STUDIES ON URICASE

IV. THE NATURE AND COMPOSITION OF SOME STABLE REACTION PRODUCTS

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In a previous paper of this series[1] a purification procedure for uricase was described and copper was shown to be the prosthetic group of this enzyme. The latter finding was supported by a study of the mechanism of oxidation of uric acid by copper ions[2]. Further, from kinetic studies of the enzymic and non-enzymic oxidation reactions, it has been possible to suggest a tentative structure for the enzyme-substrate complex[3]. The present investigation describes an improved method for the purification of uricase, and some of the intermediates and final products in the oxidation of uric acid catalyzed by the highly purified enzyme.

Although allantoin has always been considered to be the main end-product of the aerobic oxidation of uric acid in the presence of uricase, numerous other compounds have been suggested by various workers as allantoin precursors. BEHREND[4] was the first to postulate a symmetrical intermediate on the pathway from uric acid to allantoin. SCHULER AND REINDEL[5] claimed that the enzymic oxidation of uric acid by uricase gave rise to a compound which, isolated as the silver salt, had properties similar to the silver salt of the symmetrical hydroxyacetylene diureine carboxylic acid (HDC)***. This compound was synthesized by the same authors[6] by chemical oxidation of uric acid. Recently these findings were reinvestigated by KLEMPERER[7], who stated that the primary oxidation product of uric acid was an unstable compound which gave rise to either HDC or uroxanic acid. PRAETORIUS[8], using spectrophotometric techniques, described two short-lived intermediates. The first one had an absorption peak between 300 and 340 mμ and, in the absence of borate ions, was converted to a second intermediate with a absorption peak between 260 and 275 mμ, which in turn gave rise to allantoin. BENTLEY AND NEUBERGER[9] suggested that the action of uricase leads to the formation of a carbonium ion by the abstraction of an electron pair from the urate monoanion. CANELLAKIS AND COHEN[10] showed that the main product of uric acid oxidation in phosphate buffer is allantoin, whereas in borate buffer the end-products are urea, alloxanic acid and allantoin. They claimed that in the latter reaction an unstable intermediate, 5-ureido-2-imidazoline-4,5-diol-4-carboxylic acid (UIDC), is formed which decomposes to urea and alloxanic acid*.

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*** The following abbreviations will be used: BAL = 2,3-dimercapto-1-propanol; Tris = tris(hydroxymethyl)aminomethane; diol = 2-amino-2-methyl-1,3-propanediol; HDC = hydroxyacetylene diureine carboxylic acid; UIDC = 5-ureido-2-imidazoline-4,5-diol-4-carboxylic acid.

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Recently in a study on uricase[11] an increased uptake of oxygen was shown during the oxidation of uric acid by uricase in the presence of cysteine. Similarly, VAN REEN AND PEARSON[12] reported that although in the presence of BAL the rate of disappearance of uric acid is unchanged, the oxygen uptake may increase two-fold. The effect of BAL was explained as an inhibition of catalase leading to an increased oxygen uptake.

MATERIALS AND METHODS

Materials

The uric acid used for routine procedures during this investigation was an Amend Chemical Co., C.P. product. For quantitative determinations, the uric acid was freed of heavy metals as previously described[1]. Allantoin, alloxan and urea were commercial products of the highest purity available and recrystallized twice before use. Crystalline catalase was obtained from the Worthington Chemical Co. Uroxanic acid and HDC (as silver salt) were special preparations of the Aldrich Chemical Co., Inc., Milwaukee. Uric acid glycol was prepared according to the method of BILTZ[13]. Uric acid labeled at 2-^{14}C and 8-^{14}C respectively were gifts from Dr. P. P. COHEN. Uric acid 6-^{14}C was prepared by heating urea in a sealed tube with (4-^{14}C)-2,4-dihydroxy-5,6-diaminopyrimidine sulfate[14]; the latter compound was a gift of Dr. A. NEUBERGER.

Methods

Spectrophotometry was performed either manually in the Beckman Model DU spectrophotometer or automatically in the Beckman Model DK automatic scanning spectrophotometer. The microanalytical determinations were performed by the Micro-Tech Laboratories, Skokie, Illinois. The decomposition and melting points were obtained in the usual apparatus with oil as heated medium. Electrometric titrations made use of a Beckman pH meter, in a nitrogen atmosphere.

For paper electrophoresis, the Model EC 305 electrophoresis apparatus of the EC Apparatus Co., New York, and Whatman Filter Paper 3 MM, 2½ × 18½ inches were used. The distance between the electrodes is 9 inches in this particular apparatus.

0.05 to 0.1 *M* phosphate or carbonate buffers were used at the desired pH (*cf.* RESULTS). The voltage was 950 V and the time of each run 50 minutes. The samples containing between 0.03 and 0.15 mg of material were applied in small spots (diameter = 2 mm) to the paper in the center of the strip. The paper was wetted with the same buffer used for the electrophoretic run**.

The assay system for uricase, the definition of the specific activity and of the enzyme units are described in a previous paper[1]. The spray used for developing the paper electrophoresis patterns and chromatograms was based on the very sensitive method of RYDON AND SMITH[15]. It employs the chlorination of an active –NH– group to give a labile –NCl– group which then oxidizes potassium iodide to iodine—the liberated iodine being detected with starch. The solvent systems used for paper chromatography are described in the legend to Table II.

* Their paper[10] should be consulted for a more detailed discussion of the various intermediates postulated, including their structures.

** In all electrophoretic patterns migration towards the anode is indicated by movement of the spot from the starting line towards the left.

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RESULTS

Preparation of uricase

The rather laborious purification procedure described earlier[1] was reinvestigated in order to find a simpler method suitable for the large scale isolation of highly purified enzyme in good yield.

The difficulty in purifying uricase is largely due to a close association of the enzyme with certain nucleoprotein fragments. These aggregates tend to precipitate during the course of the purification procedure. The best results in separating uricase from these impurities were achieved by repeated treatment with *tert.*-butanol. Heat treatment and precipitation with 5% trichloracetic acid also proved to be effective, but did not lead to good recoveries ($\sim$ 20%). Therefore the heat and phosphate gel treatments of the earlier procedure[1] were replaced by a *tert.*-butanol treatment followed by a precipitation with acetic acid and elution of the enzyme from the precipitate with 0.1 *M* potassium bicarbonate solution.

Extraction of the acetone powder. 100 g of acetone-dried pig liver mitochondria[1] are suspended in 1000 ml of 0.02 *M* phosphate buffer pH 7.8 and extracted with constant stirring for 30 minutes at 2°. All subsequent procedures are performed at this temperature if not stated otherwise. The extract is then centrifuged for 45 minutes at full speed in the angle head (No. 845) of the International Centrifuge. The supernatant solution is discarded and the residue resuspended in 1000 ml of 0.15% sodium carbonate solution and extracted again for 30 minutes. It is important to keep the pH during extraction well above 9.0. The suspension is centrifuged for 45 minutes as before. In some preparations the supernatant solution may be turbid and a high speed centrifuge (Spinco Model L) has to be used in order to obtain a good separation. The residue is discarded.

Precipitation with ammonium sulfate and first tert.*-butanol treatment.* To each 100 ml of clear supernatant solution 30 ml of alkaline (pH 8.5) saturated ammonium sulfate solution are added. The resulting suspension is centrifuged for 20 minutes at full speed in the angle head (No. 845) of the International Centrifuge. The residue is discarded. To each 100 ml of supernatant fluid, 70 ml of alkaline saturated ammonium sulfate solution are added and the mixture is again centrifuged for 30 minutes. The residue is suspended in a small volume of 1% sodium carbonate solution, homogenized in a Potter-Elvehjem homogenizer, and diluted with the carbonate solution to a final protein concentration of 8 to 10 mg per ml. To every 100 ml of enzyme solution 15 ml of 60% aqueous *tert.*-butanol are added and the mixture kept at 38° for 30 minutes. The preparation is frozen, stored for 16 hours at —12°, and then thawed and centrifuged at high speed for 30 minutes.

Acetic acid precipitation and dialysis. To the clear supernatant solution, glacial acetic acid is added slowly until a pH of 4.2 is reached. The precipitate is centrifuged off, resuspended in 0.1 *M* potassium bicarbonate and very carefully homogenized in a Potter-Elvehjem homogenizer, to avoid foaming by the liberated CO_2. The pH is then adjusted to 7.0 by further addition of 0.1 *M* potassium bicarbonate. The suspension is centrifuged at high speed, and the residue dissolved in a solution of 1% sodium carbonate. The final protein concentration is adjusted to 8 to 10 mg per ml. The preparation is then dialyzed *vs.* 200 volumes of 0.02 *M* potassium bicarbonate. After 24 hours the dialyzed preparation is centrifuged at high speed and the supernatant solution is discarded.

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Second tert.*-butanol treatment and dialysis.* The residue is suspended in a small volume of 1% sodium carbonate solution, the protein concentration adjusted to 8 to 10 mg per ml and 15 vol. % of 60% *tert.*-butanol are added. The mixture is kept at room temperature for about 6 hours, frozen, and stored at —12° for 16 hours. The preparation is then thawed, centrifuged at high speed and the residue discarded. The supernatant fluid is dialyzed *vs.* 100 volumes of 0.01 *M* Tris versenate of pH 8.0 for 4 to 6 hours. The precipitated uricase is collected by high speed centrifugation and dissolved in a solution containing 1% sodium carbonate and 1% potassium bicarbonate.

At this point the enzyme is sufficiently pure (about 75%) for most studies. The remaining 25% of inactive protein may be removed by procedures such as the fractional ammonium sulfate precipitation described in the earlier paper[1].

Results of a typical purification are given in Table I.

TABLE I

PURIFICATION OF URICASE

	Units	*Specific activity*	*Purification*	*Recoveries in %*
Alkaline carbonate extract from 300 g PLMAP*	5720	0.17	—	—
Ammonium sulfate ppt.	5500	0.61	3.5	96
First *tert.*-butanol treatment	3380	1.22	7.0	59
Acetic acid ppt. bicarbonate solution	3280	25.70	145.0	55
Dialysis *vs.* bicarbonate	2500	37.0	210.0	44
Second *tert.*-butanol treatment	2460	66.5	370.0	43
Dialysis *vs.* Tris-versenate	2400	86.0	490.0	42

The alkaline carbonate extract is regarded as starting material. It may be added that the preparation of the mitochondria powder and the extraction of it with phosphate buffer already accomplishes about a 5 fold purification.

* Pig liver mitochondrial acetone powder.

Stability of uricase

If uricase prepared by this procedure is stored at —12° in a buffer containing 1% sodium carbonate and 1% potassium bicarbonate (pH 9.8) the enzyme does not lose any activity during the first 3 weeks, and during the following 4 weeks the activity decreases by only 10 to 15%.

When stored at 2° in 1 *M* carbonate, 1 *M* Tris, 0.5 *M* phosphate, or 0.1 *M* borate buffers all at pH 9.5, or in 0.1 *M*, 0.02 *M* or 0.002 *M* carbonate buffer at pH 9.8, the activity decreases 40 to 50% during the first 8 days. There is no significant difference between any of the above named buffers. Surprisingly enough, when uricase is stored at room temperature (28°), in the same buffers, there is also only a 50 to 60% decrease in activity during the first 8 days. Again the choice of the buffer does not appear to influence the stability of the enzyme.

Determination of the isoelectric point

A rough estimate of the isoelectric point of the enzyme was obtained from a study of its solubility at various pH values in 1 *M* bicarbonate buffer. For this purpose a 0.23% solution of highly purified uricase in this buffer was adjusted to appropriate

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hydrogen ion concentrations and its turbidity measured at 600 mμ. It was assumed that the point of maximum turbidity, *i.e.* of minimum solubility corresponded to the isoelectric point. A value of 6.3 was found by graphic interpolation of the data.

Enzymic oxidation of uric acid

The system used for the oxidation of uric acid in different buffers at various pH values was the following: 0.1 ml uricase ($\geq$ 1.2 mg protein per ml, specific activity $\geq$ 62), 0.3 ml buffer, 2.2 ml water and 0.36 ml uric acid solution (0.8%, adjusted to pH 8.5 with KOH). The buffers tested were 0.5 *M* Tris, 0.5 *M* phosphate and 0.1 *M* borate, all at pH 7.0, 8.5 and 10.0 respectively, a total of nine different conditions. The uric acid solution was added in small increments since higher concentrations ($\geq 5 \cdot 10^{-4}$ *M*) inhibit the enzyme[3]. The disappearance of each increment was followed at 293 mμ. After the oxidation was completed, the reaction mixtures were frozen and lyophilized. The dry material was taken up in a known amount of water and an aliquot was subjected to paper electrophoresis.

For the quantitative determination of the different compounds, the stained spots were carefully cut from the paper strips and titrated with standard sodium thiosulfate solution. The method was standardized against a stained spot containing a known amount of uric acid. When labeled uric acid was used, 2 aliquots were run simultaneously: one for thiosulfate titration and one for radioactivity determination. Good recoveries with, and good agreement between, both methods were observed in standardization experiments with known compounds and mixtures.

In large scale experiments the following proportions were used: 60 ml buffer, 30 ml water, and 120 units of uricase (specific activity $\geq$ 80.0). In some experiments catalase (0.5 ml of a 0.6% solution) and ethanol (2 ml of 95% ethanol, for protection of the oxidation products from catalase-mediated peroxidations) were added to this reaction mixture. Three different buffers were used in these experiments, *viz.* Tris, phosphate and carbonate, all 0.05 *M* and at pH 9.5. 120 units of purified uricase are sufficient to oxidize 100 mg of uric acid (in 20 ml of water, at pH 8.5, and added in increments) within 1 hour. The reaction mixture was stirred by a stream of oxygen and kept at a temperature of 38°. After complete oxidation of uric acid the pH was brought to 6.8 with 4% phosphoric acid. The precipitated enzyme was filtered and the filtrate lyophilized.

The dry residue was extracted with absolute ethanol in a Soxhlet extractor. The salt-free extract could be evaporated to a small volume from which the allantoin crystallized out at 0°, thus separating it from other compounds present in the extract. In an alternate procedure, used when only allantoin was to be determined, the residue was taken up in a minimum amount of water and the allantoin allowed to crystallize during one week at 2°, isolated, and recrystallized. To determine recoveries an amount of allantoin corresponding to the total uric acid added initially was taken through the whole oxidation and isolation procedure.

Oxidation products of uric acid in different buffer systems

The oxidation of uric acid in phosphate buffer at pH 7.0 resulted in a compound, which upon electrophoresis showed a mobility identical with that of allantoin. In addition to this single spot a distinct tailing towards the anode side could be noted at pH 8.5, which became still more pronounced when the oxidation of uric acid was

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performed at pH 10.0. These effects are shown in Fig. 1. R_F values on paper chromatography (see Table II) and electrophoretic patterns (see Fig. 2) were used to compare the oxidation product of uric acid obtained in phosphate buffer at pH 7.0 with several standard compounds, some of which were suggested as intermediates or end-products of enzymic uric acid oxidation[4–7,10]. From these data it can be seen that only allantoin and the oxidation product (either formed in the presence of catalase or isolated after alcohol treatment) behave similarly upon electrophoresis as well as upon paper chromatography. It may be added also that upon electrophoresis at higher pH values both compounds migrated approximately at the same rate, as a monoanion (such as urate at pH 7.0). Their mobility was 8.5 cm for the oxidation product and 9.5 cm for authentic allantoin (950 V, 50 min., in 0.1 *M* phosphate buffer of pH 10.0).

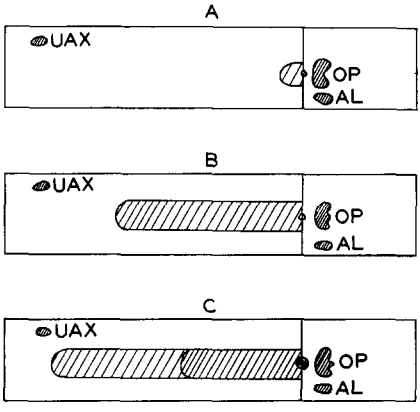

Fig. 1. Electrophoretic patterns of the oxidation products obtained by the oxidation of uric acid in phosphate at different pH values*. The amount of oxidation products applied to each strip corresponds to 0.15 mg of uric acid. AL = allantoin, UAX = uroxanic acid, OP = oxidation product. The electrophoresis was performed in 0.1 *M* phosphate buffer at pH 7.0 for 50 minutes and at 950 V. In A uric acid was oxidized in 0.1 *M* phosphate buffer at pH 7.0, in B at pH 8.5 and in C at pH 10.0.

TABLE II

R_F VALUES OF THE OXIDATION PRODUCT AND RELATED COMPOUNDS

	System A	*System B*
Alloxan	0.31	I 0.14 II 0.41
Uroxanic acid	0.17	0.17
Allantoin	0.36	0.20
Uric acid glycol	0.53	0.35
Uric acid	0.26	I 0.03 II 0.18
Oxidation product untreated	0.16	0.21
Oxidation product treated	0.36	0.20

The data were obtained by ascending paper chromatography. The solvent systems used were in system A, butanol-ethanol-H_2O (60–20–40-v/v), and in system B, butanol-glacial acetic acid-H_2O (80–20–20 v/v). After drying, the chromatograms were stained according to the chlorine-potassium iodide method. Oxidation product untreated = lyophilized reaction mixture. Oxidation product treated = treatment with Dowex-50 or extraction into methanol or ethanol of the lyophilized reaction mixture.

By applying the semi-quantitative method of titrating the different spots with sodium thiosulfate it became apparent that at pH 7.0 about 90%, and at pH 10.0

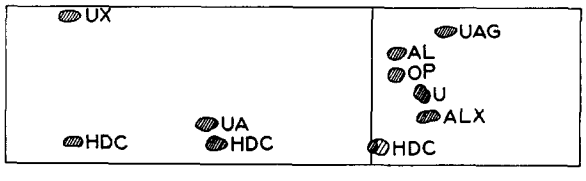

Fig. 2. Electrophoretic pattern of the oxidation product of uric acid and possibly related compounds*. UX = uroxanic acid, UAG = uric acid glycol, AL = allantoin, OP = oxidation product of uric acid, obtained in 0.1 *M* phosphate buffer at pH 7.0, U = urea, ALX = alloxan, UA = uric acid, HDC = hydroxy-acetylene diureine carboxylic acid. Of all these compounds 0.03 ml was applied to the paper strip. Electrophoresis was performed in 0.1 *M* phosphate buffer at pH 8.0 for 50 minutes and at 950 V.

* In all electrophoretic patterns migration towards the anode is indicated by movement of the spot from the starting line towards the left.

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70 to 80% of the total oxidation product was present in the allantoin-like spot. When the oxidation of uric acid was performed at pH 10.0 and the reaction mixture was brought to pH 6.8 immediately after completion of the oxidation, essentially the same electrophoretic pattern was established as in the oxidation at pH 7.0, suggesting that the formation of the by-products at the higher pH values must take place after the oxidation of uric acid to an allantoin-like compound. To support these findings labeled uric acid (2-^{14}C) was oxidized and the resulting oxidation products were applied to paper and counted after electrophoresis. Here again, at both pH 7.0 and 10.0, only one single compound was formed, as long as the oxidation mixture at pH 10.0 was brought to pH 6.8 immediately after completing the reaction. In Fig. 3 the distribution of label among the various oxidation products, formed in phosphate buffer at different pH values, is shown.

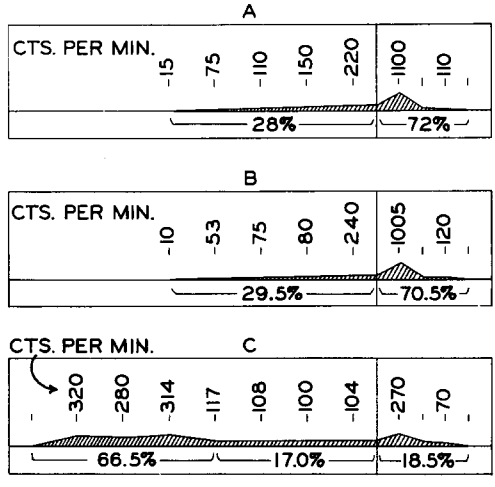

Fig. 3. Electrophoretic patterns of the oxidation product of labeled uric acid (2-^{14}C). The electrophoresis was performed in 0.1 *M* phosphate buffer at pH 7.0 for 50 minutes and at 950 V*. After drying, the paper strips were cut in pieces of adequate size and counted by means of a thin-window counter. The amount of material applied to the paper corresponded in each case to 1850 c.p.m. The recoveries after electrophoresis were in A 91%, in B 86% and in C 100%. In A the uric acid was oxidized in 0.1 *M* phosphate buffer at pH 10.0 and brought to pH 6.8 with 4% phosphoric acid immediately after the oxidation was completed. In B the uric acid was oxidized in 0.1 *M* phosphate buffer at pH 7.0 and in C at pH 10.0. All counts shown are corrected for background. The shaded area under the radioactive profile is proportioned to the amount of radioactivity in that area of the strip.

The oxidation of uric acid in Tris buffer yielded 3 different compounds. In the electrophoretic pattern one compound had the same mobility as allantoin whereas a second compound corresponded to uroxanic acid. The allantoin-like spot contributed 85% of the total oxidation products, and this proportion appeared to be relatively pH-independent. The quantitative determination of allantoin formed from uric acid showed values of 90 to 100% of the theoretical.

In borate buffer four different compounds could be detected after electrophoresis. Of these one spot had a location identical with uroxanic acid; a second and a third had mobilities similar to allantoin and urea respectively. Only about 30% of the uric acid oxidized could be recovered as allantoin.

From reaction mixtures containing 6-^{14}C uric acid not more than 7% of the initial activity could be recovered. When uric acid labeled with 2-^{14}C was oxidized in phosphate buffer 87.5 to 95.5% of the original activity could be recovered, in borate buffer 84.5 to 96.5%, and in Tris buffer 99 to 100%.

In quantitative large-scale experiments to determine the exact amount of the allantoin-like compound formed from uric acid in the presence of catalase 95 to

* In all electrophoretic patterns migration towards the anode is indicated by movement of the spot from the starting line towards the left.

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100% of the theoretical value were obtained. This compound was definitely identified as allantoin as follows: the equivalent weight found on electrometric titration was 167 (theoretical 158). The pK was 8.38 as compared to 8.30 for a highly purified allantoin sample. The decomposition points were: unknown, 230°–235°; authentic allantoin, 229°–235°; mixture of both, 229°–236°. Elementary analysis revealed C – 30.48 (theory 30.2), H – 4.01 (3.8) and N – 34.81 (35.6) % .

An attempt was made to determine the causes and conditions which lead to the formation of the small amounts of by-products during the oxidation of uric acid in phosphate buffer at pH 8.5 or higher. In two large-scale runs 1 g of uric acid was oxidized enzymically at pH 10.0, and the allantoin removed by crystallization from ethanol. The allantoin-free solution thus obtained had a yellow color. A spectrum of this solution is given in Fig. 4. On chromatography in the two solvents of Table II this yellow oxidation product showed three different spots all of which gave a positive reaction for a free ureido group with *p*-dimethyl-aminobenzaldehyde[16].

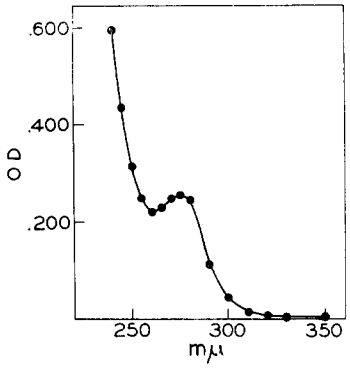

Fig. 4. Spectrum of a highly diluted sample of the yellow oxidation product in distilled water at pH 6.5.

In a control, 100 mg of allantoin were incubated in phosphate buffer, at pH 10.0, in an oxygen atmosphere with and without hydrogen peroxide but in the absence of uricase. After 24 hours a compound with absorption at 270 mμ was observed, suggesting that pure allantoin as well gives rise to similar products. No further experiments on these compounds, which are degradation products of allantoin and not intermediates in the enzymic reaction, were performed.

Oxidation of uric acid by uricase in the presence and absence of catalase

Great differences in the properties of the oxidation product were observed depending on whether the enzymic oxidation of uric acid was performed in the presence or absence of catalase.

In the absence of catalase an oxidation product was obtained which after two lyophilizations still showed a strongly positive *o*-phenylenediamine reaction[17], suggesting an α,β-diketone structure. The *p*-aminobenzaldehyde test for free ureido groups[16] was less pronounced and the rate of color development was slightly less than with a corresponding solution of allantoin. These and related findings led us to the conclusion that the primary oxidation product of uric acid was not identical with allantoin and might be an ureido-alloxan derivative[17]. However, if the oxidation product was treated with catalase in the presence of ethanol to guard against peroxidations, the *o*-phenylenediamine test became negative, whereas the *p*-aminobenzaldehyde test became more positive. Since the enzymic oxidation of one mole of uric acid leads to the formation of one mole of carbon dioxide, one mole of allantoin and one mole of hydrogen peroxide in the over-all stoichiometry, the positive *o*-phenylenediamine test even after two lyophilizations, combined with the disappearance of this reaction after catalase treatment has to be explained by complex formation between allantoin and hydrogen peroxide. The oxidation of uric acid in the presence of uricase and catalase yields allantoin which does not give an *o*-phenylenediamine reaction. It may be added that the treatment of the oxidation product with Dowex-50, or precipitation

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of the buffer phosphate ions as barium phosphate gel, or extraction of the oxidation product into methanol or ethanol was always accompanied by a loss of the *o*-phenylenediamine reaction. Upon chromatography the R_F values of these treated oxidation products were 0.36, identical with allantoin (see Table II). The untreated oxidation product on the other hand had an R_F of 0.16. Further evidence for complex formation of allantoin with hydrogen peroxide was obtained by paper electrophoresis. Uric acid was oxidized in the presence and absence of catalase, or catalase was added after complete oxidation of uric acid. The lyophilized samples were subjected to electrophoresis. The different mobilities of allantoin in the presence and absence of hydrogen peroxide can be seen in Fig. 5. It is evident that allantoin itself is formed only by the simultaneous action of uricase and catalase. Once formed, the complex of hydrogen peroxide and allantoin* is relatively stable and only slowly destroyed by a subsequent addition of catalase.

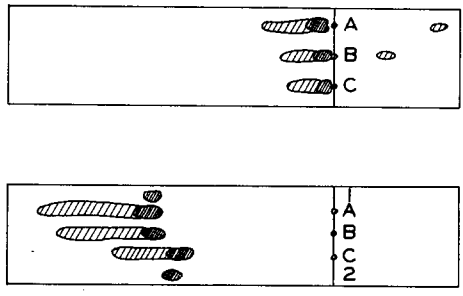

Fig. 5. Electrophoretic patterns of the oxidation product of uric acid in the absence and presence of catalase**. Each spot contained an equivalent of 0.15 mg of uric acid. All oxidations were performed in 0.05 *M* phosphate buffer at pH 10.0; neutralized to pH 6.5 with 4% phosphoric acid after the oxidation of uric acid was completed. A = catalase present during oxidation. B = catalase added after complete oxidation of uric acid. C = no catalase present. 1 = allantoin, 2 = allantoin incubated with hydrogen peroxide for 1 hour and 30 minutes at 38°. The electrophoresis was performed in 0.02 *M* phosphate buffer pH 10.6 at 950 V. In the upper pattern the electrophoresis was carried out for 15 minutes and in the lower one for 90 minutes.

Possible complex formation of uric acid and allantoin with buffer anions

Since the oxidation of uric acid in different buffer systems resulted in a variety of oxidation products, an attempt was made to determine whether these effects might be explained by the formation of complexes between buffer ions and uric acid or allantoin.

In these experiments uric acid or allantoin were incubated for 2 hours at room temperature with different buffers (0.05 *M* Tris, phosphate, borate or carbonate, or 0.05 *M* sodium chloride or distilled water both adjusted to the same pH as the buffer solutions) at different pH's. The solutions were then examined for spectral changes and changes in mobility on electrophoresis. Spectrophotometric measurements in the UV region revealed no changes for the allantoin-buffer systems compared with solutions of allantoin in distilled water at the same pH. In the case of uric acid, slight differences in the absorption maximum at 293 mμ and also at 260 mμ and in the range between 240 to 220 mμ could be noted in the uric acid-buffer spectra as compared to a uric acid solution in distilled water at the same pH (see Fig. 6). However, on electrophoresis only two buffer systems were found to affect the mobility of uric acid or allantoin. Uric acid incubated with borate buffer at pH 10.5 under the conditions indicated (Fig. 7) revealed a second slower-moving component in addition to the uric acid spot, whereas allantoin incubated with Tris buffer showed only a single

* The minor components migrating towards the cathode have not been identified. Their absence in the 90 minute pattern is probably due to their having migrated off the paper.

** In all electrophoretic patterns migration towards the anode is indicated by movement of the spot from the starting line towards the left.

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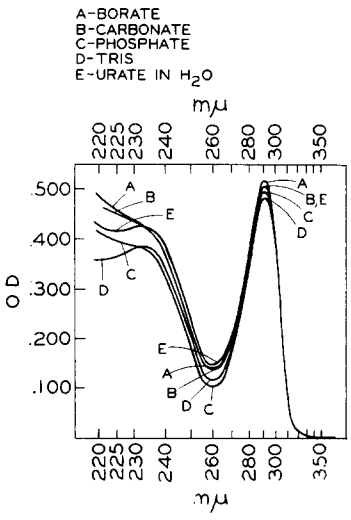
fast-moving component. It would seem, therefore, that uric acid may complex with borate anions and allantoin with Tris. Thus the possible intermediates which arise during the uricase-catalyzed oxidation of urate may form similar complexes. The variety of products formed by the action of this single enzyme of high purity may therefore be merely a reflection of the chemical environment acting upon one primary oxidation product.



← Fig. 6. Spectrum of uric acid in different buffers. Tris, borate, carbonate, and phosphate buffer all 0.05 *M*, pH 9.6. For comparison the spectrum of urate is shown in distilled water adjusted to pH 9.6 with 1 *N* KOH. The uric acid concentration was in all measurements $2.5 \cdot 10^{-5} M$, and all spectra were run in a recording spectrophotometer against a blank containing buffer alone. A, Borate; B, Carbonate; C, Phosphate; D, Tris; E, Urate in H_2O.

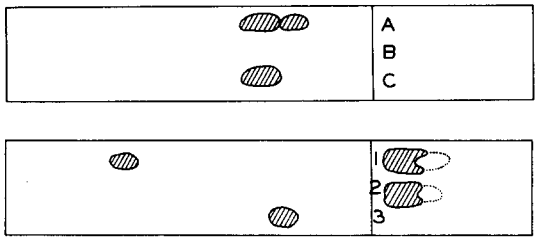


Fig. 7. Electrophoretic patterns of uric acid and allantoin in different buffers*. 0.01 *M* uric acid was incubated for 12 hours, and allantoin for 18 hours at room temperature in 0.05 *M* borate, phosphate or Tris buffer, in 0.05 *M* sodium chloride or in distilled water. The pH was 10.5 in each case. Electrophoresis was performed in 0.02 *M* phosphate buffer at pH 10.5 for 50 minutes and at 850 V. A = uric acid in borate buffer, B = borate buffer, and C = uric acid in distilled water, NaCl solution, Tris or phosphate buffers. 1 = allantoin in Tris buffer; 2 = Tris buffer alone; 3 = allantoin in phosphate, borate, sodium chloride, or distilled water.

Effect of –SH group inhibitors or activators upon uricase

ROBBINS *et al.*[11] observed an accelerating effect of cysteine upon the manometric rate of oxidation of uric acid by oxygen in the presence of uricase. In our experiments the previously described spectrophotometric assay system was used[1] in order to evaluate the possible involvement of –SH groups of uricase in the primary oxidation of uric acid. The rate studies showed that the initial rate of oxidation of uric acid was the same in the absence and presence of $2 \cdot 10^{-3} M$ cysteine, glutathione or BAL. Furthermore, the addition of *p*-chloromercuribenzoate in a concentration of $1 \cdot 10^{-4} M$ to the enzyme, even after prolonged preincubation (1 hour at 38°) did not influence the rate of disappearance of uric acid.

It seems therefore feasible to explain the accelerating effect of cysteine by way of side reactions, namely, the reduction of hydrogen peroxide or, as pointed out by VAN REEN *et al.*[12] as being due to an inhibition of catalase. If the accumulation of hydrogen peroxide leads to enzyme inhibition then its elimination should accelerate the over-all rate. In large scale oxidations it was indeed observed that addition of catalase after the rate had decreased did lead to a restoration of the original rapid rate even though the initial rate of uric acid disappearance was unaffected.

* In all electrophoretic patterns migration towards the anode is indicated by movement of the spot from the starting line towards the left.

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DISCUSSION

It has been shown that the oxidation of urate in the presence of highly purified uricase gives rise to nearly stoichiometric amounts of allantoin if the reaction is carried out in certain buffer systems. Therefore it is very likely that, as postulated by earlier investigators, the only enzymic step in the pathway from uric acid to allantoin, is the initial oxidation of uric acid to give the first unstable intermediate. Further, since the actual rate of disappearance of uric acid is the same in borate buffer as it is in phosphate buffer[3], although in the former case the intermediate is immediately destroyed, it is evident that the enzymic oxidation step is independent of the nature of the buffer. In borate buffer the product spectrum, therefore, is merely an expression of the fate of the first intermediate under the influence of the inorganic ions in the buffer system. A subsequent paper will deal in more detail with some of the properties of this and other intermediates, as well as with the kinetics of its formation and disappearance.

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SUMMARY

A new and improved method for the purification of uricase in good yield leading to a product of approximately 75% purity has been described. Using this enzyme, information was obtained about the nature and proportions of various products resulting from the enzymic oxidation of uric acid in the presence of uricase and catalase in various buffers and at various pH values. Allantoin is the main product in phosphate buffer and almost the only product in Tris buffer, while it constitutes only about 30% of the total in borate buffers.

In buffers other than borate, in the absence of catalase a product not identical with allantoin is formed in all cases. Since this product can, however, be quantitatively converted to allantoin by a variety of procedures it is probably an equimolar complex of allantoin and hydrogen peroxide.

Besides hydrogen peroxide, allantoin can also complex with certain buffer cations. Uric acid complexes with the anions of borate buffer. Complexes of this sort may profoundly influence the product picture obtained in the reaction catalyzed by uricase.

No evidence was found for the involvement of –SH groups of uricase in the oxidation of uric acid.

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