MASTER

DOE/EV/03221-63

FAVISM INDUCING AGENTS

A PULSE RADIOLYSIS STUDY OF ISOURAMIL AND CONVICINE

PROGRESS REPORT

Mordechai Chevion^{*} and Yael A. Ilan⁺

from

^{*}The Department of Cellular Biochemistry

and

⁺The Department of Physical Chemistry

The Hebrew University of Jerusalem, Jerusalem, Israel

Work carried out during the period:

December 1st, 1979 to November 30th, 1980.

Prepared for the U.S. Energy Research & Development Administration

Under Contract No. DE-AC02-76EV03221

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

MASTER

DISCLAIMER

This book was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not

COO - 3221 - 63

FAVISM INDUCING AGENTS

A PULSE RADIOLYSIS STUDY OF ISOURAMIL AND CONVICINE

PROGRESS REPORT

Mordechai Chevion^{*} and Yael A. Ilan⁺

from

^{*}The Department of Cellular Biochemistry

and

⁺The Department of Physical Chemistry

The Hebrew University of Jerusalem, Jerusalem, Israel

Work carried out during the period:

December 1st, 1979 to November 30th, 1980.

Prepared for the U.S. Energy Research & Development Administration
Under Contract No. EY-76-C-02-3221

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

SUMMARY

Isouramil and convicine, substances implicated in precipitating favic crises in glucose-6-phosphate dehydrogenase deficient individuals have been studied in N_2O -saturated aqueous solutions by pulse radiolysis, and (1) $k_{OH+isouramil} = 5 \times 10^9 M^{-1} sec^{-1}$ and (2) $k_{OH+convicine} = 1.5 \times 10^{10} M^{-1} sec^{-1}$ have been determined. The products of these reactions are addition intermediates absorbing above 300nm ($\lambda_{max} = 320$ nm). The decay of the intermediate(s) formed in reaction (1) is a biphasic one characterized by $k^f = 3.5 \times 10^3 sec^{-1}$ and $k^s = 3.5 \times 10^2 sec^{-1}$, while the decay of the corresponding intermediate for reaction (2) is characterized by a single constant ($k = 3.5 \times 10^2 sec^{-1}$).

By analogy to uracil, it is suggested that $\dot{OH}$ is added to the double bond at either positions 5 or 6 of the pyrimidine ring forming two different intermediates. Each of these intermediates loses a molecule of water indicated by the observed biphasic decay reaction (k^f and k^s). For convicine on the other hand, position 5 is blocked by the O-glucosidic bond and the addition of $\dot{OH}$ could take place only at position 6. Thus, a single intermediate is formed and its decay is a single-phase one. It has been shown that although free radicals could not be detected in the course of the reactions between isouramil and oxygen or with cellular components by electron paramagnetic resonance, isouramil can participate in a uni-electron transfer reaction and can form relatively stable intermediates. Thus, it is speculated that in the red blood cells isouramil could give rise to deleterious free radicals.

INTRODUCTION

Favism is the acute hemolytic crisis following the ingestion of broad bean (vicia faba) by glucose-6-phosphate dehydrogenase (G6PD) deficient individuals (1,2). It is assumed that the onset of the disease involves a cyclic mechanism in which a pyrimidine that is found in the bean is oxidized by molecular oxygen and then it is reduced back by cellular glutathione (GSH), (3) (scheme 1):

isouramil: R = OH

divicine : R = NH₂

G6PD deficiency has been shown to be associated with GSH instability (4); indeed, the first prominent change that takes place when red blood cells are challenged with either of these pyrimidines is the depletion of the intracellular reduced glutathione (5).

It has already been shown (6,7) that OH radicals are involved in the cyclic oxidoreduction of (dialuric acid) ~~and~~ (alloxan) which is a system very similar to scheme 1. Therefore, it may be anticipated that OH plays a role in the development of the favic crisis.

The aim of this work is to shed more light on the mechanism of action of the favism-inducing pyrimidines by studying their reactions with hydroxyl radicals. In specific, to examine whether the isouramil molecule can participate in a single electron transfer reactions indicating that during the

development of the fava crisis it could serve as a source for deleterious free radicals. In addition, the understanding of these reactions is of obvious importance in explaining biological damage caused by radiation as similar pyrimidines constitute the DNA molecule.

EXPERIMENTAL

Description of the pulse radiolysis set up was previously published (8). Pulse duration was 0.5-1.5 μ sec (200 mA, 5 Mev) and the e_{aq}^- and $\dot{OH}$ concentrations were $(5-10) \times 10^{-6}$ M. A Spectrosil cell with optical path of 1 cm was used. Appropriate light filters were used to minimize photochemical and scattered light effects.

Isouramil was prepared according to Bien et al. (9,10). Convicine was isolated from broad beans (vicia faba) (9,10) and recrystallized from ethanol-water mixture.

All other chemicals were of the highest analytical grade available and were used without any further purifications. Solutions were made using highly purified water prepared by passing distilled water through "Millipore membranes".

We used the syringe technique (11). Appropriate gases were bubbled through the solutions before irradiation. Nitrous oxide was bubbled in order to convert all the e_{aq}^- to $\dot{OH}$ radicals. Argon saturated solutions containing 0.1 M tert-butanol (in order to scavenge all the $\dot{OH}$ radicals) were used to leave the e_{aq}^- as the sole reactive species (11). The pH was adjusted between 5.3-8.0 using 0.02 M phosphate buffer.

The concentration of isouramil and convicine was $(5-10) \times 10^{-5}$ M.

The concentration of e_{aq}^- was measured spectrophotometrically at 578 nm where $\epsilon_{e_{aq}^-} = 10,600 \text{ M}^{-1} \text{ cm}^{-1}$ (11). Dosimetry for $[\dot{OH}]$ measurements was made by the ferrocyanide method (11).

RESULTS

Pulse Radiolysis of N₂O-saturated Solutions of Isouramil

Irradiation of dilute aqueous solutions yields primary radicals according to

In N₂O-saturated solutions e_{aq}^- reacts with N₂O to yield OH; hence, almost all the radicals present are OH (8).

The reaction of isouramil with OH was spectrophotometrically studied at $\lambda = 249$ and 290 nm and at pH range $5.3-8.0$; the rate constant was found to obey first order expression and to be independent of pH. From the linear dependence of the observed pseudo first order rate constant on the concentration of isouramil, $[\text{IU}] = (0.5-1.0) \times 10^{-4}$ M the second order rate constant $(5 \pm 1) \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$ could be evaluated. This reaction reached completion within $10 \mu\text{s}$. The spectrum of the product (measured at $10 \mu\text{s}$ after the pulse) is presented in Figure 1.

At longer times (millisecond region) a second reaction (equation 4) could be observed. The latter reaction represents a decay and unlike the first one (equation 3), was independent of the concentrations of isouramil and the dose. Also, the kinetic curves of the second reaction could not be fitted to a single first order rate expression. Instead, when a linear combination of two first

order rate expressions was employed (Equation 5) an excellent fit was exhibited (Figure 2) indicating that two decay reactions are taking place simultaneously.

$$A_t = A_0^f \cdot e^{-k^f t} + A_0^s \cdot e^{-k^s t} \quad (5)$$

k^f and k^s are the rate constants for the fast and slow phases of reaction 4.

Computer best fit analysis yields:

$$k^f = 3.5 \times 10^3 \text{ sec}^{-1} \text{ and } k^s = 3.5 \times 10^2 \text{ sec}^{-1}.$$

After 1 ms, the intermediate had completely decayed and the remaining substance absorbed at wavelengths under 300 nm.

Pulse-Radiolysis of N₂O-Saturated Solution of Convicine

The reaction between $\cdot\text{OH}$ and convicine was similarly studied. The second order rate constant of this reaction was found to be $1.5 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$. The difference spectrum taken at 10 μs is reminiscent of that of isouramil (figure 3); a decrease of the absorption at 274 nm with a build-up of an intermediate that absorbs at $\lambda > 300 \text{ nm}$, with a maximum at 320 nm. Quantitatively, the change in molar absorbance is only about 1/3 of that observed with isouramil.

A decay reaction similar to that observed with isouramil (Reaction 3) was found in this case as well. However, with convicine this reaction obeyed a single-phase first order expression characterized by $k = 3.5 \times 10^2 \text{ sec}^{-1}$.

Reaction of Convicine and Isouramil with e_{aq}^-

Convicine did not react with e_{aq}^- under our experimental conditions. Isouramil on the other hand, reacted with $k_{\text{obs}} = 3 \times 10^5 \text{ sec}^{-1}$.

DISCUSSION

Isouramil is implicated as being a toxic constituent leading to the precipitation of the favic crisis. The suggested mechanism of its action (2,3) involves the oxidation of isouramil by molecular oxygen and the reduction of its oxidized form by glutathione. This is a cyclic reaction consisting of two bi-electron steps.

Although it was impossible to identify pyrimidine radicals in the course of their reaction with oxygen and other cellular components by electron paramagnetic resonance (3), the question whether the first bi-electron oxidation reaction consists of two consecutive one-electron steps was still pending.

The attack of OH radical results in a one electron transfer reaction. It suggests that a one-electron oxidation product of isouramil is relatively stable and decays in the millisecond time range.

Thus the oxidation of isouramil within the red blood cell may also involve a series of reactions including the formation of free radicals. The biphasicity observed in the decay of the intermediate(s) (reaction 4) that were formed during the attack of OH radicals on the pyrimidine molecule (reaction 3) indicates that two different products had already been formed. It was found that the kinetics of the disappearance of the 5,6-double bond (reaction 2) (as was followed at 280 nm) is identical to the kinetics of the build-up of the absorbance peak at 320 nm. The possibility of radical-radical interaction in reaction 4 can be excluded as a reduction of the dose by a factor of two did not alter the rate of decay. Thus, we suggest that OH radical is added to the double bond at either position 5 or 6 of the pyrimidine ring (reaction 6). This reaction is analogous to the attack of OH radicals on the 5,6-double bond of uracil (12). In both cases, the addition intermediates exhibit a red shift in their ultraviolet spectrum as compared to the native pyrimidine, $\lambda_{\text{max}} = 320$ and 385 nm for the isouramil and uracil, respectively.

Assuming 100% efficiency of reaction 6, one can evaluate the higher limit of the molar extinction $\epsilon_{320} \sim 5000$ for the addition intermediates of isouramil.

In the case of convicine a single addition intermediate of the pyrimidine ring is observed (reaction 7). We assume that 2/3 of the OH radicals oxidize

the glucose residue of the convicine molecule while only 1/3 react directly with the pyrimidine ring. The observed rate constant for reaction 7 is $1.5 \times 10^{10} \text{ sec}^{-1}$, which is 3-fold larger than the corresponding constant for isouramil. The reason may be that when following the formation of the product (at 300 nm), k_{obs} is a sum of the rate constants of OH with all the sites on the molecule (reaction 8) (see appendix).

$$k_{\text{obs}}^{\text{formation of intermediate}} = k_{\text{OH} + \text{pyrimidine}} + k_{\text{OH} + \text{glucose}} \quad (8)$$

The evaluated second order rate constant for the formation of the intermediate (reaction 7) $k = 1.5 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$ is in agreement with the findings that

$$\begin{aligned} k_{\text{OH} + \text{IU}} &= 5 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1} \\ k_{\text{OH} + \text{glucose}} &= 10 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1} \end{aligned} \quad (13)$$

The fate of the addition intermediates can only be speculated upon. Again by analogy to uracil adducts reaction 3 can represent the elimination of a water

molecule from either carbon 5 or 6 of the pyrimidine as follows:

And from the comparison of the rate constants of reactions 9 with the corresponding constant for convicine the rate constants k^f and k^s can be assigned so that k^f represents reaction (9a) and k^s represents (9b).

It has been shown that both OH and O_2^- play a role in the reaction of dialuric acid with oxygen (6,7). As dialuric acid is structurally very similar to isouramil it is likely that OH is involved also in the decomposition of isouramil. In conclusion, we have shown that isouramil can participate in a uni-electron reaction. This may have some implications on the mechanism for the outbreak of the favic crisis, as well as on radio-damage to pyrimidine nucleic acids.

Acknowledgement

This study was supported, in part, by the United States Energy Research and Development Administration (ERDA) under contract DE-AC02-76EV03221. The authors should like to thank Profs. J. Mager and G. Czapski for encouraging this study.

References

1. J. Mager, A. Razin and A. Hershko: "Favism" in Toxic Constituents of Plant Foodstuffs (L.I. Liener, Editor) Academic Press, New York, pp.293-318. (1969).
2. J. Mager, M. Chevion and G. Gläser: "Favism" in Toxic Constituents of Plant Foodstuffs (L.I. Liener, Editor) Academic Press, New York, pp 265-294 (1980).
3. M. Chevion, T. Navok, G. Gläser and J. Mager: Favism inducing agents - the chemistry of isouramil and divicine. In preparation.
4. E. Beutler, M. Robson and E. Bittenwieser: The mechanism of destruction and protection in drug-sensitive and non sensitive erythrocytes. In vitro studies. J. Clin. Investigation 36, 617-26 (1957)
5. A. Razin, A. Hershko, G. Glaser and J. Mager: The oxidant effect of isouramil on red cell glutathione and its synergistic enhancement by ascorbic acid or 3,4 dihydroxyphenylalanine. Possible relation to the pathogenesis of favism. Israel J. Med. Sci. 4, 862-867. (1968).
6. G. Cohen and R. E. Heikkila: The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid and related cytotoxic agents. J. Biol. Chem. 249, 2447-2452 (1974).
7. J.A. Fee, R. Bergamini and R.G. Briggs: Observations on the mechanism of the oxygen/dialuric acid-induced hemolysis of vitamin E-deficient rat red blood cells and the protective roles of catalase and superoxide dismutase. Arch. Biochem. Biophys. 169, 160-167. (1975).
8. Y.A. Ilan, J. Rabani and G. Czapski: One electron reduction of metmyoglobin and methemoglobin and the reaction of the reduced molecule with oxygen. Biochim. Biophys. Acta, 446, 277-286 (1976).
9. S. Bien, G. Salemnik and L. Zamir: The structure of convicine. J. Chem. Soc. (C) (London) 496-500 (1968).
10. S. Bien, D. Amith and M. Ber: Synthesis of convicine (6-amino-5- β -glucopyranosyloxyuracil) J. Chem. Soc., Perkin Trans. I. 1089-1092 (1973).
11. M.S. Matheson and L. H. Dorfman: "Pulse Radiolysis", MIT Press (1969).
12. C.L. Greenstock, W.J. Hunt and M. Ng: Pulse radiolysis studies of uracil and its derivatives. Trans. Faraday Soc. 65, 3279-3287. (1969).
13. L.M. Dorfman and G.E. Adams (Editors) "Reactivity of the hydroxyl radical in aqueous solutions" Published by the United States Department of Commerce (NSRDS - NBS 46) 1973.

Appendix

under pseudo first order experimental conditions:

or

$$-\frac{d[A]}{dt} = k_1'[A] + k_2'[A] = (k_1' + k_2')[A]$$

solving the differential equation yields $A = A_0 \cdot e^{-(k_1' + k_2')t}$

$$\text{and hence } \frac{d[P_1]}{dt} = k_1'[A] = k_1' A_0 \cdot e^{-(k_1' + k_2')t}$$

$$\text{which yields } P_{1t} = \frac{k_1' \cdot A_0}{k_1' + k_2'} (1 - e^{-(k_1' + k_2')t})$$

$$\text{At } t = \infty (1 - e^{-(k_1' + k_2')t})$$

and this is the usual expression for the formation of P_1 by a first order reaction

$$\ln \frac{P_{1\infty} - P_{1t}}{P_{1\infty}} = -\underset{k_{\text{obs}}}{(k_1' + k_2')}t$$

$$k_{\text{obs}} = k_1' + k_2'$$

When plotting the natural logarithm of the absorbance difference against time, a straight line is obtained with a slope of $k_{\text{obs}} = (k_1' + k_2')$.

Figure Captions

Figure 1: The difference spectras measured after the oxidation of isouramil (IU) by OH[•] radicals.

a. Recorded immediately after the termination of the oxidation process (10 μsec).

o Recorded after the termination of the decay process (1 msec).

[IU] = 1×10^{-4} M; [OH[•]] = 1×10^{-5} M; pH = 6.8; [phosphate] = 0.02 M. The solutions were saturated with N₂O.

Figure 2: The change in absorbance with time: The disappearance of the transients formed in reaction 3.

The curve is a computer best fit to the experimental data points using a biphasic kinetic expression (Equation 2)

$\lambda = 320$ nm. All experimental conditions as in Fig 1.

$\Delta A_{\text{O}}^{\text{f}} = 0.043$; $k^{\text{f}} = 4000 \text{ sec}^{-1}$; $\Delta A_{\text{O}}^{\text{S}} = 0.048$; $k^{\text{S}} = 360 \text{ sec}^{-1}$.

Figure 3: The difference spectrum of convicine measured immediately after the oxidation by OH[•] radicals (10 μsec after the pulse).

[convicine] = 1×10^{-4} M; [OH[•]] = $1 \cdot 10^{-5}$ M; pH = 6.8; [phosphate] = 0.02 M. The solutions were saturated with N₂O.

Figure 1

Figure 2

Figure 3