ACTA CHEMICA SCANDINAVICA 8 (1954) 637—648

The Oxidation of Uric Acid with Horse Radish Peroxidase

K. G. PAUL and Y. AVI-DOR*

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

During the last decade the interest in the biological effects of the oxidation products of uric acid has been rapidly growing. Ascoli and Izar [1] described in 1909 an enzyme system in dog and calf liver which decomposed uric acid under aerobic conditions and resynthesized it from dialuric acid. The addition of uric acid to histamin (0.001 *M*) and impure hog kidney diamine oxidase increased the oxygen uptake [2]. The effect was attributed to a peroxidatic oxidation of the uric acid, the peroxide thus being unaccessible for catalatic decomposition. Dunn, Sheehan, and McLetchie [3] developed diabetes in rabbits by injecting alloxan, and discussed the possibility that alloxan formed *in vivo* from uric acid, might be involved in the pathogenesis of diabetes mellitus. Tipson and Ruben [4] found a substance in trichloroacetic acid extracts from a number of normal tissues which gave alloxan reactions. Alloxan was found in the liver of fasting white rat, rabbit, dog and man [5]. The injection of uric acid [6] or ribonucleic acid [7] developed hyperglycaemia in rabbits which were maintained on a diet low in cystine and methionine.

Alloxan reacts with amino and thiol groups of proteins and amino acids [8,9]. It inhibits hexosediphosphatase [10]. The structurally related ninhydrine inactivates tetanus toxin [11].

Agner [12] found that impure specimens of diphteria and tetanus toxins were inactivated by verdo-peroxidase (= myeloperoxidase) or horse radish peroxidase + hydrogen peroxide. Later on[13] he showed that pure toxins were not destroyed unless uric acid or casein hydrolysate were added. He also demonstrated that uric acid was oxidized by peroxidase + peroxide. Tuttle and Cohen [14] oxidized C^{14}-labelled uric acid with milk peroxidase and suggested tentatively that alloxan might be formed in connection with this oxidation of uric acid.

The purpose of the present investigation was to study the oxidation of uric acid with horse radish peroxidase and the conditions leading to the various possible oxidation products.

---

* Fellow of *The Israel Institute of Biological Research*, Nes-Ziona, Israel.

MATERIAL AND METHODS

Crystalline horse radish peroxidase (HRP) was taken from the same preparation as previously used [15]. Commercial preparations (B.D.H.) of uric acid, xanthin, heteroxanthin, hypoxanthin, theobromine, adenine, uracil and alloxan were used. 1-methyl, 1,3-dimethyl, 1,9-dimethyl, 3,9-dimethyl, and 1,7-dimethyl-8-acetyl uric acid were kindly supplied by Dr. U. Lagerkvist. They had been purified by him by chromatography on starch (*n*-propanol : 0.5 *M* HCl 2 : 1, v/v).

Spectrophotometric analyses were made with a Beckman DU spectrophotometer. The optical densities were corrected for absorption due to HRP and solvents and for dilutions.

The experiments were made at room temperature (20°).

1-methyl uric acid was used for most experiments because of its greater solubility than uric acid.

RESULTS

The changes in light absorption, which occur at different wavelengths during the oxidation of 1-methyl uric acid are seen in Fig. 1. At least one intermediate substance is involved in the transformation of uric acid to the final product(s); it gives a low light absorption at 285 mμ and a higher one at 230 mμ. If the reaction was interrupted after five minutes by the addition of strong HCl practically no light absorption remained above 255 mμ, *i. e.* no uric acid was left. Thus the changes in density at 285 mμ, occurring after this time, reflect only reactions concerning the intermediate(s).

The following symbols are used in the sequel to facilitate the description:

$$\text{uric acid} \xrightarrow{k_1} \text{B} \xrightarrow{k_2} \text{C}$$

where B is the intermediate and C the final compound.

Reaction 1 (0—2 min., Fig. 1) was accelerated by the add tion of another portion of HRP + H_2O_2, whereas the same addition during reaction 2 (>4 min) had no influence. Thus peroxidase catalyses only the conversion of uric acid to B.

No change in pH was noticed in experiments at pH 3.5—4 in which only dilute HCl had been used to adjust the pH.

Curves 3—5 in Fig. 2 show that the oxidation of one mole of uric acid consumes one mole of hydrogen peroxide.

Spectrophotometric experiments

Reaction 1. The initial, rapid decrease in light absorption at 285 mμ fitted in most experiments well to curves for first order reactions. However, when the oxidation of uric acid was followed in terms of remaining amounts of uric acid, agreement with neither first nor zero order reactions was found (Fig. 3). The concentrations of peroxidase and peroxide, a possible inhibition of HRP by the products B or C, and the instability of B will all influence the light absorption changes. No significance can be attributed to the abovementioned finding of an apparent first order reaction.

Some information on the influence of pH on the velocity of reaction 1 was obtained by plotting $1/t_{30\,\%}$ against pH, where $t_{30\,\%}$ is the time during

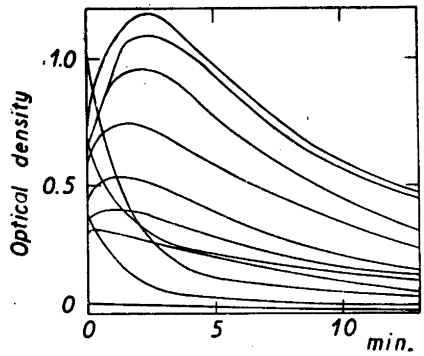


Fig. 1. Spectral changes during the oxidation of 1-methyl uric acid. The curves represent from above (at 10 min) the wave-lengths 230, 235, 240, 245, 250, 257, 270, 285, 300, and 310 mμ. HRP = 0.35 μM, 1-methyl u.a. = 82 μM, H_2O_2 = 97 μM. pH 3.55 (adjusted with dilute HCl, no buffer).

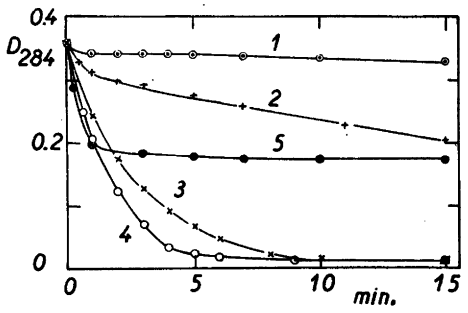


Fig. 2. Influence of $[H_2O_2]$. 1-methyl u.a. = 33 μM, HRP 0.40 μM, H_2O_2 for curves 1—5 32, 3.2 mM, 320, 32, and 16 μM. pH 3.70 (0.1 M acetate).

which the initial density at 285 mμ decreased by 30 % (Fig. 4). A few experiments were made at alkaline reaction. $t_{30\,\%}$ was roughly the same at pH 8.5 as at pH 7, while at pH 10.5 only a very slow reduction of the light absorption at 293 mμ of 1-methyl uric acid was seen.

Reaction 2. As mentioned no uric acid was left after a few minutes. Plots of

$$\log\,[(D_{5\text{min}}-D_{\text{final}})\,/\,(D_{5+t_{\text{min}}}-D_{\text{final}})]$$

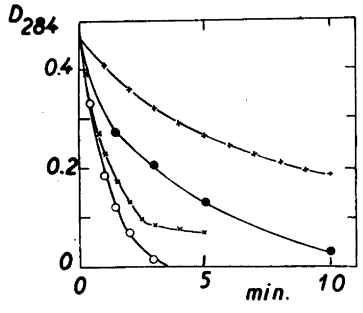


Fig. 3. Reaction 1 in terms of remaining amounts of 1-methyl u.a. + and × denote optical densities (without acidification) at pH 5.53 and 4.00. Same experiment repeated but 0.10 ml 8 M HCl added at various times to reaction mixtures of pH 5.53 (●) and 4.00 (○). HRP 0.87 μM, H_2O_2 81 μM, 1-methyl u.a. 46 μM. 0.01 M acetate buffer, volume 3.0 ml.

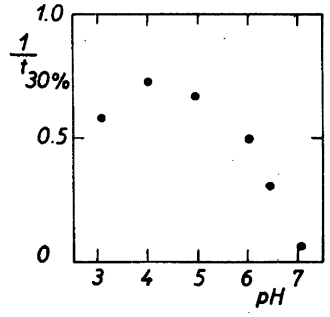


Fig. 4. Velocity of reaction 1 in terms of $1/t_{30\,\%}$ at various pH-values. HRP 0.12 μM, H_2O_2 82 μM, 1-methyl u.a. 45 μM, 0.05 M acetate.

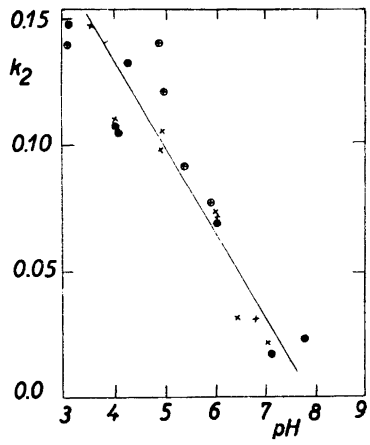


Fig. 5. Velocity constant of reaction 2.

● *235 mμ*	*HRP = 0.35 μM*	H_2O_2 *82 μM*	*1-me. u.a.*	*45—52 μM*
× *285* »	» *0.12* »	» *82* »	»	*45—52* »
⊕ *285* »	» *0.12* »	» *33* »	»	*32* »
+ *305* »	» *0.35* »	» *82* »	»	*52* »

(D = optical density) against time gave straight lines of constant and practically the same slope for all wavelengths in Fig. 1, indicating that only one compound was decomposing at this pH. Further determinations of k_2 were made at 235, 285 and, in a few cases, 305 mμ (Fig. 5). It was found that the velocity constant k_2 of the reaction B → C was independent of wavelength, ratio of H_2O_2 : uric acid and [HRP] during reaction 1 but varied with pH.

Spectrum of the intermediate B. The light absorption curve obtained by plotting the densities after 5 min. in Fig. 1 against wavelength gives the relative spectrum of B at pH 3.55 with an unknown but small contribution from C. The same experiment was then repeated, but phosphate buffer was added at 5 min. to give pH 7.60. (Fig. 6 : 1.) Compound B can obviously appear in one form ("B_1") at slightly acid reaction (pH 3.5—5) without the band at 307 mμ, and one in neutral or faintly alkaline solution ("B_2"). The latter has a lower light absorption at 230 mμ but gives a band at 307 mμ (*cf.* diagram, p. 643). The existence of the two forms was further confirmed by the following results.

1. In a series of experiments at pH 4.28 the reaction was followed at 305 mμ. After 5 min, when the density was stable and low pH was brought to values between 4.5 and 9 by means of phosphate buffers and the light absorption determined as rapidly as possible. It was then followed for a time, sufficiently long to permit the extrapolation back to $t = 5$ min. The curve obtained by plotting the density after the addition of the buffer against the resulting pH is given in Fig. 7. Thus the change from B_1 to B_2 is rapid (< 10 sec.), and its dependence upon pH seems to follow a dissociation curve with $n = 1$.

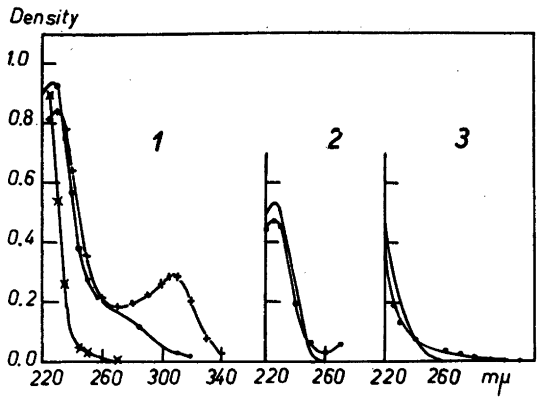


Fig. 6. 1. Relative spectra of compounds B_1 (●), B_2 (+), and B_3 (×). Spectrum of B_1 obtained from Fig. 1 (5 min.). Solutions of B_3 (pH<1) and B_2 (pH 7.60) obtained by the addition of HCl and sec. phosphate at 5 min to the same reaction mixture as used for B_1 (values corr. for dilution).
2. Spectrum of compound C_3 from solution, acidified at 2.5 min (cf. Fig. 7. Some unreacted 1-methyl uric acid but practically no C_1 in the solution). Reference curve: 79 μM 1-methyl alloxan in 0.35 M HCl.
3. Spectrum of C_1 at pH 4.05 (from 82 μM 1-methyl u.a.). Reference curve: 80 μM allantoin, pH 4.05.

2. The oxidation of uric acid was initiated as usually (pH 3.85). After 4 min pH was brought to 6.08 by means of phosphate buffer and the decreasing light absorption was followed alternatingly at 235 and 307 mμ. At both wavelengths the decrease followed a first order reaction curve with

$$k_{235\ m\mu} = 0.069\ \text{min}^{-1} \text{ and } k_{307\ m\mu} = 0.072\ \text{min}^{-1}.$$

3. In four different experiments (H_2O_2 = 81 μM; HRP = 0.53 μM; 1-methyl uric acid = 51 μM; pH 4.10, 0.05 M acetate) the changes in optical

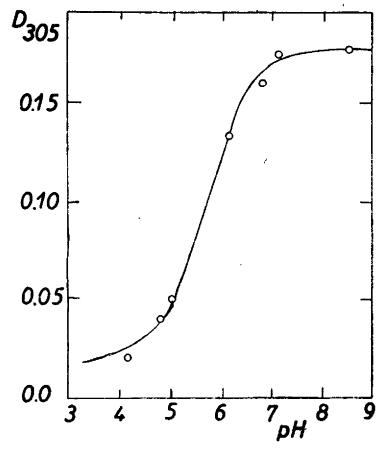


Fig. 7. Light absorption at 305 mμ after alkalinization from pH 4.18 to the values in the figure. Full drawn curve calculated for half conversion of B_1 to B_2 at pH 5.75 and n = 1. HRP 0.35 μM, H_2O_2 82 μM, 1-methyl u.a. 52 μM. Alkaline phosphate buffer added at 5 min.

Table 1. Correspondence between intermediates B_1 *and* B_2. *Reaction conditions, see text.*

	Addition of sec. phosphate min.			
	5	10	15	20
Density at 235 mμ above final level before addition of phosphate	0.342	0.200	0.110	0.060
Increase in density at 305 mμ upon the addition of phosphate	0.155	0.090	0.052	0.027
Ratio	2.23	2.22	2.12	2.22

density were registered at 235 and 305 mμ alternatingly. At 5, 10, 15, and 20 min. secondary phosphate was added to give pH 7.35, and the densities at 305 mμ determined immediately after the additions. The final levels (2 h) at 305 and 235 mμ at pH 4.10 were determined in a separate experiment. The results collected in Table 1, show that the increase in density at 305 mμ, *i. e.* formation of compound B_2 upon neutralization, is proportional to the optical density at 235 mμ at the moment of neutralization, *i. e.* to the concentration of compound B_1.

At low pH a third spectral form of the intermediate was found. 1-methyl uric acid was oxidized in the usual way to compound B, and the solutions were acidified after 3 min. with various amounts of hydrochloric acid (Fig. 8 A). At pH 4.28 and 2.33 continuously decreasing curves were found. At even lower pH the density decreased rapidly to a minimum and increased then slowly ($t_{1/2} \approx 4.5$ min). The more acid added at 3 min., the higher was the final (30 min.) level. Fig. 8 B shows that this level is proportional to the amount of compound B at the moment of acidification.

These observations are interpreted as follows. At pH 3—5 the intermediate B, at this acidity in the form B_1, yields a compound with low absorption at 235 mμ ("C_1"). The acidification of a solution of B_1 to pH 1—2 brings about two consecutive reactions. Within a very short time B_1 is converted to a form with a low light absorption at 235 mμ ("B_3"). A curve giving the inverse of $t_{1/2}$ for the rapid decrease in density at 235 mμ upon acidification *versus* pH approaches asymptotically the ordinate at pH 1. The rapid, initial decrease in density is completely reversed by an immediate (< 10 sec.) addition of an equivalent amount of alkali. In the succeeding, slower ($t_{1/2} \approx 4.5$ min.) reaction B_3 is converted to the final substance ("C_3"), which has a higher molar absorption at 235 mμ than C_1. At an intermediate pH both ways (leading to C_1 and C_3) for the decomposition of B are used.

The spectrum of compound B_3 (Fig. 6 : 1) was obtained in the same way as the spectrum of B_1. Consequently small amounts of compounds C_1 and C_3 may have been present.

The relative spectra of the final compounds C_1 and C_3 are given in Fig. 6 : 2 and 6 : 3. C_3 apparently gives the same spectrum as 1-methyl alloxan, while the spectrum of C_1 is less characteristic.

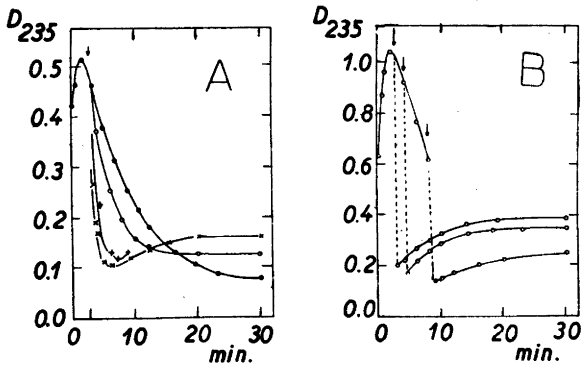
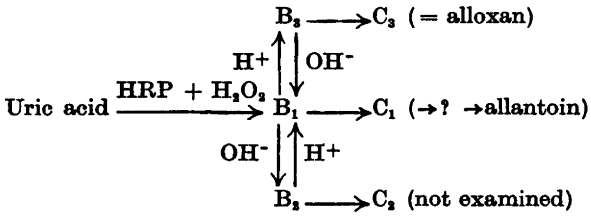


Fig. 8. Strong acidification of intermediate reaction mixture. A: HRP = 0.12 μM, H_2O_2 82 μM, 1-methyl u.a. 30 μM. pH 4.28 (●). At 3 min. HCl was added to give pH 2.33 (○) 1.56 (+) and 1.08 (×). B: HRP 0.35 μM, H_2O_2 100 μM, 1-methyl u.a. 85 μM. pH 3.55. Initial volume 3.09 ml. In three separate experiments 0.15 ml 6 M HCl were added at 2.5, 3.5 and 8 min. 235 mμ.

When 0.2 ml 6 *M* HCl was added to a Beckman cuvette, containing C_1 (pH 4.28, 0.01 *M* acetate, total volume 3.05 ml), no change in light absorption occurred except for that which was accounted for by the dilution and the effect of acidification on the acetate buffer.

The results of the spectrophotometric experiments are summarized in the diagram:



At those acidities where reaction 1 proceeds with its highest velocity, form B_1 of the intermediate is formed. B_1 is reversibly converted into forms B_2 and B_3 by basification or acidification respectively. The three forms of the intermediate B decompose non-enzymatically to the products C_1, C_2, and C_3. The two first-mentioned are not mutually convertible by changing the pH; C_2 was not studied.

In this pattern it has been assumed that B is the only intermediate between A and C. Occasionally we have seen traces of another compound at pH 4—5 when the decomposition of B_1 was followed at 285 mμ. It appeared as a plateau for 1—2 min immediately after the rapid decrease in optical density, *i. e.* 3—5 min after the addition of peroxidase. We did not, however, always trace it and it is doubtful if the plateau was significant. The difference between the spectrum of C_1 and that of allantoin (Fig. 6 : 3) is more obvious. Even if C_1 and allantoin are not identical, the changes in C_1 occur so slowly that they hardly influence the kinetic results given above.

Identification of C_1 and C_3. One mmole (168 mg) of uric acid, dissolved in an equivalent amount of lithium hydroxide (6 ml), was added in 20 portions during 3 1/2 h to 500 ml water, previously adjusted with hydrochloric acid to pH 4.1 and containing 0.27 μmoles of HRP (1.2 mg). After the tenth addition more HRP (0.13 μmoles) was added. Simultaneously to each addition of urate an equivalent amount of hydrogen peroxide was added, in all 1.01 mM. It was frequently checked spectrophotometrically that no unreacted uric acid accumulated, and pH was consistently found to be 4.1—4.2. Next day the solution was evaporated to 10 ml; a crystalline precipitate soon appeared. It was washed three times with ice-cold water and dried. Weight 70 mg. After recrystallization three times from hot water it melted at 230°—236° (Kofler-Block). The melting point of allantoin in a capillary tube has been given as 228—230° (slow heating) or 233—234° (bath preheated to 228°). Still higher melting points have been noticed on copper block [16]. (Found: C 30.63, H 3.86, N 35.40. Calc. for $C_4H_6O_3N_4$ (allantoin) (158.1): C 30.38, H 3.83, N 35.45.) C_1, or the substance deriving from it by a slow reaction (overnight) is thus allantoin. The yield of crude crystals was 44 %.

When a solution of C_3 was adjusted to pH 3.5, mixed with *o*-phenylene diamine, and illuminated with a Wood's lamp it gave a green fluorescence [17, 18], whereas compounds B_1, B_3 or C_1 gave no fluorescence under the same conditions. The increase in fluorescence during the conversion of B_3 to C_3 was followed by withdrawing samples from a solution of B_3 and matching them against a standard of 1-methyl alloxan. The final value amounted to about 50 % (Pulfrich photometer) of the value, calculated from the used amount of 1-methyl uric acid. Substance C_3 gave the same R_F-value as 1-methyl alloxan when chromatographed on starch (*n*-propanol : 0.5 *M* HCl 2 : 1 v/v) *. Moreover, since the relative spectrum of C_3 was practically the same as that of 1-methyl alloxan (Fig. 6 : 2) it seems to be clear that compound C_3 is 1-methyl alloxan. It was not possible to prepare a large amount of C_3 because of the necessary acidification. The yields of alloxan, calculated from Fig. 8 B, were found to be 78 (corr. for remaining uric acid), 80 and 56 %.

Action of HRP on other purine derivatives and uracil

Table 2 gives $t_{30\,\%}$ for uric acid and some of its methyl derivatives. The values $t_{30\,\%} = 0.3$—0.5 are not significantly different. The table confirms that the oxidizable compounds and hydrogen peroxide react in the molar ratio of 1 : 1.

No reaction could be detected spectrophotometrically at the wavelengths of maximal absorption within the range 240—270 mμ when HRP + peroxide were added, at pH 3.6 and 7, to solutions of xanthin, hypoxanthin, heteroxanthin, adenin, theobromine, and uracil.

* Dr. U. Lagerkvist.

Table 2. Oxidation of uric acid and some of its methyl derivatives. HRP 0.35 μM. H_2O_2 16.5 μM. Uric acid or derivative 29—31 μM. The reactions were followed at the wavelengths for maximal absorption (mμ). pH 3.78.

Substance	mμ	$t_{30\%}$ min.	Moles oxidized per mole of H_2O_2
Uric acid (u.a.)	284	0.4	1.1
1-methyl u.a.	284	0.5	1.0
1,3-dimethyl u.a.	286	2.5	1.0
1,9-dimethyl u.a.	285	0.5	1.0
3,9-dimethyl u.a.	292	13.5	—
1.7-dimethyl-8-acetyl u.a.	286	0.3	1.1

DISCUSSION

The highest velocity of reaction 1, as judged from the values for $t_{30\%}$, was found at pH 4—5 (Fig. 4). With increasing pH $1/t_{30\%}$ decreased, by half at pH 6.3. This value is higher than pK_1 for uric acid (5.4 [19]) and probably also for 1-methyl uric acid [20], but if it is taken into consideration that $t_{30\%}$ may only remotely reflect the velocity of reaction 1 the results of Fig. 4 may well be interpreted as showing that unionized uric acid rather than the monovalent anion is the actual subject of oxidation. Chance [21] found the decomposition of HRP-H_2O_2 Complex II by ascorbic acid (pK 4.21) to be slower above than below pH 5.3, and attributed the decrease to a change in the ascorbic acid molecule, possibly ionization. Neither of the cases is conclusive, but they indicate that (HRP + H_2O_2) reacts more easily with the uncharged donor molecule than with its ionized form. The action of peroxidases has been described [22] as a dehydrogenation, *i. e.* hydrogen atoms are removed.

Margules and Griffiths [23] have shown that uric acid can reduce ferricytochrome c without the cooperation of a dehydrogenase. Although the reaction was very slow an optimum could be found at a pH where the acid exists as the monovalent anion. The optimal pH for the action of uricase is 9.2—9.4 [24]. The sole function of this enzyme is, according to Bentley and Neuberger [25], to transfer two electrons from the mono- or divalent urate anion to oxygen.

The oxidation of cysteine by heart muscle preparation + cytochrome c has been found [26] to proceed at highest velocity at pH 8.0, whereas the maximal velocity of the reaction between ferricytochrome c and cysteine was found at pH 8.8. In the latter case the increase in velocity between pH 7 and 9 coincided with the titration curve of the sulphhydryl group of cysteine. Experiments on the oxidation of cysteine with HRP + added peroxide have shown that cysteine is more rapidly oxidized at faintly acid than at alkaline pH.

Peroxidases are, with a few exceptions, found in plants, where pH generally is lower than in tissues from at least higher animals. An acid substance AH_2 would then be oxidized in plants according to

$$AH_2 + H_2O_2 = A + 2H_2O$$

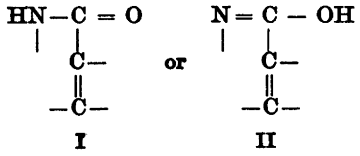
In tissues from animals the substance would be oxidized by electron transporting enzymes after protolytical release of one or several protons

$$AH_2 = A^{2-} + 2H^+$$
$$A^{2-} = A + 2\ e^-$$

As a consequence of this comparison between the modes of action of peroxidases and the exclusively electron transporting systems it must be assumed that the oxidation products, obtained when HRP and uricase act upon uric acid, at their pH optima, should become identical when brought to the same pH. Uricase oxidizes uric acid to allantoin, which also was the final product deriving from compound B_2. Moreover, B_2 and that of Praetorius' [27] intermediates, which possessed an absorption band at 325 mμ show similar spectra. His figures do not seem to exclude the possibility that the maximum could have been situated at a slightly shorter wavelength than 325 mμ.

In unsubstituted uric acid the hydrogen atoms at the positions 3 and 9 [20] (or possibly in the order 9 and 3[28]) are first dissociated. Methylation at these positions exerts the most pronounced influence on the velocity of reaction 1 (Table 2). The velocity of reaction 1 of the oxidation of a purin derivative cannot, however, be related only to the dissociations of the nuclear hydrogen atoms, since the presence of the carbonyl group at position 8 is a prerequisite for the oxidation.

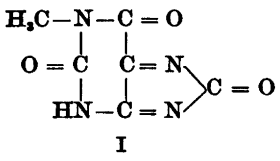
The most apparent spectral effect during the oxidation of uric acid to compound B is the vanishing of the strong band at around 285 mμ. This band is mainly caused by the chromophore [29]



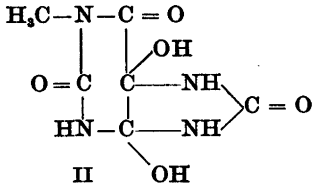
I II

It is therefore probable that the 4,5-double bond vanishes simultaneously with the loss of two hydrogen atoms. Several structures for the intermediate B will then be possible, a common feature being a number of conjugated double bonds. The structures differ by the lack of one negative charge from the one attributed by Bentley and Neuberger to the primary product from uricase action on the monovalent urate anion. The rapid transformations between the three forms of B can be accounted for by tautometric shifts or ionisations.

The following way for the decomposition of B may be worth considering. One of the possible structures of B is (I). By the uptake of water it acquires a glycol form (II).



I



II

Biltz and Max [30] suggested that (II) could be an intermediate in the oxidation of uric acid with nitric acid to alloxan and with alkaline permanganate to allantoin. The dimethyl ether of (II) has been isolated [31] after oxidation of uric acid with chlorine in methanol.

Biltz [31] has described a procedure, supposed to give (II). It gave in our hands a preparation which was spectrophotometrically stable at pH 4 (235 mμ). The addition of 6 *M* HCl (final conc. 0.2 *M* HCl) caused no immediate decrease in optical density at 235 mμ, nor did the light absorption increase slowly. At pH 9.3 (235 mμ) the initial light absorption was higher than at pH 4 and decreased by one fourth in 3 min. and was then stable. Thus our compound B cannot be identical with the substance obtained according to Biltz. Archibald [18] found that Biltz' method gave a product, from which ammonia could be liberated with urease, and which gave alloxan reactions. He concluded that Biltz' compound was not the true glycol (II) but rather some adduct of alloxan and urea, a conclusion well compatible with our spectrophotometric observations.

The rapid transformation $B_1 \rightarrow B_3$ occurs at the pH where the first proton is dissociated from hydrated alloxan [32]. A negative charge acquired by the dissociation of the hydroxylic proton at the position 5 in (II) tends to split off by decarboxylation the 6-carbon atom, corresponding to reaction 2 within the range pH 2—5. Bentley and Neuberger showed that this carbon is lost during the conversion of uric acid to allantoin by uricase. A high hydrogen ion concentration would promote this decarboxylation, *i. e.* $k_2(B_1 \rightarrow C_1)$ should increase when pH is decreased (Fig. 5). This reaction leads to allantoin. At even lower pH the dissociation of the proton is suppressed, and no decarboxylation takes place. The spectral differences between B_1 and B_3 can possibly be accounted for by this dissociation. The slow reaction $B_3 \rightarrow C_3$ then corresponds to the hydrolysis of the (7—9) urea residue.

Thus it appears unlikely that alloxan is formed in the living organism by peroxidase or uricase action. The results of Tipson and Ruben can nevertheless be explained since they extracted the tissues with 10 % trichloroacetic acid. Since neither allantoin nor uroxanic acid explain the biological observations mentioned at the beginning, it is possible that the parent substance of alloxan, *i. e.* compound B, is responsible for the effects.

SUMMARY

1. The oxidation of 1-methyl uric acid with horse radish peroxidase and hydrogen peroxide has been studied.

2. The uncharged molecule was oxidized more rapidly than the monovalent anion. The divalent anion was apparently not oxidized at all.

3. The primary oxidation product, obtained at pH 3—5, could reversibly be transformed into two other, spectrophotometrically different forms by changing the pH to > 6 ($pK = 5.75$) or ≤ 1.

4. The primary oxidation product undergoes non-enzymatic decomposition to allantoin (pH 3—6) or alloxan (pH ≤ 1). The decomposition of the alkaline form of the primary oxidation product has not been studied.

5. Experiments on uric acid, some N-methyl substituted uric acid preparations and purins, lacking the carbonyl group in position 8, showed that the presence of this carbonyl group is a prerequisite for the oxidation.

6. It is likely that the primary oxidation products of uric acid, obtained by the actions of uricase and peroxidase, should become identical when brought to the same pH. Available results seem to support this idea.

Dr. Ulf Lagerkvist has kindly supplied some methyl derivatives of uric acid. Technical assistance was given by Miss Berit Nilsson. The investigation was financially supported by *Statens naturvetenskapliga forskningsråd.*

REFERENCES

1. Ascoli, M. and Izar, G. Z. *Hoppe Seylers Z. physiol. Chem.* **58** (1908—9) 529; **62** (1909) 347.
2. Zeller, E. A. *Helv. Chim. Acta* **24** (1941) 539.
3. Dunn, J. S., Sheehan, H. L. and McLetchie, N. G. B., *Lancet* **1** (1943) 484; *J. Am. Med. Assoc.* **122** (1943) 676.
4. Tipson, R. S. and Ruben, J. A. *Arch. Biochem.* **8** (1945) 1.
5. Loubatieres, A. and Bouiard, P. *Compt. rend. soc. biol.* **145** (1951) 344.
6. Griffiths, M. *J. Biol. Chem.* **172** (1945) 853.
7. Brada, Z. *Nature* **171** (1953) 524.
8. Moubasher, R. and Othman, A. M. *J. Am. Chem. Soc.* **72** (1950) 2667.
9. Patterson, J. W., Lazarow, A. and Levey, S. *J. Biol. Chem.* **177** (1949) 197.
10. Walsh, E. O'F. and Walsh, G. *Nature* **161** (1948) 976.
11. Bricas, E. and Macheboeuf, M. *Compt. rend. soc. biol.* **141** (1947) 120.
12. Agner, K. *Nature* **159** (1947) 271.
13. Agner, K. *J. Exptl. Med.* **92** (1950) 337.
14. Tuttle, A. L. and Cohen, P. F. *Federation Proc.* **12** (1953) 281.
15. Paul, K. G. and Avi-Dor, Y. *Acta Chem. Scand.* **8** (1954) 649.
16. Hartman, W. W., Moffett, E. W. and Dickey, J. B. *Org. Syntheses, Collective Volume II*, New York 1944.
17. Kuhn, R., Reinemund, K. and Weygand, F. *Chem. Ber.* **67** (1934) 1460.
18. Archibald, R. M. *J. Biol. Chem.* **158** (1945) 347.
19. Johnsson, E. A. *Biochem. J. (London)* **51** (1952) 133.
20. Biltz, H., and Herrmann, L. *Ber.* **54** (1921) 1676.
21. Chance, B. *Arch. Biochem.* **24** (1949) 410. **40** (1952) 153.
22. Balls, A. K. and Hale, W. S. *J. Biol. Chem.* **107** (1934) 767.
23. Margules, L. and Griffiths, M. *Arch. Biochem.* **29** (1950) 225.
24. Keilin, D. and Hartree, E. F. *Proc. Roy. Soc. (London)* **119B** (1936) 114.
25. Bentley, R. and Neuberger, A. *Biochem. J. (London)* **52** (1952) 694.
26. Boeri, E., Baltscheffsky, H., Bonnichsen, R. K. and Paul, K. G. *Acta Chem. Scand.* **7** (1953) 831.
27. Praetorius, E. *Biochim. et Biophys. Acta* **2** (1948) 602.
28. Biltz, H. *Ber.* **69** (1936) 2750.
29. Cavalieri, L. F., Bendich, A., Tinker, J. F. and Brown, G. B. *J. Am. Chem. Soc.* **70** (1948) 3875.
30. Biltz, H. and Max, F. *Ber.* **54** (1921) 2451.
31. Biltz, H. *Ber.* **47** (1914) 459.
32. Richardson, G. M. and Cannan, R. K. *Biochem. J. (London)* **23** (1929) 68.

Received January 25, 1954.