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TRANSITION METALS MEDIATE ENZYMATIC INACTIVATION CAUSED BY FAVISM-INDUCING AGENTS

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SUMMARY: Enzymatic activity of purified or membrane-bound acetylcholine esterase was lost when incubated aerobically in the presence of both favism-inducing agent (isouramil or divicine) and copper ions. The requirement for oxygen could be substituted by hydrogen peroxide. Chelating agents provided total protection to the proteins. The suggested mechanism of enzymatic inactivation is analogous to that suggested earlier for the effects of superoxide and ascorbate, and involves the site-specific formation of hydroxyl radicals in the metal-mediated Haber-Weiss reaction. These findings may be relevant to the understanding of the pathogenesis of favism.

Recent studies have indicated that the common denominators in biological damage induced by superoxide radical (1), ascorbate (2,3) or paraquat (4) is the involvement of transition metal ions in a site-specific Fenton mechanism (5,6). These agents reduce the metal within a complex with a cellular component such as a protein, and subsequently, the reduced complex reacts with hydrogen peroxide yielding hydroxyl radicals which are responsible for the damage. All these agents are reducing species that liberate the required hydrogen peroxide during their oxidation.

Favism is the acute hemolytic crisis following the ingestion of broad beans in G6PD deficient subjects (7,8). Two pyrimidines, isouramil and divicine which are found in these beans, have been incriminated as the responsible toxic constituents (9-12). It has

Abbreviations used: AChE, acetylcholine esterase; Detapac, diethelenetriaminepentaacetic acid; EPR, electron paramagnetic resonance.

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been shown that treatment of erythrocytes with these agents leads to marked alterations in their physiological and biochemical characteristics, such as the shortening of their life span *in vivo*, increased susceptibility to erythrophagocytosis and decreased deformability (13). Other studies of these favism-inducing agents have shown that they are strong reducing agents which react rapidly with oxygen yielding free radicals and hydrogen peroxide (12).

This communication addresses the question whether the effects of the favism-inducing agents are also mediated by transition metal ions.

MATERIALS AND METHODS

Enzymes and Chemicals: Acetylcholine esterase from horse serum (from Sigma Chemical Co., EC 3.1.1.8 type IV, 4.5 units/mg), and ascorbic acid from Hopkins and Williams were used. Isouramil and divicine were prepared according to Bien et al. (14) and stored under nitrogen at -80°C. Stock solutions (2mM) were prepared under nitrogen in water. Once prepared, the solutions were kept on ice and used within 3 h. Decomposed divicine was prepared by overnight incubation of divicine (10mM) at 37°C in air. According to the ultraviolet absorption at 285 nm 96% of the pyrimidine was decomposed. Acetylcholine esterase activity was determined in duplicate according to the method of Ellman et al. (15).

Electron paramagnetic resonance (EPR) measurements were carried on a Varian E-4 spectrometer run at liquid nitrogen using modulation amplitude of 10 Gauss and receiver gains 100-1000.

Blood from healthy volunteers was drawn into heparinized syringes and centrifuged at 3000 RCF for 5 min. The plasma and buffy coat were removed and the cells were washed thrice with 10-15 volumes of isotonic saline solution. The packed cells were lysed in 5 mM phosphate buffer, pH8 and kept on ice for at least 30 min, to ensure complete lysis. The resulting hemolysate was centrifuged at 13,000 RCF for 15 min and membrane fragments were collected and washed at least 3 times until they were free of visible hemoglobin. The hemoglobin-free ghosts were finally suspended in 5 mM Tris buffer, pH7.4 to a protein concentration of 0.25 mg/ml.

RESULTS

Figure 1 shows the activity of purified acetylcholine esterase (AChE) as a function of exposure time to isouramil, divicine or ascorbate (1 mM, each) together with copper in air-saturated solutions. A marked decrease in the activity can be

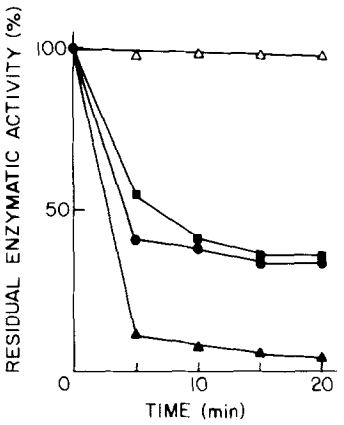

Figure 1: The effect of copper and the favism-inducing agents or ascorbate on the activity of acetylcholine esterase (AChE). The reaction mixture (RM) contained 0.1 mg/ml AChE, in phosphate buffer (5 mM pH7.4).
(●) Isouramil (1mM) + 0.02mM copper sulfate in RM
(■) Divicine (1mM) + 0.02mM copper sulfate in RM
(▲) Ascorbate (1mM) + 0.02mM copper sulfate in RM
(△) Ascorbate (1mM) + Detapac (0.01mM); no copper added in RM

seen; isouramil or divicine caused a loss of about half of the activity within 5 min of incubation, while ascorbate caused an 86% loss during that time. In control experiments (not shown), the enzyme was exposed to copper alone (0.02 mM) and a slight increase in the enzymatic activity was recorded (5-10%) (2).

Incubation of AChE with isouramil alone resulted in a 27% decrease in the enzymatic activity while divicine alone caused only a small decrease in the activity (5%). In both cases this decrease could be completely prevented by adding a chelating agent such as detapac or desferrioxamine (not shown). In fact, a slight increase (5-8%) in the activity was then exhibited. Similarly, incubation of AChE with ascorbate alone yielded a decrease in activity (15-20%), which was also totally eliminated by the addition of a chelating agent (Fig. 1) (2).

Fig. 2 shows the loss of activity of the membrane-bound AChE (from human erythrocytes) as a function of isouramil and copper concentrations. It can be seen that most of the deleterious effects were already exhibited at a concentration of 0.3 mM

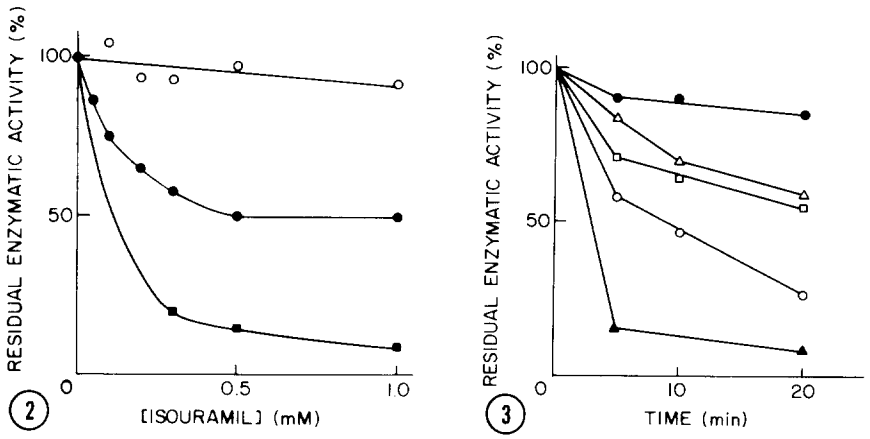

Figure 2: The residual enzymatic activity of the membrane-bound acetylcholine esterase following exposure (1h) to isouramil (IU) and copper. (○) control (no copper added) in reaction mixture (RM) containing RBC-membrane (250 µg protein/ml) phosphate buffer (5mM, pH7.4); (●) 0.02mM copper in the same RM; (■) 0.1mM copper in the same RM.

Figure 3: The effect of increasing concentrations of decomposed divicine on the (ascorbate + copper)-induced inactivation of acetylcholine esterase. The reaction mixture (RM) contained ascorbate (2mM) and copper (0.02mM) in phosphate buffer (5mM, pH7.4). (▲) RM; (○) RM + 0.25mM decomposed divicine; (□) RM + 0.5mM decomposed divicine; (△) RM + 1mM decomposed divicine; (●) RM + 2.5mM decomposed divicine.

isouramil. Also, the loss in enzymatic activity increased monotonically with copper concentration, although the dependence was non-linear; a five-fold increase in copper (from 0.02 to 0.10 mM) yielded only a 2-fold increase in the loss of enzymatic function. Incubation with isouramil but without added copper showed only a marginal loss in protein activity (Fig. 2).

The above mentioned results, as well as other experiments, showed that the inactivation caused by either pyrimidine is always smaller than that induced by ascorbate, under comparable conditions. Thus, we examined whether the decomposition products of divicine served as chelating agents. Figure 3 shows the residual AChE activity during exposure to ascorbate (2mM) and copper (0.02mM) in the presence of various concentrations of decomposed divicine. It is clearly demonstrated that the

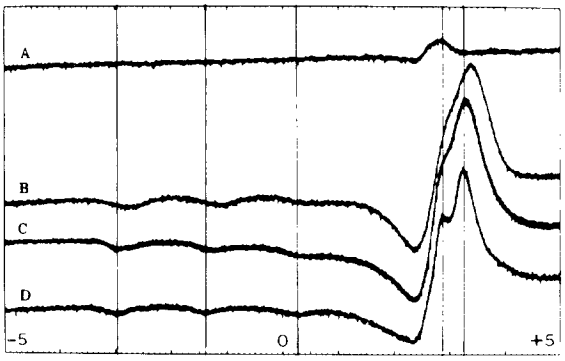

Figure 4 : The low temperature EPR spectra of copper. All spectra were recorded at liquid nitrogen with modulation amplitude of 10 Gauss. All samples contained copper sulfate (1mM) in phosphate buffer (5mM, pH7.4)
A. No additions
B. Addition of AChE (10mg/ml)
C. Addition of AChE (10mg/ml) together with decomposed divicine (5mM)
D. Addition of decomposed divicine (5mM).

decomposed pyrimidine provided protection to the enzyme, possibly by chelating of the copper ions.

Figure 4 shows the low temperature EPR spectra of copper(II) in various systems. It can be seen that copper formed a complex with the decomposed divicine (trace D), and with AChE (trace B). In the presence of both AChE and decomposed divicine (trace C), the spectral features were similar to those in trace D, indicating that now the copper was bound to the decomposed pyrimidine.

DISCUSSION

We have used the inactivation of a protein, purified or membrane-bound acetylcholine esterase, as a simplified model for the mechanism of biological damage in the hemolytic crisis in favism. The results show that loss of enzymatic function is dependent on the presence of a favism-inducing agent (isouramil or divicine) together with copper or iron (not shown) ions. Chelating agents totally protected against the deleterious effects. Enzymatic inactivation was also dependent on oxygen,

though this requirement could be substituted by hydrogen peroxide (not shown). The results indicate that these pyrimidines can induce biological damage in a mechanism which is analogous to the "site specific, metal mediated, Haber-Weiss" reaction earlier suggested for the damage caused by ascorbate or superoxide in the presence of transition metal ions (1-3).

Isouramil and divicine are reductones (16) which react quickly with molecular oxygen yielding oxygen-derived free radicals and hydrogen peroxide (11,12). They can also reduce Cu(II) ions to the corresponding cuprous complexes which can, in turn, produce the deleterious hydroxy radicals in a site specific Fenton reaction (2). These radicals are assumed to be the harmful species in the system. Being also effective chelating agents, these pyrimidines or their decomposition products act also in an additional pathway. From EPR spectroscopy, it is obvious that they compete for the binding of the copper and simultaneously reduce the rate of protein inactivation.

These results may explain the deleterious role of isouramil or divicine in favism. The possible involvement of available redox-active transition metal ions in this pyrimidine-induced site specific mechanism may provide an explanation for the apparently bizarre and unpredictable onset of the favic crisis in susceptible subjects who are exposed to the broad beans (7,8). Thus the nutritional state and the physiological availability of transition metal ions may play a crucial role in the pathogenesis of this hemolytic crisis.

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