Chemical Analysis and Hemolytic Activity of the Fava Bean Aglycon Divicine†

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Divicine is an unstable aglycon metabolite of the fava bean pyrimidine β-glucoside vicine. Divicine has long been thought to be a mediator of an acute hemolytic crisis, known as favism, in susceptible individuals who ingest fava beans (*Vicia faba*). However, a recent report has questioned the chemical identity of the divicine that was used in most of the studies on divicine hemotoxicity. The present study was undertaken to examine the hemolytic potential of synthetic divicine. Divicine was synthesized and its identity and purity were confirmed by HPLC, mass spectrometry, and NMR spectroscopy. The stability and redox behavior of divicine, under physiological conditions, were examined by HPLC and cyclic voltammetry. The data indicate that divicine is readily oxidized under aerobic conditions; however, it was sufficiently stable at pH 7.4 to permit its experimental manipulation. When ^{51}Cr-labeled rat erythrocytes were exposed in vitro to the parent glucoside, vicine (5 mM), and then readministered to rats, no decrease in erythrocyte survival was observed. In contrast, erythrocyte survival was dramatically reduced by in vitro exposure to divicine (1.5 mM). These data demonstrate that divicine is a direct-acting hemolytic agent and thus may be a mediator of the hemolytic crisis induced by fava bean ingestion.

Introduction

Vicine and convicine are pyrimidine β-glucosides present in fava beans (*Vicia faba*) that are hydrolyzed upon digestion to the unstable aglycons, divicine and isouramil, respectively. Divicine and isouramil have been implicated in the onset of the potentially life-threatening hemolytic anemia, known as favism, in individuals who are genetically deficient in erythrocytic glucose-6-phosphate dehydrogenase (G6PD)[1] activity (*1–3*). Although the mechanism of toxicity is unknown, it has been suggested that divicine and isouramil induce oxidative damage within red blood cells by a mechanism that involves the formation of free radicals produced during the autoxidation of these aglycons (*1, 4–6*).

Most of the research on favism has been done with vicine and its aglycon metabolite, divicine (Figure 1). Two problems, however, have hampered efforts to link these compounds with the onset of favism. First, since divicine is not commercially available, it has typically been prepared from vicine by either enzyme hydrolysis (*2*) or acid hydrolysis (*7–9*) immediately prior to experimental use. Since the commercial availability of vicine is limited, the small amounts available have been used primarily for in vitro studies and direct assessment of divicine hemotoxicity in vivo has not been practical. Second, Pedersen et al. (*10*) have recently reported that the acid hydrolysis procedure, which has been the most commonly used method for generating divicine from vicine, yields a deaminated derivative of divicine. These investigators thus questioned the validity of much of the work in the area since it had been performed using divicine preparations of unknown structure and purity.

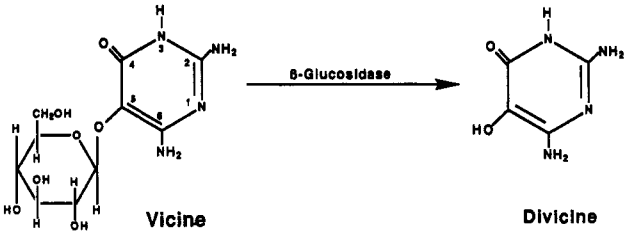

Figure 1. Enzyme-catalyzed hydrolysis of vicine to divicine.

To overcome these problems, we have utilized a synthetic method (*11*) to prepare gram amounts of divicine. This paper reports on the chemical characteristics of the synthetic divicine, on its stability and redox behavior, and on its direct hemolytic activity. The data indicate that chemically defined divicine is a direct-acting hemolytic agent and that it is practical to synthesize and store divicine in sufficient quantity and purity to examine the mechanism underlying its hemolytic response in vivo.

Materials and Methods

Animals. Male Sprague-Dawley rats (100–120 g) were purchased from Charles River (Raleigh, NC) and were maintained on food and water *ad libitum*. Animals were acclimated for 1 week to a 12-h light–dark cycle prior to their use.

Chemicals. Vicine [2,6-diamino-5-(β-D-glucopyranosyloxy)-4(1*H*)-pyrimidinone] was purchased from Serva Biochemicals (Westbury, NY) and was judged to be >99% pure by HPLC-UV. $Na_2{}^{51}CrO_4$ in sterile saline (1 mCi/mL, pH 8) was purchased from New England Nuclear (Billerica, MA). All other chemicals were reagent grade and were used without further purification.

Divicine [2,6-diamino-5-hydroxy-4(3*H*)-pyrimidinone] hemisulfate was synthesized from 6-hydroxy-2,4,5-triaminopyrimidine

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[1] Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; LSIMS, liquid secondary ion mass spectrometry; PBSG, phosphate-buffered saline with glucose; GSH, reduced glutathione.

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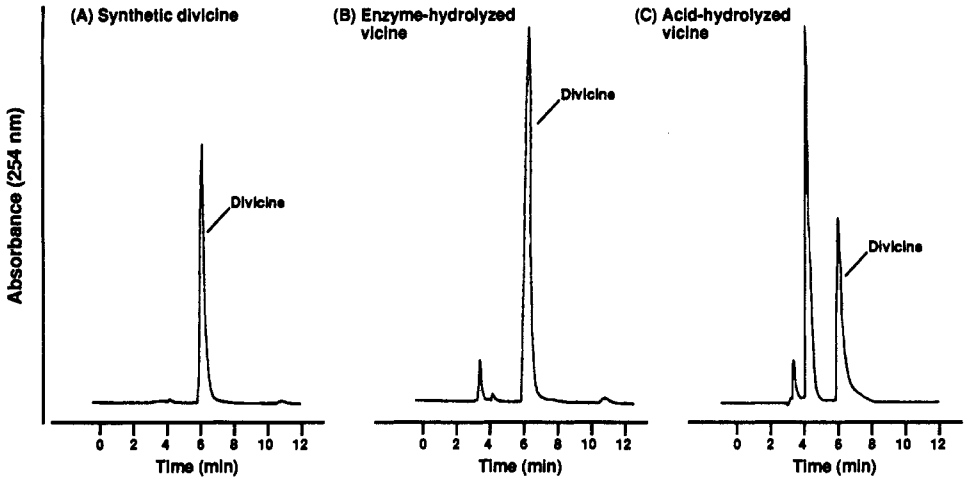

Figure 2. Cation-exchange HPLC elution profile of (A) synthetic divicine, (B) divicine prepared by enzymatic hydrolysis of vicine (enzyme-hydrolyzed vicine), and (C) divicine prepared by acid hydrolysis of vicine (acid-hydrolyzed vicine).

sulfate (Aldrich Chemical Co., Milwaukee, WI) as described by Bailey et al. (*11*). Divicine hemisulfate was converted to divicine hydrochloride with barium chloride, collected by lyophilization, and stored under argon at −20 °C to minimize oxidation.

Divicine was also prepared by both enzymatic and acid hydrolysis of vicine as described previously (*2*, *7*). Briefly, enzymatic hydrolysis of vicine (15 mM) was carried out with β-glucosidase (5 mg/mL; β-D-glucoside glucohydrolase from almonds; EC 3.2.1.21; Sigma Chemical Co., St. Louis, MO) in 1 mL of argon-purged NaH_2PO_4 (50 mM, pH 5.0) at 37 °C for 1.5 h. Acid hydrolysis of vicine (15 mM) was carried out in argon-purged hydrochloric acid (1 N) at 100 °C for 15 min. The concentration of the reduced pyrimidine aglycon was determined by measuring the reduction of ferriphenanthroline (*12*).

Instrumentation. Chromatography was performed on a Waters HPLC system (Waters Associates, Milford, MA) consisting of a Model 6000A pump, a Model U6-K universal injector, a Resolve C_{18} precolumn, and a RCM 8 × 10 radial compression module equipped with a Waters Radial-Pak Partisil SCX cation-exchange cartridge (8 × 100 mm). The samples were eluted with helium-purged 35 mM ammonium citrate/1 mM EDTA (pH 3.3) at a flow rate of 1.5 mL/min (*11*) and detected on a Waters Model 440 absorbance detector at 254 nm.

Liquid secondary ion mass spectrometry (LSIMS) data were acquired in the first stage of a JEOL HX110/HX110 tandem mass spectrometer operating with a mass resolution of 1500. To determine the molecular weight of enzymatically-prepared divicine, an aliquot of the enzyme hydrolysis mixture (10 μL) was loaded onto a sample probe and dried with a heat gun. Glycerol (1–2 μL) was then added to the residue and gently stirred prior to insertion of the probe into the mass spectrometer. Protonated molecular ions were generated by bombardment with 10-keV Cs ions from a cesium ion gun. The molecular weight of synthetic divicine was then determined under the same conditions that were used for the analysis of enzymatically-prepared divicine.

^1H (300 MHz) and ^{13}C NMR spectra (74.6 MHz) were recorded on a Varian Gemini 300 (broad-band) spectrometer at ambient temperature. Carbon chemical shifts are reported in ppm (δ) and are referenced to residual dimethyl sulfoxide-d_5 (δ 39.51). Divicine hemisulfate was dissolved in argon-purged dimethyl sulfoxide-d_6 (20 mg in 0.7 mL) and immediately analyzed. Under these conditions, divicine hemisulfate was sufficiently soluble and stable (<20% oxidation in 6 h) for the acquisition of the spectrum (1 h).

Stability and Electrochemistry Experiments. To examine the stability of divicine, synthetic divicine (1 mM) was dissolved in argon-purged phosphate-buffered saline with glucose (PBSG) (110 mM NaCl, 20 mM Na_2HPO_4, 4 mM KH_2PO_4, 10 mM D-glucose; pH 7.4) and maintained under argon at 37 °C. Aliquots (5 μL) were removed at T_0 and at various intervals thereafter for analysis by HPLC-UV. Divicine peak heights were measured and are expressed as a percentage of the peak height at T_0. Stability was also measured following addition of an anaerobic solution of divicine (500 μL) to aerobic PBSG (4 mL) in order to reproduce the conditions used for administration of divicine to erythrocyte suspensions. The incubation was then maintained under air at 37 °C, and aliquots were removed and analyzed by HPLC-UV as described above.

Cyclic voltammetry experiments were performed using a Bioanalytical Systems (West Lafayette, IN) CV-27 voltammograph, C-1 A/B cell stand, and a Model RXY recorder. Standard solutions of divicine (5 mM) were prepared in argon-purged PBSG. Samples were scanned at a rate of 150 mV/s under argon atmosphere at room temperature using a glassy-carbon working electrode, platinum auxiliary electrode, and Ag/AgCl reference electrode.

Measurement of the Hemolytic Response. The ability of divicine to induce a direct hemolytic response was examined as previously described (*13*). Briefly, ^{51}Cr-labeled rat erythrocytes were exposed in vitro to the test compounds for 2 h at 37 °C under aerobic conditions prior to their readministration to isologous rats. Vicine and synthetic divicine were dissolved in argon-purged PBSG before addition to erythrocyte suspensions (4 mL, 40% hematocrit). Divicine was prepared enzymatically as described above and immediately added to the erythrocyte suspensions in a volume of concentrated PBSG (5×) sufficient to keep the solution isotonic with respect to the erythrocytes. Prior to readministration, the cells were centrifuged (5 min at 2000*g*), resuspended in PBSG (45 mL), and again centrifuged. The extent of erythrocyte hemolysis that had occurred during the incubation was determined for each treatment by measuring the amount of radioactivity that had been released into the incubation supernatant. The washed cells were then resuspended in PBSG to the original hematocrit and administered iv to isologous rats. A 75-μL blood sample (T_0) was taken from each rat into heparinized capillary tubes via the orbital sinus 5 min after administration of the labeled cells. Serial blood samples (75 μL) were then taken from each rat at designated intervals for 7 days. At the end of the experiment, all of the samples were counted in a well-type γ counter, with the counts per minute above background for each sample expressed as percentage of the T_0 sample. Statistical significance was determined with the use of Student's *t* test.

Results

Chemical Analysis of Divicine. The purity of synthetic divicine was examined by cation-exchange HPLC as described by Bailey et al. (*11*) (Figure 2A). Synthetic divicine had a UV purity of >99% and an HPLC retention time (6.0 min) that was identical to that of divicine

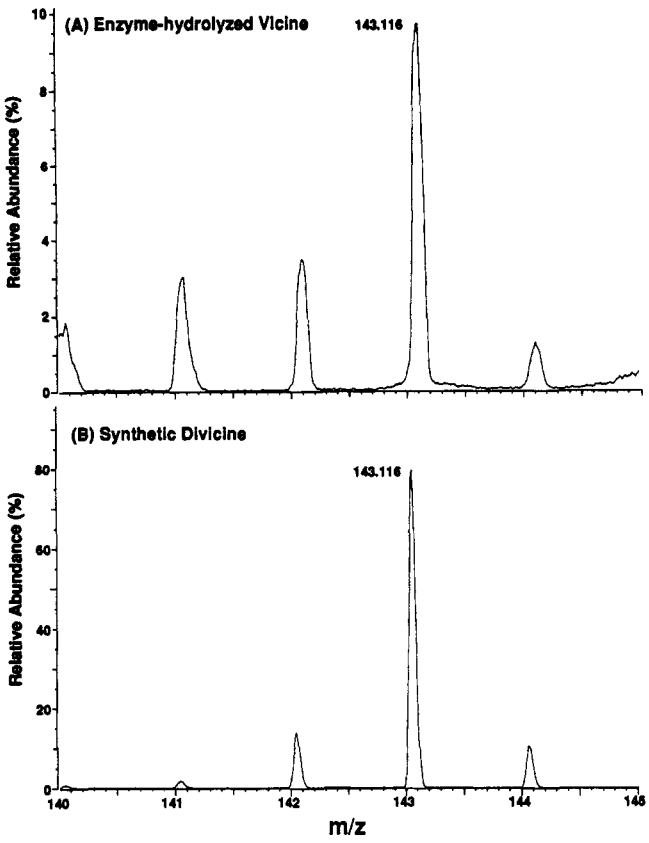

Figure 3. LSIMS mass spectrum of (A) divicine prepared by enzymatic hydrolysis of vicine (enzyme-hydrolyzed vicine) and (B) synthetic divicine.

prepared enzymatically from vicine (Figure 2B). Moreover, synthetic divicine cochromatographed with the enzyme hydrolysis product on a C_{18} reversed-phase HPLC column and on a normal-phase cellulose TLC plate (data not shown). In contrast, divicine generated from vicine by acid hydrolysis (Figure 2C) yielded an elution profile showing multiple peaks consistent with the acid-catalyzed degradation of divicine proposed by Pedersen et al. (*10*).

LSIMS analysis of synthetic divicine and enzymatically-generated divicine (Figure 3) indicated that the molecular weight for both protonated aglycon molecular ions was 143.116. This value is in agreement with the calculated value (143.12) for the structure of divicine proposed by Bendich and Clements (*14*).

NMR analysis was performed in an attempt to gain additional information on the structure of divicine. The proton spectrum of synthetic divicine hemisulfate showed a single broad peak at δ 6.0 but was not structurally informative due to rapid proton exchange. The carbon spectrum of divicine (Figure 4) showed four signals. The signal at δ 150.05 had a half-height peak width of 8.0 Hz. We have tentatively assigned this signal as C_2 on the basis of the assumption that the increased peak width of this signal is due to the quadrapole broadening of the three nitrogens bonded to C_2 (*15*). The signals at δ 111.53, 149.14, and 156.78 had half-height peak widths that ranged from 2.3 to 3.0 Hz, which were not sufficiently different to make unequivocal assignments based on quadrapole broadening of adjacent nitrogens.

Stability and Electrochemical Activity of Divicine. Divicine is known to undergo rapid oxidation in oxygen-saturated solutions (*4*). The kinetics of this oxidation are markedly influenced by pH; i.e., the stability of divicine

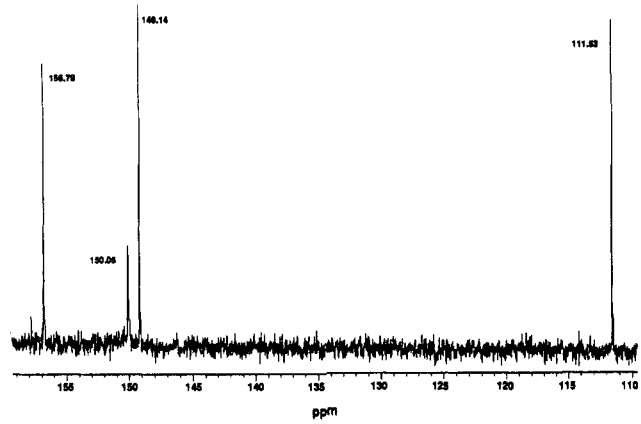

Figure 4. ^{13}C NMR spectrum of synthetic divicine hemisulfate in dimethyl sulfoxide-d_6. Chemical shifts are referenced to residual dimethyl sulfoxide-d_5 (δ 39.51).

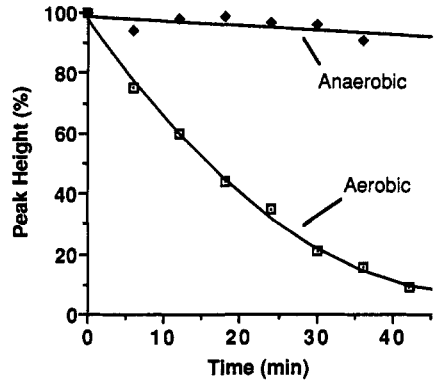

Figure 5. Stability of synthetic divicine in phosphate buffer (pH 7.4) at 37 °C under aerobic (□) and anaerobic (◆) conditions.

decreases exponentially as pH increases above 5.5 (*4*). Therefore, the stability of divicine was monitored at pH 7.4 to determine whether divicine would degrade under the experimental conditions used to administer it to erythrocyte suspensions. As shown in Figure 5, divicine was stable at pH 7.4 in the absence of oxygen; however, once divicine was added to PBSG and incubated under air at 37 °C, degradation occurred with a $t_{1/2}$ of ca. 17 min. As shown in Figure 6A, the disappearance of divicine was accompanied by the appearance of oxidative degradation products that eluted from the HPLC at 3.5 (peak 1), 4.0 (peak 2), and 6.5 min (peak 3). Addition of a small amount of a reducing agent (sodium hydrosulfite) to the divicine solution eliminated peak 3, but had no effect on peaks 1 and 2 (Figure 6B).

Examination of the electrochemical behavior of divicine, using cyclic voltammetry, indicated that when an excitation potential scan was initiated in the positive direction at −250 mV, two peaks were observed (Figure 7). Peak A represents the oxidation of divicine (E_{pa} = 35 mV). When the scan was reversed in the negative direction at +0.5 V, a peak appeared at B, which represents the reduction of the product formed at A (E_{pc} = −125 mV). Together, these data are consistent with the presence of a partially reversible redox couple and probably correspond to a two-electron oxidation of divicine to its quinone derivative.

Hemolytic Activity of Vicine and Divicine. To examine the ability of vicine or divicine to induce a direct hemolytic response, the compounds were incubated in vitro with ^{51}Cr-labeled rat erythrocytes for 2 h at 37 °C before readministration of the labeled cells to isologous rats. As shown in Figure 8, exposure of red cells to the vehicle

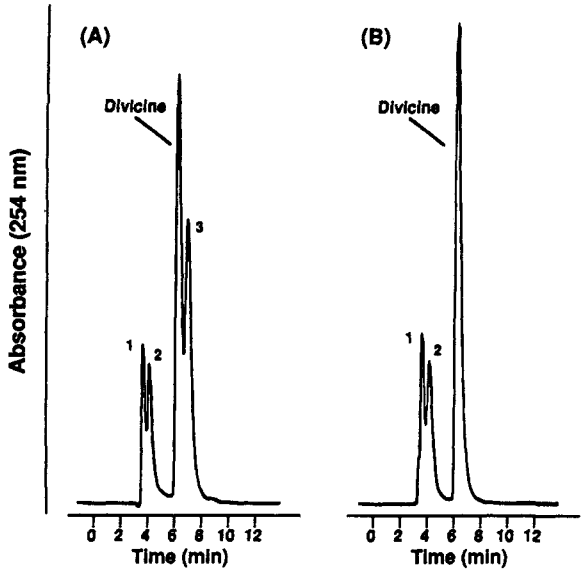

Figure 6. Cation-exchange HPLC elution profile of (A) synthetic divicine following aerobic incubation in phosphate buffer (pH 7.4) for 40 min at 37 °C and (B) the same sample following addition of sodium hydrosulfite.

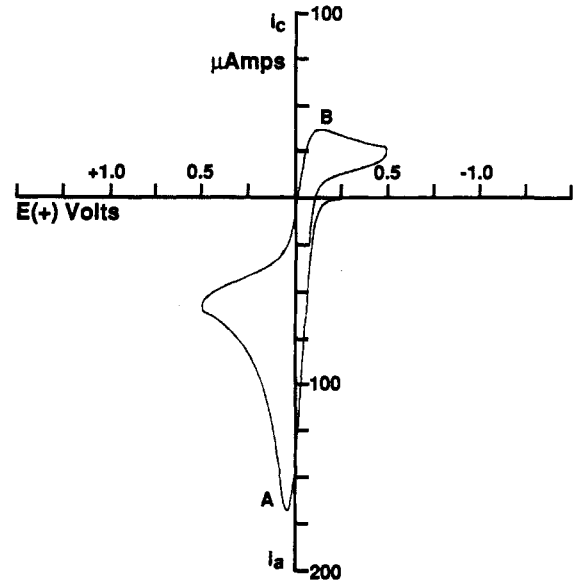

Figure 7. Cyclic voltammogram of synthetic divicine (5 mM) in PBSG (pH 7.4). Working electrode, glassy carbon; reference electrode, Ag/AgCl; auxiliary electrode, platinum; scan rate, 150 mV/s. A, anodic peak; B, cathodic peak.

(PBSG) induced a gradual decline in blood radioactivity that is thought to reflect primarily the normal removal of senescent erythrocytes from the circulation by the spleen (*23*). Exposure of the labeled erythrocytes to vicine (5 mM) also did not induce a significant increase in the rate of removal of blood radioactivity as compared to that of the vehicle control.

In contrast, direct exposure of the labeled cells to either synthetic divicine (1.5 mM) or enzymatically-prepared divicine (1.5 mM) dramatically increased the rate of removal of radioactivity from the blood as compared to the vehicle control (Figure 8). Two phases of removal were readily apparent: an early phase of rapid removal of damaged cells (ca. 70–85%) within the first 24 h, and a late phase of slow removal of senescent (presumably undamaged) erythrocytes. Of importance, the amount of radioactivity released into the incubation supernatants was not significantly different among the treatment groups

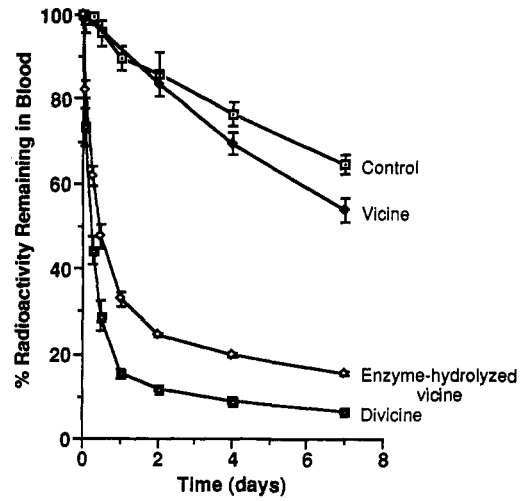

Figure 8. Erythrocyte survival in vivo after in vitro exposure to the vehicle (□), 5 mM vicine (◆), 1.5 mM synthetic divicine (■) or 1.5 mM enzyme-hydrolyzed vicine (◇). ^{51}Cr-Labeled rat erythrocytes were incubated with the test compounds in PBSG for 2 h at 37 °C. The erythrocytes were then washed and administered iv to isologous rats. T_0 blood samples were taken 5 min after administration of the labeled cells. Data points are means ± SD (n = 5).

and represented less than 1% of the total radioactivity in the blood. These data indicate that divicine hemotoxicity is not associated with a direct chemical lysis of the red cells.

Discussion

Although favism has been studied for several decades, the identity of the toxic constituent(s) of fava beans has not been firmly established and the mechanism of favic hemolysis is not yet understood (for reviews, see refs 1 and 16). It is well-known that susceptibility to favism is associated with a genetic deficiency in erythrocyte G6PD activity, which decreases the ability of the red cell to maintain adequate levels of reduced glutathione when challenged by an oxidative stress. Since this "glutathione instability" is also known to be associated with the enhanced sensitivity of G6PD-deficient individuals to the hemolytic anemia induced by certain arylamine drugs (*3*), it has been suggested that the mechanisms underlying favism and drug-induced hemolytic anemia may be similar (*1, 2*).

The fava bean pyrimidine aglycons divicine and isouramil were initially implicated as mediators of favism primarily because they were observed to deplete reduced glutathione when added to suspensions of G6PD-deficient human erythrocytes (*2*). Although many studies on these pyrimidines have since been published, strengthening this relationship, the evidence is still only circumstantial. Thus while autoxidation of divicine and isouramil in acellular systems yields hydrogen peroxide, which can potentially induce oxidative damage within erythrocytes (*4–6*), the fate of divicine and isouramil in the red cell is not known. In vitro exposure of G6PD-deficient human erythrocytes to divicine also has been reported to alter red cell morphology (i.e., membrane cross-bonding) in a manner similar to that observed in erythrocytes withdrawn from patients undergoing favic crises (*17*). However, the significance of this morphological change for the hemolytic event is unclear.

A direct examination of divicine and isouramil hemotoxicity has not previously been practical due to the

difficulty in obtaining sufficient amounts of these compounds for in vivo studies. Neither isouramil nor its parent glucoside, convicine, are commercially available, and only limited amounts of divicine can be prepared for immediate experimental use by hydrolysis of vicine. Alternative synthetic methods for the preparation of divicine and isouramil exist in the literature (*18–20*); however, these methods are relatively complex and have not been utilized by most of the investigators in this field.

In order to obtain a sufficient amount of divicine for a direct test of its hemolytic activity, we utilized the synthetic method of Bailey et al. (*11*). This method yielded a single product with chromatographic properties (Figure 2A) and a molecular weight (Figure 3B) that was identical to divicine prepared enzymatically from vicine. In contrast, when the acid hydrolysis procedure was used to prepare divicine from vicine, a mixture of products was obtained (Figure 2C), which supports the suggestion by Pedersen et al. (*10*) that studies utilizing this method may be of questionable validity.

Further attempts to elucidate the structure of synthetic divicine by NMR analysis were of only limited success (Figure 4). Although the carbon spectrum of synthetic divicine was consistent with the proposed structure of divicine, definitive structural information could not be obtained under the present experimental conditions. Since divicine can (theoretically) exist in several tautomeric forms (*14*), it would be of interest to know what form of divicine predominates at physiological pH. Further studies will be necessary, perhaps by utilizing derivatization of divicine functional groups, in order to fully elucidate the structure of divicine.

It has been suggested that divicine induces toxicity as a result of its autoxidation within the red cell by a process involving the formation of free radicals derived from divicine and/or molecular oxygen (*4–6*). Cyclic voltammetry experiments (Figure 7) directly demonstrated that divicine readily undergoes a reversible oxidation at physiological pH. Nevertheless, divicine is reasonably stable in solution provided that oxygen is excluded (Figure 5), which permits its convenient experimental handling. These data support the suggestion that divicine undergoes a two-electron oxidation to its quinone derivative, generating hydrogen peroxide and other active oxygen species (*4–6*). However, the nature of this reaction within red cells and its significance with respect to divicine hemotoxicity remain to be determined.

Of importance, these studies provide unequivocal evidence that divicine is a direct-acting hemolytic agent in rats (Figure 8). Incubation of divicine, prepared either synthetically or by enzyme hydrolysis of vicine, with ^{51}Cr-tagged red cells in vitro caused damage in the red cells such that they were removed very rapidly from the circulation after their readministration to isologous rats. The capacity of divicine to induce "premature aging" of red cells in the in vitro incubates indicates that extra-erythrocytic metabolism, such as that of the liver, is not obligatory for the hemolytic activity of this aglycon. It is also noteworthy that the hemotoxicity of divicine was expressed in normal rat red cells, that is, in red cells that have normal G6PD activity. This observation raises the possibility that a favic response can be induced by divicine in the rat, providing a useful experimental model to resolve the mechanism underlying favism.

The concentration of divicine used in the present hemotoxicity study (1.5 mM) may be considered to be high relative to that expected in the blood of favic patients, where hemotoxicity has been claimed in response to as little as one bean (*3*). It is not yet known whether the apparent resistance of the rat red cells is associated solely with the presence of normal G6PD capability or with the intervention of additional factor(s). Additional studies in progress will more fully characterize the concentration dependence for the hemolytic response induced by divicine.

The data also confirm that the parent glucoside, vicine, is not directly hemotoxic, but must be converted to divicine in order to induce toxicity. Overall, the data are consistent with the hypothesis that favism is initiated by microbial β-glucosidase-mediated hydrolysis of the parent glucosides in the GI tract and that the absorbed pyrimidine aglycons are directly hemotoxic (*21*). However, these experiments do not distinguish whether divicine induces a premature splenic sequestration or a direct vascular lysis of the damaged cells. Direct vascular lysis of osmotically fragile red cells seems unlikely since the release of radiolabel from the red cells during in vitro incubation was always very low (<1%) and was not significantly different between control and divicine-treated cells.

Of mechanistic interest, the hemolytic response induced by divicine was characterized by an immediate, very rapid phase of removal of erythrocytes from the circulation, followed by a very gradual phase which was even slower than that seen in the control (vehicle-treated) incubates (Figure 8). The rapid initial phase is considered to represent the removal of divicine-damaged red cells whereas the slow phase reflects the normal removal of younger, otherwise undamaged, erythrocytes as they become senescent. This sharply defined biphasic response contrasts markedly with the more moderate and gradual removal of erythrocytes observed after exposure to hemolytic arylamine metabolites, such as those of aniline and dapsone (*13, 22*). The marked difference in the pattern of removal of the damaged red cells in these two situations suggests that the mechanism underlying favism may be significantly different from that of arylamine-induced hemolytic anemia.

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