Isolation of Crystalline Divicine from an Acid Hydrolysate of Vicine and a Crystalline Decomposition Product of Divicine and Some Properties of These Compounds

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An improved procedure was developed for the production of the unstable compound divicine (DV, 2,6-diamino-5-hydroxy-4(3*H*)-pyrimidinone) from vicine [2,6-diamino-5-(β-D-glucopyranosyloxy)-4-(1*H*)-pyrimidinone]. Optimal hydrolysis and isolation conditions were established. The final product, which was whitish in color and had a crystalline structure, yielded a single chromatographic peak following HPLC analyses with no traces of vicine, glucose, or the decomposition product, referred to as pre-DV. The molar absorbency coefficient was 12 500 M^{-1} cm. The compound was unstable in aqueous solution, but its stability was greatly improved in the presence of degassed solvents, reducing reagents, low temperature, and low pH values. The dried form of DV was stable for at least several months at −20 °C. Maximum solubilities were obtained at low pH values and high temperatures. In addition, a stable, pale yellow decomposition product (pre-DV) was isolated in crystalline form. Other intermediate decomposition products were identified by HPLC analysis.

Divicine [DV, 2,6-diamino-5-hydroxy-4(3*H*)-pyrimidineone] is the aglycon of vicine [2,6-diamino-5-(β-D-glucopyranosyloxy)-4(1*H*)-pyrimidineone] (Mager et al., 1980; Windholz et al., 1983). Vicine and an analogous compound, convicine, are found in faba beans (*Vicia faba*) and have been strongly implicated in the human metabolic disease favism (Mager et al., 1980; Chevion et al., 1982; Arese et al., 1981; Yannai and Marquardt, 1985; Arbid and Marquardt, 1986). Favism is a hemolytic anemia caused by the consumption of faba beans and occurs in glucose-6-phosphate dehydrogenase deficient humans (Mager et al., 1980).

The consumption of faba beans or vicine and convicine has also been shown to depress egg size, to markedly increase plasma lipid concentrations in the laying hen, and to reduce fertility and hatchability of eggs (Muduuli et al., 1982). In addition, intravenous injections of DV (Yannai and Marquardt, 1985) or intraperitoneal injections of vicine and convicine into rats (Arbid and Marquardt, 1986) cause many adverse metabolic effects including death. Death appears to be the result of asphyxiation due to the loss of the functional properties of the red blood cell. DV is produced in the gastrointestinal tract by hydrolysis of vicine (Frohlich and Marquardt, 1983; Hegazy and Marquardt, 1984) and is absorbed into the blood. It then reacts with oxygen to form the superoxide free radical (Albano et al., 1984) by catalyzing a one-electron shuttle between reducing compounds such as glutathione and oxygen (Chevion et al., 1982; Albano et al., 1984).

The oxidized form of isouramil, which is analogous to DV, has been shown to elicit a persistent hypoglycemia in rats. Its effects are considerably more rapid and extensive than those of alloxan (Rocic et al., 1985). Oxidized DV would probably have a similar effect. In addition, DV and isouramil have been shown to have antimalaria activity. (Golenser et al., 1983; Clark et al., 1986).

Different procedures have been developed to synthesize DV (Davoll and Laney, 1956; McOmie and Chesterfield, 1956; Chesterfield et al., 1964; Zavyalov and Pokhvisneva, 1973; Bailey et al., 1982) or to produce it from vicine by either enzymatic hydrolysis (Herissey and Cheymol, 1931; Mager et al., 1965) or acid hydrolysis (Levene and Senior, 1916; Lin and Ling, 1962). All of these procedures have disadvantages, which limits the availability of DV for biological studies. Preparation of DV by chemical synthesis involves a considerable number of steps not readily carried out while DV produced by enzymatic synthesis has only been used to produce limited supplies of DV for immediate in vitro studies as the compound under the condition of production has a very short half-life. DV produced by acid hydrolysis using the procedure of Levene and Senior (1916) is of low yield and purity. Both Levene and Senior (1916) and Bendich and Clements (1953) reported that DV was extremely unstable and that it was difficult to obtain a product of constant composition.

The availability of a simple method for the production of a stable and highly active form of DV from vicine would greatly assist in the further studies of this very interesting compound. DV is not commercially available. The objectives of this study were to systematically investigate factors affecting the yield and purity of DV when produced by acid hydrolysis with the goal of developing a simple procedure for the production of a product with high yield and purity and a long shelf life. In addition the influence of pH, temperature, and reducing reagents on DV stability and the former two factors on DV solubility were to be investigated. This latter information would assist in the effective use of DV. The decomposition product of DV when treated with air was also to be isolated. This study was facilitated by the ready availability of crystalline vicine (Marquardt et al., 1983).

MATERIALS AND METHODS

Vicine was prepared by the procedure described by Marquardt et al., (1983). All other chemicals were from Fisher Scientific Co. Ltd., Winnipeg, from Sigma Chemical Co., Ltd., St. Louis, MO, or as given in Marquardt and Frohlich (1981).

Preliminary Preparation of DV. DV was initially prepared by a procedure similar to that utilized by Levene and Senior (1916) and Lin and Ling (1962). Vicine (5 g) was hydrolyzed in 2 N H_2SO_4 (50 mL) in a boiling water bath for 10 min. The hydrolysate was placed in an ice bath, 125 mL of degassed absolute ethanol was added, and DV was allowed to precipitate. After 1 h the precipitate was collected by centrifugation at 20000*g* for 15 min at 3 °C, washed with 35 mL each of ethanol (95%), ethanol–0.1

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N HCl (3:1), ethanol (95%), and acetone, and dried under nitrogen. An initial experiment established the optimal hydrolysis time.

Preparation of Enzymatically Hydrolyzed DV. Vicine (82.5 mg) was made up to 50 mL in a solution containing 0.05 M Na_2HPO_4, 0.05 M acetic acid, 5 mM EDTA, and 20 mM β-mercaptoethanol (pH 4.8), and 20 mg of β-glucosidase (Type II from almonds; Sigma) was added to 5 mL of the preparation. The sample was degassed by bubbling with oxygen-free argon, incubated for 1 h at 37 °C, placed on ice, diluted with 30 volumes of cold 6% perchloric acid, and analyzed for DV by HPLC (Marquardt and Frohlich, 1981).

DV for determination of the molar absorption coefficient was prepared in a similar manner except two hydrolysis times were employed (1 and 2 h) and the initial concentrations of vicine were 33 and 16.5 mg/100 mL. The sample was diluted with 12 volumes of cold, degassed perchloric acid (6%) and filtered (25 mm cellulose acetate, 0.45-μm pore size; Millipore, Bedford, MH), and absorbance at 281 nm was determined against the appropriate blanks in a Model DU-8 spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

Standard Procedure for Preparing DV. Vicine (4 g) was transferred to a glass tube (16 × 125 mm) containing 10 mL of hot 3 N H_2SO_4 for 20 min. The tube was placed in a boiling water bath prior to the addition of vicine so as to preheat the acid to 98 °C. The hydrolyzed sample was transferred to an ice bath, allowed to stand for 30 min for DV precipitation to go to completion, transferred quantitatively to centrifuge tubes by rinsing with 3 mL of degassed water, and centrifuged at 20000*g* for 25 min at 3 °C. The sample was drained well so as to minimize carryover of acid. The precipitate was resuspended, mixed, and successively recentrifuged and resuspended with the following solvents: 4 mL of water, 4 mL of 0.05 N HCl, 4 mL of 0.0025 N HCl, and 4 mL of 0.000 25 N HCl. An acetone washing was also used if the sample was dried in vacuo at 25 °C. All solvents were degassed by bubbling an oxygen-free inert gas (argon) through the solution followed by sonication. The preparation was generally placed in a freezer at −75 °C for 1 h and then in a freeze-drier overnight. DV was pulverized with the aid of a mortar and pestle, and the product was stored in a darkened and sealed vial at −20 °C.

The total amount of sample hydrolyzed can be increased by increasing the number of hydrolysis tubes. The volumes and amounts of vicine hydrolyzed per tube should remain constant so as to ensure proper heat transfer. Samples can be pooled after hydrolysis. A total of three isolations were carried out, with the amount of vicine used with each isolation being 8 × 4 g. Crystalline DV was also prepared by maximally solubilizing DV in degassed water containing 10 mM dithiothreitol (DTT) at 70 °C followed by slow cooling to 2 °C. The final pH of the solution was 2.5. Copious amounts of crystals formed within 1 h, and these were photographed.

In the development of the procedure, different hydrolysis conditions were studied. In the first trial, vicine (0.3 g/mL) was hydrolyzed for different time periods in 2 N H_2SO_4 in a boiling water bath at 98 °C (Figure 2). In the second trial, different concentrations of vicine were hydrolyzed in 3 N H_2SO_4 for 20 min at 98 °C (Figure 3). In the final trial, vicine (0.4 g/mL of H_2SO_4) was hydrolyzed for 20 min in the presence of different concentration of H_2SO_4 (Figure 4). In these studies the samples were assayed immediately after hydrolysis by HPLC (Marquardt and Frohlich, 1981). Samples were diluted to the appropriate concentration with cold-degassed 6% perchloric acid prior to injection.

Preparation of Pre-DV (Yellow DV). Yellow DV was prepared on the basis of the observation that a compound with pale yellow crystals formed when an aqueous solution of DV (400 mg/100 mL of 0.05 M phosphate buffer, pH 7.0) was left overnight under air. This compound, referred to as yellow-DV or pre-DV, was harvested by centrifugation (20000*g* for 10 min), resuspended in 20 volumes of water, and recentrifuged. The washing procedure was repeated three times with water and once with acetone. The acetone-extracted precipitate was dried under nitrogen, pulverized, and stored in sealed containers at 2 °C. This compound was used in combination with purified DV to assist in the identification of the HPLC peaks.

The solubility of the pre-DV at 25 °C was determined in 0.1 N HCl by procedures described below for DV except the amount of compound in solution was estimated spectrophotometrically with a molar absorbency coefficient at 250 nm of 3000 M^{-1} cm. The assumed molecular weight was 142. Crystalline pre-DV for photography was prepared by dissolving a maximum amount of pre-DV in water at 90 °C, diluting it 3-fold with water, and allowing the solution to slowly cool to 2 °C. Copious crystals formed within 24 h and were photographed by procedures described below. A second form of oxidized DV can be produced by incubation of DV in 0.1 N HCl under aerobic conditions for 24 h. The crystalline material was also photographed.

DV Stability. DV was prepared by enzymatic hydrolysis of vicine as described in the previous section except that β-mercaptoethanol was omitted from one of the two preparations and the pH of hydrolyses was 5.5. After complete hydrolysis, the preparation was mixed with an equal volume of water or water plus 20 mM β-mercaptoethanol and the DV solutions were incubated in duplicate at 1, 25, and 50 °C for 5, 30, and 120 min. Aliquots were diluted with cold 6% perchloric acid containing 10 mM β-mercaptoethanol, filtered, and assayed immediately for DV as described previously (Marquardt and Frohlich, 1981).

For the pH studies, DV was prepared as described above and was then mixed with equal volumes of 0.2 N HCl, H_2O, 0.2 M Tris (pH 8.1), and 0.2 N NaOH (pH 13) with and without 20 mM β-mercaptoethanol for 30 min at 23 °C. The final respective pHs of the solutions were 1.8, 5.5, 8.1, and 12. In another study, DV was prepared in the same manner, diluted with 12 volumes of 6% perchloric acid plus 10 mM β-mercaptoethanol, and incubated for 30, 60, and 120 min at 2 and 25 °C.

Solubiltiy of DV. A severalfold excess of DV prepared by the standard procedure was added to the degassed solution to be tested for solubility. The solution was mixed for 2 h, the mixer was stopped, the nonsoluble material was allowed to settle for 2 min, and an aliquot was removed and passed through a 13-mm cellulose acetate filter having a pore size of 0.45 mM (Millipore). Appropriate dilutions were made with 6% perchloric acid, and the amount of DV in solution was determined by HPLC analysis (Marquardt and Frohlich, 1981).

In the first study a solution containing 0.2 M acetic acid, 0.2 M sodium phosphate, 5 mM EDTA and 30 mM β-mercaptoethanol was prepared having pH values of 2.2 (2.2), 3.6 (3.1), 4.8 (4.5), 6.0 (5.6), and 8.4 (7.2). Values in parentheses indicate pH after solubilization of DV. The pH of DV suspended in water was also adjusted with 1.0 M HCl so that the final pH values were 2.1, 1.0, and 0.1. In the second study DV was solubilized in a HCl solution

so that the final pH was 2.7 after solubilization of DV. The pH 3.6 buffer from the previous study was also used. The solubility of DV at 2, 25, 60, and 80 °C was determined in water (pH 3.7) and at 25 and 60 °C with the pH 3.6 (3.1) buffer solution. A standard preparation of DV having a molar absorbency coefficient of 12 500 M cm^{-1} was used as a reference standard. A minimum of duplicate sets of analysis were carried out for all studies in this paper.

Analyses of DV and Pre-DV (Yellow DV). The ultraviolet absorbance spectra of a solution of DV that was prepared by the standard procedure was determined in degassed 0.1 N HCl and in degassed 0.05 M phosphate buffer (pH 7.0) plus 4 mM DTT using the DU 8 spectrophotometer. Ultraviolet scans were also carried out on DV when incubated for different time periods in degassed pH 7.0 buffer (Figure 5). In addition, the time course effect of incubating the sample under air or DTT (4 mM final concentration) was followed. A fresh stock solution of DV was prepared several times per day in degassed 0.1 N HCl (200 mg/100 mL). This solution was stable at 2 °C over a period of several hours. The stock solution was generally diluted 100-fold with the appropriate solution immediately before initiation of the study. A solution of the crystalline pre DV (2 mg/100 mL) was mixed with 0.1 N HCl at 50 °C, cooled to 25 °C, and directly scanned. A stock solution of DTT (640 mM) in water was prepared each day. The final pH of all solutions was that of the diluting solution.

HPLC analysis was carried out on two different columns. Initially, the studies involved a reversed-phase column using conditions and equipment as described by Marquardt and Frohlich (1981). Injection volume was 20; flow rate, 2 mL/min; chart speed, 1 cm/min; optical density setting, 0.2; and temperature, ambient. Peaks were monitored at 280 nm. In the final series of studies DV and the intermediate decomposition products were resolved with a strong cation exchanger having sulfonic acid functionality. Analysis was as described by Marquardt and Frohlich (1981) except the HPLC stainless steel column (250 × 4.6 mm (i.d.)) was prepacked by the manufacturer with Ultrasil-CX (Beckman) and the elution solution was 10 mM hexanesulfonic acid and 25 mM ammonium phosphate buffer (final pH 2.0). The sample was monitored at 250 and 282 nm in an ISCO Model 1840 absorbance monitor (Lincoln, NB), and the chromatograms and peak areas were recorded with the aid of Model 3390 A Hewlett-Packard (Palo Alto, CA) integrator.

DV stock solution, which was the same as for the ultraviolet scans, was diluted 20- or 40-fold with degassed eluting buffer (pH 2.0) or 4-fold with degassed 0.05 M phosphate buffer (final pH 6.5) and then 5- or 10-fold with the eluting buffer. DV prepared in the phosphate buffer (final pH 6.5) with or without DTT (4 mM) was incubated at 25 °C for different time periods, diluted with the eluting buffer, and injected into the column. Air was bubbled through samples as required. Crystalline pre-DV (2 mg/100 mL) was mixed with the eluting buffer at 50 °C, cooled to 25 °C, and injected directly into the column. Peaks were identified on the basis of elution profiles of pure DV, pre-DV, decay patterns of oxygen-treated solutions of DV, and effect of the reducing reagent on oxygen-treated DV at 250 and 282 nm. For data presented in Figure 6 the column flow rate was 1.8 mL/min at 25 °C and the chart speed was 0.5 cm/min. The amount of sample injected in 20 μL was 0.4 μg of pre-DV (C and D) or an amount equivalent to 1 μg of DV (A, B, E, G, I) or 2 μg of DV (F, H, J).

Glucose analysis on the final product was carried out with the glucose oxidase method following the instructions

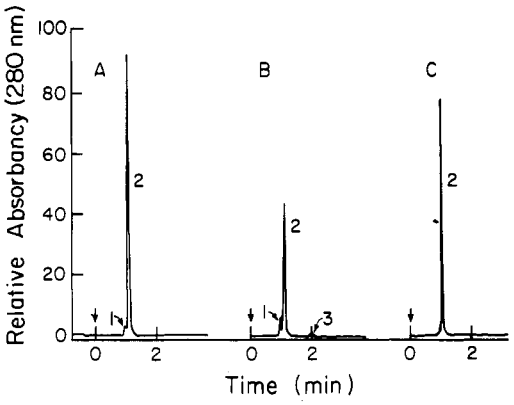

Figure 1. Comparative reversed-phase HPLC elution profiles of equal amounts of (1.3 μg in 20 mL) DV prepared enzymatically (A), by acid hydrolysis using a method as outlined in preliminary studies (B), and by acid hydrolysis using the standard procedure developed in this paper (C). Peaks 1–3 represent pre-DV, DV, and vicine, respectively. Arrow indicates time of injection.

of the manufacture (Bulletin 510, Sigma). The biological potency of DV in degassed saline (0.9%) and in oxygen-treated saline were compared with the procedure of Arbid and Marquardt (1986). In this procedure DV or pre-DV was incubated with red blood cells from the rat, the blood was hemolyzed, and the change in the ratio of absorbancy at 576 and 628 nm was determined. Blood glutathione concentrations were also determined (Beutler et al., 1963). Microscopic observations of crystalline DV and pre-DV were carried out by using a Cambridge Stero Scan Mark 2A electron microscope at 10 kV. The crystals were suspended in acetone, transferred by Pasteur pipet to an aluminum stub, allowed to air-dry, and subsequently coated with 1000 nm of gold in a Balzer Union spatter coater. Representative photographs were taken.

RESULTS AND DISCUSSION.

Preliminary Preparation. An initial study demonstrated that optimal yields of DV under the conditions indicated in Materials and Methods were obtained after 15 min of hydrolysis. Using this time, a yield of 0.4 g of crude DV was obtained from 1 g of vicine. The preparation had a DV absorbance peak per unit of dry matter that was approximately 50% of that of enzymatically produced DV (Figure 1A,B). It contained approximately 2% vicine and some pre-divicine (pre-DV). Pre-DV, as shown by HPLC analysis, is the small heat-stable peak that occurs prior to DV and is probably a decomposition product of DV. This compound coelutes from the reversed-phase column with the oxidized form of DV (quinone form). These two forms of DV, as shown subsequently, can be readily distinguished. The disadvantage of the procedure is that it yields a product of low purity. Levene and Senior (1916) and Bendich and Clements (1953) also reported that it was difficult to obtain a hydrolytic preparation of a high purity and of a constant composition, using a similar procedure, due to the extreme instability of DV.

DV produced by this procedure was biologically active as shown by its ability to promote the oxidation of glutathione in the red blood cell and to convert hemoglobin into its ferryl species as determined by the change in hemoglobin absorbency ratios at 576 nm relative to 628 nm. Although the purity of the DV preparation was low, as indicated by the above tests, its stability when dried and stored in the dark at −20 °C in a sealed container was excellent as the amount of DV essentially remained constant over a period of several months. Similar stabilities were obtained with all DV preparations. The preliminary results demonstrate that DV can be prepared by acid

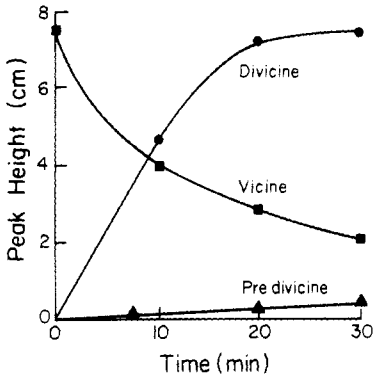

Figure 2. Influence of hydrolysis time on the production of DV and pre-DV and on the degree of hydrolysis of 0.3 g/mL vicine in 2 N H_2SO_4 at 98 °C and on the production of DV and pre-DV.

hydrolysis of vicine but that improvements in the method would be desirable.

Optimization of the Hydrolysis Procedure. An initial study demonstrated that the degree of vicine hydrolysis and yield of DV and pre-DV were markedly influenced by concentration of vicine, the concentration of acid, and hydrolysis times. It was also observed that DV precipitated out of solution when the original concentration of vicine in the hydrolysate was 2 g/10 mL rather than 1 g/10 mL. The inclusion of 10 mM EDTA, 50 mM glutathione, or both into the hydrolysis medium did not affect the yield of DV. These compounds as shown subsequently and as shown by Chevion et al. (1982) and Navok et al. (1984) protect DV from oxidative decomposition in the presence of low concentrations of divalent cations and oxygen. These studies, however, were carried out with biological systems in which incubation temperatures and the concentration of DV were much lower than that used in the current studies. Overall, these observations suggested that the ethanol precipitation step could possibly be eliminated from the procedure and that the conditions of hydrolysis should be modified.

The first study in this series (Figure 2) demonstrated that optimal hydrolysis time was between 20 and 30 min with regards to yield of DV but that the lowest amount of pre-DV occurred in the 20-min sample as compared to the 30-min sample. The concentration of unhydrolyzed vicine was relatively high and probably would be further reduced by increasing the concentration of H_2SO_4. Vicine in the hydrolysate, however, did not affect the purity of the final product as vicine remained in solution while DV precipitated out of solution. This separation does not occur with the Levene and Senior (1916) method as ethanol precipitates both vicine and DV.

Results in Figure 3 show that the yield of DV is markedly influenced by the concentration of vicine in the hydrolysate. Increasing the concentration of vicine from 0.1 to 0.4 g/mL (4-fold increase) resulted in a 12.5-fold increase in yield of DV. The increase in DV yield when the concentration of vicine was doubled from 0.2 to 0.4 g/mL, however, was not nearly as dramatic, being 2.5-fold. The optimal amounts of vicine would seem to be between 0.2 and 0.4 g/mL of 3 N H_2SO_4. Pre-DV, in contrast to DV, remained essentially constant on an absolute basis but decreased on a relative basis when the amount of vicine in the hydrolysates was increased from 0.2 and 0.4 g/mL H_2SO_4. These results show that the lowest relative concentration of pre-DV was obtained with the highest concentration of vicine. The amount of unhydrolyzed vicine also increased dramatically when the higher concentrations of vicine were hydrolyzed. Complete hydrolysis of vicine would presumably be achieved by increasing the concentration of acid or hydrolysis time.

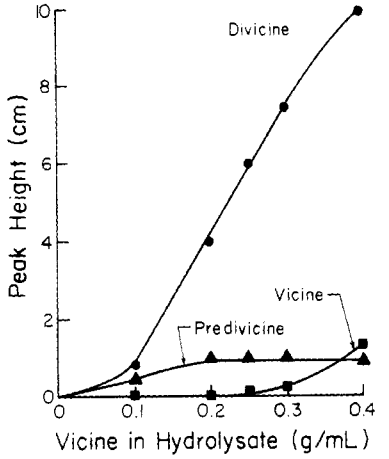

Figure 3. Influence of the concentration of vicine on the degree of hydrolysis of vicine in 3 N H_2SO_4 for 20 min at 98 °C and on the concentration of DV and pre-DV.

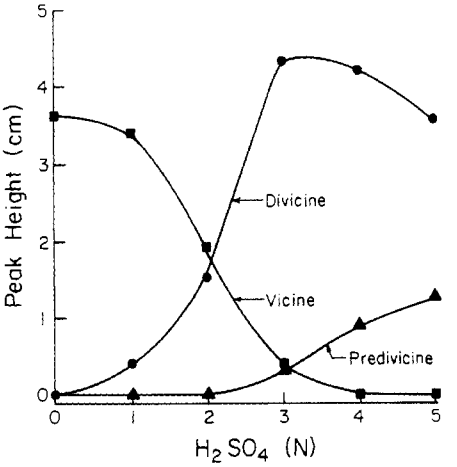

Figure 4. Influence of the concentration of H_2SO_4 at 98 °C for 20 min on the degree of hydrolyses of vicine (0.4 g/mL) and the concentration of DV and pre-DV.

The results in Figure 4 demonstrate that the concentration of acid markedly influenced the degree of hydrolysis of vicine and the yield of DV and pre-DV. Maximal yields of DV were obtained when the concentration of H_2SO_4 was 3 M, which also yielded the lowest relative amounts of pre-DV. The amount of unhydrolyzed vicine was slightly more than 10%. Further increases in the amount of vicine above 0.4 g/mL are not feasible because of solubility problems. In all of these studied standard error values of duplicate analysis were less than 6% of the mean value. The results suggest that optimal conditions for hydrolysis of vicine are 0.4 g/mL of 3 N H_2SO_4 for 20 min at 98 °C and have provided the basis for the standard procedure.

Stability of DV. DV was almost completely stable over a 2-h period at 1, 25, and 50 °C when incubated in a pH 5.5 buffer that was of the same composition as that used for the enzymatic hydrolysis studies. However, DV values decreased by 6, 37, and 96%, respectively, at 1, 25, and 50 °C over a 30-min period when incubated in the same buffer minus β-mercaptoethanol.

DV when incubated at 23 °C for 30 min in the presence of β-mercaptoethanol decreased from 4 to 8% at pH values between 1.8 and 8.1 and by 32% at pH 13. In the absence of β-mercaptoethanol, the amount of DV remaining under the same conditions were as follows: pH 1.8, 92%; pH 5.5, 64%; pH 8.1, 0%; pH 13, 0%. DV in 6% perchloric acid–10 mM β-mercaptoethanol decreased in a linear

manner over time with the values being 6%/h at 2 °C and 19%/h at 23 °C. Overall the results demonstrate that DV is considerably more stable at a low as compared to a high pH, that a reducing reagent such as mercaptoethanol protects DV from decomposition, particularly at a high pH, and that the process of decomposition accelerated with increasing temperatures. DV decomposition also appears to be accelerated with increasing concentration of acid. Chevion et al. (1982) also reported that both pH and reducing reagents affect DV stability. Samples for DV analysis should, therefore, be assayed within a short time period, the solution should be kept at a low pH, oxygen should be removed from the solution, and, if necessary, a reducing reagent should be added. Chelating agents should be added if metal ions are present (Navok and Chevion, 1985). Under these conditions DV stability in aqueous solution is reasonably good.

Solubility of DV. The solubilities of DV and the final pH values at 25 °C for solutions given in the Materials and Methods were as follows: 4.9 mg/mL, pH 2.2; 4.3 mg/mL, pH 3.1; 3.2 mg/mL, pH 4.5; 2.3 mg/mL, pH 5.6; 2.2 mg/mL, pH 7.2. The solubility of DV in dilute HCl was 3.1 mg/mL, pH 2.1; 4.5 mg/mL, pH 1.0; and 11.7 mg/mL, pH 0.1. The solubilities of DV in the pH 2.7 HCl solutions at different temperatures were 1.5 mg/mL, 2 °C; 2.4 mg/mL, 25 °C; 6.4 mg/mL, 60 °C; and 14.6 mg/mL, 80 °C. The solubilities of DV in the buffered solution (pH 3.1) were 3.9 mg/mL, 25 °C and 13.8 mg/mL, 60 °C. DV crystallized within 30–60 min when the temperature of a saturated solution was lowered from 60 to 25 °C. A saturated solution of DV at 80 °C, in contrast, precipitates out of solution within 1 or 2 min when cooled to 25 °C. Maximum solubility was observed at 80 °C in the pH 2.7 HCl solution, with the value being 14.6 mg/mL (103 mM). Higher solubilities could probably be obtained if more concentrated HCl and higher incubation temperatures were used.

The results demonstrate that the solubility of DV increases with decreasing pH values and increasing temperatures. Very little has been reported on the solubility of DV. It has been reported that 1 g of DV dissolves in 100 mL of boiling water and 300 mL of cold water (Windholz et al., 1983). The latter value of 3.0 mg/mL is more than that obtained in our study as the solubility of DV at the pH of water (5.6–7.7 and 25 °C) was 2.2–2.3 mg/mL. In the physiological pH range, the solubility of DV appears to be minimal, being approximately 2.3 mg/mL (16.2 mM).

Spectrophotometric and HPLC Analysis of DV and DV Degradation Products. The ultraviolet spectrum of DV in 0.1 N HCl exhibited a single absorption peak at 282 nm that did not change over a period of 30 min (curve 1, Figure 5). Although the addition of DTT (1–10 mM) to DV in 0.1 N HCl or in the pH 7.0 solution did not shift the absorbance peak, it caused a slight decrease in absorbancy and a broadening of the spectrum, with a shoulder being formed at 251 nm. This may be attributed to the formation of a DV–DTT complex in a manner analogous to the formation of a DV–glutathione complex reported by Chevion et al. (1982).

DV in degassed, pH 7.0 buffer decayed over time with the concomitant appearance of a peak at 250 nm (curves 2–4, Figure 5). DDT when present in the incubation solution prevented this decay. The addition of DTT to an aliquot of the mixture after 5 min (curve 2, Figure 5) resulted in the rapid disappearance of the band at 253 nm with the restoration of the 282-nm peak (curve 1, Figure 5). The addition of reducing reagent to an aliquot of the sample after 20 min (curve 3, Figure 5) resulted in only

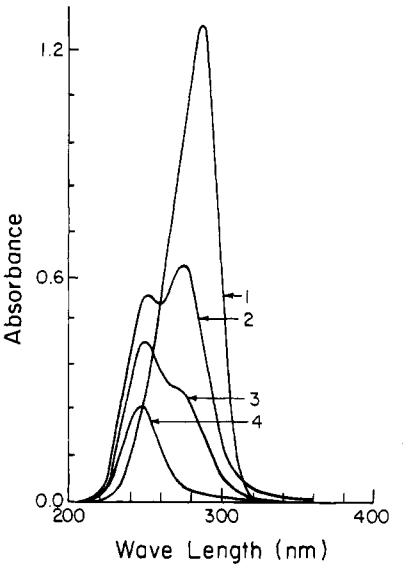

Figure 5. Typical ultraviolet absorption spectra of DV as influenced by pH of buffer and incubation time. Curve 1 represents DV in 0.1 N HCl. The other curves represent DV in degassed pH 7.0 phosphate buffer when incubated at 25 °C without DTT and scanned 5 min (curve 2), 20 min (curve 3), and 100 min (curve 4) after dilution. The absorbency maximum for curve 1 was 283 nm for DV in 0.1 N HCl and 282 nm in pH 7.0 buffer; curve 2, 278 and 253 nm; curve 3, 251 nm; and curve 4, 250 nm.

partial restoration of the 282-nm peak, and after 1 h and 40 min (curve 4, Figure 4) it had no effect on size or shape of the curve. Also, there were no further changes in curve 4 (Figure 5) in the absence of DTT over the next 2 h. Pre-DV yielded a scan similar to that seen in curve 4 (Figure 5) suggesting that the two may be the same compound. The rate of decay of DV observed in Figure 5 was accelerated when the solution was treated with air or oxygen and was greatly reduced when the diluting solution was degassed 0.1 N HCl. These results indicate the presence of at least three compounds: DV, which has an absorbancy maximum of 282 nm, and two compounds having maximal ultraviolet absorbance in the region of 250–253. One compound, according to Chevion et al. (1986), is the oxidized (quinone) form of DV. It is reversibly converted in the presence of a reducing reagent to reduced DV (hydroquinone form), and the other is a decomposition product not affected by reducing reagents. Chevion et al. (1982) reported that the absorbance maximum of DV and oxidized DV were 285 and 245 nm, which is higher than that observed in this study. They also concluded that oxidized DV is converted to a decomposition product(s) having a maximum absorbancy value of less than 230 nm and that this may be attributed to the opening of the ring structure of DV. In contrast, the current results indicate that the end product of decomposition is a yellow form of DV (pre-DV) with an absorbancy maximum of greater than 230 nm (250 nm).

HPLC analysis using a reversed-phase (Figure 1C) or a cation-exchange column (Figure 6A) demonstrated that the purified preparation of DV yielded only one peak. However, when monitored at 250 nm, DV exhibited a main peak plus a shoulder (Figure 6B), indicating the possible presence of a contaminating decomposition product since this compound was not present in vicine. This compound, as discussed subsequently, appears to be an unstable intermediate formed from DV. HPLC analyses of crystalline pre-DV at 250 (Figure 6D) and 282 (Figure 6C) nm yielded a single symmetrical peak that was eluted at 1.9 min compared to 4.3 min for DV (Figure 6A,B).

The height of the DV peak (peak 2) in the pH 6.5 buffer

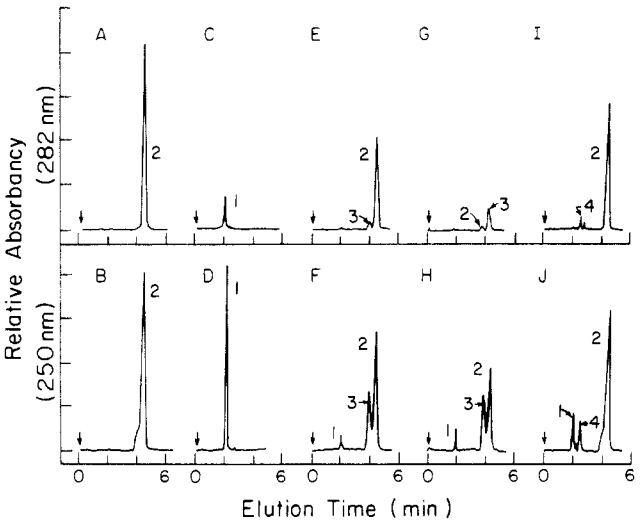

Figure 6. Comparative HPLC elution profiles of DV and related compounds on a cation-exchange column. The compounds were monitored at two wavelengths (A, C, E, G, and I at 282 nm and B, D, F, H, and J at 250 nm). Compounds injected: A and B, DV in pH 2.0 eluting buffer; C and D, crystalline pre-DV in eluting pH 2.0 buffer; E and F, DV in eluting buffer and after a 10-min incubation at pH 6.5; G and H, DV in eluting buffer and after a 20-min incubation at 6.5; I and J, as in G and H only DTT was added to a final concentration of 4 nM upon completion of the 20-min incubation period, and the mixture was reincubated for an additional 10 min. Peak numbers for compounds: 1, pre-DV, 2, DV; 3, oxidized DV; 4, DTT.

following dilution with the eluting buffer and HPLC analysis progressively decreased over a 20-min period at both 282 nm (Figure 6A,E,G) and 250 nm (Figure 6B,F,H). Oxidized-DV (peak 3, Figure 6 E–H), in contrast, was not present in freshly diluted DV (Figure 6A,B) but remained constant in the 10- and 20-min samples (Figure 6F,H). Pre-DV (Figure 6D, peak 1), which was also not present in the zero time sample (Figure 6B), doubled in area between 10 and 20 min (Figure 6F,H). After 60 min all absorbance associated with peaks 2 and 3 at both wavelengths disappeared, with the concomitant increase in absorbance of peak 1 (pre-DV) at 250 nm. The complete disappearance over time of peaks 2 and 3 (DV and oxidized DV) plus that associated with the shoulder of DV indicates that the contaminant in the DV peak was transitory in nature and probably is an unstable intermediate decomposition product of DV. During the final stages of decomposition, peak 2 (DV) disappeared before peak 3 (oxidized DV). The rate of spontaneous decomposition was also accelerated when the sample was treated with air or oxygen, but the pattern remained the same.

The addition of an excess of reducing reagent such as DTT (4 mM) to the DV solution after 20-min incubation greatly increased the area of peak 2 (DV) and decreased that of peak 3 (oxidized DV) (Figure 6I,J compared to G and H). This was particularly pronounced at 250 nm. The addition of DTT to a sample that contained only peak 1 or to crystalline pre-DV had no effect on peak area. These results demonstrate that DV (peak 2) is reversibly converted into oxidized DV (peak 3) and possibly a third intermediate and that oxidized DV or the intermediate compound is irreversibly converted into a stable compound (pre-DV, peak 1). HPLC analyses of a concentrated sample of DV (10 vs 1 μg of DV/20 μL) that was incubated in the pH 7.0 buffer for 20 min revealed the presence of a third transitory peak that was eluted at 2.8 min. The change in elution times from the cation-exchange column from 3.80 to 2.80 min for the transitory intermediate to 1.90 nm for pre-DV could be explained on the bases of successive losses of amino groups from oxidized DV. It may also be concluded that HPLC analysis at different wavelengths is a highly effective means of monitoring DV and its decomposition products of which there appears to be several intermediate compounds. Additional research, however, must be carried out to establish the nature of these reactions and the identity of the intermediates.

General Properties of DV. The average dry matter yield of DV was 8 g from 32 (8 × 4) g of vicine. The yield was 54%, assuming a molecular weight of vicine of 304 and DV of 142 (Mager et al., 1980). The average molar absorbency coefficient of DV was 12 500 M^{-1} cm with a typical ultraviolet absorption spectrum as shown in Figure 5 and HPLC elution profiles as shown in Figures 1 and 6. The product was whitish in color, had a crystalline rectangular barlike structure (Figure 7), contained essentially no pre-DV, did not contain any vicine or the other hydrolysis product, glucose, and as discussed previously was biologically active.

The molar absorbency coefficient at 282 nM of 12 500 M^{-1} cm in an acid medium (0.1 N HCl or 6% perchloric acid) was lower than that determined from the enzymatically hydrolyzed vicine (14 800 M^{-1} cm) and that reported by Bailey et al. (1982) (14 900 M^{-1} cm) and Chesterfield et al. (1964) (15 850 M^{-1} cm) but similar to that of Davoll and Laney (1956) (12 900 M^{-1} cm). If it is assumed that the true molar absorbency coefficient is 14 900 M^{-1} cm, then DV prepared by the current procedure would be 84% pure, the balance possibly being nonultraviolet absorbancy decomposition products or ionically bound HCl or H_2SO_4. Overall the yield and purity of the product are good considering the instability of DV and the rather vigorous treatment that it was subjected to during preparation. More extensive washing of the sample was not carried out as it was shown in a separate study that the yield and purity of the sample decrease if washed excessively, possibly due to oxidation of DV.

Pre-DV. Pre-DV was purified as described in Materials and Methods. It had HPLC elution and ultraviolet absorption patterns as described previously and appears to be an end product of DV decomposition. This product cannot be reversibly converted to DV. It is pale yellow, has a crystalline structure as shown in Figure 7, and has very low solubility in 0.1 N HCl (1.6 mg/100 mL). It appears to be much more soluble at pH 7.0. The molar absorbency coefficient, assuming a molecular weight of 142, which is the same as DV, was estimated to be 3000 M^{-1} cm at 250 nm. A second estimate was obtained by comparing the ratio of absorbancy at the absorption maxima of DV to that of pre-DV in Figure 6 and multiplying this value by 12 500 M^{-1} cm. In this calculation it is assumed that the reaction goes to completion. The value obtained was 2600, which is in reasonably good agreement with the other value. Pre-DV was biologically inactive by the tests described in Materials and Methods. The molecular weight, structure, and other properties of this compound were not determined.

A second form of pre-DV was also produced in 0.1 N HCl. The crystalline structure as shown in Figure 6 was different from that produced by the oxidized form of pre-DV produced at pH 7.0. Also, the HPLC elution times were different, being 1.67 min as compared to 1.90 min for pre-DV. In addition, this compound was only slightly soluble in 0.1 N HCl and considerably less soluble than that of pre-DV in 0.1 N HCl.

CONCLUSIONS

A simple method has been developed for the preparation

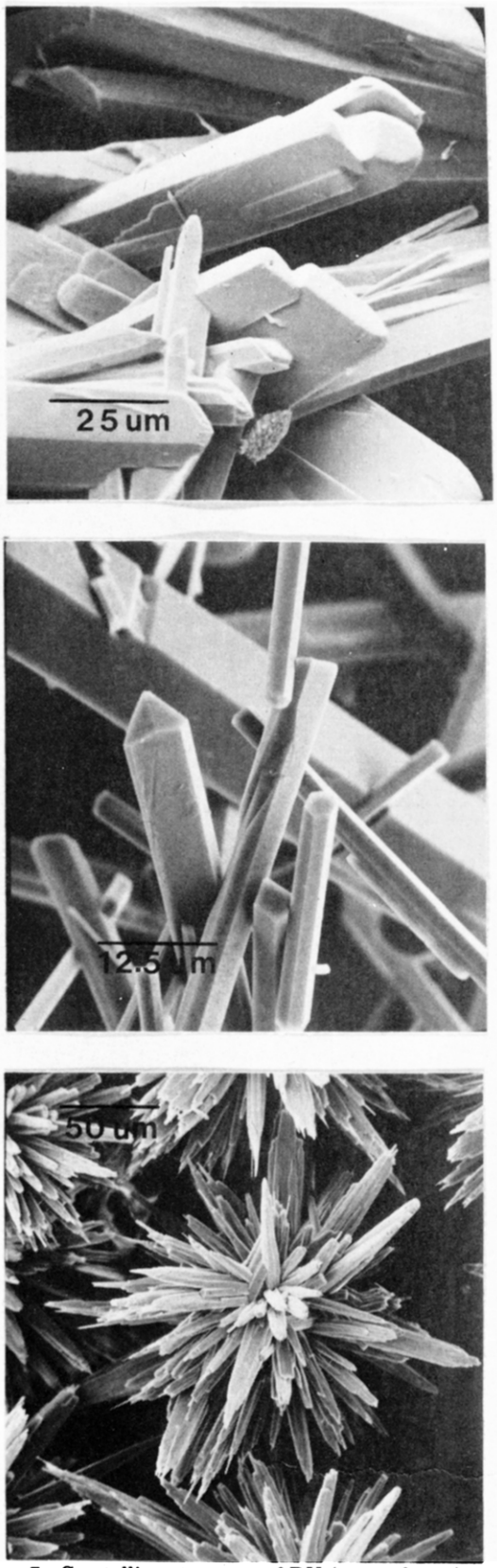

Figure 7. Crystalline structure of DV (upper frame), pre-DV (formed at pH 7, middle frame), and a second form of pre-DV (formed in 0.1 N HCl, lower frame). The crystals were photographed at a magnification of 1000-, 2000-, and 425-fold, respectively.

of DV of relatively high purity considering the instability of DV. The solubility, stability, elution profiles, from cation and reverse-phase columns and some of the ultraviolet absorption properties in the presence of oxygen and a reducing reagent were established. DV in the dry form at 2 °C was stable for several months. A stable decomposition product of DV, which was pale yellow and referred to as pre-DV, was also isolated in crystalline form. In addition, HPLC analysis using a cation-exchange column was shown to be a highly effective procedure for resolving reduced DV, oxidized DV, possible transitory intermediates, the final decomposition product (pre-DV), and the parent compound vicine. These studies should provide a basis for further studies on the identity and nature of intermediate breakdown products of DV and provide a simple procedure for the production of DV for biological studies.

ACKNOWLEDGMENT

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Registry No. DV, 32267-39-3; vicine, 152-93-2.

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Development of (Phenoxyphenoxy)- and (Benzylphenoxy)propyl Ethers as Potent Insect Juvenile Hormone Mimetics

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We prepared as insect juvenile hormone (JH) mimetics (4-phenoxyphenoxy)- and (4-benzylphenoxy)alkyl ethers in which the oxime moiety of the (4-phenylphenoxy)alkanaldoxime *O*-ether compounds we have already developed is replaced by a simple ether function. The activity against a mosquito, *Culex pipiens*, was examined. Structural factors, especially steric ones, that concern variations in potency were common to the two series of compounds. On the basis of this finding, we optimized the structure as has been done for the earlier oxime compounds, developing 3-(4-benzylphenoxy)propyl and 3-[4-(3-methylphenoxy)phenoxy]propyl isobutyl ethers, the most potent members of the class. The oxygen function was equivalent or somewhat better than the oxime with respect to expression of JH-mimetic activity. A methyl branch at the 2- or 3-positions of the propyl moiety lowered the potency dozens of times, suggesting a disturbance of the steric fitting to the receptor or the taking on of the active conformation.

Based on the receptor map for insect juvenile hormones (JHs) drawn based on results of quantitative structure–activity relationship analysis of the terpenoid 2,4-dodecadienoate JH mimics (Nakayama et al., 1984), we have prepared new classes of terpenoid JH mimics, undecen-2-one oxime *O*-ethers and undecen-2-yl carbamates (Nakayama et al., 1985). The activity of these mimics was comparable to or stronger than that of JH I, methyl (2*E*,6*E*)-*cis*-10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate, against *Culex pipiens* (the common mosquito), *Chilo suppressalis* (the rice-stem borer), and *Musca domestica* (the house fly), but less potent than that of methoprene [isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate], one of the most active JH-mimetic compounds known. To obtain higher activity, we have then transformed the terpenoid structure to a nonterpenoid one within the framework of the receptor map, resulting in the development of the ω-(4-phenoxyphenoxy)- and ω-(4-benzylphenoxy)alkanaldoxime *O*-ether series of JH mimics. The activity against *C. pipiens* of the most active member, 3-[4-(3-methylphenoxy)phenoxy]propionaldoxime *O*-isopropyl ether, exceeded that of methoprene (Niwa et al., 1987).

The structure–activity profile of the new class of compounds is in accord with that of the previous terpenoid oximes. Moreover, the position of the oxime *O*-ether function in a molecule has been found important for high activity, 3-(4-phenoxyphenoxy)- or 3-(4-benzylphenoxy)-propionaldoxime *O*-ethers being dozens of times more potent than the corresponding acetaldoxime *O*-ethers. Recently, propionaldoxime *O*- 2-(4-phenoxyphenoxy)ethyl ethers has been reported, where the oxime function is built in the structure in the reverse of our oxime ethers (Ohsumi et al., 1985). In the same run of assays with our compounds, they were highly active, pI_{50} (M) against *C. pipiens* being 9.50 (Niwa et al., 1987). Previously, the effect of various functions has been examined in the phenoxyphenoxy type of compounds to show that those having a carbamate or ester group are highly active against some species of insects (Karrer and Farooq, 1981). Although the position specificity has not been systematically investigated, it is suggestive that a potent compound is obtainable with a variety of functional groups if they are properly located in the molecule. In this study, we prepared a series of compounds with the simplest functional group, ether, and optimized the structure as has been done for the oxime

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