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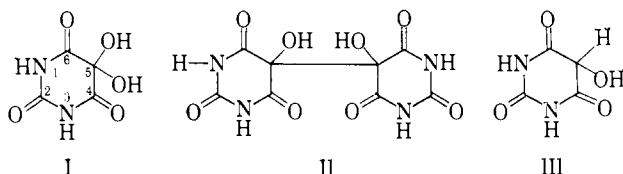
Polarographic Study of the System Alloxan-Alloxantin-Dialuric Acid

BY WILLIAM A. STRUCK AND PHILIP J. ELVING

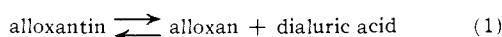
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Earlier investigations of the polarography of the reversible oxidation-reduction system, alloxan-alloxantin-dialuric acid, have been fragmentary or have embodied observations which have not been supported by the present study, in which the electrochemical reduction and oxidation of the three compounds involved have been examined polarographically, coulometrically, and by controlled potential electrolysis over the normal pH range with polarographic, spectrophotometric, and chemical examination of the reaction products. Alloxan exhibits two pH-dependent reduction waves at the dropping mercury electrode (d.m.e.). Wave I involves the kinetically controlled 2e reduction to dialuric acid. Wave II (observed only between pH 4.6 and 6.0) is apparently due to a further reduction. Above pH 6, alloxan rapidly rearranges to alloxanic acid, which is not reducible at the d.m.e. Alloxantin gives a combined anodic-cathodic wave at the same potential as wave I of alloxan; the diffusion-controlled anodic portion is many times larger than the kinetically controlled cathodic portion. Since under the experimental conditions used alloxantin is only very slightly dissociated (in contradiction to earlier literature), the cathodic portion of the combined wave is due to reduction of alloxantin rather than to alloxan formed by dissociation. Dialuric acid is isolated as the product of the controlled potential electrolysis of alloxantin. Contrary to the literature, no other cathodic processes have been observed for alloxantin except for a reduction wave very near the hydrogen discharge, which is similar to alloxan wave II and is apparently due to reduction of dialuric acid produced by the first wave process. Dialuric acid is oxidized to alloxan in an anodic wave at the same potential as cathodic wave I of alloxan and the combined wave of alloxantin. There is also evidence for a cathodic wave similar to wave II of alloxan.

Investigation of the system, alloxan (I)-alloxantin (II)-dialuric acid (III), as an oxidation-reduction system has been sparse in comparison with the extensive biological and biochemical literature on alloxan itself. Furthermore, the few investigations reported were undertaken at such widely separated time intervals and for such diverse purposes, that there is a general lack of clarity in the definition of this system.



For example, on the basis of potentiometric studies of the dissociation of alloxantin

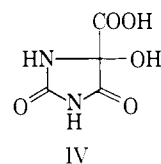


Hill and Michaelis¹ state that alloxantin does not exist to any appreciable extent in solution, whereas Richardson and Cannan² conclude that the existence of alloxantin itself is favored. A well-documented method for the preparation of alloxantin involves mixing solutions of alloxan and dialuric acid.³ Such a procedure, as well as a study involving solubility measurements,⁴ supports the latter conclusion.

The observations^{5,6} that reduction of alloxan with excess H₂S always produces dialuric acid and that alloxantin can be produced from alloxan only by limiting the total H₂S to the required stoichiometric amount, suggest that the oxidation-reduction potentials of the alloxantin-dialuric acid system is less negative than is generally assumed in the preparation of alloxantin from alloxan with excess H₂S. The success of such a procedure⁷ for the formation of alloxan from the oxidation of uric acid consequently can be attributed only to the enormous sensitivity of dialuric acid in solution to air oxidation⁸ and the slight solubility of alloxantin.

Investigation of the system is further complicated by the rearrangement of alloxan to alloxanic acid (IV),

which begins at *ca.* pH 4.5 and is very rapid at pH 7.^{2,9,10} The initial brief polarographic investigation¹¹



of the system attributed to its behavior that has not been confirmed in the present study.¹² Ono, Takagi, and Wasa,¹³ in a study primarily concerned with tricarbonyl compounds related to dehydroascorbic acid, showed that alloxan is reversibly reduced in a kinetically controlled step, presumably to dialuric acid, although no specific evidence was adduced for the latter point; they did not study the pH dependence of this wave, alloxan wave II, or the alleged¹¹ reduction waves of alloxantin and alloxanic acid.

Alloxan

Alloxan exhibits two well-defined cathodic waves at the dropping mercury electrode (d.m.e., Table I). Above pH 6, rearrangement to alloxanic acid is so rapid that the disappearance of alloxan is apparent while the polarogram is being run. Below pH 4.6, wave II is not resolved from the hydrogen discharge. Since the height of wave II is approximately ten times that of wave I, both alloxan concentration and pH must be carefully chosen in order to show both waves clearly on the same polarogram, *e.g.*, as in Fig. 1. Detailed study of each wave is best made on separate solutions of appropriate pH and alloxan concentration.

Wave I.—The upper limit of the pH range is determined by the base-catalyzed alloxanic acid rearrangement, which becomes very rapid above pH 5.6. The half-wave potentials ($E_{1/2}$) in acetate and McIlvaine buffers fall on a single straight line (Fig. 2A), described by the equation

$$E_{1/2} = 0.060 - 0.031\text{pH} \quad (2)$$

The pH dependence of 0.031 per pH unit is very close to the value expected for a reduction involving two electrons and one proton, and suggests that wave

- (1) E. S. Hill and L. Michaelis, *Science*, **78**, 485 (1933).
- (2) G. M. Richardson and R. R. Cannan, *Biochem. J.*, **23**, 68 (1929).
- (3) R. S. Tipson, *Org. Syn.*, **33**, 3 (1953).
- (4) E. Billman and J. Bentzon, *Ber.*, **51**, 522 (1918).
- (5) R. S. Tipson and L. H. Cretcher, *J. Pharm. Sci.*, **40**, 399 (1951).
- (6) R. S. Tipson and L. H. Cretcher, *J. Org. Chem.*, **16**, 1091 (1951).
- (7) E. D. Korn, "Methods in Enzymology," Vol. IV, S. P. Colowick and N. O. Kaplan, Ed., Academic Press, New York, N. Y., 1957, p. 634.
- (8) G. M. Richardson, *Biochem. J.*, **26**, 1959 (1932).

- (9) D. Seligson and H. Seligson, *J. Biol. Chem.*, **190**, 647 (1951).
- (10) I. M. Sarasohn, Ph.D. Dissertation, University of Delaware, 1959.
- (11) G. Sartori and A. Liberti, *Ric. sci. Suppl.*, **16**, 313 (1946).
- (12) There is some uncertainty about the unspecified reference potential used in ref. 11. We have assumed it to be the s.c.e., based on other reports from the same laboratory at about the same time.
- (13) S. Ono, M. Takagi, and T. Wasa, *Bull. Chem. Soc. Japan*, **31**, 364 (1948).

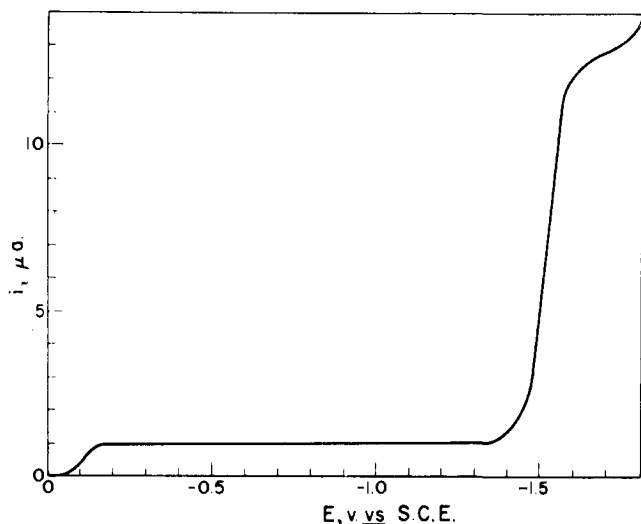


Fig. 1.—Polarogram of alloxan (2 mM) at pH 5.6. Curve constructed by combining the average current traces of separate polarograms of wave I and wave II.

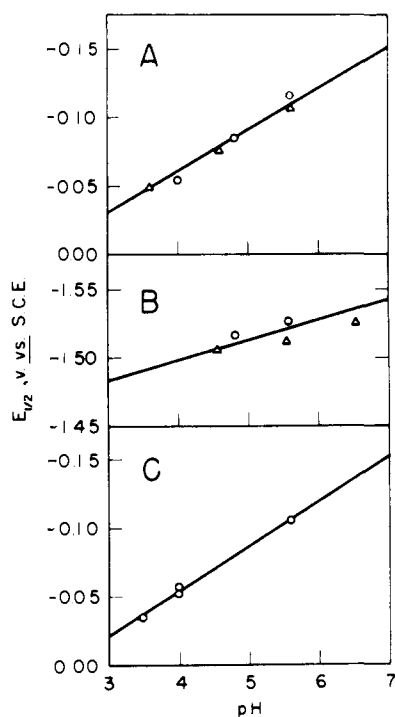


Fig. 2.—Variation with pH of $E_{1/2}$: A, wave I of alloxan; B, wave II of alloxan; C, combined wave of alloxantin; acetate buffer, circles; McIlvaine buffer, triangles.

I of alloxan is due to the reduction of alloxan ($pK_a = 7.2$, 10)² to the anion of dialuric acid ($pK_a = 2.8$)² in a single step.

The limiting current of wave I is essentially independent of the height of mercury column; the apparent diffusion current constant, calculated from this current, is abnormally small for a presumed 2e process. These observations point strongly to a kinetically controlled process, in agreement with the temperature dependence of the wave reported by Ono, *et al.*¹³

Wave II.—The second alloxan reduction wave can only be observed in the narrow pH range of 4.6 to 6.6 as previously mentioned. The variation of $E_{1/2}$ with pH is described by the equation (Fig. 2B)

$$E_{1/2} = -1.44 - 0.015\text{pH} \quad (3)$$

The proportionality of the limiting current to the square root of the mercury height and the more or less

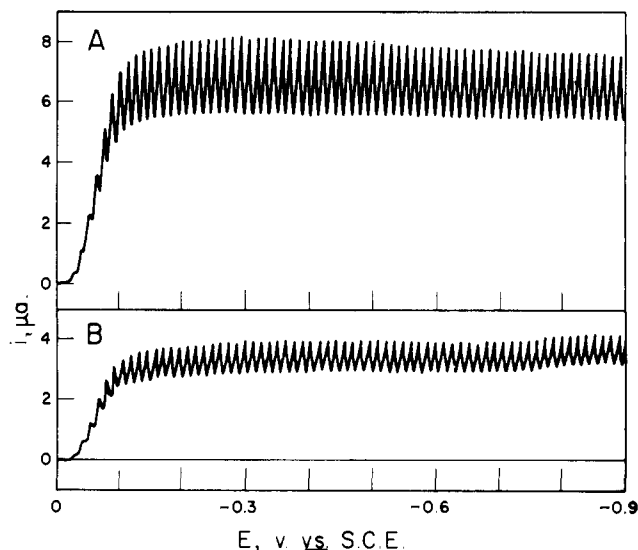


Fig. 3.—Effect of air oxidation on wave I of alloxan (originally 10 mM; pH 4.0, acetate buffer): A, initial polarogram; B, final polarogram after 72 hr. treatment with air, followed by deaeration.

typical values of the diffusion current constant indicate a diffusion-controlled process.

There is some evidence for the occurrence of an adsorption process; the erratic drop behavior almost always seen at potentials more negative than the $E_{1/2}$ of wave II may be due to film formation on the mercury.

TABLE I
POLAROGRAPHIC BEHAVIOR OF ALLOXAN

pH ^a	$E_{1/2}$, v.	i_l , μA .	I^b	Slope ^c	h , ^d cm.	i ratio ^e
Wave I ^f						
3.6 M	-0.048	4.60	0.167	1.5	70	1.01
	-0.058	4.56	.259	1.24	50	
4.0 A	-0.055	6.28	.285	1.22	70	1.02
	-0.062	6.18	.350	1.22	50	
4.6 M	-0.077	4.12	.187	1.22	70	1.02
	-0.075	4.04	.229	1.34	50	
4.8 A	-0.088	6.08	.276	1.51	70	1.02
	-0.093	5.94	.336	1.33	50	
5.6 A	-0.113	5.90	.254	1.60	70	1.13
	-0.113	5.24	.300	1.33	50	
5.6 M	-0.106	4.72	.215	1.36	70	0.96
	-0.107	4.90	.278	1.40	50	
Wave II ^g						
3.6 M	Wave obscured by hydrogen discharge					
4.0 A	Wave obscured by hydrogen discharge					
4.6 M	-1.506	7.30	3.45	1.27	70	1.13
	-1.498	6.45	3.82	0.94	50	
4.8 A	-1.517	7.75	3.64	1.06	70	1.17
	-1.513	6.60	3.92	1.27	50	
5.6 A	-1.527	Data not reliable;				
		decomposition			70	
5.6 M	-1.512	Data not reliable;				
		decomposition			50	
6.6 M	-1.525	Data not reliable;				
		decomposition			70	

^a A denotes acetate buffer; M, McIlvaine buffer; ionic strength = 0.5 M. ^b Diffusion current constant, $I = i_l / C m^{2/3} t^{1/6}$. (It should be noted that this is an apparent diffusion current constant since it is calculated from i_l .) ^c Slope = $0.056 / (E - 1/4 E^{3/4})$. ^d Height of mercury column. Ratios of the uncorrected heights are $(70/50) = 1.40$ and $(70/50)^{1/2} = 1.18$. ^e Ratio of the limiting currents at heights of 70 and 50 cm. ^f Alloxan concentration = 10.0 mM. ^g Alloxan concentration = 1.0 mM.

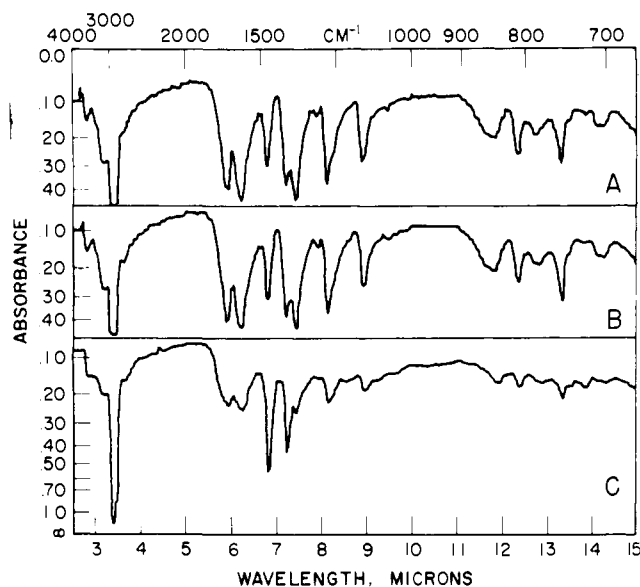


Fig. 4.—Infrared spectra (Nujol mulls) of electrolytic reduction products of alloxan and alloxantin: A, authentic sodium dialurate hemihydrate; B, product from reduction of alloxan; C, product from reduction of alloxantin.

Polarographic Waves due to Air Oxidation of Alloxan.—There is a total absence of any wave in the potential region (*ca.* -0.7 to -0.8 v.) in which a wave was previously reported¹¹ as wave II of alloxan. We have not been able to observe the latter wave or to attribute it in any conclusive way to oxidation products of alloxan. The only change noted on passing a slow stream of air through a 0.01 *M* alloxan solution in pH 4.0 acetate buffer for periods up to 72 hr. was a diminution of the apparent alloxan concentration (Fig. 3). Since polarograms run at intermediate time intervals showed no other wave, the possible appearance and subsequent disappearance of an intermediate species that could account for the reported wave seems remote. The final polarogram in Fig. 3 shows an extremely faint wave at *ca.* -0.7 v., which might possibly be the latter wave.

Coulometry.—Exhaustive electrolysis of 100 ml. of a 0.01 *M* alloxan solution in pH 4.0 acetate buffer liberated silver (200.3 mg.) in a series-connected silver coulometer equivalent to 1.9 electrons per molecule of alloxan. At the end of the electrolysis, a large quantity of solid crystalline material, which had accumulated in the cathode compartment, was filtered, washed free of buffer under nitrogen, and dried overnight in a vacuum desiccator over Drierite. The infrared spectrum of this material (Fig. 4) and its behavior on attempted melting point determination are identical with those of the sodium salt of a commercial sample of dialuric acid. The spectrum also corresponds closely to that of the free acid.⁶

Alloxanic Acid.—By careful choice of pH, the rearrangement of alloxan to alloxanic acid can be followed polarographically by observing the disappearance of the characteristic alloxan waves. Since the only product of the rearrangement is alloxanic acid, polarograms showing no further change are, in effect, polarograms of alloxanic acid.

Polarograms of an alloxan solution run 20 min. apart at pH 7.6, at which pH the conversion is sufficiently rapid that the decrease in the limiting current of alloxan wave I can be observed while the potential is scanned, clearly show that alloxanic acid gives no reduction wave at the d.m.e. at pH 7.6 (Fig. 5). To eliminate the possibility of a pH effect, the com-

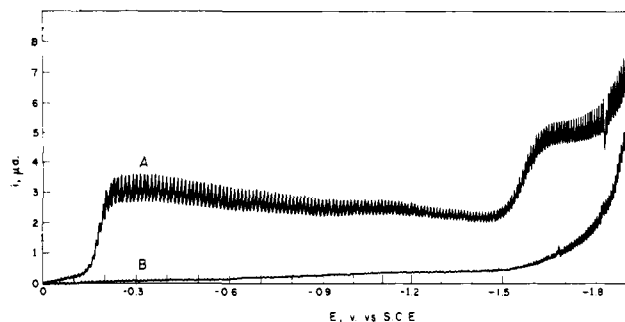


Fig. 5.—Polarograms of alloxan in pH 7.6 McIlvaine buffer: A, within 5 min. after initial dissolution; B, 20 min. later.

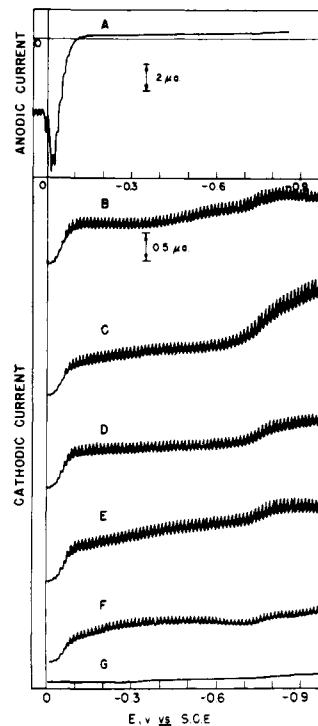


Fig. 6.—Polarographic behavior of alloxantin (originally $1mM$) treated with air (pH 4.0): A, initial behavior (completely anaerobic); B, 30 min. treatment with air; C, 1 hr. treatment; D, 6 hr. pretreatment; E, 19 hr. pretreatment; F, 72 hr. pretreatment; G, background, similarly aerated and deaerated.

pletely rearranged solution was acidified to pH 3 with phosphoric acid and polarographed; there was still no evidence for a reducible species. Thus, we are forced to conclude that the reduction wave previously reported¹¹ for alloxanic acid was an artifact.

Alloxantin

Alloxantin shows a combined cathodic-anodic wave, whose cathodic portion is very small compared to the anodic portion (Fig. 6A). This is the only readily observable cathodic process and contradicts the previous assertion¹¹ that alloxantin exhibits a reduction wave at about -0.7 v. (the same potential as the alleged alloxan wave II). Alloxan does indeed exhibit a second reduction wave but, as has been discussed, at much more negative potential, *viz.*, *ca.* -1.5 v. By subtracting background at a pH higher than 4.8, a very small wave at *ca.* -1.5 v. can be observed for alloxantin as it should, if wave II of alloxan represents the further reduction of dialuric acid resulting from wave I.

The data on the combined alloxantin wave (Table II) show that with polarization toward increasing negative potential, $E_{1/2}$ for alloxan wave I and the combined

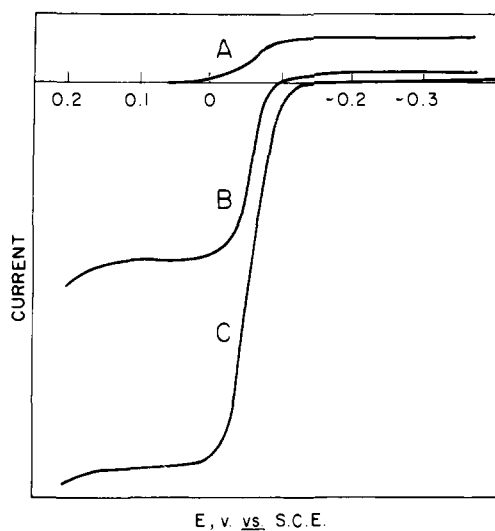


Fig. 7.—Polarograms at equivalent concentrations in pH 4.0 acetate buffer: A, 0.5 mM alloxan ($E_{1/2} = -0.055$); B, 0.25 mM alloxantin ($E_{1/2} = -0.058$); C, 0.5 mM dialuric acid ($E_{1/2} = -0.056$). Figure constructed from the average current traces of separate polarograms.

alloxantin wave are essentially identical (Fig. 7). The pH dependence of the alloxantin wave (Fig. 2C)

$$E_{1/2} = 0.078 - 0.031\text{pH} \quad (4)$$

has exactly the same slope as that for alloxan wave I but a slightly different intercept. The latter may be due to the fact that the alloxan data were obtained in both acetate and McIlvaine buffers and the alloxantin data only in acetate buffers.

TABLE II
POLAROGRAPHIC BEHAVIOR OF ALLOXANTIN

pH ^a	Concn., mM	$E_{1/2}$, v.	Anodic, i_1 , μA .	I^b	Slope ^c	h , cm. ^d	i ratio ^e
3.5	0.3	-0.038	1.69	2.46	1.60	70	1.26
3.5	0.3	-0.036	1.34	2.49	1.37	50	
4.0	1.0	-0.063	5.68	2.48	Maximum present	70	1.18
4.0	1.0	-0.063	4.44	2.48	Maximum present	50	
4.0	0.455	-0.052	2.63	2.55	1.55	70	
4.0	1.0	-0.058	5.12	2.25	Maximum present	70	
5.6	0.3	-0.106	1.57	2.29	1.64	70	1.21
5.6	0.3	-0.088	1.29	2.40	1.45	50	

^a Acetate buffer. ^b Diffusion current constant: $I = i_1 / C m^{2/3} t^{1/6}$. (It should be noted that this is an apparent diffusion current constant since it is calculated from i_1 .) ^c Slope = $0.056 / (E_{1/2} - E_{2/2})$. ^d Height of mercury column. Ratios of the uncorrected heights are $(70/50) = 1.40$ and $(70/50)^{1/2} = 1.18$. ^e Ratio of the limiting currents at heights of 70 and 50 cm.

The slope of 0.031 v. per pH unit again suggests the involvement of one proton and two electrons in the reduction of alloxantin to dialuric acid. This is as expected; since alloxantin is a dimeric species, its reduction to dialuric acid requires one electron per molecule of dialuric acid, compared with two for the reduction of alloxan.

The cathodic portion of the combined alloxantin wave is apparently under kinetic control. This conclusion is based mainly on the fact that this cathodic portion is very much smaller than the anodic portion, and is of the same order of magnitude as alloxan wave I, which is kinetically controlled.

Macroscale Electrolysis.—Recrystallized alloxantin dihydrate (160 mg. or 0.0005 mole), dissolved in 100 ml. of pH 4.0 acetate buffer, was electrolyzed exhaustively

at -0.40 v., which corresponds to a potential on the limiting current of the cathodic portion of the wave; strictly anaerobic conditions were observed in all operations. Near the end of the electrolysis, solid material separated, whose infrared spectrum was identical with that of the sodium dialurate isolated from the 2e reduction of alloxan (Fig. 4).

Polarography of Air-Oxidized Alloxantin.—In connection with the cathodic waves reported¹¹ for alloxan and alloxantin, which could not be found in the present investigation, it seemed pertinent to investigate the polarographic behavior of alloxantin after prolonged treatment with air (Fig. 6).

On bubbling air through a 1 mM alloxantin solution in pH 4.0 acetate buffer at room temperature, all of the alloxantin is oxidized in less than an hour largely to alloxan. More interesting, however, is the appearance and subsequent disappearance of additional cathodic waves, which are not very sharply delineated. After 30 min. exposure, two new waves appear of $E_{1/2}$ ca. -0.5 and -0.75 v.; the latter wave exhibits a broad maximum (Fig. 6B). After 1 hr., the -0.5 v. wave has shifted to -0.3 v. and diminished slightly; the -0.75 v. wave has shifted to ca. -0.8 v. and increased fivefold in size. The latter shift and growth may actually be due to the appearance of an overlapping third wave at somewhat more negative potential; the appearance of the wave (Fig. 6C) supports this possibility.

After treatment with air for almost 6 hr. (Fig. 6D), the -0.3 v. wave has almost disappeared; the -0.75 v. wave has also diminished greatly, but still has the appearance of two unresolved waves. After 19 hr. (Fig. 6E), waves are still visible at ca. -0.35 and -0.75 v., the latter again exhibiting a flat maximum. After 72 hr., the polarographic curve (Fig. 6F) shows a diminished alloxan concentration, a residuum of the -0.3 and -0.75 v. material, and a new, poorly defined wave running from -0.9 to -1.2 v.

The background curve of pH 4 acetate buffer, saturated with air and then deaerated in the same way as the alloxantin solutions (Fig. 6G), shows that removal of oxygen from the air-saturated solutions prior to polarography was complete and, consequently, that none of the waves observed are due to reduction of oxygen.

The appearance of the wave at ca. -0.75 v. could explain the second cathodic wave previously ascribed¹¹ to alloxantin; this wave first appears with a limiting current of ca. $0.2 \mu\text{A}$., increases to ca. $1.3 \mu\text{A}$., and finally diminishes to ca. $0.2 \mu\text{A}$.. This behavior, combined with the appearance, disappearance, and reappearance of maxima, and the apparent shifts of $E_{1/2}$ (which may be due to additional unresolved waves), suggests a complex pattern of oxidation in which certain species are intermediates in the pathway from alloxantin to alloxan, and others are terminal products at either the same or higher oxidation states than alloxan.

The over-all conclusion is that alloxantin exhibits no cathodic processes at the d.m.e. other than the small cathodic portion of the combined wave (Fig. 6A), which is due to the reduction of alloxantin to dialuric acid, and a small, ill-defined wave near the hydrogen discharge. The cathodic wave for alloxantin alleged¹¹ to occur at ca. -0.7 v., where wave II of alloxan is also alleged¹¹ to occur, has not been observed. However, alloxantin on air oxidation produces several species that might account for this wave.

Dialuric Acid

Dialuric acid shows an anodic wave at the same potential as cathodic wave I of alloxan and the com-

bined alloxantin wave. $E_{1/2}$ in pH 4.0 acetate buffer is -0.056 v. when polarization is toward increasingly negative values, and -0.047 v. when scanned in the opposite direction. On a molar basis, the anodic limiting current is approximately the same as for alloxantin; since alloxantin is dimeric as compared with alloxan or dialuric acid, alloxantin involves 1e per four-carbon unit as compared to 2e per four-carbon unit for the other two species (Fig. 7).

The dialuric acid oxidation wave shows no cathodic component under the conditions that result in a combined wave for alloxantin. This is as expected and furnishes good evidence for the effective exclusion of oxygen, since solutions of dialuric acid are extremely efficient oxygen absorbers.

Mechanism of Oxidation-Reduction

Dissociation of Alloxantin in Solution.—The polarographic behavior of alloxantin in the potential region of wave II of alloxan (*ca.* -1.5 v.) suggests that alloxantin under the conditions of these experiments is only slightly dissociated (eq. 1). It has been shown (Table I and Fig. 1) that the process giving rise to the large alloxan wave II (diffusion current constant >3) may be the reduction of dialuric acid produced in the wave I process and that alloxantin is reduced to dialuric acid at a potential on the limiting current portion (cathodic) of the combined alloxantin wave. Thus, the further reduction of dialuric acid (or of alloxan itself beyond the dialuric acid stage) represented by alloxan wave II at *ca.* -1.5 v. should also be observable as a second reduction wave of alloxantin. This is indeed the case, but with an important quantitative difference; alloxantin wave II occurs at the same potential as alloxan wave II, but can only be detected by subtracting out background, since it is only one-tenth the magnitude of alloxan wave II; *e.g.*, i/C , the ratio of current to concentration, in pH 5.6 acetate buffer is 7 for alloxan wave II and 0.7 for alloxantin wave II.

If alloxantin were largely dissociated at the concentrations used, as indicated in the earlier literature,¹ the alloxan present as a result of such dissociation should give a wave II for alloxantin completely comparable to wave II of alloxan. The observation of a wave of only 0.1 the current of alloxan wave II indicates that the alloxantin cannot be largely dissociated.

The polarographic behavior of the combined alloxantin wave (Fig. 6A) suggests a means of determining the equilibrium tendency in the dissociation of alloxantin. Taken by itself, the combined wave does not allow any conclusion on this point, *i.e.*, the anodic portion could equally well represent the oxidation of x moles of alloxantin to alloxan in a 1e step as that of $x/2$ moles of dialuric acid to alloxan in a 2e step. However, if the concentration of alloxan in the equilibrium represented by eq. 1 could be appreciably increased, the shift in equilibrium would result in only a vanishingly small concentration of dialuric acid; consequently, if the anodic wave were largely due to dialuric acid formed by dissociation of alloxantin, it should diminish appreciably in the presence of a large excess of alloxan, whereas if it were principally due to undissociated alloxantin, it should not diminish and conceivably could increase.

Accordingly, a 10.0-ml. solution of 0.45 mM alloxantin in pH 4.0 acetate buffer, prepared under strict anaerobic conditions, was polarographed. The solution was then made 4 mM in alloxan by the addition of 6.4 mg. of alloxan monohydrate (dissolution and mixing were accomplished in the cell by a stream of nitrogen). On repolarographing, no appreciable change in the anodic limiting current was noted despite the

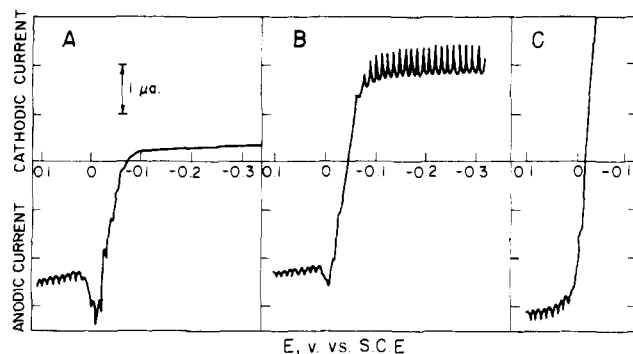


Fig. 8.—Effect of added alloxan on anodic portion of combined wave of alloxantin in pH 4.0 acetate buffer: A, 0.45 mM alloxantin; B, 0.45 mM alloxantin, 4 mM in added alloxan; C, 0.45 mM alloxantin, 40 mM in added alloxan.

10-fold excess of alloxan compared with the original alloxantin concentration, indicating that the original anodic wave was due to undissociated alloxantin. Increasing the alloxan concentration to 40 mM and repolarographing showed some changes attributable to the high alloxan concentration, *e.g.*, disappearance of the anodic maximum, but the anodic wave not only did not diminish but actually increased. However, because of the high alloxan concentration and the disappearance of the maximum, no significance in respect to the equilibrium should be attached to this increase. Polarograms obtained at several intervals up to 2 hr. remained unaltered, indicating that slow attainment of equilibrium is not a factor.

The data are summarized in Table III and Fig. 8. In principle, an experiment of this kind should permit calculation of the equilibrium constant for the dissociation process of eq. 1. Unfortunately, the small difference between the limiting currents of the last and first polarograms, which should represent the amount of alloxantin dissociated, and the abnormalities introduced into the final polarogram by the relatively high alloxan concentration completely negate the validity of such calculation. Nevertheless, the general conclusions must be that, under the conditions of pH and ionic strength involved, the behavior of alloxantin in solution is not due to dialuric acid or alloxan produced by its dissociation and, more specifically, the anodic wave of alloxantin is due principally to undissociated alloxantin.

TABLE III
BEHAVIOR OF ANODIC WAVE OF ALLOXANTIN IN THE PRESENCE OF EXCESS ALLOXAN

Alloxantin, mM	Added alloxan, mM	Anodic i_l , μ a.	$E_{1/2}$, v.
0.455	0	2.39	-0.045
.455	4	2.22	-0.043
455	40	3.2 ^a	

^a See text for additional changes.

These results indicate that in a pH 4 acetate buffer of 0.5 M ionic strength the association constant must be considerably higher than 21, the value reported by Richardson and Cannan.² At the initial alloxantin concentration used in the present studies, *ca.* 0.45 mM, a constant of 21 requires more than 98% dissociation, which is clearly not the case. For even 50% dissociation at a concentration of 1 mM, the association constant would have to be 10^8 . The fact that addition of a 100-fold molar excess of alloxan to a solution of alloxantin causes no diminution in the anodic wave of alloxantin (Fig. 8), also indicates that the as-

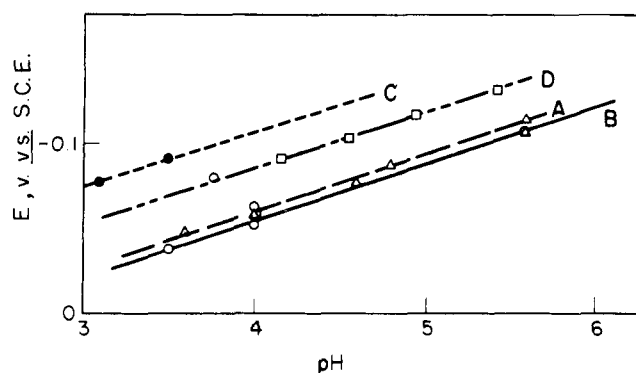


Fig. 9.—Variation of E_0 and $E_{1/2}$ with pH for alloxan-alloxantin-dialuric acid system: A, $E_{1/2}$ of alloxan (present investigation); B, $E_{1/2}$ of alloxantin (present investigation); C, $E_{1/2}$ of alloxan (Ono, *et al.*¹³); D, E_0 of dialuric acid (Richardson and Cannan²).

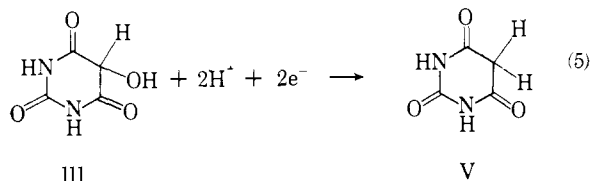
sociation constant for alloxantin under the experimental conditions employed must be very large.¹⁴

Obviously, the statement¹ that alloxantin does not exist in solution to any appreciable extent, must be rejected.

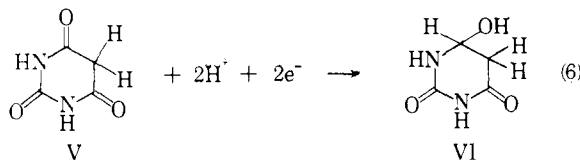
Alloxan Wave I Reduction.—Based on macroscale electrolysis and coulometry, pH dependence, and identification of the reduction product, the process represented by alloxan wave I is a 2e reduction to dialuric acid. Furthermore, the polarographic process has the characteristics of a completely reversible system, with the reduction current being under kinetic control. The rate-controlling process involved may be the dehydration of the hydrated carbonyl (*gem*-glycol) at C-5 of alloxan, analogous to the well-known behavior of formaldehyde at the d.m.e.¹⁵

The direct reduction of alloxan to dialuric acid in a single 2e step has not been explicitly demonstrated heretofore, although Ono, *et al.*,¹³ assume that this occurs and Sartori and Liberti¹¹ conclude without presenting any evidence that this is the case.

Alloxan Wave II Reduction.—The nature of the reduction process represented by wave II of alloxan can be surmised. The diffusion current constant of 3 to 4 (Table I) is of the proper magnitude for a 2e process such as would be observed in the reduction of dialuric acid (III) to barbituric acid (V)

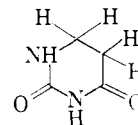


However, the process may proceed further, *e.g.*



The possible occurrence of the latter process is indicated by the fact that reduction of dialuric acid at a lead cathode at an unspecified potential gave dihydrouracil (VII) as the principal product; reduction

of alloxan also gave dihydrouracil.¹⁶ Thus, alloxan wave II may represent a step or steps in a sequence leading from dialuric acid to reduced products such as V, VI, or VII.



Role of Alloxantin in Reduction of Alloxan.—The role of alloxantin in the oxidation-reduction sequence cannot be unequivocally identified. Since alloxantin in solution under our experimental conditions is largely undissociated, it is important to know if alloxantin is reduced at the same potential as alloxan. If it is not, the reduction of alloxan directly to dialuric acid can only be successful if the association of alloxan and dialuric acid to form alloxantin is very slow. If the association is rapid, alloxantin would be the end product and the reduction of alloxan would involve only one electron; this has already been shown not to be true by macroscale electrolysis at a potential corresponding to a point just on the crest of the first alloxan wave and identification of dialuric acid as the reduction product. Thus, either the association of dialuric acid and alloxan is very slow, or alloxantin itself is reducible at the same potential as alloxan. In the latter case, there is no way of showing the existence of alloxantin during the reduction of alloxan except perhaps as a transitory species; in fact, there may be no point in the attempt.

Macroscale reduction of alloxantin established that solutions of the latter are reducible at the same potential as alloxan. However, this may be due to direct reduction of alloxantin or to reduction of alloxan produced by dissociation. Similarly, it is readily apparent from a polarogram of alloxantin (Fig. 6A) that the major phenomenon is anodic, and that it cannot be deduced by inspection whether the small cathodic portion is due to reduction of alloxan produced by dissociation of alloxantin or to reduction of alloxantin itself. The small size of the cathodic wave may indicate that it is due to alloxan formed by dissociation.

In light of the uncertainties involved, it is impossible at the present time to decide whether or not alloxantin, as such, ever exists in solution during the electrolytic reduction of alloxan to dialuric acid.

Evidence for Reversibility.—Several observations indicate that the reduction of alloxan and alloxantin to dialuric acid are part of a reversible process. The half-wave potentials of the reduction and oxidation waves of the three species involved are essentially identical. For example, in pH 4.0 acetate buffer, when the potential is scanned toward increasing negative values, $E_{1/2}$ for the cathodic wave I of alloxan is -0.055 v.; for the combined wave I of alloxantin, -0.058 v.; and for the anodic wave of dialuric acid, -0.056 v. (Fig. 7). This $E_{1/2}$ as well as those at other pH values (Fig. 2A), correspond closely to the E_0 values observed² in the potentiometric titration of dialuric acid with ferricyanide, when adjusted to a common reference electrode basis. In fact, the agreement of our half-wave potentials with the potentiometric E_0 values is somewhat better than with Ono's half-wave potentials¹³; the three sets of values (Fig. 9) lie on parallel lines with an extreme variation of 0.05 v., with the E_0 values falling between the two sets of half-wave potentials.

(14) Dr. Louis Meites in a private communication has indicated that the data of Fig. 7 and 8 can be used to estimate an association constant in the range of $(6 \text{ to } 15) \times 10^3$. Unfortunately, the conditions under which the present experiments were done do not permit an accurate estimation.

(15) I. M. Kolthoff and J. J. Lingane, "Polarography," 2nd Ed., Interscience Publishers, New York, N. Y., 1952, pp. 652 ff.

(16) J. Tafel and L. Reindl, *Ber.*, **34**, 3286 (1901).

The accumulated evidence of the present investigation and the work of Ono¹³ point to kinetic control of the reduction of alloxan and alloxantin to dialuric acid and diffusion control of the oxidation of dialuric acid and of alloxantin.

Experimental

Chemicals.—The purity of alloxan monohydrate (Distillation Products) and dialuric acid hydrate (Calbiochem) was shown by elemental analysis and infrared spectrometry. Alloxantin dihydrate (Distillation Products) was recrystallized from boiling water in a nitrogen atmosphere. The infrared spectra of these three compounds (Nujol mull) corresponded exactly to those in the definitive work of Tipson and Cretcher.⁶

The composition of sodium dialurate hemihydrate, prepared by recrystallizing dialuric acid from boiling pH 4 acetate buffer (all operations conducted under nitrogen), was verified by elemental analysis. Its infrared spectrum is very similar to that of free dialuric acid, except for a general shift of bands to lower frequencies in the fingerprint region.

Anal. Calcd. for $C_4H_3N_2NaO_4 \cdot 0.5H_2O$: C, 27.4; H, 2.0; N, 16.0; Na, 13.1. Found: C, 27.0; H, 2.3; N, 15.9; Na, 13.1. (Attempted melting point: see under "Isolation of Electrolysis Products.")

Buffer solutions were prepared from analytical reagent grade chemicals. Acetate buffers contained only HOAc and NaOAc. McIlvaine buffers were adjusted to constant ionic strength with KCl.¹⁷

Nitrogen used for deoxygenation was purified by successive passage through vanadous chloride solution, distilled water containing marble chips to neutralize any HCl carried over, and copper wool maintained at 450–500°.

Solutions of alloxantin and dialuric acid, which are highly susceptible to air oxidation,⁸ were prepared by weighing solid material into clean, dry volumetric flasks from which the air had been displaced by nitrogen, and then adding thoroughly deaerated buffer solution. Dissolution was accomplished by heating on a steam bath while a slow stream of nitrogen was led through the solution. Final volume adjustment was made after cooling in a 25° constant temperature bath. At no time were such solutions exposed to air. Transfer to the polarographic cell was done by using nitrogen-filled pipets and delivering the solution by positive nitrogen pressure into a nitrogen-filled cell.

Apparatus.—Polarograms were recorded on a Leeds & Northrup Type E Electro-Chemograph, using a water-jacketed H-cell,¹⁸ maintained at $25.0 \pm 0.1^\circ$ and containing a saturated calomel reference electrode (s.c.e.). Damping of the polarograph was equivalent to galvanometer performance. All potentials mentioned are referred to the s.c.e.

The capillary used (marine barometer tubing) had a flow, in distilled water (open circuit) at 25°, of 2.45 mg./sec. at 70 cm., and 1.57 mg./sec. at 50 cm. The drop times were all within the normally accepted range.

The cell resistance, measured with a General Radio Type 650 A impedance bridge, was always below 300 ohms; consequently half-wave potentials were not corrected for iR drop.

The cell used for macroscale electrolysis and coulometry has been described by Meites.¹⁹ The mercury cathode (quiescent area of ca. 44 cm.²) was stirred concurrently with the solution during electrolysis. A graphite anode was used. Bridge and anode compartment electrolytes were the same buffer as that used to dissolve the substrate; these were deoxygenated prior to electrolysis and were protected from air during electrolysis.

Hydrazine was used as an anodic depolarizer. The cell was connected in series with a silver coulometer and a Fisher-controlled potential electro-analyzer, which was the power source. The potential between mercury cathode and reference electrode (Beckman s.c.e.) was monitored with a Rubicon Type B-1 potentiometer. Infrared spectra (Nujol mulls) were obtained with a Perkin-Elmer Model 137B spectrophotometer. The pH was measured with a Leeds & Northrup No. 7664 pH meter.

Polarographic Procedure.—Alloxan solutions were usually prepared by dilution of a relatively concentrated stock solution with the appropriate buffer. In the case of alloxantin and dialuric acid, solubility limitations did not permit this, and exactly weighed samples were dissolved anaerobically in requisite volumes of buffer as previously described.

Approximately 10 ml. of test solution was transferred to the H-cell, deoxygenated with nitrogen for about 10 min., and then polarographed. No maxima suppressors were used. A portion of the buffer solution was treated identically to obtain the background current. When appropriate, the latter was subtracted from the total current; $E_{1/2}$ and i_1 were determined graphically from the average of the recorder trace.

Coulometric Procedure.—Limited solubility of alloxantin did not permit pre-electrolysis of buffer followed by addition of a relatively concentrated stock solution. However, since residual currents with thoroughly deoxygenated buffer were of the order of 30 μ a., it was concluded that pre-electrolysis could be omitted. Thus, for both alloxan and alloxantin, 100 ml. of solution prepared anaerobically was introduced into the cell through which nitrogen had been passing for some time. After further deaeration, mercury was introduced, the coulometer was connected, and electrolysis was begun at a potential on the crest of the wave (alloxan wave I or alloxantin combined), which was -0.4 v. vs. s.c.e. in all experiments. Nitrogen was passed slowly through the cell during electrolysis.

Isolation of Electrolysis Products.—Sodium dialurate, which precipitated on exhaustive electrolysis of either alloxan or alloxantin, was isolated with minimum exposure to air by means of a simple positive pressure filter. A 3.5-cm. diameter medium porosity glass Büchner funnel, fitted to a filter flask, was capped with a tight two-hole rubber stopper, one hole of which was used for the introduction of nitrogen at about 4 p.s.i. pressure and the other for the introduction of the electrolysis product. The entire system was thoroughly flushed with nitrogen prior to use.

The electrolysis product was removed as a suspension from the cell by means of a nitrogen-filled pipet, whose contents were then delivered into the filter with positive nitrogen pressure; filtration was completed under such pressure and the crystalline product was washed free of buffer components with several portions of deaerated water. The entire funnel was then placed over anhydrous $CaSO_4$ in a nitrogen-filled vacuum desiccator, which was evacuated for 18 hr.

Materials thus isolated were hemihydrates, whose infrared spectra (Nujol mulls, Fig. 4) were identical with the sodium salt of authentic dialuric acid similarly handled (see Chemicals). They did not melt upon heating, but behaved identically with authentic sodium dialurate. A very faint pink color developed at 170°; at 280°, a gradual tanning began, which progressed gradually to a light brown color; heating was discontinued at 315° with no sign of melting.

It was not possible to obtain the weight of product from these operations; the experimental technique, which was primarily designed to protect the product from exposure to air, was incompatible with quantitative transfer of material.

Acknowledgment.—The authors thank the U. S. Atomic Energy Commission, The Upjohn Company, and the Horace H. Rackham School of Graduate Studies of The University of Michigan, which helped support the work described.

(17) P. J. Elving, J. M. Markowitz, and I. Rosenthal, *Anal. Chem.*, **28**, 1197 (1956).

(18) J. C. Komyathy, F. Malloy, and P. J. Elving, *ibid.*, **24**, 431 (1952).

(19) L. Meites, *ibid.*, **27**, 1116 (1955).