Biochimica et Biophysica Acta, 570 (1979) 371—387

BBA 68826

INVESTIGATION OF THE ENZYMIC AND ELECTROCHEMICAL OXIDATION OF URIC ACID DERIVATIVES

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(Received February 14th, 1979)
(Revised manuscript received June 6th, 1979)

Key words: Uric acid; Peroxidation; N-methyl uric acid; (Electrochemistry)

Summary

The electrochemical oxidation of a number of N-methylated uric acids at the pyrolytic graphite and gold electrodes has been compared to their enzymic oxidation with type VIII peroxidase and H_2O_2. Spectral, electroanalytical and kinetic evidence supports the conclusion that for all compounds the electrochemical and enzymic reactions proceed by identical mechanisms.

Introduction

The oxidation of uric acid in the presence of various enzyme systems has been studied by many investigators. In the presence of uricase/O_2 [1—3], peroxidase/H_2O_2 [4,5], methemoglobin/H_2O_2 [6] and cytochrome *c* [7] the major organic product at low pH is alloxan while at around pH 7 the major product is allantoin. Paul and Avi-Dor [4] found that the neutral form of 1-methyl uric acid is oxidized in the presence of horseradish peroxidase and H_2O_2 via an intermediate species which exhibited ultraviolet absorption at around 307 nm at pH 7.6. This intermediate apparently decomposed to alloxan or allantoin in a first-order reaction. At pH 6.1 the observed first-order rate constant was $1.2 \cdot 10^{-3}\ s^{-1}$. A mechanism was proposed for this reaction where 1-methyl uric acid (I, Eqn. 1) was oxidized in a $2\,e - 2\,H^+$ reaction to give an intermediate diimine (II, Eqn. 1); this was proposed to be the ultraviolet-absorbing intermediate. By uptake of two molecules of water the diimine was

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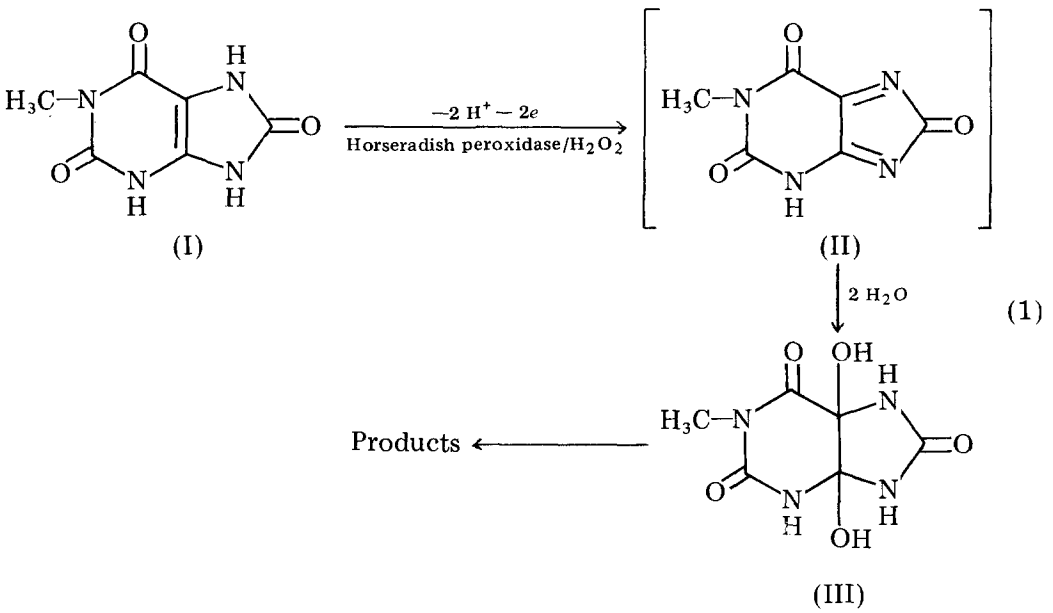
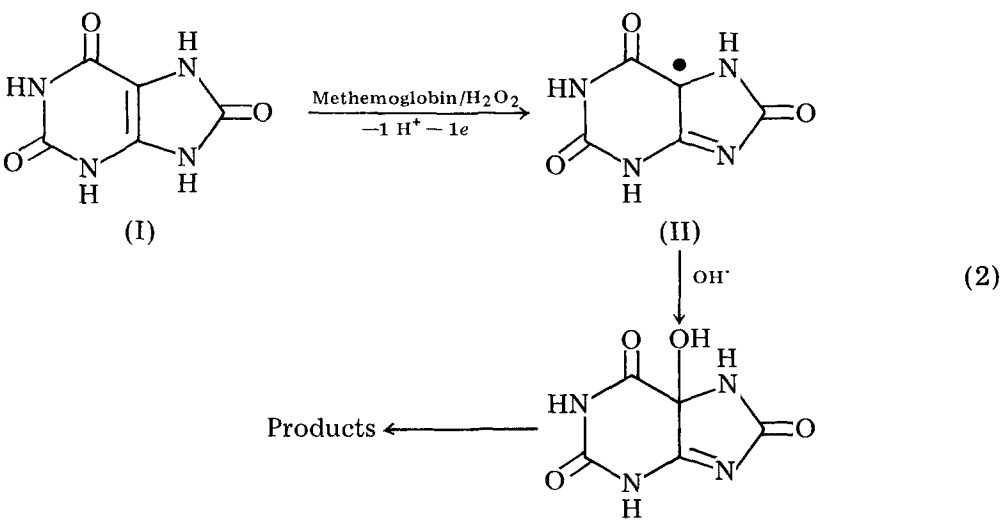
proposed to give a 4,5-diol derivative of 1-methyl uric acid (III, Eqn. 1) which decomposes to the ultimate products



(I) (II) (III) (1)

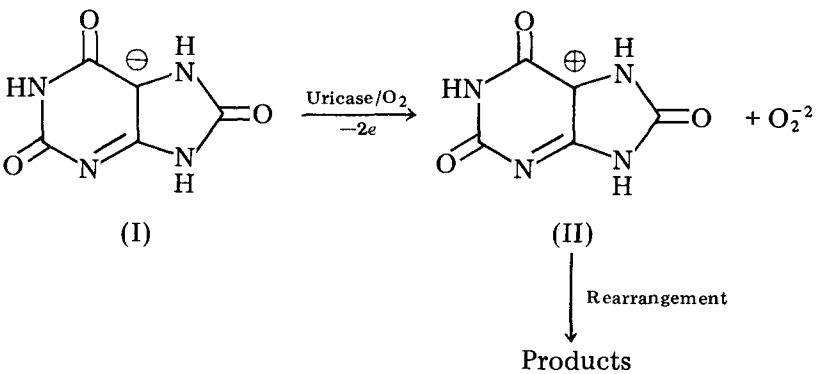
Canellakis et al. [5] also proposed that uric acid-4,5-diol is an intermediate species formed upon oxidation of uric acid in the presence of various peroxidase enzymes. Howell and Wyngaarden [6], however, precluded the mechanism proposed by Paul and Avi-Dor [4] as one of general applicability based on the susceptibility of uric acid derivatives methylated at N-7 or N-9 to oxidation in the presence of methemoglobin/H_2O_2 or horseradish peroxidase/



(I) (II) (III) (2)

H_2O_2 but the failure of these enzyme systems to oxidize 1,3,7,9-tetramethyl uric acid. Thus, an alternative mechanism was proposed in which the initial oxidation step involved a dehydrogenation of the uric acid (I, Eqn. 2) at N-9 or N-7 catalyzed by an enzyme-H_2O_2 complex with one oxidizing equivalent, i.e. a $1e$-$1H^+$ reaction. The resulting product was proposed to be a urate radical (II, Eqn. 2) which was hydroxylate by a hydroxyl radical derived from H_2O_2 to give an imine-alcohol intermediate (III, Eqn. 2). The latter species was then proposed to rearrange to give, ultimately, allantoin.

There have been extensive studies on the oxidation of uric acid in the presence of uricase and O_2 [8—14]. Bentley and Neuberger [14] concluded that the monoanion of uric acid (I, Eqn. 3) is oxidized in the latter system in a $2e$ reaction to a carbonium ion (II, Eqn. 3) which undergoes several rearrangements giving, finally, allantoin.



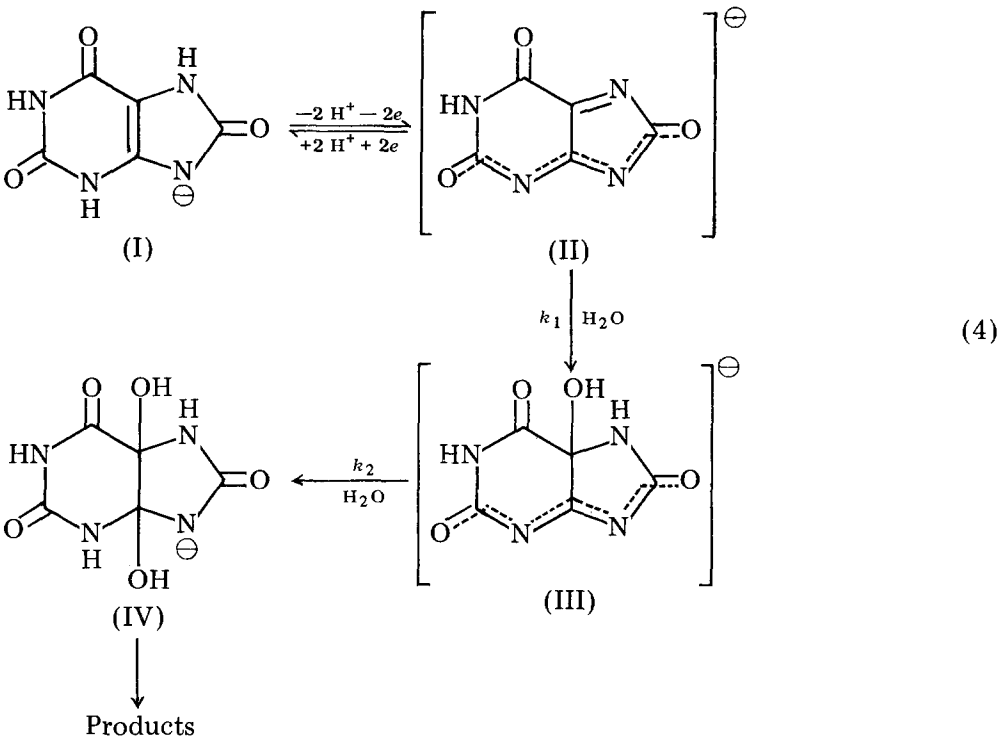
(3)

Thus, many different mechanisms have been proposed to explain the biological oxidation of uric acid. Many different structures have been proposed for intermediate species. The only factor in common agreement is that the final products of the enzyme-catalyzed reaction are alloxan and allantoin, depending upon pH, along with CO_2.

For several years this laboratory has been investigating the mechanism of electrochemical oxidation of purines including uric acid [15—17] in the expectation that these studies might provide valuable insights into the biological oxidation of such compounds. By using a variety of sophisticated electroanalytical methods it has been established [18] that at approx. pH 7 the electrochemical oxidation of uric acid (I, Eqn. 4) at both the pyrolytic graphite electrode and gold electrode proceeds by an initial $2e$-$2H^+$ oxidation of the monoanion of uric acid to give a very unstable diimine primary product (II, Eqn. 4).

The diimine primary intermediate is very unstable and is rapidly attacked by water in a first-order reaction to give an imine-alcohol (III, Eqn. 4), the second intermediate in the reaction. The apparent rate constant for the latter reaction was found to be 32.5 s^{-1} at pH 8.0. At higher and lower pH values this rate constant appeared to increase significantly [18]. The primary, diimine intermediate is also very readily reducible back to uric acid, a process which may be observed under fast-sweep cyclic voltammetric conditions. The imine-alcohol intermediate (III, Eqn. 4) is more stable than the diimine species from which it

is derived and it may be observed via its ultraviolet absorption band, λ_{max} =



(4)

302—304 nm at pH 7—9.3, by means of thin-layer spectroelectrochemical experiments using an optically transparent gold minigrid electrode. The imine-alcohol also undergoes a first-order hydration reaction to give uric acid-4,5-diol (IV, Eqn. 4) with an observed rate constant of $3.5 \cdot 10^{-3}$ s^{-1} at pH 7—9.3. The imine-alcohol is also electrochemically reducible but at much more negative potentials than is its precursor diimine. In a subsequent report [19] the oxidation of uric acid in the presence of horseradish peroxidase and H_2O_2 was studied under identical solution conditions to those employed for the electrochemical investigations. It was observed that during the peroxidase-catalyzed oxidation of uric acid an intermediate species appears which absorbs in the same spectral region (i.e. 302—304 nm at pH 7) and decomposes with the same apparent first-order rate constant as the imine-alcohol intermediate generated electrochemically. These observations suggest that the intermediates formed and their reactions in both the electrochemical and peroxidase-catalyzed reactions are the same. On the basis of studies of the electrochemical oxidation of uric acid [18] the actual structures of the proposed intermediate species (i.e. II, III, IV, Eqn. 4) were still uncertain. Accordingly, a further study has been carried out into the electrochemical oxidation of N-methyl derivatives of uric acid [20]. It was found that N-methylation of uric acid does not alter the basic electrooxidation reaction but it does affect the potential for the primary electron transfer reaction, the number of protons involved in the reaction and some physicochemical properties of intermediate species. The observed effects of the site of N-methylation on the stability and/or ultraviolet absorption of

the intermediate species were those expected on the basis of their structures shown in Eqn. 4.

The work reported here was undertaken to study the oxidation of various N-methylated uric acid derivatives with peroxidase/H_2O_2 and to compare the observed behavior with that observed upon electrochemical oxidation of these molecules.

Experimental

Chemicals. *N*-methyl uric acid derivatives were synthesized according to published methods [20] or obtained from Adams Chemical Co., Round Lake, IL. Horseradish peroxidase type VIII, Rz ≈ 3.2, and catalase were obtained from Sigma. Catalase had an activity of 2000 units/mg at 25°C. Buffer solutions were prepared from sodium phosphates and had an ionic strength of 0.5 M.

Apparatus. Optical measurements utilized either a Harrick rapid scan spectrophotometer and signal processing module (Harrick Scientific Co., Ossining, NY) or a Hitachi-Perkin-Elmer Model 124 Spectrophotometer. Repetitive spectral sweeps utilizing the rapid scan spectrophotometer were recorded on a Tektronix Model 5031 dual-beam storage oscilloscope equipped with a Tektronix Model C-70 camera. Absorbance versus time curves were recorded on a Hewlett-Packard Model 7001A X-Y recorder or a Sargent Model SRG recorder.

Ultraviolet studies of the enzymic oxidation of uric acid derivatives utilized 1.0 cm optically matched quartz cells. Optically transparent thin-layer electrochemical cells were similar to those described by Murray et al. [21] and Heineman et al. [22] and utilized a gold minigrid as the optically transparent electrode. The gold minigrid employed had 100 wires/inch and a transmittance of approx. 50%. The quartz microscope slides used to construct the cell were obtained from Esco Optics Products, Oak Ridge, NJ. Details of the cell design and construction have been presented elsewhere [23].

The construction of gold (0.2 cm^2) and pyrolytic graphite (0.03 cm^2) electrodes has been described in detail in an earlier report [18]. Linear and cyclic-sweep voltametry were carried out with an instrument based on conventional operational amplifier design [24]. A two compartment cell was employed for voltametric experiments which contained a saturated calomel reference electrode (SCE) and a mercury pool counter electrode. All potentials are referred to the SCE electrode at 25°C.

Procedure for enzymic oxidation of uric acids. Stock solutions of type VIII horseradish peroxidase and H_2O_2 were prepared in phosphate buffer (pH 7.1) having an ionic strength of 0.5 M. Usually, 0.70 ml of the appropriate uric acid dissolved in phosphate buffer, pH 7.1, ionic strength 0.5 M, and 0.70 ml of the peroxidase solution were transferred to a 1.0 cm quartz spectrophotometer cell. The oxidation of the uric acid derivative was initiated by addition of 0.70 ml of the H_2O_2 solution. Changes in the ultraviolet absorption during the enzymic oxidation were monitored with the rapid scan spectrophotometer. When approximately 95% of the substrate had been oxidized the reaction was terminated by addition of 0.7 ml of the same buffer solution (pH 7.1) containing 0.7 mg of catalase. This amount of catalase was sufficient to very

rapidly remove H_2O_2 from the reaction mixture.

Thin-layer spectroelectrochemistry. Thin-layer spectroelectrochemical studies were carried out using the same buffer systems utilized for enzymatic studies. The experimental procedures have been described previously [18,20]. Rapid scan spectrophotometric studies of the electrochemical and enzymic oxidation of uric acid derivatives covered the same spectral region, i.e. 220–375 nm.

Results

In order to be able to compare the enzymic and electrochemical oxidation of N-methylated uric acids it was necessary to employ a peroxidase/H_2O_2 as the enzyme system. The other enzyme widely used to oxidize uric acid is uricase but this enzyme is virtually specific for uric acid. Peroxidases, such as horseradish peroxidase, are known to be active towards N-methylated uric acids [4,6]. An earlier report from this laboratory [19] established that the type VIII isoperoxidase enzyme isolated from horseradish peroxidase exhibits quite high activity for uric acid and hence this isoenzyme was used in the present study.

Paul and Avi-Dor [4] have reported that horseradish peroxidase oxidizes the monoanionic form of 1-methyl uric acid (pK_a = 5.75 [25]) only very slowly. However, we have found that the rate of oxidation of all *N*-methyl uric acids, with the exception of the 1,3,7,9-tetramethyl derivative, proceeds rapidly at pH 7 where all compounds are in their monoanionic form. Accordingly, most studies were carried out at pH 7.

Some typical spectra obtained during the electrochemical and enzymatic oxidation of 1-methyl uric acid at pH 7.1 are presented in Fig. 1. Curves 1 in Figs. 1A and B correspond to the spectrum of 1-methyl uric acid before initia-

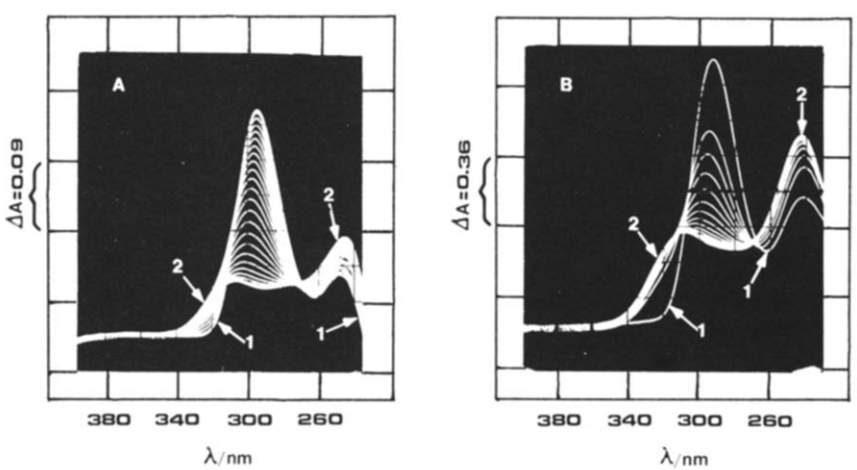


Fig. 1. Oxidation of 1-methyl uric acid in phosphate buffer, pH 7.1, ionic strength 0.5 M (A) at a gold minigrid electrode at a potential of 0.5 V; concentration of 1-methyl uric acid 2 mM and (B) in the presence of type VIII peroxidase (0.28 μM) and H_2O_2 (200 μM); concentration of 1-methyl uric acid 100 μM. Curve 1 is the spectrum of 1-methyl uric acid before oxidation. Curve 2 is that when essentially all 1-methyl uric acid has been oxidized. Repetitive scans of 4.7 s.

tion of the oxidation. At pH 7.1, 1-methyl uric acid exhibits two absorption bands with λ_{max} values at 292 nm and 236 nm. Upon initiation of the oxidation, either enzymically or under thin-layer spectroelectrochemical conditions, the band at 292 nm systematically decreases, whereas the band at approx. 236 nm increases in height (Fig. 1A and B). Simultaneously, a new peak appears between about 310 and 340 nm. With increasing time this new absorption band increases and reaches a maximal value corresponding to about curve 2 in Figs. 1A and B, and then decreases. The spectra shown in Fig. 1 clearly indicate that a new absorbing species is formed during both the electrochemical and enzymic oxidation of 1-methyl uric acid and that the λ_{max} values for this species occur at 306—308 nm and 236—240 nm. The spectrum of this species and its disappearance with time is shown more clearly in Fig. 2A and B. The spectra shown in Fig. 2A and B were obtained after all 1-methyl uric acid has been oxidized. Clearly the species responsible for the band at 306—308 nm must be an unstable intermediate formed in the electrochemical (Fig. 2A) or enzymic (Fig. 2B) oxidation reactions. Rapid scan spectrophotometric studies of the thin-layer spectroelectrochemical and peroxidase-catalyzed oxidation of 7-methyl uric acid and unsubstituted uric acid [18,19] gave similar results, i.e. an unstable intermediate was formed which exhibited an ultraviolet absorption band at longer wavelengths than did the parent uric acid. The ultraviolet spectra of the intermediate formed enzymically or spectroelectrochemically were essentially identical.

In the case of 3-methyl, 9-methyl, 1,3-dimethyl, 3,7-dimethyl, 7,9-dimethyl and 1,3,7-trimethyl uric acids, electrochemical and enyzmic oxidation gave an intermediate species which absorbed at shorter wavelengths than the major ultraviolet peak of the parent compound. The oxidation behavior of 3,7-dimethyl uric acid at pH 7 is typical of this group of compounds. Upon electrochemical (Fig. 3A) or peroxidase-catalyzed (Fig. 3B) oxidation of 3,7-dimethyl uric acid the characteristic ultraviolet absorption band at 295 nm decreases. Simultaneously the absorbance of the oxidizing solution increases between about 280 and 240 nm (Fig. 3A and B). This absorption reaches a

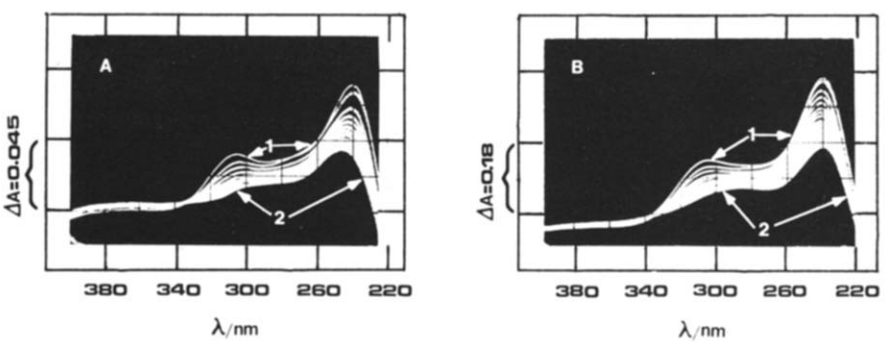


Fig. 2. Variation with time of the ultraviolet spectrum of the intermediate formed upon oxidation of 1-methyl uric acid in phosphate buffer, pH 7.1, (A) electrochemically at a gold minigrid electrode; initial concentration of 1-methyl uric acid 2 mM, and (B) enzymatically with type VIII peroxidase (0.28 μM) and H_2O_2 (200 μM); initial concentration of 1-methyl uric acid 100 μM. Curve 1 corresponds approximately to the point where all 1-methyl uric acid has been oxidized. Curve 2 is the spectrum after 31.5 min. Repetitive scans of 189 s.

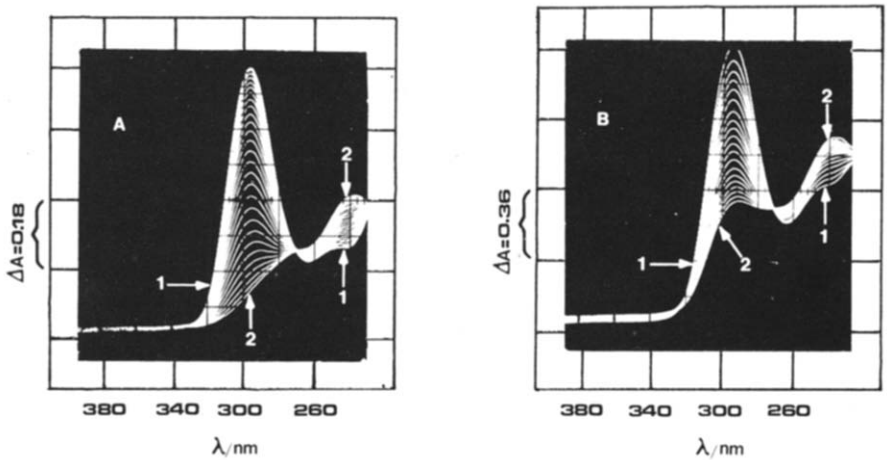


Fig. 3. Oxidation of 3,7-dimethyl uric acid in phosphate buffer, pH 7.1, ionic strength 0.5 M (A) at a gold minigrid electrode at a potential of 0.6 V; concentration of 3,7-dimethyl uric acid 4 mM; repetitive scans of 4.7 s, and (B) in the presence of type VIII peroxidase (0.06 μM) and H_2O_2 (200 μM); concentration of 3,7-dimethyl uric acid 100 μM; repetetive scans of 2.35 s. Curve 1 is the spectrum of 3,7-dimethyl uric acid before oxidation. Curve 2 in (A) corresponds to the point when all 3,7-dimethyl uric acid has been oxidized; curve 2 in (B) is that when approximately 70% of 3,7-dimethyl uric acid has been oxidized.

maximal value approximately corresponding to curve 2 in Fig. 3A and B and then it decreases. The decrease in absorbance observed between 280 and 240 nm occurs even when the electrolysis or enzyme reaction is terminated at approximately the time corresponding to curve 2 in Fig. 3A and B. This clearly implies that upon both electrochemical and enzymic oxidation and unstable intermediate is formed which absorbs between approx. 280—240 nm and which decomposes spontaneously at pH 7 when the oxidation reaction is terminated.

Such spectral studies of the electrochemical and peroxidase-catalyzed reactions suggest that the oxidation of uric acid and its 1-methyl and 7-methyl derivatives give the same unstable intermediate which absorbs at a longer wavelength than the parent compound. In the case of the 3-, 9-, 1,3-, 3,7-, 7,9- and 1,3,7-methylated derivatives of uric acid, electrochemical and enzymic oxidation appears to give the same intermediate but, for these systems, the intermediate absorbs at shorter wavelengths than the main band of the parent compound.

1,3,7,9-Tetramethyl uric acid was not oxidized in the presence of type VIII peroxidase/H_2O_2. This compound is electrochemically oxidized but thin-layer spectroelectrochemical studies gave no evidence for formation of an ultraviolet-absorbing intermediate.

Kinetic measurements

The kinetics of the decomposition reaction of the ultraviolet-absorbing intermediate generated upon electrochemical oxidation of N-methylated uric acids was studied by thin-layer spectroelectrochemistry. These studies were carried out at pH 7—8 where the intermediate species is most stable [18—20]. The procedure employed was to apply a constant potential to an optically transparent gold minigrid electrode in a thin-layer quartz spectoelectrochemical

cell. The absorbance was monitored at a wavelength corresponding to maximal absorbance of the intermediate species with minimal interference from the starting compound. When the absorbance at the selected wavelength reached its maximal value the electrolysis was terminated and the absorbance versus time decay was recorded. Similar experiments were carried out at several different wavelengths. For all compounds which give an ultraviolet-absorbing intermediate plots of log $A-A_\infty$ versus time, where A is absorbance, were linear indicating that the decomposition reaction follows first-order kinetics. The rate constants determined were independent of the wavelength used to monitor the decay of the intermediate. The measured first-order rate constants are presented in Table I. The rate constants reported in Table I were independent of the concentration of the starting compounds. The rate constants reported were also found to be the same at pH 7 and 8. At both higher and lower pH values the apparent first-order rate constants appeared to increase and have been discussed elsewhere [20].

Similar kinetic studies were carried out on the ultraviolet-absorbing intermediate species formed upon oxidation of uric acids with the type VIII peroxidase/H_2O_2 system. Preliminary experiments revealed that the measured kinetics of decay of the ultraviolet-absorbing intermediate in fact reflected a combination of the oxidation of the substrate molecule and formation and decomposition of the intermediate species. Thus, in order to obtain meaningful kinetic data on the reaction of the ultraviolet-absorbing intermediate it was necessary to terminate the peroxidase-catalyzed oxidation of the methylated

TABLE I

OBSERVED FIRST-ORDER RATE CONSTANTS FOR REACTION OF THE ULTRAVIOLET-ABSORBING INTERMEDIATE FORMED ON ELECTROCHEMICAL OXIDATION OF *N*-METHYL URIC ACID DERIVATIVES AT A GOLD MINIGRID ELECTRODE

All measurements made in phosphate buffers (pH 7—8) having an ionic strength of 0.5 M. k, the quoted rate constant, is the average value obtained from several runs at concentrations of the starting uric acid derivative ranging from 1 to 4 mM. n.d., no intermediate species could be detected.

Compound	λ_{max} for ultraviolet-absorbing intermediate (nm)	k (s^{-1})
Uric acid *	302	0.0035 ± 0.0005 **
1-Methyl uric acid	308	0.0018 ± 0.0004
3-Methyl uric acid	<270	0.0041 ± 0.0004
7-Methyl uric acid	310	0.0024 ± 0.0002
9-Methyl uric acid	<280	0.015 ± 0.004
1,3-Dimethyl uric acid	<300	0.0019 ± 0.0003
3,7-Dimethyl uric acid	<280	0.0050 ± 0.0008
7,9-Dimethyl uric acid	<300	<0.001 ***
1,3,7-Trimethyl uric acid	<280	0.0010 ± 0.0004
1,3,7,9-Tetramethyl uric acid	n.d.	

* Data from Ref. 19.

** Mean value ± standard deviation.

*** The reaction of this intermediate was so slow that diffusion of 7,9-dimethyl uric acid into the thin-layer cell interfered with the kinetic measurements.

uric acid derivative at a point in the reaction where the absorbing intermediate was approximately at its maximal concentration but where all of the methylated uric acid had not been oxidized. This was accomplished by terminating the enzymic oxidation of the uric acid derivative by addition of a large excess of the enzyme catalase (see Experimental). This enzyme rapidly removes H_2O_2 from the reaction mixture and hence terminates the peroxidase-catalyzed reaction. The first-order rate constants observed for decomposition of the ultraviolet-absorbing intermediate at pH 7.1 under the latter conditions are presented in Table II. The data presented indicate that the rate of reaction of the ultraviolet-absorbing intermediate is independent of both the enzyme and substrate concentration. The observed rate constants were also found to be independent of the wavelength employed in the range where the intermediate absorbs. The results shown in Tables I and II clearly indicate that electrochemical and peroxidase oxidation of N-methylated uric acids, and uric acid itself [19], gives rise to an intermediate species having identical spectral and kinetic properties.

Extensive studies in this laboratory of the electrochemical oxidation of uric acid derivatives [18—20] support the view that the ultraviolet-absorbing intermediate is an imine-alcohol species. In the case of uric acid this imine-alcohol has the structure III (Eqn. 4). Accordingly, it seems reasonable to conclude, on the basis of the present study, that the same imine-alcohol intermediate is formed during the peroxidase-catalyzed oxidation of these uric acid derivatives.

Voltametry of N-methylated uric acids undergoing enzymic oxidation

A cyclic voltamogram of uric acid at pH 7.1 at a rough pyrolytic graphite electrode [18] is presented in Fig. 4. The first peak observed is oxidation peak I_a. Having scanned peak I_a then on the reverse, negative-going sweep reduction peak I_c is observed which forms a quasi-reversible couple with peak I_a. At more negative potentials a second reduction peak II_c is observed. Peaks I_c and II_c cannot be observed unless oxidation peak I_a is first scanned. The electrode reactions responsible for the various peaks which appear in the cyclic voltamogram may be described in terms of Eqn. 4 [20]. Thus, peak I_a is due to the $2e\text{-}2H^+$ oxidation of uric acid (I, Eqn. 4) to the unstable diimine species (II, Eqn. 4). Peak I_c is due to reduction of the unstable diimine back to uric acid. Reduction peak II_c is due to reduction of the imine-alcohol intermediate (III, Eqn. 4). It might be noted that reduction peak I_c is only observed for uric acid derivatives which are not substituted at N-7. This is so because 2e oxidation of a 7-substituted derivative give rise to a diimine which carries a positive charge at N-7 such that a very rapid attack by water occurs giving the corresponding imine-alcohol [20].

The spectroelectrochemical and enzymic studies described earlier in this report support the view that the ultraviolet-absorbing intermediate generated electrochemically and enzymically is an imine-alcohol. This conclusion has been confirmed by oxidizing uric acid and various N-methylated derivatives with type VIII peroxidase/H_2O_2 and detecting formation of the imine-alcohol, i.e. reduction peak II_c, by means of voltametry at the rough pyrolytic graphite electrode. These experiments were carried out by placing equimolar concentrations of substrate and H_2O_2 into an electrochemical cell. Then, a suitable

TABLE II

OBSERVED FIRST-ORDER RATE CONSTANTS FOR REACTION OF THE ULTRAVIOLET-ABSORBING INTERMEDIATE FORMED ON OXIDATION OF N-METHYLATED URIC ACIDS IN THE PRESENCE OF TYPE VIII PEROXIDASE/H_2O_2 IN PHOSPHATE BUFFER

Phosphate buffer: ionic strength 0.5 M and pH 7.1. Enzyme concentration based on a molecular weight for type VIII peroxidase of 40 000. In all experiments the concentration of H_2O_2 was 200 μM. n.o., this compound is not oxidized by Type VIII peroxidase/H_2O_2.

Compound	λ_{max} for ultraviolet-absorbing intermediate	Concentration (μM)		k (s^{-1})	
		Substrate	Enzyme		
Uric acid	302	200	0.17		0.0035
		100	0.17		0.0039
		100	0.34		0.0037
		20	0.17		0.0039
				mean	0.0037 ± 0.0004
1-Methyl uric acid	308	150	0.50		0.0016
		100	0.50		0.0018
		100	0.20		0.0017
		50	0.50		0.0018
				mean:	0.0017 ± 0.0002
3-Methyl uric acid	<270	200	0.40		0.0045
		100	0.40		0.0042
		100	0.65		0.0043
		50	0.40		0.0044
				mean:	0.0043 ± 0.0003
7-Methyl uric acid	310	150	0.38		0.0024
		100	0.38		0.0023
		100	0.46		0.0024
		100	0.23		0.0022
		50	0.38		0.0024
				mean:	0.0024 ± 0.0002
9-Methyl uric acid	<280	200	0.55		0.018
		100	0.55		0.014
		100	0.16		0.017
		50	0.55		0.019
				mean:	0.017 ± 0.002
1,3-Dimethyl uric acid	<300	200	0.24		0.0020
		100	0.24		0.0017
		100	0.12		0.0020
		50	0.24		0.0019
				mean:	0.0019 ± 0.0002
3,7-Dimethyl uric acid	<280	200	0.65		0.0046
		100	0.65		0.0053
		100	0.35		0.0054
		50	0.65		0.0046
				mean:	0.0050 ± 0.0004
7,9-Dimethyl uric acid	<300	200	0.42		0.0007
		200	0.15		0.0009
		100	0.42		0.0009
				mean:	0.0008 ± 0.0002
1,3,7-Trimethyl uric acid	<280	200	0.65		0.0009
		100	0.65		0.0013
		100	0.38		0.0010
		50	0.65		0.0006
				mean:	0.0010 ± 0.0004
1,3,7,9-Tetramethyl uric acid	n.o.				

* Data from Ref. 19.

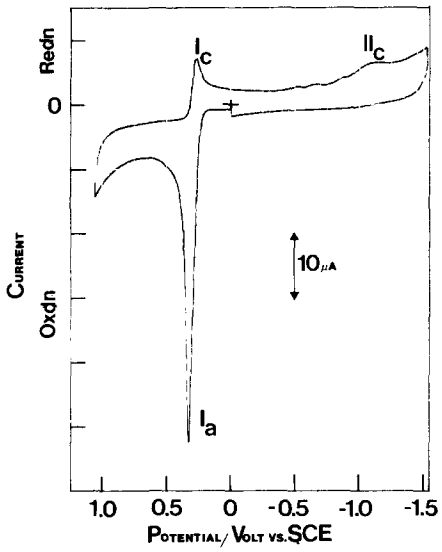
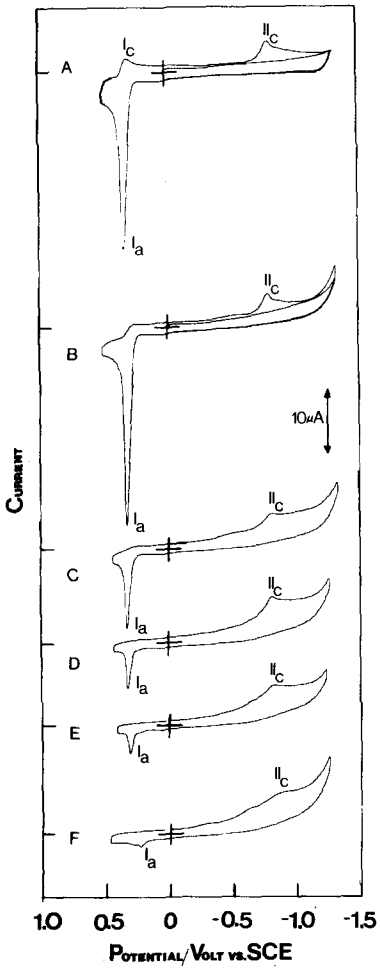


Fig. 4. Typical cyclic voltamogram of uric acid (1 mM) in phosphate buffer, pH 7.1, ionic strength 0.5 M at a pyrolytic graphite electrode (area = 0.03 cm^2). Sweep rate 200 $mV \cdot s^{-1}$.

amount of type VIII peroxidase enzyme was added and voltamograms at a rough pyrolytic graphite electrode were recorded with the following sweep pattern: 0 → —1.3 V → 0.6 V → 0 V. Preliminary experiments were carried out to observe the effect of peroxidase and H_2O_2 on the voltametric behavior of the uric acid derivatives studied. In the case of 7-methyl, 3,7-dimethyl, and 1,3,7-trimethyl uric acids neither the enzyme nor H_2O_2 had any observable effect on the cyclic voltametric behavior of these compounds. However, in the case of uric acid and its 1-methyl, 3-methyl, 9-methyl, and 1,3-dimethyl derivatives the presence of H_2O_2 caused a significant decrease of reduction peak I_c. The latter effect may be seen by reference to Fig. 5. A cyclic voltamogram of 9-methyl uric acid at pH 7.1 at a rough pyrolytic graphite electrode is shown in Fig. 5A where it may be observed that after sweeping oxidation peak I_a a small reduction peak I_c is observed followed by reduction peak II_c at more negative potentials. In the presence of an equimolar concentration of H_2O_2 it is noted in Fig. 5B that reduction peak I_c is absent. The cyclic voltamogram shown in Fig. 5C was obtained just after addition of type VIII peroxidase to the 9-methyl uric acid/H_2O_2 mixture. Fig. 5D—F, were obtained 0.5, 1 and 2 min after initiation of the enzymic oxidation. It is clear from these cyclic voltamograms that in the absence of the enzyme/H_2O_2 system reduction peak II_c is not observed if the first potential sweep is toward negative potentials (Fig. 5A and B). However, after initiation of the enzymic oxidation reaction reduction peak II_c is clearly observed on the first negative-going sweep (Fig. 5C—F). It is also clear that after about 0.5 min oxidation peak II_c is relatively small (Fig. 5C); after 1 min oxidation peak II_c has grown (Fig. 5D) and after even longer periods of time reduction peak II_c decreases (Fig. 5E and F) and ultimately disappears.

Similar behavior was observed with other N-methylated uric acids. For example, peak II_c is not observed on the initial sweep towards negative poten-



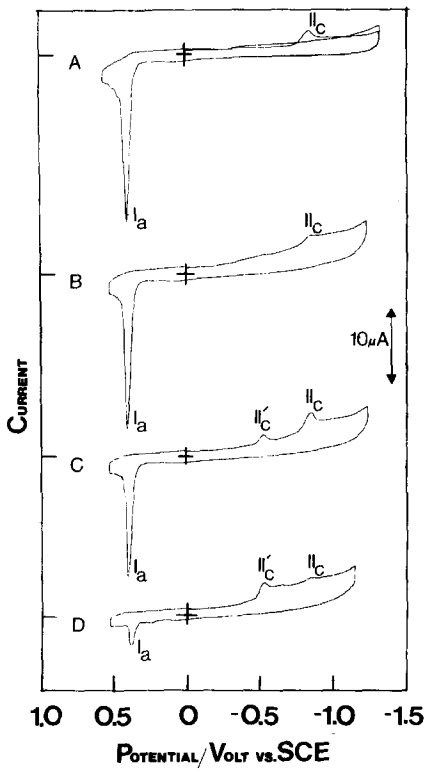


Fig. 5. Cyclic voltamograms of (A) 9-methyl uric acid (400 μM) in phosphate buffer (pH 7.1) having an ionic strength of 0.5 M; (B) with the addition of H_2O_2 (400 μM), and (C—F) after addition of H_2O_2 (400 μM) and type VIII peroxidase (0.30 μM). Voltamogram (C) was taken within a few seconds of initiation of the enzymic oxidation, (D) after 0.5 min, (E) after 1 min, and (F) after 1.5 min. Sweep rate in all instances: 200 mV · s^{-1}. Sweep pattern: 0 → —1.3 V → +0.5 V → 0.

Fig. 6. Cyclic voltamograms of (A) 7-methyl uric acid (300 μM) in phosphate buffer (pH 7.1) having an ionic strength of 0.5 M; (B—D) with the addition of H_2O_2 (300 μM) and type VIII peroxidase (0.30 μM). Voltamogram (B) was taken within a few seconds of initiation of the enzymic oxidation, (C) after 1 min, and (D) after 7 min. Sweep rate: 200 mV · s^{-1}. Sweep pattern 0 → 1.25 → +0.5 → 0.

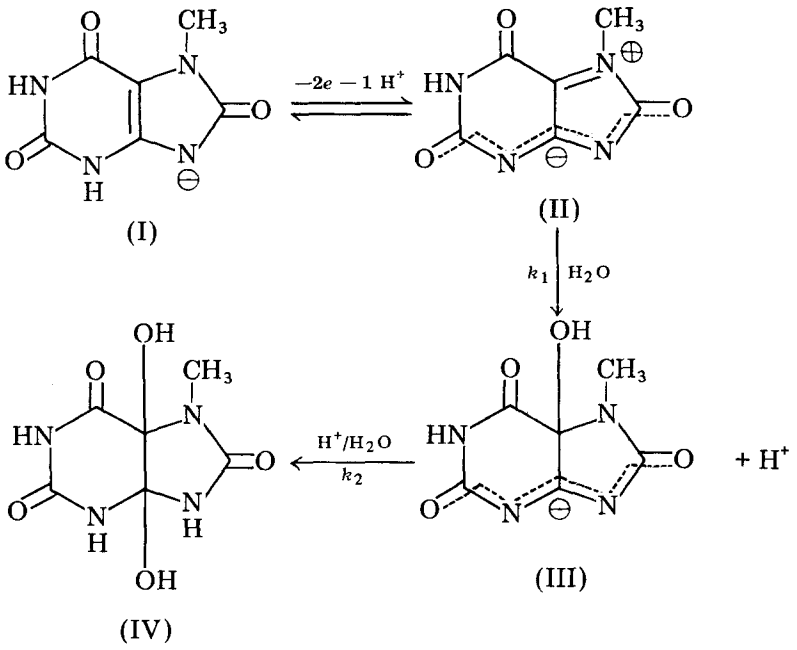
tials for 7-methyl uric acid in the absence of peroxidase/H_2O_2 (Fig. 6A); it is necessary to first sweep oxidation peak I_a. However, after initiation of the enzymic reaction peak II_c is observed on the initial, negative-going sweep (Fig. 6B). With increasing time, peak II_c increases (Fig. 6C) reaches a maximal value and then decreases (Fig. 6D) and disappears. In the case of 7-methyl ruic acid and other 7-methylated derivatives it may be noted that after the enzymic oxidation has proceeded for some time a new reduction peak (peak II'_c, Fig. 6C and D) appears. This peak may also be observed in the absence of the enzyme

system provided the sweep towards positive potentials is extended well past peak I_a to a second ill-defined peak which appears with these compounds. Previous work suggests that the second, more positive electrooxidation peak II_a is due to oxidation of the imine-alcohol intermediate to a reducible product which gives rise to peak II'_c [20]. The extact nature of the peak II_a and peak II'_c reactions, however, is not yet understood but is under current investigation.

Conclusions

The results reported here indicate that when uric acid and its N-methyl derivatives are oxidized either electrochemically or with type VIII peroxidase, intermediate species are formed which have identical physicochemical properties regardless of whether they are generated electrochemically or enzymically. Thus, the ultraviolet-absorption spectra, the kinetics of the decomposition reaction of the intermediate and the electrochemical properties of the intermediate species generated electrochemically or enzymically are the same.

In a previous report [20] it was demonstrated that N-methylation of uric acid does not change the basic electrochemical reaction scheme but only affects the potential required for and number of protons involved in the primary oxidation reaction and the stability of intermediate species. It was shown that all examined *N*-methyl uric acid derivatives are electrooxidized at pyrolytic graphite and gold electrodes in a 2*e* reaction to a diimine species. The reaction scheme shown in Eqn. 4 is applicable to uric acid at pH 7 where it exists as a monoanion [25,26]. Thus the primary electrochemical step is a $2e$-$2H^+$ reaction to give the anionic diimine species II (Eqn. 4). This species is very short-lived in homogeneous solution and is rapidly attacked by water ($k_1 = 32.5\ s^{-1}$ at pH 8 [18]) to give an imine-alcohol (III, Eqn. 4). The reason that reduction peak I_c, due to electrochemical reduction of the diimine to uric acid, can be observed on relatively slow cyclic voltametry of uric acid at a rough pyrolytic graphite electrode (Fig. 4) is due to the fact that the diimine is very strongly adsorbed at the rough electrode surface and is less rapidly attacked by water in the adsorbed state [18]. The imine-alcohol species can exist in at least four resonance forms represented by structure III (Eqn. 4). This structure has a more extensively delocalized π-electron system than even the parent uric acid anion (I, Eqn. 4) with the result that it absorbs radiation at somewhat longer wavelengths than uric acid. The imine-alcohol is the intermediate which, it is believed, may be observed upon thin-layer spectroelectrochemical and peroxidase-catalyzed oxidation of uric acid [19]. Hydration of the imine-alcohol then forms a diol (IV, Eqn. 4) which subsequently decomposes to allantoin and CO_2 [16,17]. The electrochemical and enzymic oxidation of N-methylated uric acids may be readily rationalized on the basis of a similar mechanism and will be illustrated for two representative compounds. 7-Methyl uric acid (I, Eqn. 5) is electrooxidized in a $2e$-$1H^+$ reaction at pH 7 [20] which should give the zwitterionic diimine II (Eqn. 5) which would be very rapidly attacked by water to give the corresponding imine-alcohol (III, Eqn. 5). Cyclic voltametry of 7-methyl uric acid gives no evidence for reduction peak I_c after having scanned peak I_a even at sweep rates greater than $100\ V \cdot s^{-1}$, i.e. the primary $2e$-$1H^+$ oxidation product is very reactive in accord with structure II

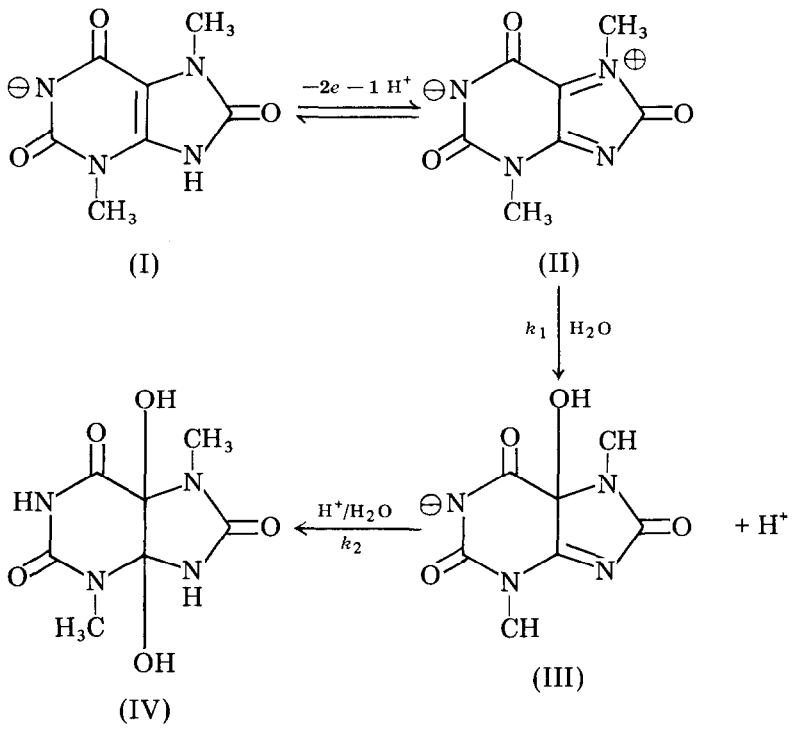


(5)

(Eqn. 5). On the other hand, thin-layer spectroelectrochemistry and rapid scan spectrophotometric studies of the enzymic oxidation of 7-methyl uric acid reveals the formation of an ultraviolet absorbing intermediate having a λ_{max} at longer wavelengths than for the parent compound (Tables I and II). Such behavior supports structure III (Eqn. 5) for the imine-alcohol. Again, the imine-alcohol is hydrated to a diol (IV, Eqn. 5) which decomposes to the observed products.

Electrochemical oxidation of 3,7-dimethyl uric acid (I, Eqn. 6) again proceeds by a $2e$-$1H^+$ reaction [20]. This should give again a highly unstable zwitterionic diimine (II, Eqn. 6) which upon hydration would give an imine-alcohol having structure III (Eqn. 6). Both electrochemical and enzymic oxidation of 3,7-dimethyl uric acid gives an ultraviolet-absorbing intermediate but, in contrast to that formed from uric acid and 7-methyl uric acid and discussed above, this intermediate absorbs at shorter wavelengths than the parent compound (Tables I and II). This appears to be so because the imine-alcohol formed from 3,7-dimethyl uric acid (III, Eqn. 6) is not as extensively conjugated as that formed from uric acid and 7-methyl uric acid hence it is expected to absorb at shorter wavelengths. The observed behavior of the intermediates formed on electrochemical and enzymic oxidation of all the uric acid derivatives may be readily rationalized using the same arguments. The experimental evidence reported conclusively demonstrates that the ultraviolet-absorbing imine-alcohol intermediate may be generated by electrochemical or peroxidase-catalyzed oxidation of uric acids. It is, therefore, quite reasonable to conclude that in the case of the enzymic oxidation the imine-alcohol is derived from the same precursor detected in the electrochemical oxidation, i.e. a diimine. Unfortunately, there is no simple way to observe the putative

diimine precursor formed under enzymic conditions because it is too unstable. It can only be detected in the electrochemical oxidation reaction because when



(I) (II) (III) (IV) (6)

formed at the rough pyrolytic graphite electrode surface it is stabilized somewhat as a result of its strong adsorption at the electrode surface and hence may be detected by cylic voltametry.

The conclusion of Howell et al. [9] that because 7-methyl and 9-methyl uric acids are oxidized whereas 1,3,7,9-tetramethyl uric acid is not in the presence of horseradish peroxidase and hence that an unsubstituted N-7 or N-9 position is necessary for the enzyme-catalyzed oxidation to occur is not supported by the results reported here. This is so because 7,9-dimethyl uric acid is readily oxidized in the presence of type VIII peroxidase (Table II). The most likely reason for the failure of peroxidase to oxidize 1,3,7,9-tetramethyl uric acid is related to the fact that this compound is the most difficult of all the methylated uric acids to oxidize [20]. Thus, at pH 7 the peak potential for electrochemical oxidation of 1,3,7,9-tetramethyl uric acid is 0.92 V. 1,3,7-Trimethyl uric acid has a peak potential at pH 7 of 0.513 V and all other methylated uric acids have peak potentials between 0.30 and 0.45 V. It is our conclusion, therefore, that the failure of peroxidase to oxidize 1,3,7,9-tetramethyl uric acid is due to the large amount of energy required to carry out this reaction rather than to a mechanistic difficulty.

We believe that the results reported here and in earlier communications [18—20] support the view that the electrochemical and type VIII peroxidase/H_2O_2 oxidation of uric acids proceed, in a chemical sense, by identical mechanisms.

Acknowledgement

Financial support for this work was provided by the National Institutes of Health through Grant No. GM-21034-04.

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