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Electrolytic Oxidation of Uric Acid: Products and Mechanism*

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ABSTRACT: Alloxan is the dominant product of the chemical oxidation of uric acid under strongly acid conditions; allantoin is the corresponding product for less acidic to alkaline conditions; separate reaction paths have generally been postulated to account for this difference. Investigation of the electrolytic oxidation of uric acid under moderately acidic conditions indicates the presence of a common path, which eventually diverges to produce both alloxan and allantoin in comparable amounts. To this extent, the mechanism of electrolytic oxidation bears a resemblance to the mechanism proposed for the enzymatic oxidation of uric acid. Uric acid gives a well-defined anodic voltammetric wave at a graphite electrode. When uric acid is electrolytically oxidized in dilute acetic acid at large graphite electrodes, 2.2 faradays are passed, and 0.25 mole CO_2, 0.25 mole of a precursor of allantoin, 0.75 mole urea, 0.3 mole parabanic acid, and 0.3 mole alloxan simultaneously appear per mole of uric acid oxidized. At any stage during electrolysis, the sum of the moles of allantoin precursor and urea equals the moles of uric acid oxidized. This material balance and the stability of the allantoin precursor indicate that the production of urea is associated with the pathway(s) that produce alloxan and parabanic acid.

These and other facts indicate a mechanism whereby uric acid is oxidized in a 2*e* process to a primary short-lived dicarbonium ion intermediate, which undergoes three simultaneous transformations: (1) hydrolysis to the allantoin precursor, (2) hydrolysis to alloxan and urea, and (3) further oxidation and hydrolysis leading to parabanic acid and urea. The nonintegral number of electrons involved are accounted for by the formation of parabanic acid. The primary oxidation intermediate ultimately produces both allantoin and alloxan, suggesting that this intermediate may be common to all uric acid oxidations and that the ultimate product heretofore considered to be typified by either allantoin or alloxan (but not both) is most likely controlled by experimental conditions. A major conclusion is that the electrochemical oxidation more nearly resembles the enzymatic oxidation than the chemical oxidation.

Uric acid gives a well-defined anodic voltammetric wave at the graphite electrode (Smith, 1962). Macroscale electrolysis is accompanied by complete disappearance of the characteristic ultraviolet absorption bands, indicating that oxidation must have occurred at the 4,5 double bond (see subsequent discussion).

In virtually all earlier chemical investigations of uric acid[1] oxidation, two fundamentally separate and distinct mechanisms are implied, one occurring under strongly acid conditions and the other under weakly acid, neutral, and alkaline conditions (Biltz and Schauder, 1923). The most characteristic product of the latter conditions is allantoin, but many other products have been isolated (Brandenberger, 1956); however, there is no report of alloxan ever having been identified. Similarly, strongly acid conditions produce alloxan as the most characteristic oxidation product with no apparent involvement of species that could lead to products encountered when allantoin is the characteristic product. The present investigation indicates a possible common primary oxidation intermediate that can subsequently produce either allantoin or alloxan, analogous to the mechanism suggested (Canellakis and Cohen, 1955; Paul and Avi-Dor,

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[1] To facilitate reading of the text, structural formulas of the principal compounds discussed are given in Figures 1 and 5. The formulas used are commonly accepted ones; allowance must be made for alteration owing to keto-enol and acid-base equilibria.

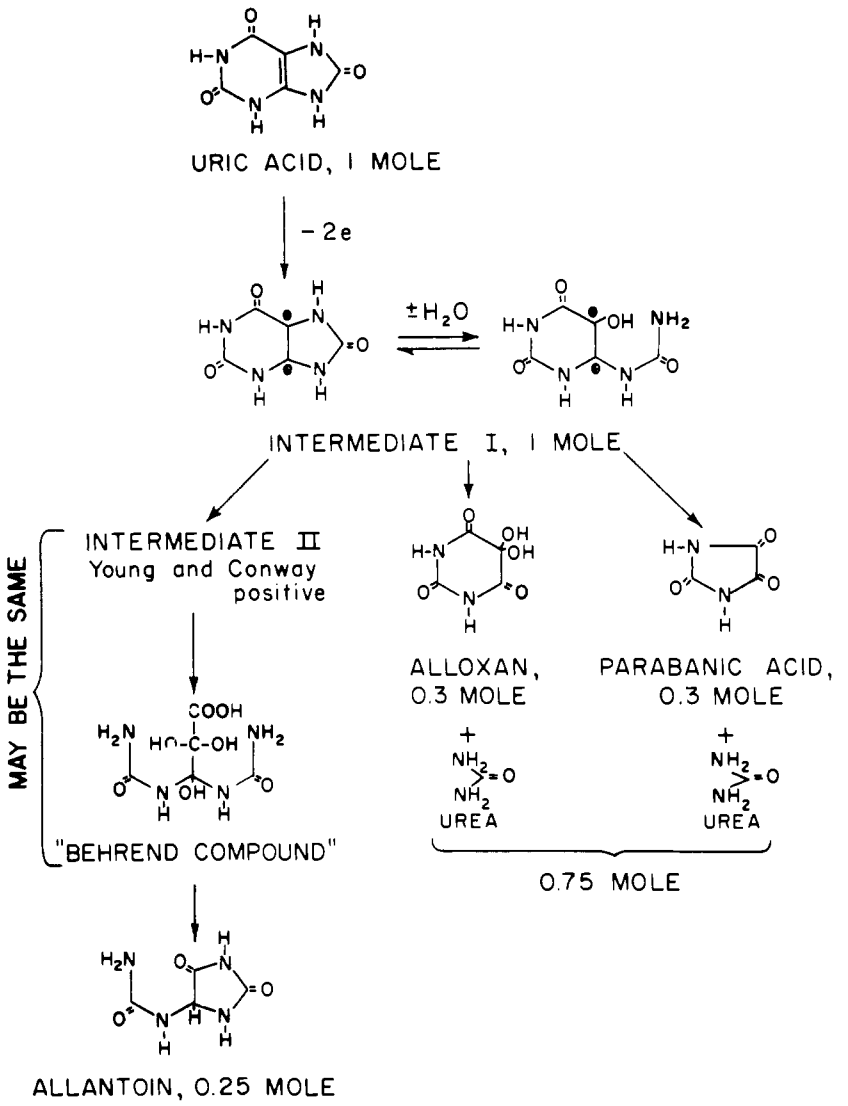

FIGURE 1: Proposed pathways for electrolytic oxidation of uric acid in acetic acid solution and rapid transformations

1954; Soberon and Cohen, 1963) for the enzymatic oxidation of uric acid.

Investigation of the electrolytic oxidation of purines, prior to the present study, is limited to a single report (Fichter and Kern, 1926), which was done at a time when there was little appreciation of the importance of controlled potential electrolysis, so that considerable oxygen generation may have occurred concomitantly with anodic oxidation. Consequently, this prior work has little or no direct bearing on the present investigation, even though its results are suggestive in retrospect.

The experimental evidence, subsequently described in detail, points to a mechanism whereby uric acid in acetic acid solution is electrochemically oxidized in a 2*e* process to some primary intermediate whose exact nature can only be surmised. This intermediate can react further by three different paths (Figure 1): (1) transformation, most likely by hydrolysis, to the allantoin precursor; (2) hydrolysis to alloxan and urea; and (3) further electrolytic oxidation and hydrolysis leading ultimately to parabanic acid and urea. The relative amounts of the three products, allantoin precursor, alloxan, and parabanic acid, are most likely controlled by the relative rates of the three processes.

Results and Discussion

Although the slight solubility of uric acid in all but strongly basic media seemed at first to be a serious obstacle, it was found that suspensions could be electrolyzed uneventfully if given sufficient time. Acetic acid was used as background electrolyte because of the favorable anodic potential range it affords (Smith, 1962); in addition, its removal by lyophilization (freeze-drying) is practical.

Voltammetric and Coulometric Behavior. Uric acid gives a well-defined anodic wave with a peak current at the stationary graphite electrode in 1 M acetic acid

(Figure 2). The half-peak potential of +0.58 v indicates that uric acid is fairly readily oxidizable. The ratio of peak current to concentration at a 6-mm electrode, 28.9 μa/mM, suggests by comparison to comparable data on hydroquinone and ferrocyanide (Smith, 1962) the involvement of two electrons per molecule of uric acid. The wave shifts to less positive values with increasing *p*H; $E_{p/2}$ is 0.45 v at *p*H 3.7 and 0.33 at *p*H 5.7 in acetate buffer (Smith and Elving, 1962). The shift in $E_{p/2}$ of 0.06 v per *p*H unit suggests the participation of the same number of hydrogen ions in the electrode reaction as electrons, viz., two per molecule of uric acid.

Coulometry at an anode potential of +0.8 v showed that *ca.* 2.2 electrons are removed per molecule of uric acid; the oxidation of some of the uric acid to parabanic acid accounts for the nonintegral value. The detailed examination of the composition of the solution produced on electrolytically oxidizing uric acid at a fixed anode potential, as well as the changes in composition with time observed during the course of electrolysis, allowed the identification and quantitative determination of both the ultimate products and the probable intermediates.

Lyophilization of Oxidation Products. Lyophilization of the total reaction product solution from the electrolytic oxidation of uric acid results in the complete removal of water and acetic acid, and production of a fluffy white powder with a faint pink cast, which is very hydroscopic and extremely water soluble, to give a neutral solution. When this material is heated in a melting-point capillary, it swells and froths somewhat at 115–130°, and fairly abruptly turns to an intense red violet. The red-violet material has spectral properties in acid, base, and neutral solution identical to murexide, which is usually made by heating together alloxan or alloxantin and a source of ammonia (Whitmore, 1951), e.g., urea.

Allantoin was isolated from the total oxidation solids by reconstituting the lyophilized material in a very small volume of water and cooling to near 0°. Identification was accomplished on the basis of melting point, mixture melting point with authentic allantoin, and comparison of infrared spectra. The maximum yield obtained was 11% based on uric acid.

Production of Carbon Dioxide. Production of allantoin and certain other compounds such as parabanic acid from uric acid requires the loss of CO_2. The 0.20–0.25 mole of CO_2 isolated per mole of uric acid electrolyzed can be accounted for by the production of parabanic acid from intermediate I (Figure 2). The other oxidation products can thus be considered intermediates that have not lost CO_2. Such a hypothesis is tenable since the "Behrend compound" (Figure 1) is widely accepted as a reasonably stable intermediate.

Paper Chromatography of Oxidation Products. The most generally useful detection agents were bromcresol green (positive reaction indicates the presence of an acidic material of pK_a below *ca.* 6) and Ehrlich's reagent (positive test indicates urea or a compound having a free ureido grouping).

The presence of urea in the oxidation product solution was unmistakable in any solvent system and technique used; its R_F values in seven different solvent systems were identical to those of authentic urea. Similarly, a spot acidic to bromcresol green was repeatedly observed.

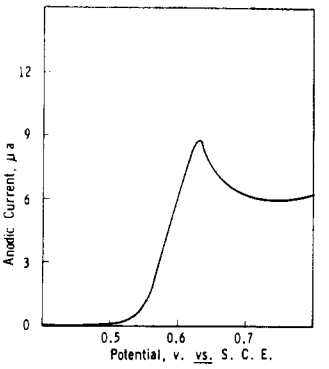

FIGURE 2: Voltammogram of 0.3 mM uric acid in 1 M HOAc at the stationary graphite electrode.

Since alloxanic acid was never detected on chromatograms run using acidic systems, its identification when using systems containing NH_3 was correctly attributed to decomposition caused by NH_3 (Sarasohn, 1959). In this sense, it is significant evidence for the presence of alloxan, whose rearrangement to alloxanic acid is base catalyzed.

Ion-Exchange Behavior of Oxidation Products. Recognition of urea and acidic material in the oxidation product solution suggested passage of the latter over an anion-exchange resin (OH^- form) to isolate urea. An appropriate volume of oxidation product solution was slowly passed through a column of Dowex 2-X8, followed by a thorough water wash. During the several hours required, a very small amount of free alkali bled from the column. The total effluent was lyophilized; the infrared spectrum of the solid residue was identical to that of USP urea, indicating that urea was the only neutral or basic species present in the oxidation product solution. After correction for the free alkali present, the yield was 62 ± 3% based on 1 mole of urea generated per mole of uric acid. Since, formally, uric acid can produce 1 or 2 moles of urea, it cannot be concluded from such an experiment alone whether the yield was 62% or 31%.

Since paper chromatography also showed that only urea was present in the column effluent, all other materials in the oxidation-product solution must be either acidic or capable of being transformed into acidic materials on the basic column. This view is consistent with the final conclusions as to the nature of the oxidation products.

The total acidic components retained by the anion-resin column were displaced with 1 M HCl. The strippings, after lyophilization, gave a small amount of water-insoluble material, which was identified as oxaluric acid by melting point and infrared absorption.

Quantitative Characterization of the Course of Electrolysis of Uric Acid. Paper chromatographic and ion-exchange evidence clearly indicate that, in addition to the urea, allantoin, and oxaluric acid, which have been

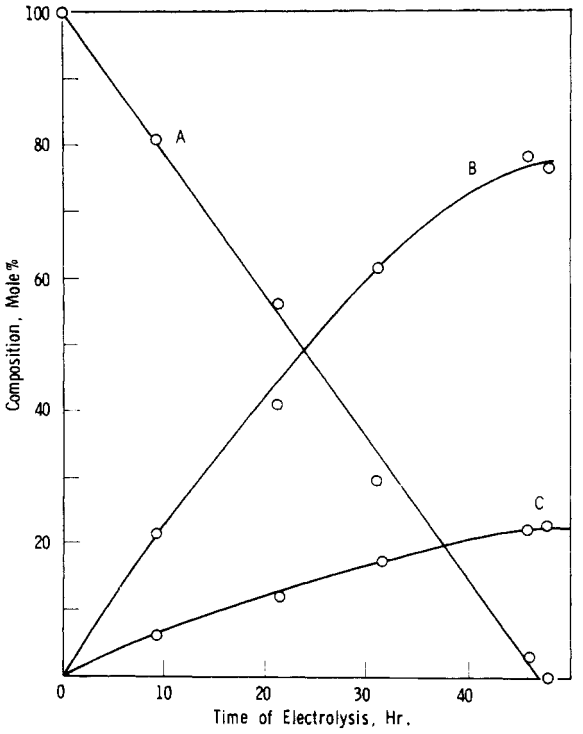

FIGURE 3: Change in composition of a uric acid suspension in 1 M HOAc solution during electrolysis. (A) Unoxidized uric acid; (B) urea produced; (C) allantoin precursor produced.

isolated from the electrolytic oxidation of uric acid in sufficient purity and quantity to allow unequivocal identification, additional species exist in the product solution. For this reason, as well as to determine at what stage of the total process the various species appear, the course of an electrolysis as a function of time was followed analytically on the basis of assays for (a) unreacted uric acid, (b) "allantoin precursor" (cf. subsequent discussion) in terms of allantoin, (c) urea, and (d) electroreducible material as measured polarographically. This was considered the most feasible approach because the appearance of several products indicated that the primary oxidation intermediate must be a relatively short-lived species, whose identity could best be postulated on the basis of the nature and distribution of the ultimate products.

The results (not including polarographic data) of thus monitoring the electrolysis of 2 mmoles of uric acid, suspended in 100 ml of 1 M HOAc (Figure 3), show that the sum of the moles of allantoin precursor and of urea present at any time during the electrolysis equals the number of moles of uric acid oxidized. Thus, allantoin precursor and urea account for all of the uric acid, if one assumes that that portion of the uric acid which does not produce allantoin precursor produces only 1 mole of urea, and that none of the urea arises from decomposition of allantoin precursor.

Since the allantoin precursor, as measured by the Young and Conway (1942) assay, is stable for weeks in HOAc solution, none of the urea can arise by its decomposition. The major remaining problem was then to account for the uric acid which is not transformed to the allantoin precursor; i.e., since the urea measured accounted for half of the formally possible urea or available nitrogen, the remaining nitrogen still had to be accounted for. The answer resulted from identification and determination of the species in the oxidation product solution, which were electroreducible at the dropping mercury electrode.

Preliminary studies established that electrochemical oxidation of uric acid produced electroactive material, which gave at least one well-defined cathodic wave. Polarograms of aliquots taken during the course of electrolysis show the development of two *p*H-dependent cathodic waves of $E_{1/2}$ at *ca.* −0.7 and −1.5 v, the second of which was obscured by the hydrogen discharge below *ca.* *p*H 4.6 (Table I; Figure 4).

TABLE 1: Variation of $E_{1/2}$ with *p*H for Waves I and II of Electrolytic Oxidation Products of Uric Acid.

	$E_{1/2}$ (v)	
*p*H	Wave I	Wave II
1.0[a]	−0.57	Obscured by H_2 discharge
2.1[b]	−0.66	Obscured by H_2 discharge
3.3[b]	−0.72	Obscured by H_2 discharge
3.9[b]	−0.74	Obscured by H_2 discharge
4.4[b]	−0.79	Obscured by H_2 discharge
5.1[b]	−0.82	−1.50
6.4[b]	−0.89	−1.53
5.4[c]	−0.84	−1.43

[a] Mixed sulfate-acetate buffer. [b] Mixed McIlvaine-acetate buffer. [c] Mixed ammonia-acetate buffer.

As the electrolysis proceeds, wave I, while unmistakably clear, shows some irregularities, very likely owing to the low product concentration and the high buffer phosphate concentration (Struck and Elving, 1964a); however, it is fairly well defined by the end of the electrolysis. The material accounting for wave I was identified polarographically as parabanic acid by comparing the two in acetate medium where well-defined waves are obtained, e.g., identity of both $E_{1/2}$ and wave slope (Table II). The parabanic acid concentration in the oxidation-product solution was equivalent to 0.3 mole per mole of uric acid electrolyzed. Since equimolar amounts of parabanic acid and urea would form from oxidation of uric acid, this accounts for 0.3 mole of the 0.75 mole of urea measured.

Parabanic acid was also identified in the oxidation products by paper chromatography and by its instability at relatively high *p*H. In *p*H 6.4 mixed phosphate-acetate

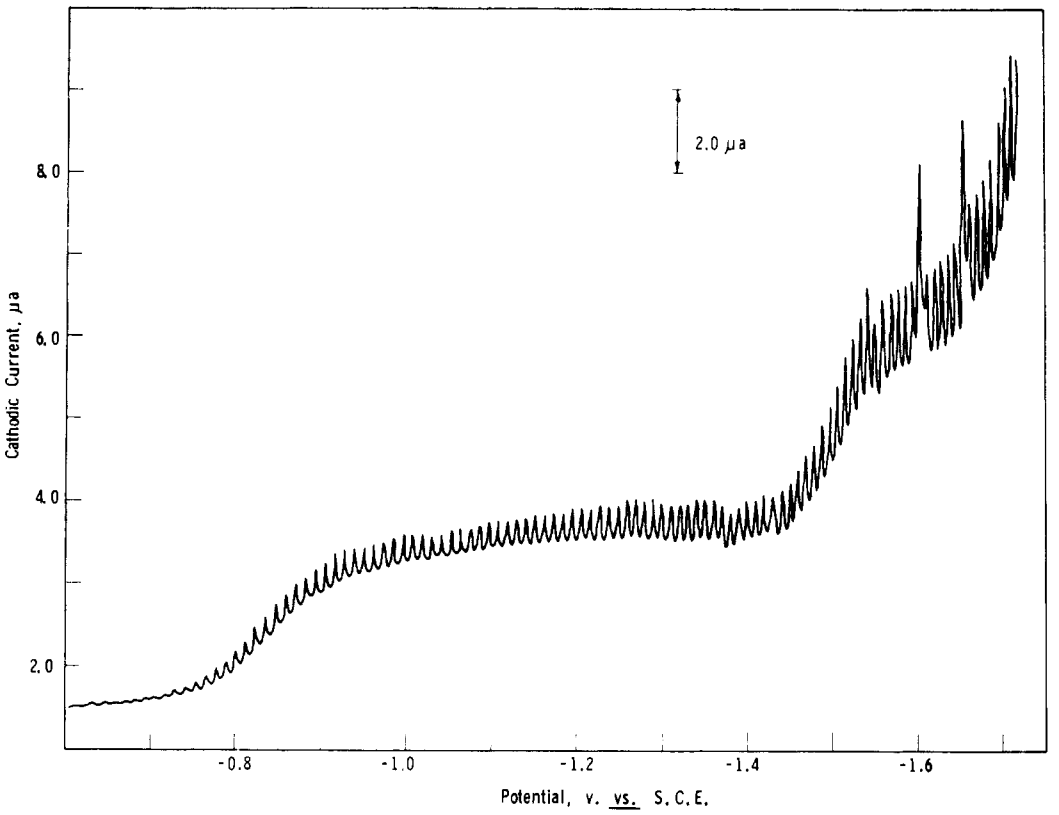

FIGURE 4: Polarogram of solution of electrolytic oxidation products of uric acid at *p*H 5.1. (McIlvaine buffer containing some acetate.) Concentration, 2.0 mM in terms of initial uric acid.

TABLE II: Comparison of Polarographic Behavior of Parabanic Acid and of the Oxidation Product of Uric Acid in Acetate Buffer.

	$E_{1/2}$ (v)		Slope of Wave[a]	
*p*H	Reaction Product	Parabanic Acid	Reaction Product	Parabanic Acid
4.0	−0.75	−0.75	1.09	1.08
4.8	−0.77	−0.78	1.03	1.02
5.6	−0.81	−0.84	1.06	0.97

[a] Slope = $0.056/(E_{1/4} - E_{3/4})$.

buffer, wave I disappears upon standing overnight at room temperature; this is consistent with the well-known base-catalyzed hydrolysis of parabanic to oxaluric acid (Andrews and Sell, 1955). The disappearance of wave II under these conditions is discussed in the following paragraphs in connection with alloxan.

While wave I of alloxan ($E_{1/2} = -0.1$ v) occurs at an unusual potential, which is therefore useful for identification, its magnitude is unusually small (*ca.* 0.1 of "normal" for a 2*e* process) (Struck and Elving, 1964b); therefore this wave can easily be overlooked in dilute solution. Reexamination of the product solution at a higher concentration showed that a wave having the same $E_{1/2}$ as wave I of alloxan was indeed present. Because of overlap with the hydrogen discharge, wave II of alloxan can be observed only above *p*H 4.6; above *p*H 6, alloxan rapidly rearranges to polarographically nonreducible alloxanic acid (Struck and Elving, 1964b). Thus wave II of the oxidation-product solution was considered to be alloxan wave II.

The concentration of alloxan in the oxidation product solution, determined from the limiting current of wave II, was equivalent to 0.3 mole of alloxan per mole of electrolyzed uric acid; this accounts for another 0.3 mole of urea. Additional evidence for the presence of alloxan is afforded by treating the oxidation product solution with cysteine, which in acid solution reduces alloxan to dialuric acid, generating the characteristic absorption maximum of the latter at 270 mμ.

Material Balance. Estimation of all definitively identified products from the electrolysis of 1 mole of uric acid (Figure 1) gives the following summation: allantoin precursor, 0.25 mole; urea, 0.75 mole; parabanic acid, 0.3 mole; alloxan, 0.3 mole. Thus, in terms of allantoin precursor and urea, 100% of the uric acid is accounted for; in terms of the products other than urea, 85% is accounted for.

Within the framework of the assumptions and imponderables involved in the analytical data, the present

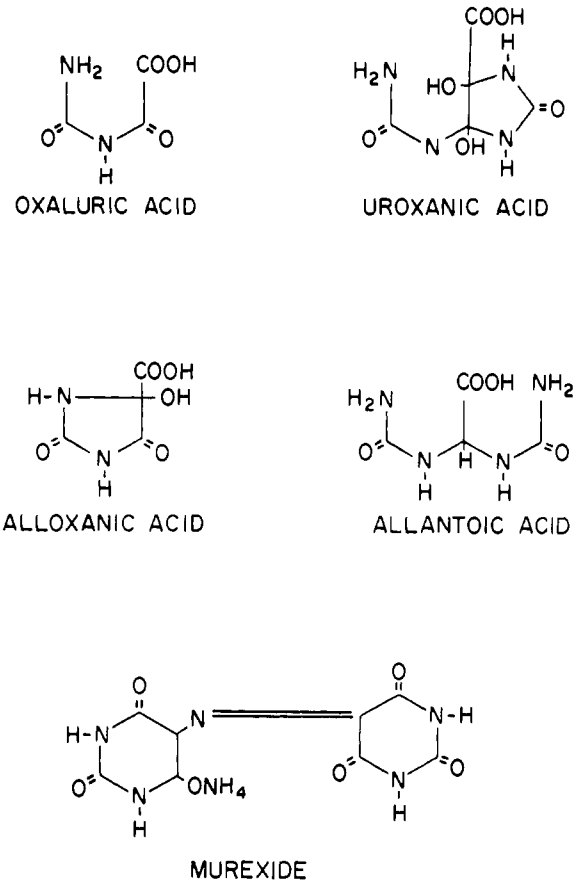

FIGURE 5: Structural formulas of certain compounds discussed in the text. See also Figure 1 and footnote 1.

summation is considered to account for all of the uric acid electrolytically oxidized; a more detailed quantitative description of the oxidation is probably not justified, e.g., the molar response of the allantoin precursor in the Young and Conway (1942) assay may not be identical to that of allantoin itself.

Rationalization of Behavior on Ion-Exchange Columns. The behavior of the uric acid oxidation product solution on ion-exchange resins can be rationalized on the basis of the identified products (Figure 1). When the product solution is passed over an OH^--form strong-base anion resin, HOAc should be retained. At the high column *p*H, alloxan should be immediately transformed to alloxanic acid, which would be retained. Parabanic acid should be both retained and rapidly hydrolyzed to oxaluric acid, which would also be retained. The allantoin precursor would most likely be converted to uroxanic acid, which would also be retained. Thus, only urea should appear in the column effluent.

Appearance of oxaluric acid in strong acid column strippings indicates the possible existence of species of a higher oxidation state than allantoin or alloxan. The presence of parabanic acid accounts for the appearance of oxaluric acid and for the involvement of more than two but fewer than three electrons in the electrolytic oxidation.

Nature and Properties of Reaction Intermediates

Nature and Properties of Intermediate I. The following evidence indicates that the primary oxidation intermediate (intermediate I, Figure 1) must be short-lived. When aliquots of an oxidized solution are removed, diluted, and examined polarographically, both alloxan and parabanic acid are found. Since transformation to these species must therefore be rapid, transformation to the allantoin precursor must be comparably rapid in order for the latter species to appear on a relatively comparable basis. Such rapid conversion of a primary intermediate is consistent with the observations of Paul and Avi-Dor (1954), who studied the oxidation of uric acid with horseradish peroxidase. Parabanic acid is considered to arise from further oxidation of intermediate I rather than from oxidation of alloxan, because alloxan is not oxidized at the graphite electrode under the experimental conditions used in the preparative electrolysis.

The ultraviolet spectra of pyrimidines and purines are due mainly to the —C=C—C=N— or —C=C—C=O chromophore of the pyrimidine ring, depending on the particular substituents (Bergman and Dikstein, 1955; Cavalieri and Bendich, 1950; Cavalieri *et al.*, 1948). For uric acid, the latter chromophore would involve the 4,5,6 —C=C—C=O grouping. Destruction of this chromophore by electrolytic oxidation (only end absorption is observed in the product solution) must therefore involve the 4,5 double bond. Removal of two electrons from uric acid in such a way that the product can give ultimately both alloxan and allantoin also requires that the pyrimidine ring be left intact. If it can be assumed by analogy to chemical studies (Cavalieri and Brown, 1948) that allantoin arises via a symmetrical intermediate but that alloxan does not, then intermediate I must be unsymmetrical in order to produce alloxan. On this basis, the product of the primary oxidation step can be written as a dicarbonium ion (Figure 1), whose immediate hydrolysis to a species such as that shown in Figure 1 is formally allowable, but no further transformations are consistent with the experimental evidence. The alternative hydrolysis at the 4-carbon is not likely, because Brandenberger (1956) has shown that, in the formation of allantoin, bonds are never broken between the 4-carbon and the 3- and 9-nitrogens.

Evidence for Intermediate II. If the Young and Conway (1942) assay were measuring free allantoin in the system studied, there would be little need to postulate the existence of intermediate II, other than by analogy to chemical studies. However, a considerable body of evidence in the present investigation indicates that free allantoin does not exist in the oxidation product solution, and that the Young and Conway assay is measuring an allantoin precursor.

The sequential appearance of allantoin on repeated lyophilizations strongly suggests its formation during the lyophilization process. The CO_2 directly evolved during electrolysis (0.25 mole per mole of uric acid) nicely accounts for the measured production of parabanic acid; since formation of allantoin during electrolysis would have released more CO_2, this also indicates that free allantoin does not exist in the oxidation product

solution. The concept of an allantoin precursor also gains considerable support from analogy to the alkaline permanganate oxidation of uric acid (Biltz and Schiemann, 1926), in which the present studies have demonstrated the existence of an allantoin precursor (see under Experimental).

The existence of an uncharacterized material common to both permanganate and electrolytic oxidation products of uric acid is also suggested by the precipitation from both solutions of insoluble material with Ag(I). Schuler and Reindel (1932, 1933) based their identification of the "Behrend compound" on analysis of such silver precipitates from permanganate oxidations. The following materials do *not* precipitate with Ag(I) under the experimental conditions used in the present study: alloxan, alloxanic acid, allantoin, allantoic acid, and parabanic acid.

Uniqueness of Intermediate I. As has been indicated, studies of the chemical oxidation of uric acid have implied the existence of mutually exclusive mechanisms leading either to allantoin or to alloxan. The present investigation has demonstrated the plausibility of postulating an intermediate, which can undergo transformation to either alloxan or allantoin. This suggests that oxidation by any method may produce a common intermediate corresponding to intermediate I in Figure 1, and that its subsequent transformations are determined by experimental conditions.

The possibility of such a general mechanism is also suggested by the results of several studies on the enzymatic oxidation of uric acid. The data of Canellakis and Cohen (1955) on the oxidation of uric acid by uricase can be interpreted in such a way as to require an intermediate common to both alloxan and allantoin generation. In addition, Paul and Avi-Dor (1954) and Soberon and Cohen (1963) present evidence and postulate mechanisms requiring a primary intermediate which can produce either alloxan or allantoin when uric acid is oxidized with various peroxidases. The latter authors, in fact, postulate a mechanism essentially the same as that proposed in the present study.

Experimental

Chemicals. Uric acid, murexide, alloxan monohydrate, allantoin, *p*-dimethylaminobenzaldehyde, phenylhydrazine hydrochloride, 2,3-butanedione monoxime, and 2-butanone were obtained from Eastman; 1-cysteine from Nutritional Biochemicals; 1-butanol, and oxalic, acetic, and formic acids from Mallinckrodt; allantoic acid from K & K Laboratories; parabanic acid from Mann Research Laboratories; urea and Superoxol from Merck, and Dowex 2-X8 from Dow. Potassium alloxanate was prepared following Behrend and Zieger (1915) and uroxanic acid following Brandenberger (1959); details are available (Struck, 1963).

Oxaluric acid was prepared following the suggestion of Biltz and Schauder (1923): 3 g alloxan monohydrate, dissolved in 10 ml 30% hydrogen peroxide, was kept at 45–50° for several hours (higher temperature causes overoxidation); white crystals precipitated. The entire reaction mixture was then kept overnight at 5°. The crystals were filtered, washed with several portions of cold water (5°), then with 95% EtOH, and dried at 100°. Yield: 1.64 g; mp 210° (decomp), reported (Andrews and Sell, 1955) 208–210° (decomp). Infrared spectrum corresponded in all prominent bands to that published (Chouteau, 1953).

The following development solvent systems were used in paper chromatography (ratios indicated in parts by volume; NH_3 and HCl = concentrated aqueous solution): 1-BuOH-HOAc-H_2O (12:3:5); 1-BuOH-HN_3-H_2O (86:5:9); EtOH-NH_3-H_2O (18:1:1); 1-BuOH-H_2O (43:7); 2-PrOH-HCl-H_2O (195:50:55); 2-butanone-acetone-H_2O (40:2:1:6). The standard detection reagents used are described in the references indicated: Ehrlich's reagent (Smith, 1960), bromcresol green (Smith, 1960), and bromphenol blue (Reio, 1960).

Anion-exchange resins (Dowex 2-X8) were converted to the OH^- form from the Cl^- form in which they were received by passing 1 M NaOH slowly through a column of the resin until the effluent no longer gave a test for Cl^-. The column was then washed with distilled water until the effluent was neutral.

Buffer solutions, prepared from reagent grade chemicals, had an ionic strength of 0.5 M. Acetate buffers contained only HOAc and NaOAc. McIlvaine buffers were adjusted to constant ionic strength with KCl (Elving *et al.*, 1956). Nitrogen used for deoxygenation was purified and subsequently equilibrated with water by passing it successively through vanadous chloride solution, distilled water containing marble chips to neutralize HCl, copper wool maintained at 450–500°, and water containing marble chips.

Apparatus. Polarograms were recorded on a Leeds & Northrup Type E Electro-Chemograph, using damping equivalent to galvanometer performance and a water-jacketed H-cell (Komyathy *et al.*, 1952) maintained at 25.0 ±0.1°, which contained a saturated calomel reference electrode in one leg. All potentials are referred to the saturated calomel electrode. Since the cell resistance, measured with a General Radio Type 650A impedance bridge, was always below 300 ohms, half-wave potentials were not corrected for potential drop due to cell resistance. The dropping mercury electrode capillary (marine barometer tubing) had normal m and t values.

The graphite electrodes were United Carbon Products Co. 6-mm diameter spectrographic rods (density U-1), impregnated with ceresin wax (Morris and Schempf, 1959), and coated with Krylon clear lacquer. For voltammetry, a new surface was exposed for each run by removing a very thin layer of graphite from one end by means of a small lathe equipped with an end-cutting tool. For macroscale electrolyses, 76-mm lengths of impregnated graphite rods were carefully scraped with a knife on all surfaces in order to have the maximum available area.

The electrolysis cell was a 150-ml spoutless beaker equipped with a gas-tight rubber stopper carrying a large gold foil working cathode (32 cm^2), three 6-mm impregnated graphite rods connected together as a working anode, and a Beckman fiber-type saturated calomel

electrode. Stirring was accomplished by a magnetic stirrer, using a Teflon-coated stirring bar. A Fisher controlled-potential Electro-Analyzer automatically controlled the anode potential as compared with the saturated calomel electrode. The latter potential was checked with a Rubicon Type B-1 potentiometer.

The silver coulometer used consisted of a tantalum crucible cathode, containing 1 M $AgNO_3$, into which a porous clay finger, which functioned as a diaphragm, dipped. A No. 10 silver wire anode was immersed in $AgNO_3$ solution inside the clay finger.

The *p*H was measured with a Leeds & Northrup No. 7664 *p*H meter. Infrared spectra (Nujol mulls) were obtained with a Perkin-Elmer Model 137B spectrophotometer, and ultraviolet and visible spectra with a Beckmann Model DU spectrophotometer, using 1-cm glass-stoppered quartz cells. Melting points were obtained with a Fisher-Johns apparatus. Lyophilization was accomplished using a Cenco Hyvac-7 vacuum pump connected by 25-mm diameter glass tubing through a trap cooled with solid carbon dioxide–2-propanol to the lyophilization vessel (a round-bottom 250- to 1000-ml Pyrex flask), on whose wall solutions were shell-frozen.

For horizontal (circular) paper chromatography, an 18.5-cm circle of Whatman No. 1 filter paper was supported on a 150- × 15-mm Petri dish containing the solvent system used. A wick attached to the center of the paper dipped into the solvent and served to feed solvent. During development, the paper was covered with a second Petri dish. Ascending paper chromatography was performed in 15- × 46-cm battery jars covered with glass squares and containing stainless steel frames, which supported two strips of Whatman No. 1 filter paper (11 × 41 cm) in each jar. Developed chromatograms were dried in a stream of heated air obtained from a Model HG-201 Heat Gun (Master Appliance Corp.). The ultraviolet lamp used to inspect chromatograms was a Model L Mineralight (Ultraviolet Products, Inc.).

Polarographic Procedure. The *p*H of test solutions, which generally employed buffer systems as supporting electrolytes, was checked prior to polarography. About 10 ml was transferred to the sample leg of the H-cell, deaerated with purified nitrogen for not less than 8 minutes, and then polarographed. No maximum suppressors were used. A portion of the buffer solution was treated identically to obtain a background curve. Values of $E_{1/2}$ and i_d or i_l were determined graphically, utilizing the average of the recorder trace.

Voltammetric Procedure. Solutions were treated as for polarography, except that a nitrogen sweep was not employed when the potentials scanned were more positive than the cathodic oxygen waves. Just prior to use, the freshly lathed graphite road was dipped for 1 minute into a 0.002% Triton X-100 solution and then rinsed with a portion of the test solution; this procedure ensured prompt wetting and rapid equilibration of the electrode.

Macroscale Electrolysis Procedure. Uric acid was oxidized as a suspension in 1 M HOAc with the anode potential generally controlled at *ca.* 1.0 v; in the coulometric runs, the anode potential was maintained at 0.8 v in order to minimize background electrolysis. For most runs, 336 mg (2 mmoles) of uric acid was suspended in 100 ml of 1 M HOAc. Exhaustive electrolysis of this quantity required about 48 hours. Completion of electrolysis was indicated by complete dissolution of all suspended material and by the replacement of the characteristic ultraviolet absorption of uric acid (ϵ_{max} = 230 and 283 in acid) by end-absorption completely devoid of maxima. At the end of an electrolysis, a dilution of 1:500 in 2 M H_2SO_4 gave an absorbance of 1.0 at 224 mμ with no other features.

Coulometric Procedure. Prior to use, the clean, dry coulometer crucible was tared; the coulometer was assembled, filled with electrolyte, and connected in series with the electrolysis cell. At the end of electrolysis, electrolyte was removed by suction (medicine dropper), and the crucible was washed with several small portions of water followed by 95% EtOH and finally by acetone. After brief drying at 80°, the crucible plus deposit was weighed. In a typical experiment, exhaustive electrolysis of 0.998 mmole uric acid deposited 237.4 mg silver, equivalent to 2.21 meq; background electrolysis at the same anode potential of 0.8 v for the same length of time deposited no silver.

Paper Chromatographic Procedures. Aqueous solutions of reference compounds were generally prepared at 1 mg/ml concentration; slightly soluble materials, e.g., oxaluric acid, were used as saturated solutions. Alloxan was prepared in dilute HOAc for stabilization; potassium alloxanate was dissolved in dilute HOAc to obtain the protonated species.

Solutions of standards and unknowns were applied via a small loop of No. 26 platinum wire, which deposited about 5 μl on the paper. When it was desired to load spots heavily, the applied solution spot was dried between additions. All spots were dried prior to development. The platinum loop was cleaned between samples by heating it strongly in a Meker burner flame.

For circular chromatography, standards and unknowns were spotted on the perimeter of a 2-cm diameter circle, drawn about the wick, at about 120° from each other so that after development, which usually took about 4 hours, separated material appeared in three roughly equal well-separated sectors.

For ascending chromatography, standards and unknowns were spotted in four equidistant locations along a line 76 mm from the end, that would be immersed in the development solvent, to give four parallel "tracks." Immersion depth of the paper was about 13 mm. Small solvent batches were freshly mixed each day; occasionally the same batch was used on 2 successive days. Development time was usually overnight, totalling about 16 hours. No particular precautions were taken to equilibrate the system prior to use. Suspending the air-dried chromatogram in a rising column of steam for 10–15 minutes was helpful in removing last traces of HOAc. Apparently no harmful effects were produced by steaming.

Identification reagents were applied by the dip technic (Smith, 1960). About 25 ml of reagent, usually diluted with acetone to increase volatility, was placed in a 150- × 15-mm Petri dish. The dried chromatogram was

drawn rapidly through the solution and then placed on a clean sheet of white filter paper for drying and observation. Identification was based primarily on parallel behavior of standard and unknown on the same sheet of paper, rather than on R_F values.

In some instances sequential application of detection reagents was employed. For example, spots fluorescent in ultraviolet radiation were marked in pencil. The chromatogram was then drawn through bromcresol green and dried, and acidic spots were marked. If compounds containing a free ureido group were present, subsequent treatment with Ehrlich's reagent gave intense-yellow spots on a pale-yellow background.

Occasionally, difficulty was encountered in removing all HOAc from chromatograms to be examined with bromcresol green, even with the use of steam. In these cases, it was sometimes helpful to expose the dried chromatogram, completely yellow from treatment with bromcresol green, to fumes of a dilute NH_3 solution. Often, before the entire chromatogram became blue, a transient yellow spot could be located.

Isolation Procedures. Allantoin was most conveniently isolated by lyophilization of a total product (100 ml) from the electrolysis of 1 or 2 mmoles of uric acid to a liquid volume of 2–3 ml, and storage of the latter in the refrigerator (4°) for several days. The material, which precipitated, was filtered, washed with very small portions of 0–5° water, dried at 50°, and weighed. Typical yield: 10 mg (7%) from 1 mmole (168 mg) uric acid, mp 223° (decomp), reported (Hartmann *et al.*, 1933) 230–231° (decomp). The infrared spectrum was identical to that of authentic allantoin. The special appearance of allantoin on repeated lyophilization is illustrated by the following: When the total lyophilized solids from an oxidation are reconstituted in 2–3 ml of cold water, a small amount of insoluble allantoin appears; on lyophilization of the resulting filtrate, more allantoin can be separated, e.g., a solution from the electrolysis of 168 mg uric acid gave 2 mg allantoin from the first lyophilization and 8 mg from the second. On other occasions, as much as a total of 16 mg allantoin was isolated from the electrolysis of 1 mmole of uric acid.

Oxaluric acid was obtained in small yield (*ca.* 5%) when ion-exchange columns, following the separation of urea (see following paragraph), were stripped with 1 M HCl. The total strippings were evaporated to a very small volume *in vacuo* at room temperature. The small amount of insoluble material produced was filtered, washed with several small portions of ice water, then with 95% EtOH, and dried at 60°. The mp was 198–200° (decomp). The infrared spectrum was identical to that of authentic oxaluric acid.

To isolate urea, 50 ml of the total oxidation product solution from 1 mmole of uric acid was slowly passed (1 drop/sec) through a column of Dowex 2-X8 ion-exchange resin (OH^- form), which occupied about three-fourths of the total volume of a standard 50-ml buret. Distilled water was then passed through the column until a total of 125 ml of effluent had been collected; this effluent was lyophilized to complete dryness. Yield, 50 mg. The infrared spectrum was identical to that of USP urea. Free alkali in the product was estimated by dissolving in water, adding phenolphthalein, and titrating dropwise with standard 0.10 N HCl, whose weight was measured; 0.995 g 0.1 N HCl solution was required, which is equivalent to 4.6 mg NaOH. The net yield is therefore 35.4 mg, or 62% based on 1 mole urea per mole uric acid.

A small portion of urea similarly isolated in a separate experiment was treated with concentrated HNO_3, filtered, washed, and dried, to give urea nitrate. The mp was 149–152°. The infrared spectrum was identical to that of authentic urea nitrate, similarly prepared from USP urea.

Murexide was formed by heating in a melting-point capillary at 120–130° the lyophilized total solids from a uric acid oxidation until all action ceased (frothing and color development began at 114°, but did not occur at an appreciable rate below 120°). The resulting intensely red product showed no further changes up to 160°, at which point the color faded and general decomposition ensued. Spectra, on which identification was based, were obtained by crushing the melting-point capillary in a small vessel, dissolving the contents in water, filtering, and diluting to a suitable volume with dilute NaOH solution.

After removal of oxaluric acid from the anion-column strippings (see earlier paragraph) the filtrate was lyophilized to total dryness, and finally dried at 60°. The residues so obtained were extremely hygroscopic and eluded all attempts at identification.

Isolation and Measurement of Carbon Dioxide. An electrolysis vessel (200-ml spoutless beaker) was closed with a tightly fitting rubber stopper carrying an inlet tube for nitrogen, an outlet tube, a connecting wire to a gold working cathode, a Beckman fiber-type saturated calomel electrode, and two impregnated graphite rods as working anodes; the latter were notched along their entire length to increase area. The reaction train consisted of (in sequence) a nitrogen cylinder, a scrubbing tower containing soda lime followed by Ascarite, a water saturator, the electrolysis cell (N_2 inlet tube submerged), drying tower (Drierite followed by Dehydrite), CO_2(s)-2-propanol trap (for HOAc condensation), a tared Pregl-type absorption tube filled with Ascarite, and a guard tube containing Dehydrite; the glass parts were connected by Tygon tubing. In one experiment, 84.4 mg (0.5 mmole) uric acid, electrolyzed at +1.4 v to completion, gave 4.6 mg CO_2, equivalent to 0.21 mole/mole uric acid. In another experiment, 169 mg. uric acid gave 9.8 mg CO_2, equivalent to 0.22 mole CO_2/mole uric acid.

Analytical Examination of Course of Electrolysis. Uric acid (2 mmoles; 335 mg), suspended in 100 ml 1 M HOAc, was electrolyzed at an anode potential of +1.1 v. The following analytical methods were used to determine the components indicated:

(1) For residual uric acid by ultraviolet absorption, exactly 1.00 ml of suspension (solution at completion of electrolysis) was withdrawn and delivered into a 100-ml volumetric flask; *ca.* 80 ml 2 M H_2SO_4 was added to dissolve the solid material; the solution was made up to

volume with 2 M H_2SO_4. Near the end of the electrolysis this solution was of a suitable concentration for direct measurement; early in the reaction further dilution with 2 M H_2SO_4 was required. The uric acid concentration was calculated from $\epsilon_{283} = 11{,}500$ in acid solution (determined by Cavalieri and Bendich (1950) and confirmed in the present investigation).

(2) For the determination of urea and allantoin precursor, 5.00 ml of suspension was transferred to a 12-ml centrifuge cone. After centrifugation, 1.00 ml of the clear supernatant solution was diluted to 50.0 ml (dilution "A"); aliquots ranging from 5.0 to 2.0 ml from beginning to end of the electrolysis, respectively, were used for the assay described in the next paragraph. The solids in the apex of the centrifuge cone were resuspended in the remaining supernatant liquid and returned to the electrolysis cell. Three 1.00-ml portions of 1 M HOAc used to rinse the sampling pipet and the centrifuge cone were also added to the cell, thus keeping the total volume in the latter constant since these 3 ml replaced the 1 ml each withdrawn for ultraviolet, allantoin precursor and urea, and polarographic examination.

Allantoin precursor in terms of allantoin was determined by the Young and Conway (1942) adaptation of the Rimini-Schryver (Schryver, 1910) reaction, involving estimation of glyoxylic acid resulting from acid hydrolysis of allantoic acid, which is produced from allantoin by alkaline hydrolysis. Glyoxylic acid produces a red color with phenylhydrazine and potassium ferricyanide. Essentially no interference is given by creatine, creatinine, hydantoin, guanidine, uracil, guanine, oxalic acid, xanthine, hypoxanthine, glycerol, aldehydes other than glyoxylic acid, alloxan, alloxantin, and glycerine (Young and Conway, 1942). Uric acid gives a positive test equivalent to 0.125 allantoin on a molar basis, which was considered too small to be of significance in our studies. Parabanic acid can be eliminated as a possible interference because of its oxidation state; it cannot by any hydrolytic step produce glyoxylic acid.

Uroxanic acid gives a positive test equivalent to 70–75% allantoin on a molar basis, but does not require the initial alkaline hydrolysis step, thus behaving like allantoic acid, which also gives a positive test in the assay when the initial alkaline step is omitted.

The filtrate from a cold alkaline permanganate oxidation (Brandenberger, 1959) of uric acid gave a positive reaction in the complete assay equivalent to more than 50% of the reacting uric acid on a molar basis. Since it is unlikely that such a filtrate contains any allantoin, and since such a filtrate can be manipulated further to produce good yields of allantoin (up to 70%), it is highly probable that the species giving the positive reaction is an intermediate or precursor of allantoin. Thus, the only likely positive interference in the Young and Conway assay for allantoin is such an allantoin precursor. Consequently, material reacting positively to the allantoin assay during and subsequent to an electrolytic oxidation of uric acid has been referred to as "allantoin precursor," rather than as "allantoin."

Two identical aliquots of dilution "A," equivalent to 0–0.1 mg allantoin, were placed in graduated 35-ml test tubes and, if less than 5.0 ml, diluted to 5.0 ml. One was treated with 1 ml 0.5 M NaOH, held in a boiling-water bath for 7 minutes, rapidly cooled, and acidified with 1 ml 0.5 M HCl plus 5 drops in excess. The other aliquot was directly acidified with 5 drops of 0.5 M HCl. One ml phenylhydrazine hydrochloride solution (20 mg/15 ml water; freshly prepared daily) was added to each tube, which was placed in boiling water for exactly 2 minutes, and immediately cooled to incipient freezing in an ice-rock salt bath (3–4 minutes at −10°). Cold (−10°) concentrated HCl (3 ml) was added, followed by 1 ml freshly prepared $K_3Fe(CN)_6$ solution (250 mg/15 ml water). After the material was mixed and allowed to stand for 40 minutes to permit full color development, the absorbance of the solutions was measured at 515 mμ, using 2 M HCl as a reference. Reagent blank corrections were nil. The concentration of allantoin precursor (or allantoin) was deduced by reference to an absorbance-concentration curve obtained with pure allantoic acid.

(3) Urea was determined by measuring photometrically the yellow color produced when urea is heated with 2,3-butanedione monoxime in the presence of an oxidizing agent (the Fearon (1939) reaction, using the method described by Rosenthal (1955)).

Usually two aliquots of dilution "A" (4.0 ml early in the electrolysis and 2.0 ml at the end) were diluted to 5.0 ml in graduated 35-ml test tubes. Three ml As(V) solution (10 ml concentrated HCl saturated with As_2O_5 and then diluted to 35 ml with the acid) and 1 ml 2,3-butanedione monoxime solution (2.5% w/v in 5% HOAc) were added to each tube. After mixing and dilution of the As(V) solution to 10.0 ml with water, the tubes were closed with rubber stoppers containing a small vent hole and heated in boiling water for 30 minutes. After cooling the tubes the absorbance was measured at 470 mμ against water as a reference. Parallel reagent blanks were run and appropriate corrections were made. Urea concentration was estimated from a standard curve based on USP urea similarly treated. To accommodate small daily variations in response, two urea standards (prepared fresh daily) of different concentrations were run with each group of unknowns.

Interference by allantoin or allantoin precursor was estimated by carrying known amounts of allantoin through the procedure. Allantoin also has its maximum absorption at 470 mμ where its absorbance is 0.31 that of urea on a molar basis. Apparent urea concentrations were corrected for allantoin interference by an appropriate subtraction based on the concentration of allantoin precursor determined by the Young and Conway assay.

(4) For polarographic estimation of parabanic acid and alloxan, 1.0 ml of the supernatant solution obtained on centrifuging a portion of the electrolyzed reaction mixture was diluted to 10.0 ml with 0.5 M *p*H 6.6 McIlvaine buffer. (The *p*H of the resulting solution was 5.1, since the HOAc in the sample exceeded the capacity of the buffer.) This solution was immediately transferred to the polarographic cell, deaerated, and polarographed from −0.5 to −1.8 v. A typical polarogram is

shown in Figure 4. The parabanic acid concentration was estimated by comparing the wave I limiting current with a limiting current–concentration curve of authentic parabanic acid in the same buffer mixture at the same *p*H. Alloxan concentration was similarly estimated from wave II.

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