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PRELIMINARY COMMUNICATION

DETECTION OF A FREE RADICAL INTERMEDIATE FROM DIVICINE OF VICIA FABA

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Vicine (2,6-diamino-4,5-dihydroxypyrimidine-beta-glucoside) occurs in relatively high amounts in broad bean seeds (Vicia faba) (1). Upon hydrolysis of the beta-glucosidic bond, the unstable aglycone divicine is released. Divicine is believed to be responsible for the acute hemolysis observed in glucose-6-phosphate dehydrogenase (G6PD)-deficient subjects after consumption of fresh fava beans (2,3). Recently Chevion et al. (4) have shown that, in the presence of oxygen, divicine is rapidly oxidized with the simultaneous formation of H_2O_2. The addition of reduced glutathione (GSH) reduces the oxidized pyrimidine back to its original state, allowing it to react once again with oxygen, hereby establishing a cyclic reaction producing H_2O_2 and consuming GSH (4).

Since this mechanism shows some similarities with the oxidative cycle of alloxan (5), in which a free radical intermediate has been detected (6), we have investigated the possibility that a free radical intermediate occurs during the autoxidation of divicine.

Materials and Methods

Vicine was obtained from Serva, Heidelberg, FRG. Beta-glucosidase and GSH were purchased from Sigma U.K., Poole, Dorset, U.K. Divicine was produced as follows: a mixture containing 10 mM vicine and 10 mg/ml beta-glucosidase in 0.1 M phosphate buffer, pH 6, was deaerated by flushing 10 min with nitrogen and then incubated 60 min at 37°C in a nitrogen atmosphere. Aliquots of 0.2 ml of such a mixture were transferred to a flat cell and allowed to react with oxygen at room temperature directly in the cavity of the ESR spectrometer.
ESR analysis was carried out at room temperature using a Varian Associates E 3 spectrometer. The instrument settings were: modulation amplitude 0.25 G; modulation frequency 100 kHz; sweep rate 16 min; time constant 1 sec.

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Results and Discussion

The reaction of divicine with atmospheric oxygen produces a free radical species that can be detected by ESR spectroscopy, as shown in Fig. 1. The ESR spectrum has hyperfine structure consisting of 15 equally spaced lines with hyperfine coupling constants of 0.5 G. These features became more evident when the pH of the incubation mixture was increased up to 9 by the addition of minimal amounts of 1 mM NaOH in the flat cell. On the contrary, practically no signal was observed when the pH was lowered below 5 with 1 mM HCl. The pH changes apparently did not modify the ESR spectra, apart from their intensity, as judged by the trace overlapping, suggesting that the same radical species was still observed. In the better resolved spectra the intensity ratios of the single lines appeared not to follow a Tartaglia's triangle scheme (7), in agreement with the presence of two non-equivalent amino groups in the molecule. The low intensity of the ESR signal does not permit an exact definition of the weaker components of the spectrum against the background noise so that it is not possible to make a definitive assignement.

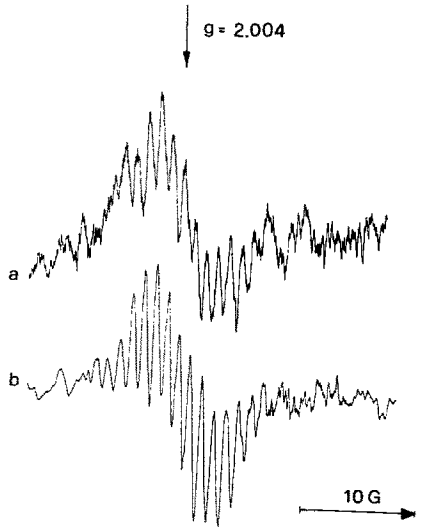

Fig. 1 : ESR spectrum formed during the oxidation of divicine at pH 6 (a) The spectrum in (b) with a reaction mixture as in (a), but after the pH was increased up to 9.

The instrument gain was 8 x 10^4 for the spectrum (b). For the other instrument settings, see Materials and Methods section for details.

The ESR spectrum was relatively stable with time, showing a 20 percent decrease in the intensity after 15 min when maintained in buffer at pH 6. No signal was seen if beta-glucos= idase was omitted from the incubation mixture or if divicine was maintained in a nitrogen atmosphere, indicating that the release of the pyrimidine aglycone and its reaction with oxygen are essential for the radical formation.

Recently it has been shown that divicine can readily be oxidized by atmospheric oxygen with a characteristic shift in the absorption spectrum from 285 nm to 245 nm and the stoichiometric production of H_2O_2 (4). This reaction is compatible with the formation of a semiquinone radical species in analogy to that observed with alloxan (6). The addition of 1 mM GSH to the flat cell completely suppressed the signal (not shown), in agreement with the finding that GSH reacts rapidly with the partially oxidized pyrimidine, forming a complex absorbing at 305 nm (4).

These results suggest that the free radical species observed by ESR spectroscopy is consistent with the oxidized form of divicine reported by Chevion et al. (4) that has an absorbance maximum at 245 nm. Previous attempt to detect the presence of a radical intermed= iate by the same authors probably failed because of the low signal intensity and the high noise/signal ratio. In our conditions the steady state concentration of the radical, as estimated by the ESR signal intensity, was likely quite low. Nevertheless, according to the cyclic redox reaction suggested by the above authors (4) even a minimal amount of radical could be sufficient for the formation of large quantities of H_2O_2.

The presence of a semiquinone free radical intermediate supports the hypothesis that glucosides of Vicia faba cause hemolytic crises in patients with G6PD-deficiency as a consequence of the oxidative stress induced in the red cell by the cyclic oxidation-reduction of the pyrimidine aglycone. In fact, during these reactions large amounts of GSH are oxidized to GSSG because of both pyrimidine reduction and the formation of H_2O_2 (1,9) . The absence of G6PD critically affects the normal regeneration of GSH by blocking the formation of NADPH from the pentose phosphate pathway (8). Under such conditions red cells will rapidly undergo peroxidative reactions leading to irreversible alterations and sequestration by the reticuloendothelial system.

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