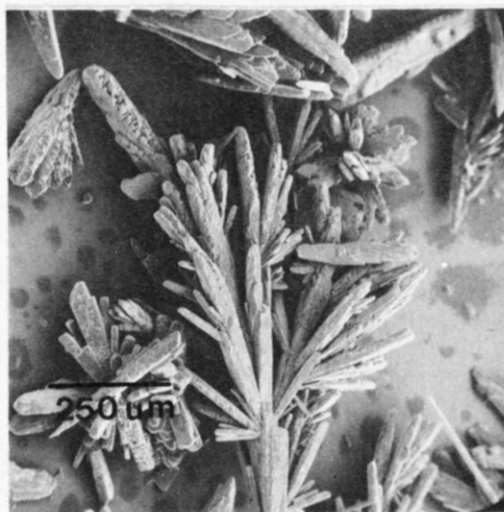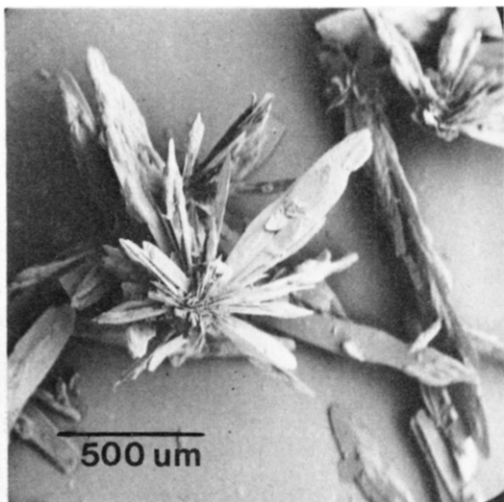
Figure 1. Crystalline structure of IU (upper frame), X (middle frame), and Y (lower frame).

Figure 2. HPLC elution patterns of CV, IU-red (red), and Y at 220 (lower frame) and 281 nm (upper frame) on a cation-exchange column.

Figure 3. Typical ultraviolet absorption spectra of IU, IU decomposition products (frames A and B), and IU treated with air (frame C). Key: frame A, IU-red (reduced), X-red, and Y in 0.1 N HCl; frame B, X-red, X-ox (oxidized), and X-ox plus DTT in 0.1 N HCl; frame C, IU in pH 7.0 buffer and aerated for 0, 5, 10, and 25 min.

280/220 nm ranging from a high of 3.8 for IU, to 0.4 for X (reduced), to a low of <0.01 for Y (Table I). The molar absorbency coefficients of the three compounds were also very different, with the values being 13 000 M^{-1} cm for IU at 281 nm in 0.1 N HCl and 2600 M^{-1} cm at 271 nm for X (reduced form) in 0.1 N HCl assuming the molecular weight was the same as the parent compound (143 Da). This value will have to be adjusted once the correct mo-
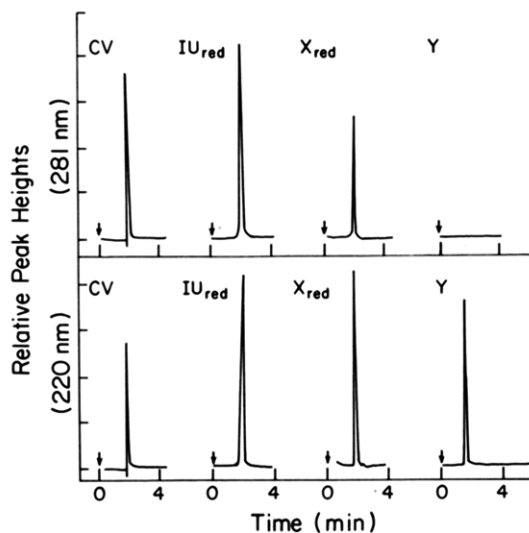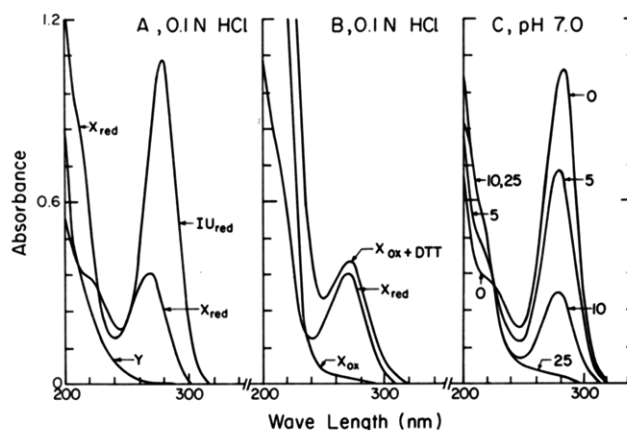
lecular weight of X has been established. An extinction coefficient could not be estimated for Y as it did not have an absorbency peak in the ultraviolet region.

The calculated purity of IU assuming that the pure compound had a molar absorbency coefficient of 13 600 M^{-1} cm (Davoll and Laney, 1956) was 96%. The overall yield of IU was approximately 40%.

Compound X as indicated previously appears to exist in two forms, an oxidized (X-ox) and a reduced form (X-red) (Figure 3B). This conclusion is based on the observation that treatment of the isolated form of X with oxygen in an acid media results in the near-complete disappearance of absorbance at 271 nm, whereas the addition of DTT to the oxygen-treated samples restores the 271-nm absorbency band. The process is repeated upon successive treatments with oxygen and DTT. The results indicate that reversible oxidation–reduction forms of X are produced in an acid medium and that these reactions can be monitored at 271 nm. The oxidized form of X at 220 nm also yielded a single symmetrical peak following HPLC analysis (chromatogram not shown). Both forms of X, in contrast to the differences that were observed in absorbency at 271 nm, have similar HPLC elution times (1.80 and 1.82 min, respectively). Compound X-ox at pH 7.0 is unstable.

Compound Y, which is produced by oxidative decomposition of IU or X at pH 7.0, appears to be the end product of the reaction as it is not converted into other products in the presence of oxygen or DTT.

These studies have demonstrated that four compounds can be prepared from CV by acid hydrolyses (IU, X-red, X-ox, Y) and that they can be readily identified on the basis of their HPLC elution patterns, ultraviolet absorbency spectra, and reaction with oxygen or a reducing reagent at either an acid or neutral pH.

Oxidative Decomposition of IU. The ultraviolet absorption spectrum of IU in an oxygen-free solution of a pH 7.0 phosphate buffer (Figure 3C) also exhibited a single absorption peak with an absorption maximum at 281 nm. The spectrum at pH 7.0 was stable for several minutes in deaerated solutions, but in the presence of oxygen (air), the 281-nm absorption rapidly declined and completely disappeared. This was associated with the concomitant increase in end absorption (<220 nM). The decline in absorption of an IU solution at 281 nm in the presence of oxygen was prevented by reducing reagents such as DTT. Also absorption at 281 nm increased somewhat if the reducing reagent was added after the initiation of the reaction of IU with oxygen up to a time period of at least 20 min. Preliminary results demonstrated that the same net effect was obtained with both oxygen and air.

Chevion et al. (1982) reported that the ultraviolet spectrum of an aqueous solution of IU (pH 7.0), when in an atmosphere of nitrogen, also exhibited a single absorption peak at 280 nm. In the presence of oxygen, the 280-nm absorption band declined with the concomitant appearance of a new absorption band at 255 nm. The latter species showed a transient build-up and then decayed, with a concomitant increase in end absorption (<230 nm). They also reported that excess reducing reagents prevented the decline in absorbance at 280 nm on exposure to oxygen. Also if the reducing reagent was added after the initiation of the reaction of the pyrimidine with oxygen while the band at 255 nm was still present, this absorption band rapidly disappeared, concomitant with the restoration of the 280-nm band. On the basis of this and comparative data on divicine, they concluded that IU existed in two forms (reduced or hydroquinone and oxidized or quinone), which undergo irreversible oxidation–reduction reactions in the presence of molecular oxygen and reducing reagents.

The results from the current study are only in partial agreement with those of Chevion et al. (1982) as a second absorption peak for the oxidized form of IU could not be demonstrated (Figure 3C). Their proposal that IU existed in two forms was, nevertheless, confirmed by HPLC analysis on samples that were treated with oxygen (air) and the reducing reagent, DTT. Results from the current study demonstrated that freshly prepared IU had an elution time of 1.96 min (Figure 4A; Table I) and when treated with oxygen for 5 min yielded three peaks (Figure 4B). There were two major peaks having elution times of 1.82 and 1.96 min and a minor peak having an elution time of 1.52 min. Treatment with oxygen for 20 min (Figure 4C) yielded a major peak having an elution time of 1.82 min, a much much larger peak at 1.52 min, and no detectable peak at 1.96 min. The time course changes continued until all absorbency associated with the peaks that were eluted at 1.82 and 1.96 min had disappeared with the concomitant increase in absorbency associated with the 1.52-min peak. The reducing reagent, DTT, prevented the conversion of IU into compounds that had elution times that were different from 1.96 min. Also, the addition of DTT to all
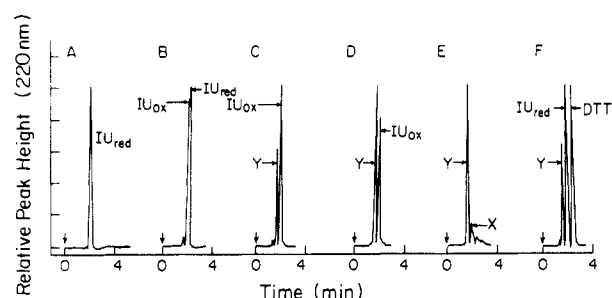

Figure 4. Chromatographic elution patterns at 220 nm on a cation-exchange column of a pH 7.0 solution of IU when treated with air over a period of 210 min at 25 °C. Key: frame A, 0 time; B, 5 min under air; C, 20 min under air; D, 60 min under air; E, 210 min under air; F 20 min under air followed by DTT addition to a final concentration of 4 mM.

samples that had been treated with oxygen for a period of from 5 to 60 min caused near-complete disappearance of the peak at 1.82 min and the concomitant increase in the peak at 1.96 min (see Figure 4C vs 4F for the 20-min sample). The compound having an elution time of 1.52 min, which coeluted with the subsequently described compound Y, was not affected by DTT. These results suggest that IU-red had an elution time of 1.96 min, IU-ox an elution time of 1.82, and compound-Y on elution time of 1.52 min. The results also demonstrate that there was successive oxidation of IU-red to IU-ox and IU-ox to Y. DTT prevents and reverses the conversion of IU-red to IU-ox but had no effect on Y.

As indicated above, the results from the ultraviolet scan, in contrast to those of Chevion et al. (1982), suggest that either the absorbancy maxima of the two forms of IU (IU-ox and IU-red) were nearly the same or they decayed in such a manner that the concentration of IU-red is always high relative to that of IU-ox. HPLC analysis would indicate that the latter is not the case (Figure 4 A–C). The net effect is that the two forms of IU decay in a manner such that they cannot be distinguished spectrophotometrically, presumably because the two forms have similar absorbancy maximas.

Although X was the end product when prepared from CV in concentrated acid, it is unstable at pH 7.0 and may be an intermediate in the conversion of IU to Y. The time course studies for the oxidative decomposition of IU to Y as shown in Figure 4 demonstrated that X could not be detected during the early stages of the reaction but appeared to be detected after all of the IU-red and most of the IU-ox had decomposed. For example, IU in the pH 7.0 buffer when treated with air for 210 min yielded a small peak (Figure 4E) that had an elution time of 1.82 min, which is the same as that for both IU-ox and X. This compound, however, was not IU-ox as the addition of excess DTT at several concentrations (2–10 mM) had no effect on elution times but prevented its further decomposition. However, continued incubation of the sample in air and in the absence of DTT resulted in its conversion to Y. These results indicate that X-red, which has the same properties, was formed in small amounts at pH 7.0 and that it could only be detected by HPLC analysis after most of the coeluting IU-ox had been converted to Y. The same pattern occurred at time periods of 120 and 180 min except these samples contained residual IU-ox.

On the basis of these studies, it is not clear whether X is an intermediate in the oxidative decomposition of IU at pH 7.0 or if it forms as a minor side reaction. IU at pH 7.0, in contrast to its behavior at a low pH, may be directly converted to IU-ox, which often appears to decompose to Y without going through the intermediate X step. If X
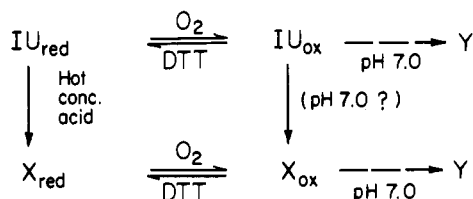
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Figure 5. Hypothetical decomposition of isouramil.

is indeed an intermediate, then IU-red would be converted to IU-ox, which would be rapidly converted to X-ox and ultimately to Y. Additional research will be required to solve this problem. A hypothetical scheme for the oxidative decomposition of IU is outlined in Figure 5.

Solubility, Stability, and Some Biological and Chemical Properties of IU. IU prepared in this study not only had a molar absorbency coefficient similar to that reported by Davoll and Laney (1956) for chemically synthesized IU but also reacted with $FeCl_3$, oxygen, and DTT and had biochemical activities that were identical with those reported by other researchers (Bendich and Clements, 1953; Mager et al., 1965; Chevion et al., 1982; Winterborun et al., 1986) including its ability to promote the autocatalytic oxidation of glutathione in vitro and the conversion of hemoglobin to its ferryl species. The biological effect, both in vivo and in vitro, of IU that was prepared by the acid hydrolysis procedure is presented elsewhere (Arbid and Marquardt, 1988).

In addition, IU prepared by the current procedure also yielded ultraviolet spectra, HPLC elution patterns, and biological effects in vitro identical with those obtained when IU was prepared by enzymatic hydrolysis. The enzymatic procedure (data not shown) has been a standard method for the preparation of IU (Mager et al., 1965, 1980; Arese et al., 1981). The major disadvantage of this procedure is that only small quantities of IU can be prepared at relatively low concentrations and that IU must be used within a short time of its preparation.

The average solubility (±SE) values for IU at pH 3.7 in water and the pH 7.2 sodium phosphate buffer were similar and average 0.6 ± 0.1 mg/mL at 1 °C and 1.7 ± 0.4 mg/mL at 25 °C. Solubility equilibrium values were reached within 5 min, and they did not change over 25 min. IU was insoluble in acetonitrile and essentially insoluble in methanol. Similar studies have not been reported.

The stability of IU in aerated and deaerated buffers and in buffers containing a reducing reagent were followed at different temperatures. The percent decrease (±SE) values in IU concentrations when incubated in oxygen-free solutions at 25 °C for 20 min and for 5 h were 56 ± 4 and 95 ± 5 in the pH 7.2 sodium phosphate buffer; 2 ± 2 and 24 ± 6 in water (final pH 3.7); 5 ± 3 and 45 ± 0 in the column eluting buffer (pH 2.0); and 23 ± 3 and $70 \pm 5\%$ in 6% perchloric acid (pH 0.4). Analysis at 1, 2, 3, and 4 h demonstrated that there was a linear decrease in IU concentration over time. The same solutions when treated with oxygen were much less stable. The percent loss values of IU (±SE) at 25 °C when oxygen was bubbled through the solution for 10 and 20 min were 100 ± 2 and 100 ± 3 in the pH 7.2 sodium phosphate buffers; 79 ± 3 and 94 ± 4 in water (final pH 3.7); 27 ± 4 and 50 ± 4 in the column eluting buffer (pH 2.0); and 56 ± 3 and 89 ± 5 in 6% acid (pH 0.4). Reducing reagents such as DTT, as indicated elsewhere in the paper, protect IU against oxidative decomposition at all pH values. The stability of IU in degassed water (pH 3.7) was followed at different temperatures over time periods of up to 2 h. There appears to be a linear decrease in IU concentration over time at all temperature values. The percent IU (±SE) values lost

after 2 h were 4 ± 3 at 0 °C, 10 ± 2 at 25 °C, 20 ± 1 at 40 °C, and 34 ± 4 at 60 °C. In general, IU is more stable in the absence of oxygen and presumably divalent cations (Novok and Chevion, 1984) and in the presence of reducing reagents, in the lower pH range and at low temperatures. These results are in agreement with those reported by other researchers (Mager et al., 1980; Chevion et al., 1982).

IU in powder form was stable for several months when stored at -20 °C in the absence of moisture in a darkened and sealed container. However, in a moist, oxygen-containing atmosphere it is converted from a pale yellow-light brown to a deep purplish reddish color, which indicates oxidative changes.

Conclusions. A simple method has been developed for the preparation of relatively pure IU in crystalline form from CV by acid hydrolysis. The purity of the preparation and its biological activity were similar to that of IU produced by either enzymatic hydrolysis or chemical synthesis. A prerequisite for high yields and purity of IU is that the concentration of both CV and acid be high and that the hydrolysis be complete within a relatively short period of time. Several different tests confirmed that the preparation was IU and that it did not contain any other ultraviolet-absorbing compounds. HPLC analysis confirmed previous reports (Chevion et al., 1982) that IU exists in two forms (reduced and oxidized) and that these were reversibly interconverted in the presence of molecular oxygen and a reducing reagent such as DTT.

CV when hydrolyzed at high temperatures in acid and IU when subjected to oxidation at different pH values were converted into several intermediate breakdown products having different ultraviolet absorbancy patterns and different HPLC elution profiles. Two intermediate breakdown products were isolated, compounds X and Y. Compound X was the intermediate that was formed during acid hydrolysis of CV. This acid-stable compound crystallized in the acid solution and was isolated by washing with water. It was yellow and had HPLC and ultraviolet absorption properties and reactions with reducing reagents and oxygen that were collectively different from those of the other compounds. Two forms of X were identified: a reduced form (X-red) and an oxidized form (X-ox). These forms can be reversibly interconverted at low pH in the presence of molecular oxygen and reducing reagents. X-red was the form that was isolated. The reduced form of X was stable at pH 7.0 in the presence of reducing reagent but was converted into the oxidized form in the presence of oxygen, which in turn rapidly decomposed into a new compound Y. Y was the final product of IU decomposition and can readily be produced from IU following treatment with oxygen at a neutral pH. The properties of this compound are distinct from those of the other compounds, some of which are summarized in Table I. A hypothesis sequence of reactions in the conversion of IU to Y has been given.

Although X was not identified, the properties of X-ox and X-red are the same as those of dialuric acid and alloxan, both of which undergo reversible oxidation-reduction reactions (Patterson et al., 1949). IU could be readily converted to dialuric acid simply by deamination at the pyrimidine carbon 6. Preliminary studies in our laboratory using nuclear magnetic resonance spectrometry indicate that compound X-red is dialuric acid and compound X-ox is alloxan (Pitura et al., unpublished data). These observations are also consistent with the biological data of Rocic et al. (1985) who reported that isouramil when administered to rats produced diabetes in a manner similar to that for alloxan. They did not, however, establish

whether IU was directly responsible for the diabetogenic effects or whether it became biologically active after being converted to alloxan. These results would suggest that the consumption of faba beans not only causes the human disease favism (Mager et al., 1980) but may also induce diabetogenic effects.

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