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MECHANISM OF URICASE ACTION

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Summary

Uricase (urate:oxygen oxidoreductase, EC 1.7.3.3) exposes a positional and steric specificity in the enzymic conversion of urate to allantoin. C-2 of urate was recovered as C-2 of allantoin. By the consecutive oxidation and hydrolysis reactions a levorotatory intermediate was formed, presumably (—)-2-oxo-4-hydroxy-4-carbohydroxy-5-ureido-imidazoline. The absorption and optical rotation dispersion spectra of the intermediate were established. In the presence of borate buffer, the intermediate was transformed to (+)-alloxanate. The decay of the former compound depends on general base and acid catalysis. *RS*-(±)-allantoin was formed by chemical decarboxylation and *S*-(+)-allantoin by enzymic decarboxylation.

Introduction

Uricase (urate:oxygen oxidoreductase, EC 1.7.3.3) catalyzes the oxidation of uric acid to allantoin, carbon dioxide and hydrogen peroxide, but in the presence of borate buffer alloxanate and urea are the main products [1]. Since various uricases have been obtained in pure and homogeneous form [2], the above reaction must be ascribed to one enzyme. A reaction mechanism accounting for the data was presented [3]. The urate anion, deprotonated at N-9, is the most probable substrate for uricase at physiological pH values [4]. Bentley and Neuberger [5] established that the enzymic oxidation involves a two-electron transfer to molecular oxygen, which is transformed into hydrogen peroxide.

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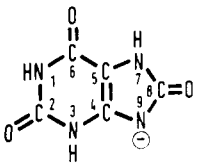
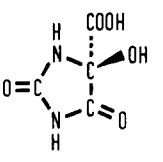
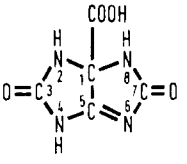
Various lines of evidence led to the proposition of a symmetric intermediate for the chemical and enzymatic oxidation of uric acid: (a) both the carbon dioxide evolved and the carbohydroxy group of alloxanate are derived from C-6 of uric acid [3,5]. A ring contraction by a rearrangement reaction was postulated to occur; (b) only 3-methylallantoin was formed during the chemical oxidation of both 1- and 7-methyluric acid and only 1-methyl-allantoin was obtained from both 3- and 9-methyluric acid [6]; (c) an equal distribution of the ^{15}N-label of [1,3-^{15}N]uric acid among the ureido- and hydantoinmoiety of allantoin, formed by non-enzymic oxidation, was reported by Cavalieri and Brown [7]. The symmetric intermediates were supposed to be 1-carbohydroxy-2,4,6,8-tetraaza-3,7-dioxo-4-ene-bicyclo · (3,3,0) · octane (the so-called 'Compound I') [3,8], and/or 1-carbohydroxy-2,4,6,8-tetraaza-3,7-dioxo-5-hydroxy-bicyclo · (3,3,0) · octane (the so-called 'Behrend compound' or 'hydroxy-acetylene-diureido-carboxylic acid') [1,5,9,10].



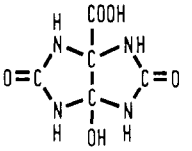
urate



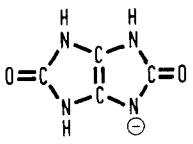
(+)-alloxanic acid



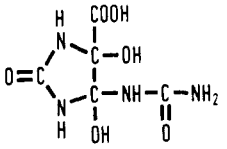
compound I



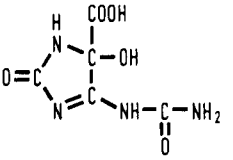
III



compound II



IV



V

According to the reaction sequence proposed by Mahler [3], Compound I is converted into racemic allantoin via a decarboxylated symmetrical Compound II ($\Delta^{1,5}$-ene-2,4,6,8-tetraaza-3,7-dioxo-bicyclo · (3,3,0) · octane) in the absence of borate buffer. In the presence of borate alloxanate is formed via 2-oxo-4,5-dihydroxy-4-carbohydroxy-5-ureido-imidazolidine (Compound IV) [1].

However, the following observations do not fit into the given model: (i) C-5 of urate is completely converted into C-4 of allantoin during oxidation of urate by potassium permanganate in alkaline solution [6,11—13]; (ii) uricase converts urate into optically active *S*-(+)-allantoin [14,15]; (iii) Canellakis and Cohen [1] observed that the C-2 atom of urate is recovered completely in alloxanate on hydrolysis of the IV-borate complex prepared in borate buffer by uricase-catalyzed oxidation, whereas C-8 is recovered in urea. This hydrolysis was performed by addition of phosphate buffer to the isolated complex. Since very rapid exchange of a proton may be expected to occur between N-4 and N-N-6 of Compound I, Mahler's proposal for the recovery of C-2 of urate in alloxanate is hardly tenable.

The steric and positional specificity of the uricase reaction is investigated now. Evidence is presented that, in addition to Compound IV (which is one of the intermediates in Mahler's scheme), not Compound II, but the dehydrated

form of Compound IV (2-oxo-4-hydroxy-4-carbohydroxy-5-ureidoimidazoline (Compound V)), may be involved in the production of both alloxanate and allantoin, thus being a common intermediate in the urate degradation.

Materials and Methods

Bacterial strains. Bacillus fastidiosus strain SMG 83, kindly supplied by H. Kaltwasser (Laboratory of Microbiology, University of Saarbrücken, F.R.G.) served as source of pure uricase (specific activity 53 units/mg protein) which was prepared as described previously [16]. The urease-negative *B. fastidiosus* strain C.6.A., described earlier [15], was used for experiments on the degradation of [2-^{14}C]urate.

Substrate solutions. To obtain buffered substrate solutions uric acid was dissolved in 0.1 N NaOH and the pH was lowered to 7.2—7.4 with concentrated buffer solution. The substrate solutions were kept at 30°C to prevent crystallization of uric acid, and were freshly prepared every day.

Degradation of [2-^{14}C]urate. Incubation mixtures (1 ml) contained 80 μmol phosphate buffer (pH 6.6), 88 nmol [2-^{14}C]urate (56.7 Ci/mol, obtained from Radiochemical Centre Amersham, Buckinghamshire, U.K.) and urate-grown cells of *B. fastidiosus* C.6.A., the protein content of which was equivalent to 145 μg protein. The mixtures were aerated at room temperature and 10-μl samples were placed on dry Sartorius cellulose nitrate filters (11307). The filters were dried immediately at 60°C and counted by use of a Packard Liquid Scintillation Counter.

Polarimetric measurements. Optical rotations were measured at 365, 436, 546 and 578 nm in a Perkin Elmer, model 141, polarimeter. 10-ml mixtures were incubated in 100-ml Erlenmeyer flasks in a shaking (120 rev./min) waterbath at 30°C. Samples were poured into 10 cm quartz cuvettes, temperature-controlled at 30°C, and were measured immediately.

Optical rotation dispersion measurements. Optical rotation dispersion spectra were recorded with a Jasco Optical Rotation Dispersion Recorder model ORD/UV-5 in a 1 cm quartz cuvette. Reaction mixtures contained 1 μmol uric acid and 15 μg of protein per 5 ml.

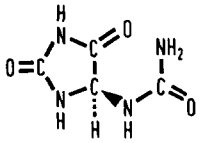
Spectrophotometric measurements. Spectra were recorded with a Cary 118 C spectrophotometer. Reaction mixtures containing $7.5 \cdot 10^{-4}$ M uric acid at the start of the reaction were compared to similar mixtures in which the substrate was absent.

Results

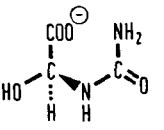
Degradation of [2-^{14}C]urate

It was commonly [3,5,7,10,13,17] accepted that C-2 of urate is distributed equally among both the hydantoin and the ureido moiety of allantoin, when urate is degraded enzymatically in the absence of borate ions. Since the experimental and analytical procedures used did not preclude the introduction of artefacts we tested the degradation of [2-^{14}C]urate by whole cells of *B. fastidiosus*. This organism contains *S*-(+)-allantoinase (allantoin amidohydrolase, EC 3.5.2.5) which converts only one of the stereoisomers of

allantoin to allantoate, which is subsequently converted by allantoate amidohydrolase (allantoate amidohydrolase (decarboxylating), EC 3.5.3.9) into *S*-(—)-ureidoglycolate, ammonia and carbon dioxide [15]. Since the absolute



S(+)-allantoin



S(-)-ureidoglycolate

configurations of *S*-(+)-allantoin and *S*-(—)-ureidoglycolate are known [18,19], the fate of the carbon atoms of the hydantoin and ureido moiety of allantoin can be followed in this process, if a urease-negative strain of *B. fastidiosus* is used. When C-2 of urate appears in the hydantoin ring of allantoin by the action of uricase, it will be excreted as CO_2 by these bacteria, and, when this atom will be present in the ureido moiety of allantoin, it will be converted to urea. We found that 94% of the label of [2-^{14}C]urate disappeared from the incubation mixture as gaseous carbon dioxide within 10 min. Thus C-2 urate appeared to be converted to C-2 of the hydantoin ring of allantoin, and, con-

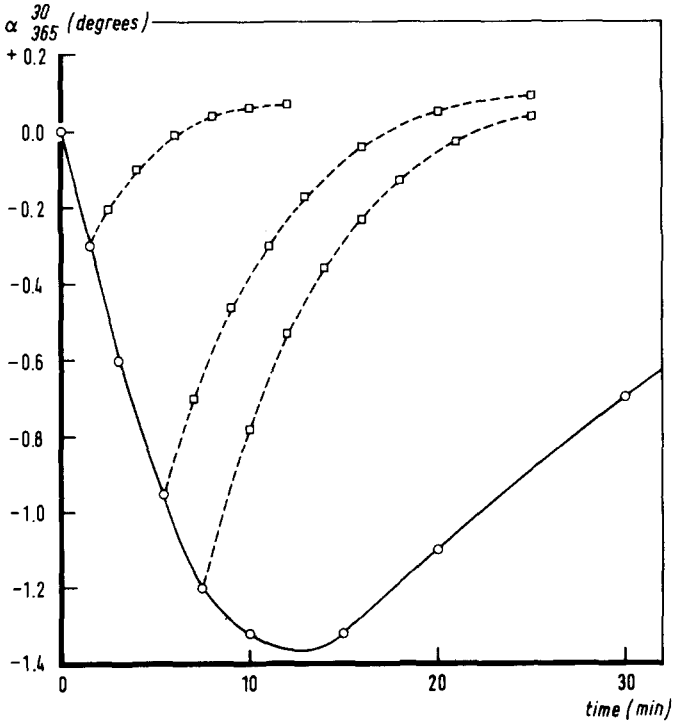


Fig. 1. Production of levorotatory intermediate by uricase and disappearance of this intermediate after aeration has been stopped. The reaction mixture at 30° C contained per ml 15 μmol urate, 75 μmol phosphate buffer (pH 7.5) and 0.38 mg uricase. ——, alteration of the optical rotation at 365 nm in a reaction mixture supplied with oxygen by shaking. - - - - - -, alterations after transfer of a sample to a cuvette which was closed to prevent the entry of air. The increase of the decay rate of the intermediate in time is due to a slight acidification which is caused by the production of the intermediate.

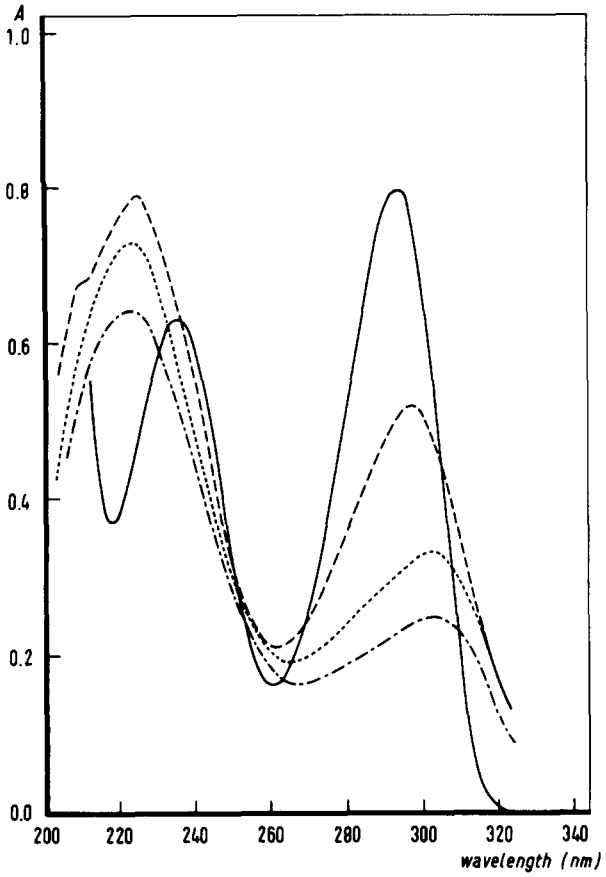


Fig. 2. Spectra of a reaction mixture in which uric acid ($6 \cdot 10^{-5}$ M) is degraded by uricase (50 μg/ml). The compounds were present in 0.1 M Tris-HCl buffer (pH 7.5) and were incubated at room temperature. The spectra were recorded after 0 (———), 4 (- - - - -), 8 (· · · · ·) and 10 (— · — · —) min of incubation.

sequently, the six-membered ring of urate was converted into the five-membered one of allantoin by the action of uricase.

Optical rotation during uricase action

The production of *S*-(+)-allantoin by uricase [15] in well-aerated reaction mixtures was accompanied by the temporary accumulation of an intermediate with a strongly negative optical rotation (Fig. 1). When the aeration was stopped, this intermediate was no longer produced and disappeared ($t_{1/2}$ about 180 s after 1.5 min and $t_{1/2}$ about 270 s after 7.5 min). Then a slightly positive rotation became detectable. The ratio of the positive optical rotations at 365, 436, 546 and 578 nm was 1 : 0.65 : 0.37 : 0.34 which result corresponds well with that measured for optically active allantoin [18]. When borate buffer (10^{-2} M) was added at the moment at which the oxygen supply was stopped, the levorotatory intermediate disappeared much faster ($t_{1/2}$ about 40 s). Under these circumstances also a dextrorotatory compound was formed but the ratio of the optical rotations at 365, 436, 546 and 578 nm was 1 : 0.57 : 0.31 :

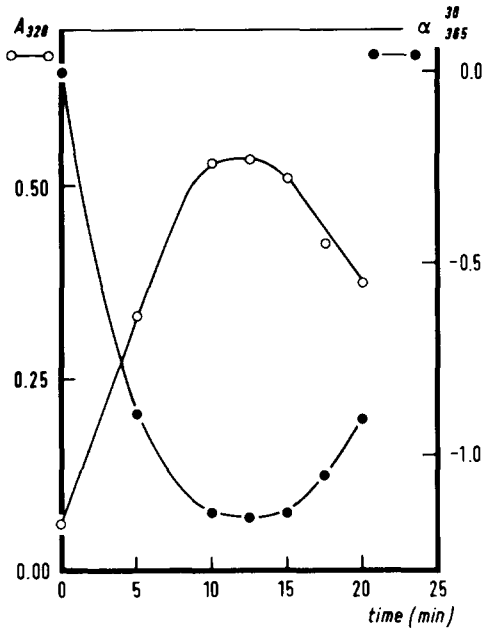


Fig. 3. Changes in the optical rotation at 365 nm and in the absorption at 320 nm. The conditions applied for incubation are as described in Fig. 1. For spectrophotometric measurements samples were diluted 20-fold in 0.1 M Tris-HCl (pH 7.5) containing oxonate (10^{-4} M), a potent competitive inhibitor of uricase, and measured immediately.

0.27, which values differ substantially from those observed for allantoin. Most probably (+)-alloxanate was formed.

Spectrophotometrical measurements

The enzymatic degradation of urate was reported to proceed via a number of unstable intermediates, whose presence may be detected by study of the ultraviolet absorption spectrum [3,20]. In neutral solutions (pH 7.0 to 7.5) urate exhibits absorption maxima located at 292 nm and 235 nm with molar absorption coefficients being $12.0 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ and $10.2 \cdot 10^3\ M^{-1} \cdot cm^{-1}$, respectively. At the same wavelengths the molar absorption coefficients of allantoin are extremely low and amount to zero and $0.33 \cdot 10^3\ M^{-1} \cdot cm^{-1}$, respectively [21]. During urate degradation by uricase at pH 7 to 8, spectral peaks were observed at 302 nm, as was reported previously by others [3,20,22], and at 222 nm (Fig. 2). The peaks appeared both in the presence and in the absence of borate buffer and disappeared on further incubation. Both peaks are attributable to one intermediate, on basis of the following observations: (a) the intensity of both spectral peaks exhibited a similar course in time, when measured at pH 8.0 as well as at pH 7.0 (data not shown); (b) in the presence of borate ions the disappearance rate of the spectral peaks was much higher, but again identical for both peaks (data not shown). The time-dependent changes in the spectral peaks coincided with those observed for the optical rotation of the levorotatory intermediate (Fig. 3). Hence, the spectral peaks at 222 nm and 302 nm might be attributed to this levorotatory compound.

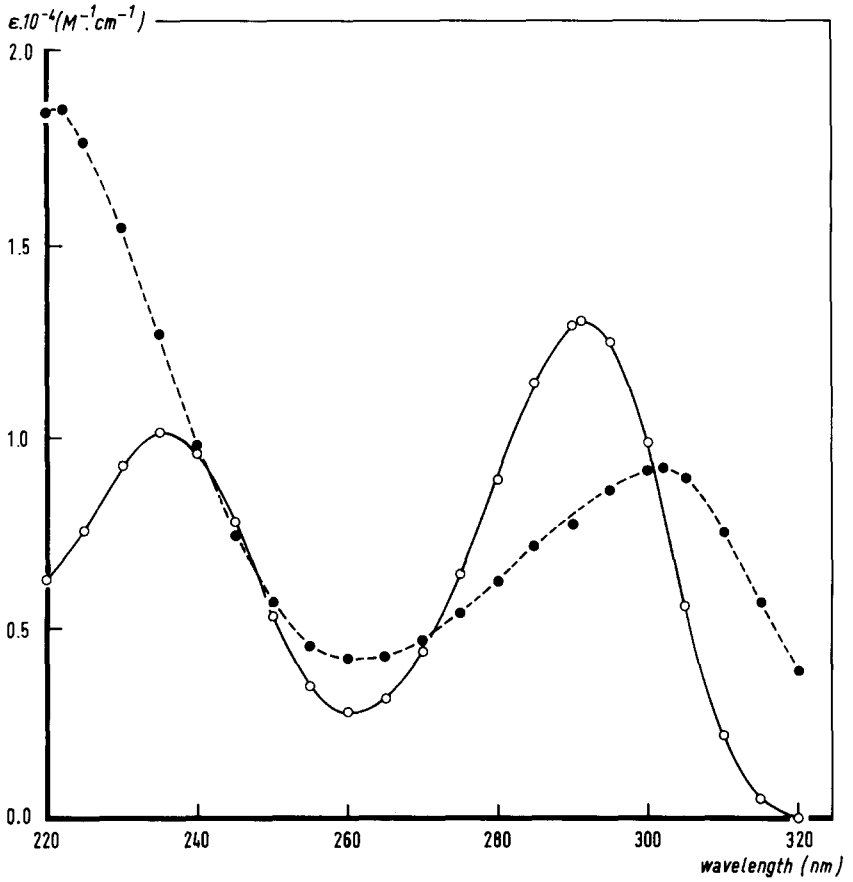


Fig. 4. Molar absorption spectra of urate (———) and the intermediate formed (- - - - -). Spectral scans were made of urate in 0.1 M Tris-HCl buffer (pH 7.0) and of reaction mixtures containing per ml 0.075 μmol uric acid and 50 μg uricase in 0.1 M Tris-HCl buffer, at various time intervals. Simultaneously the residual amount of urate was determined according to Mahler [23]. The intermediate does not interfere in this determination since this compound is hydrolyzed rapidly under the acidic conditions applied. The contribution of urate to the absorption was subtracted from the recorded spectra. The contribution of allantoin to the absorption spectrum could be neglected, because of its low absorption coefficient. The values given for the intermediate are approximations, since part of it will be degraded at the moment of the measurement. To avoid this interference as much as possible short incubation periods and relatively high amounts of uricase were applied. The molar absorption spectrum of urate differs a little from that given in the literature due to the effect of rapid scanning.

The spectrum of the intermediate was determined from scans taken during urate degradation by uricase (Fig. 4). A weak absorption peak at about 275 nm was observed when the uricase reaction was performed at pH 9.0 in 0.1 M glycine buffer (Fig. 5).

Kinetic experiments

The disappearance of the intermediate under various conditions was studied spectrophotometrically at 320 nm to avoid interference by uric acid, allantoin and alloxanate. The compound was formed by the action of uricase on urate and the enzymatic reaction was stopped by the addition of oxonate (final concentration 10^{-4} M), when appropriate amounts of the intermediate were

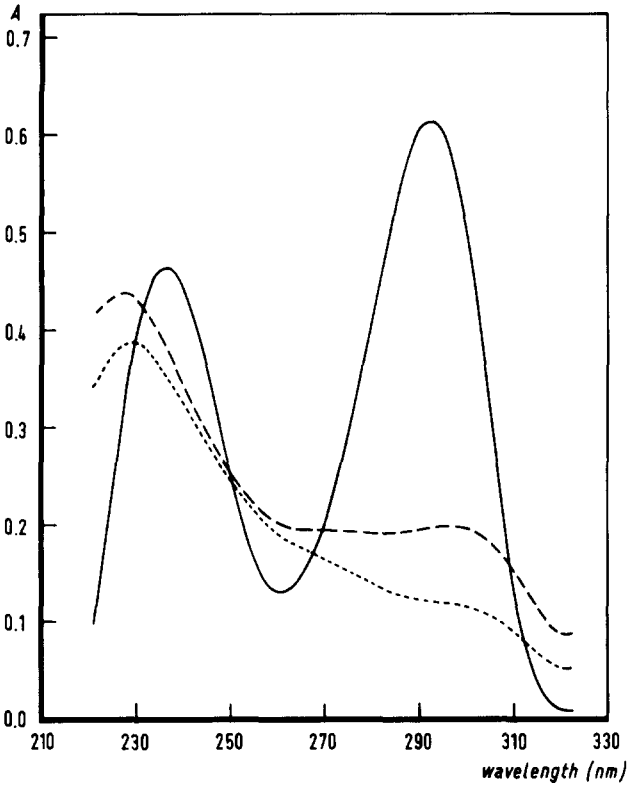


Fig. 5. Spectra recorded during urate degradation by uricase in 0.1 M glycine buffer (pH 9.0). Further conditions are given in Fig. 2. The spectra were taken before addition of the enzyme (———) and 3 min (- - - - - -) and 5 min (· · · · · ·) after addition.

present. Oxonate does not interfere during the measurements. The decomposition of the intermediate appeared to obey first-order kinetics.

The catalytic action of borate buffer was only weakly influenced by pH (Fig. 6). The reaction rate was linearly dependent on the concentration of this buffer, tested from 0.05 to 0.75 M (data not shown), and obeys the equation:

$$v = k_b \cdot (\text{boric acid}) \cdot (\text{intermediate})$$

in which v is the rate of decomposition and k_b is 0.21 $l \cdot mol^{-1} \cdot min^{-1}$. Similar results were reported by Pitts and Priest [8] but they found a saturation behaviour for boric acid in tests exerted at 36° and 46°C.

The rate-pH profiles in the other buffers indicate that both protons and hydroxide ions catalyze the reaction. However, the reaction rates were not linearly dependent on the concentration of these ions. Moreover, the concentration of the buffering substances strongly affected the reaction rate (Table I). This was not due to alterations of ionic strength of the solution since the addition of 0.5 M KCl did not exert any effect. These results strongly suggest that the decomposition reaction displays a general acid and base catalysis in these buffers. The rate-concentration profiles of the decomposition of the intermediate in phosphate buffers expose a complex effect based on two actions of phos-

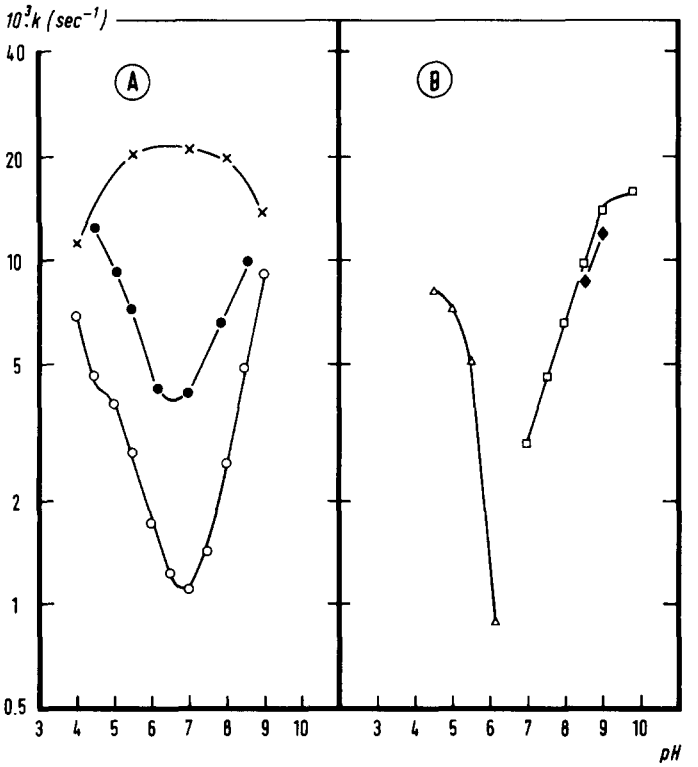


Fig. 6. Rate-pH profiles of the decomposition of the intermediate in various buffers. The intermediate was produced in a reaction mixture containing per ml 10 μmol or urate and 200 μmol of Tris-HCl (pH 7.5 to 7.7). Incubation was at 30°C and 120 rev./min in a shaking water bath. After 5 min 0.1 ml was added to 2.9 ml of the desired buffer, in which oxonate (10^{-4} M) was present. Absorption at 320 nm was recorded immediately. (A) The profile measured in 0.1 M phosphate buffer (○———○), 0.4 M phosphate buffer (●———●) or 0.01 M borate together with 0.1 M phosphate or 0.1 M Tris-HCl buffer (X———X). (B) The pH profiles recorded in 0.2 M acetate buffer (△———△), 0.1 M Tris-HCl buffer (□———□) and 0.2 M glycine/NaOH buffer (♦———♦).

TABLE I

INFLUENCE OF BUFFER CONCENTRATION ON THE HALF-LIFE TIME OF THE INTERMEDIATE

The conditions are as described in Fig. 6.

Buffer	pH	Half-life time (s) at the indicated buffer molarity *		
		0.1 M	0.2 M	0.4 M
Acetate	5.0	110	100	100
Acetate/0.5 M KCl	5.0	—	100	100
Tris-HCl	7.4	200	105	70
Tris-HCl	8.0	100	55	32
Tris-HCl/0.5 M KCl	8.0	—	55	—
TEA **/HCl	8.0	250	185	160
Tris-HCl	8.5	70	45	29
Glycine/NaOH	8.5	205	105	70

* The relation between the half-life time and the reaction constant is given by the formula $k = \ln 2/t_{1/2}$.
** Triethanolamine.

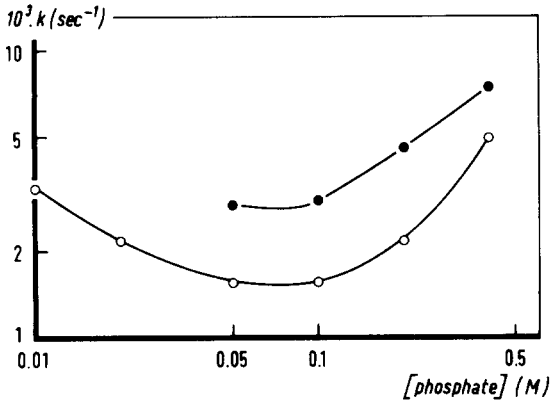


Fig. 7. Rate constant of the decomposition of the intermediate as a function of the phosphate concentration at pH 7.5 (○———○) and pH 5.5 (●———●). The measurements were performed as described in Fig. 6.

phate ions. Phosphate ions appeared to protect the intermediate against the catalytic action exerted by protons and hydroxide ions (Fig. 7). This protective effect may be due to the formation of a complex between phosphate and the intermediate. The optical rotation dispersion spectra, which will be discussed below, yielded additional evidence for the occurrence of such a complex. Secondly, the effect observed at concentrations above 0.1 M indicate that phosphate buffers exert a general acid and base catalysis like other buffering substances.

Optical rotation dispersion measurement

ORD-spectra were recorded during urate degradation at pH 8.0 in three buffer systems (Fig. 8). Levorotatory peaks (at about 320 and 260 nm), dextrorotatory peaks (at about 290 and 230 nm) and isosbestic points with nearly zero rotation (at 307 and 272 nm) were recorded when a Tris-buffered system was used. The shifts with time to higher wavelengths (from 225 to 237 nm) observed in the ultraviolet region are probably due to the formation of *S*-(+)-allantoin [18].

The differences between the spectra in Tris-HCl buffer and Tris-HCl/borate buffer may result from the catalytic action exerted by boric acid on the decomposition of the intermediate. The contribution of this compound to the spectrum between 275 and 325 nm disappeared rapidly. The final positive rotation peak shifted to a higher value (248 nm instead of 237 nm) and an overall positive rotation at higher wave lengths was observed when borate buffer was present in the reaction mixture. Both phenomena were due to the formation of (+)-alloxanate instead of *S*-(+)-allantoin under these conditions.

The variations of the ORD-spectra in 0.1 M phosphate buffered reaction mixtures were quite different and indicate a secondary reaction of the intermediate with phosphate ions. Addition of phosphate (final concentration 0.05 M) and oxonate to a reaction mixture in which the uricase reaction had proceeded in Tris-HCl buffer caused a shift from the normal spectrum, ascribed to the levoratatory intermediate, to that observed in phosphate buffer.

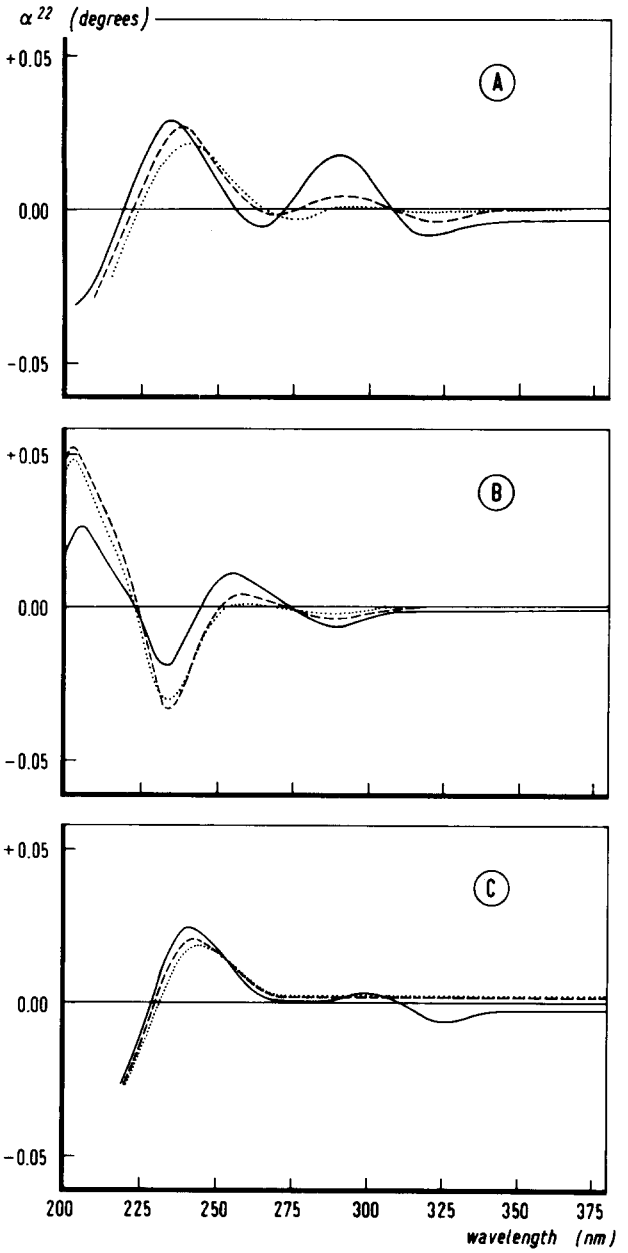


Fig. 8. ORD-spectra recorded during urate degradation by uricase. The reaction mixtures (5 ml) contained 1 μmol of urate and 15 μg of uricase in the indicated buffer system. ORD scans were started 5.5 (———), 10 (- - - - - -) and 15 (· · · · · ·)min after the start of the experiment. Scanning time for one spectrum (from 375 nm to 200 nm) was 2.5 min. The following buffer systems were used: (A) 0.05 M Tris-HCl (pH 8.0), (B) 0.1 M phosphate buffer (pH 8.0), (C) 0.05 M Tris-HCl (pH 8.0) supplied with 0.01 M borate buffer at the start of the uricase action. Oxonate (10^{-4} M) was added at 5 min after the start of the reaction to stop the uricase action.

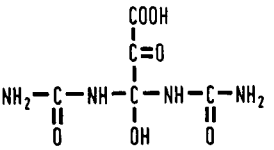
Discussion

The enzymic oxidation of uric acid by uricase involves an oxidative, a decarboxylative and a hydrolytic step, in all of which a high degree of positional and stereochemical specificity is maintained [1,14,15].

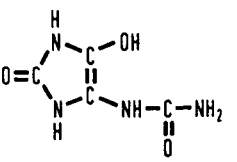
In this study we demonstrate the in vivo conversion of C-2 of urate into C-2 of allantoin, and the occurrence of a strongly levorotatory intermediate which is transformed into dextrorotatory products. Under physiological conditions *S*-(+)-allantoin is formed, but in the presence of boric acid another dextrorotatory compound is produced, which is most probably identical to (+)-alloxanate [1]. The measured half-life time of the levorotatory intermediate corresponds to that reported by Mahler et al. [3] for the decomposition of Compound I. However, the polarimetric data presented now yield evidence that the most stable intermediate in the uricase reaction is optically active thus excluding all symmetric compounds previously proposed as likely candidates. On basis of the polarimetric and spectrophotometric results and the possible reaction sequences (—)-2-oxo-4-hydroxy-4-carbohydroxy-5-ureidoimidazoline ((—)-Compound I) is the only serious candidate for the levorotatory intermediate. The absorption peak located at about 300 nm can be attributed to this compound which contains a conjugated carbonyl function which may enlarge to the lone electron pair on N-6 of the ureido moiety and the carbonyl function on C-7. The general acid-base catalyzed disappearance of (—)-Compund V is most probably due to an initial hydration of the compound followed by further conversions of the product. The only other possible acid-based catalyzed reaction should be a decarboxylation, but this reaction seems more likely specifically acid-base catalyzed. General acid-base catalysis was also observed in phosphate buffers, but phosphate exerted also a stabilizing effect against the hydrolytic decay of Compound V. This effect is probably due to the formation of an ester-complex between phosphate and Compound V.

The next series of events are supposed to occur during the degradation of urate by uricase. Uricase catalyzes the transfer of two electrons from urate to molecular oxygen under formation of hydrogen peroxide. As a result an unstable carbonium ion is created, which may be directed by the enzyme specifically to (—)-Compund V. This may occur along different ways. By an intramolecular rearrangement a new bond may be formed between N-1 and C-5 of the carbonium ion, while the bond between N-1 and C-6 is broken. Thus, 1-carbohydroxy-2,4,6,8-tetraaza-3,7-dioxo-4-ene-bicyclo · (3,3,0) · octane (Compound I) is formed which may deliver (—)-Compound V either by a regiospecific hydrolysis of the C-1 to N-8 bond or by a hydration/hydrolysis/dehydration sequence in which 1-carbohydroxy-2,4,6,8-tetraaza-3,7-dioxo-5-hydroxy-bicyclo · (3,3,0) · octane (Compound III) and one of the steroisomers of 2-oxo-4,5-dihydroxy-4-carbohydroxy-5-ureido-imidazoline (Compound IV) would participate. Alternatively, diureido-hydroxypyruvate could be formed from the carbonium ion and (—)-Compound V is produced by a subsequent specific ring closure. The first pathway given is most plausible because of its simplicity.

Uricase is supposed to be involved also in the stereospecific conversion of (—)-Compound V into *S*-(+)-allantoin, in which Compound IV and the enol-



diureido-hydroxypyruvate



enol-form of allantoin

form of allantoin may be intermediates in the hydration, decarboxylation and tautomerization reactions. The latter intermediate was postulated to be involved in the racemization of allantoin [21]. The weak absorption band observed at about 275 nm (Fig. 5) may be due to a temporary accumulation of Compound IV, which lacks the conjugated system. A similar reaction sequence, but lacking stereospecificity in the hydratation of (—)-Compound V and the conversion of the enol-form of allantoin, is supposed to occur in the rather rapid chemical degradation of (—)-Compound V to *R*S-allantoin in the absence of boric acid. In the presence of boric acid (considering the effect of pH on the catalytic action and the pK_a value (9.24) of boric acid it is justified to attribute specific effects to boric acid) (+)-alloxanate was formed both in the presence and absence of an active uricase. *cis*-Diols can react with boric acid; the primary adduct can react again to the so-called spiro-compounds. One of the stereoisomers of Compound IV contains such a vicinal diol structure and is, therefore, a likely candidate to be involved in a reaction with boric acid as was proposed earlier by Canellakis and Cohen [1]. In such a complex Compound IV is protected against the normal decomposition to allantoin. Upon a nucleophilic attack of a hydroxide ion or, in view of the pH dependence of the reaction (Fig. 6), more likely a water molecule, the ureido moiety will behave as a leaving group and alloxanate is formed. In these chemical reactions the specific configuration around C-4 is conserved and is identical in (—)-Compound V, Compund IV and (+)-alloxanate. This explains that only one of the isomers of alloxanate is formed.

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