THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

I. ELECTROCHEMICAL OXIDATION OF BIOLOGICALLY IMPORTANT XANTHINES

II. ELECTROCHEMICAL REDUCTION OF ALLOXAN, METHYL- AND DIMETHYL ALLOXAN IN AQUEOUS AND ACETONITRILE SOLUTIONS

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I. ELECTROCHEMICAL OXIDATION OF BIOLOGICALLY IMPORTANT XANTHINES

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DEDICATION

To the many, beginning with my parents, who have entered my life and by the Spirit of their lives have strengthened faith, renewed hope and taught love:

"And man the stumbler and finder, goes on,
man the dreamer of deep dreams,
man the shaper and maker,
man the answerer."*

*Lines from "Man will never write" by Carl Sandburg.

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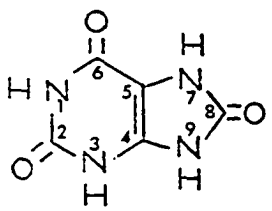
PART I

ELECTROCHEMICAL OXIDATION OF BIOLOGICALLY IMPORTANT XANTHINES

INTRODUCTION

Statement of Problem

Electrochemical oxidation of uric acid,[1] 2,6,8-trioxypurine (1), at the pyrolytic graphite electrode (p.g.e.) follows a mechanism common to that for the enzymatic oxidation of uric acid. Similar electrochemical oxidation of adenine,[2] 6-aminopurine (2), indicates that



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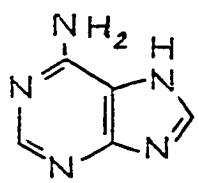

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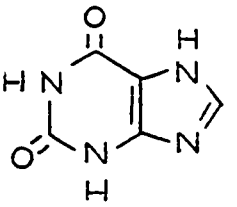
initially the oxidation path is the same as that of enzymic oxidation, followed by further oxidation and fragmentation. This study was therefore initiated to investigate whether the N-methylated derivatives of xanthine, 2,6-dioxypurine (3), exhibited a similar behavior upon electrochemical oxidation at the p.g.e.



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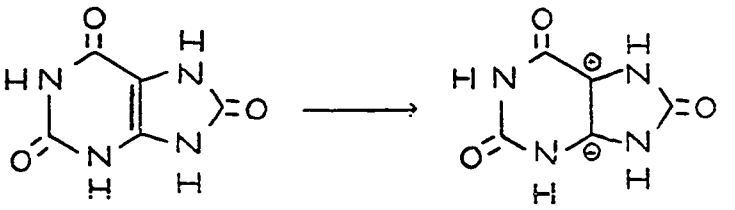
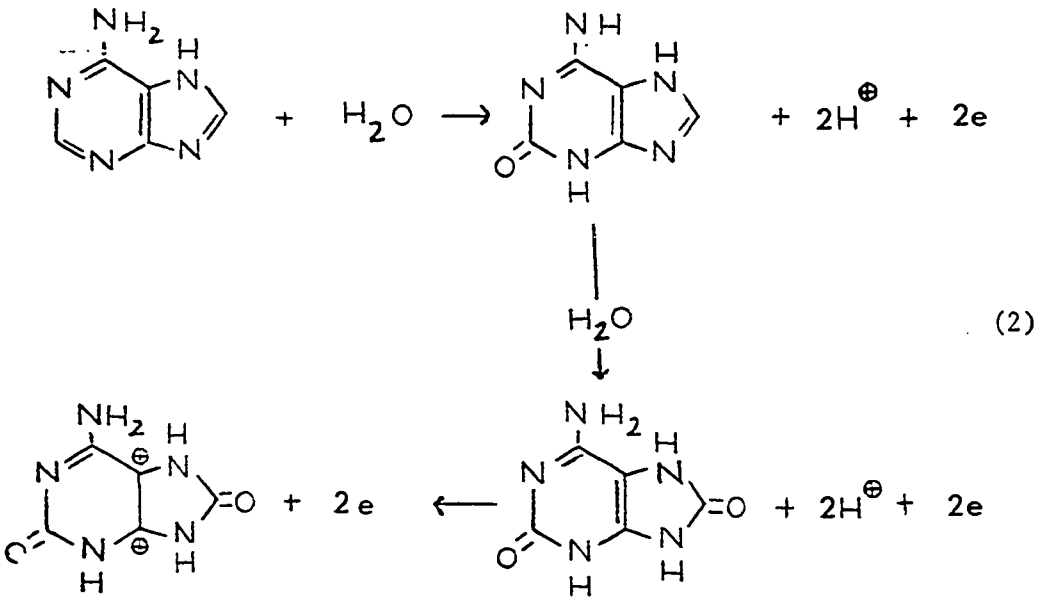
Struck and Elving[1] had postulated a primary oxidation of uric acid by a two-electron process yielding an intermediate dicarbonium ion (Equation 1). The studies of Cankellakis and Cohen,[3] Paul and Avi-Dor[4]



(1)

and Soberon and Cohen[5] also suggest such an intermediate upon enzymic oxidation of uric acid.

Dryhurst and Elving[2] reported that the primary electrochemical oxidation of adenine proceeded by way of two two-electron oxidations at the 2- and 8-positions to the oxy- and dioxyadenine, followed by the two-electron oxidation of dioxyadenine to a dicarbonium ion as represented by Equation 2. In both cases, subsequent hydrolysis and oxida-



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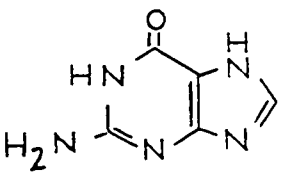
tion reactions occurred, all products of which were identified and totalled up to the quantity of parent compound oxidized.

While this present work was in progress, the electrochemical oxidation of guanine (4) was completed and is reported to also initially



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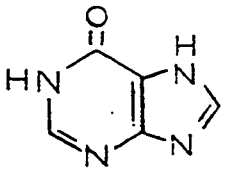
follow the enzymatic oxidation path.[6] Furthermore, Dryhurst[7] has reported the detection of a short-lived electrochemically reducible oxidation product, appearing as an almost totally reversible couple for uric acid, adenine, xanthine, hypoxanthine (5), 2,8-dioxyadenine (6), isoguanine (7) and guanine under the conditions of rapid sweep cyclic



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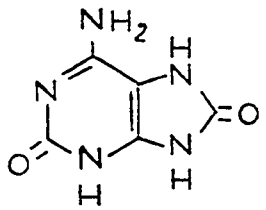

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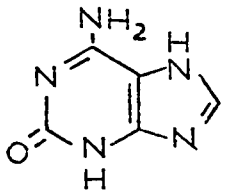

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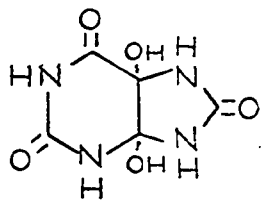
voltammetry. This product is related to the oxidation of the $-C_4=C_5-$ double bond giving $-\overset{OH}{C_4}-\overset{OH}{C_5}-$, the 4,5 diol, since it is observed that with decreasing pH the peak potential for oxidation of the purines shifts in the positive direction. This suggests that the primary electrochemical product is not the dicarbonium ion as noted in the early work on uric acid and adenine, but rather the dihydroxy or 4,5-diol species (8) which results from concerted removal of two electrons and attack



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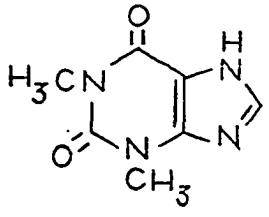
by water. Subsequent reduction occurs on the return sweep again producing the $-C_4=C_5-$ double bond structure.

This similarity of initial electron transfer processes between electrochemical oxidations at the p.g.e. and enzymic oxidations is a potential bridge to understanding both the mechanisms and specificity of enzyme oxidations and may perhaps provide a means to understanding the physiological and pharmocological behavior of the compounds.

Attention is given to all the naturally-occurring xanthines, i.e., all the N-methylated combinations with the exclusion of N_9 methylation, but the most thorough investigation centers around the three xanthines commonly ingested via beverages, namely: 1,3-dimethylxanthine (theophylline) (9), 3,7-dimethylxanthine (theobromine) (10) and 1,3,7-trimethylxanthine (caffeine) (11).



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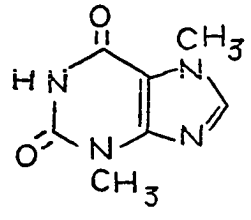

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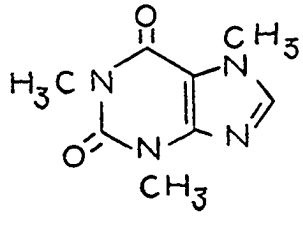

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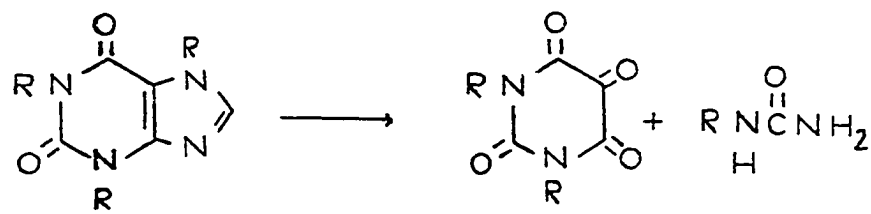
Chemistry of the Xanthines

Xanthine was found in the pancreas gland by Scherer[8] and has

been identified in tea,[9] cow's milk,[10] human urine[11] and in sugar beets.[12] Runge[13] discovered caffeine in coffee in 1820, Woskresensky[14] found theobromine in cocoa beans in 1841 and Kossel[15] extracted theophylline from tea leaves in 1888. 1-Methylxanthine is the most important purine constituent in human urine with 7-methylxanthine ranking second.[16] These, along with 1,7-dimethylxanthine and 3-methylxanthine are reportedly present in human urine only after intake of coffee and/or large doses of theobromine, theophylline and caffeine.[17] 7-Methylxanthine has also been isolated from yeast.[18]

Fischer established the true structure of xanthine,[19] caffeine,[20] theobromine,[21] theophylline,[22] 7-methylxanthine[23] (heteroxanthine) and 1,7-dimethylxanthine (paraxanthine).

Caffeine is oxidized by moist chlorine to dimethyl alloxan and methyl urea, theobromine to methyl alloxan and methyl urea and theophylline is oxidized by potassium chlorate-hydrochloric acid to dimethyl alloxan and urea.[16] The general reaction is illustrated by Equation 3. Caffeine in strong contrast to the other methylated xanthines is unstable in alkali producing first caffeidenecarboxylic acid (12) and then caffeidine (13) as illustrated in Equation 4.[16]



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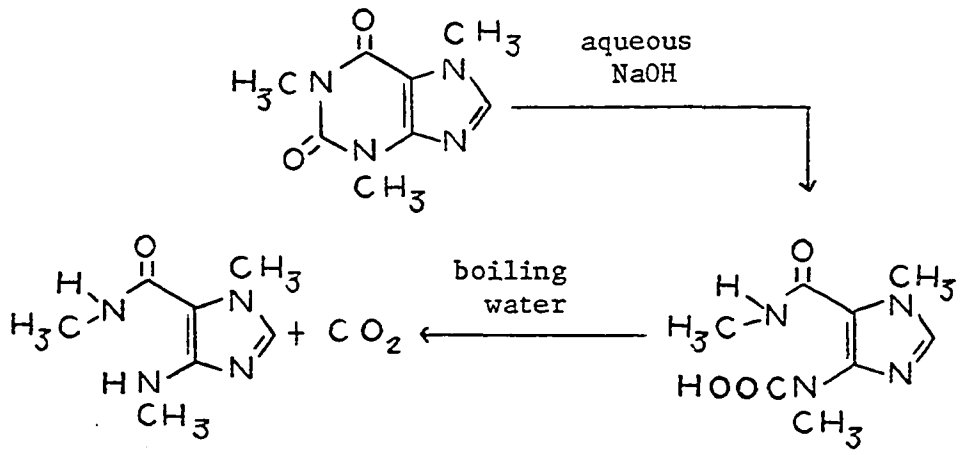
N-Methylated Xanthine alloxan urea

R = CH_3 or H



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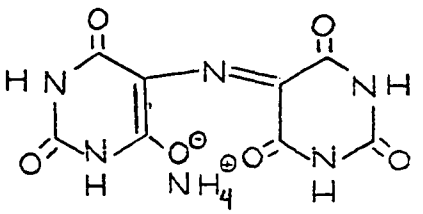
The formation of a purple color due to the ammonium salt of purpuric acid (14), when ammonia is added to the solution of an oxida-



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tion reaction, is a characteristic reaction of uric acid and related purines and is known as the murexide reaction.[16]

Some of the properties of the N-methylated xanthines are summarized in Table 1. Cavalieri and co-workers[24] conclude from the correlation of spectral shift with pH that in the various xanthines, the sequence of the three dissociable hydrogen atoms is N_3, then N_7 and finally N_1.

Caffeine cannot enolize or ionize, i.e., its spectrum remains unchanged and the characteristic selective absorption can be ascribed to carbonyl functions. In theophylline below pH ca. 7, the 6-oxygen

Table 1

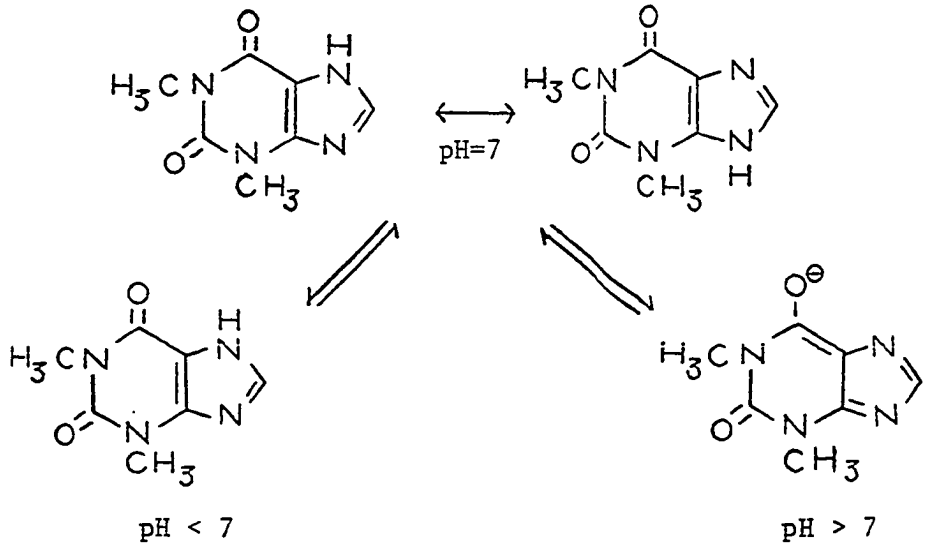
Properties of the N-Methylated Xanthines

Xanthine: Position of N-Methyl Groups	m.p.[26,27] °C	Solubility in Water	λ_{max} mμ		pK[a]	Reference
			Neutral	Basic		
1-	(dec.)	sparingly	266	241 276	7.7 12.05	23,24
3-	227-229 (dec.)	350 parts boiling water	271.5	278	8.8	24,25
7-	380 (dec.)	sparingly	269	291	7.0 10.7	24,25
1,3-	268	120 parts cold water	272	278	8.7	24,25
1,7-	298-299	sparingly	269	290	8.8	24,25
3,7-	290 subl.	2000 parts cold water	273	---	9.9	24,25
1,3,7-	236.5	45.6 parts cold water	273	273	---	23,25

[a] pK for the dissociation $R = H^+ + R^-$.

does not exist to any appreciable extent as a hydroxyl group, in neutral solution the hydrogen resonates between N_7 and N_9 and in basic solutions the major contribution is from the anion. These structures and equilibria are illustrated in Equation 5. Similar equilibria for 3-methylxanthine are represented in Equation 6. Theobromine also exists in equilibrium as an anion (15) in basic solutions. These four xanthines therefore are placed in a single class as regards their spectral characteristics.[24]

Xanthine, 1-methylxanthine, 7-methylxanthine and 1,7-dimethyl-



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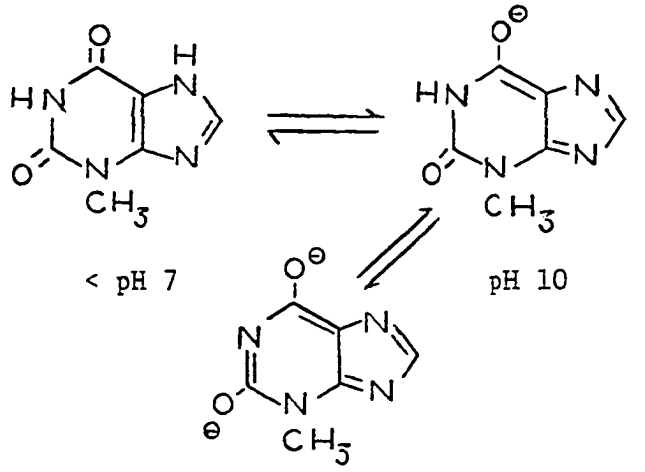

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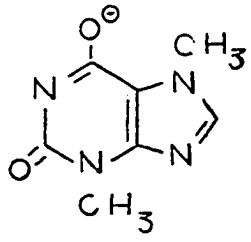

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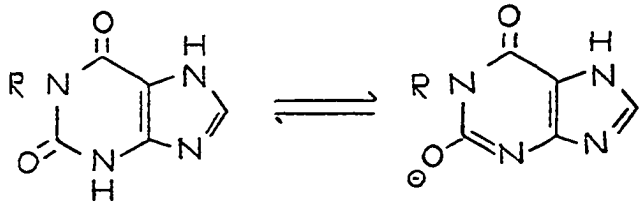
xanthine reveal a different spectral pattern and their anion equilibria can be represented as in Equation 7.



pH 7 pH 13 (7)

R = CH_3 or H

Pharmocological Significance

1,7-Dimethylxanthine possesses marked antithyroid activity.[28] The methylated xanthines are extensively used as diuretics, i.e., an agent that increases the flow of urine, in both humans and a wide variety of animal species.[29] The measured diuretic activity of each is summarized in Table 2.[30]

Caffeine is most noted for its stimulation of the central nervous system (C.N.S.) and is used to allay drowsiness and fatigue and to increase the capacity for intellectual effort. Such effects appear after the intake of 150 - 250 mg caffeine, the equivalent of two

Table 2

Diuretic Activity of the Xanthines

Compound	Oral Dosage mg /100 g	Diuretic[a] Activity
1-Methylxanthine	5	0
3-Methylxanthine	5	43
7-Methylxanthine	5	90
1,3-Dimethylxanthine	5	348
3,7-Dimethylxanthine	5	187
1,3,7-Trimethylxanthine	5	123
1,7-Dimethylxanthine	5	34
Xanthine	5	0

[a] Determined in rats: Values derived by arbitrary planimetric method from urine volumes collected over an 8 hr period.

Table 3

Relative Potencies of the Xanthines

Xanthine	C.N.S. Stimulation	Respiratory Stimulation	Coronary Dilatation	Cardiac Stimulation	Skeletal Muscle Stimulation
Caffeine	1[a]	1	3	3	2
Theophylline	2	2	1	1	3
Theobromine	3	3	2	2	1

[a] 1 = most potent.

cups of coffee or tea. The human toxic level is 10 g.[29] The relative potencies of the three xanthines used as stimulants are given in Table 3.[31]

Extensive studies by Starr and co-workers[32] on the comparative cardiovascular respiratory and metabolic effects of caffeine and theophylline indicate that (1) theophylline has the most active cardiovascular effects, e.g., increases cardiac output 25.8% compared to 14.8% for caffeine, (2) increases metabolic rate (7.8% compared to 6.9%) and (3) increases the respiratory rate 2.7% compared to a 4.1% decrease for caffeine. As a result of both central stimulation and action on heart and vascular musculature, theophylline is used clinically to increase cardiac output and to alleviate bronchial asthma.[29]

Xanthine Metabolism

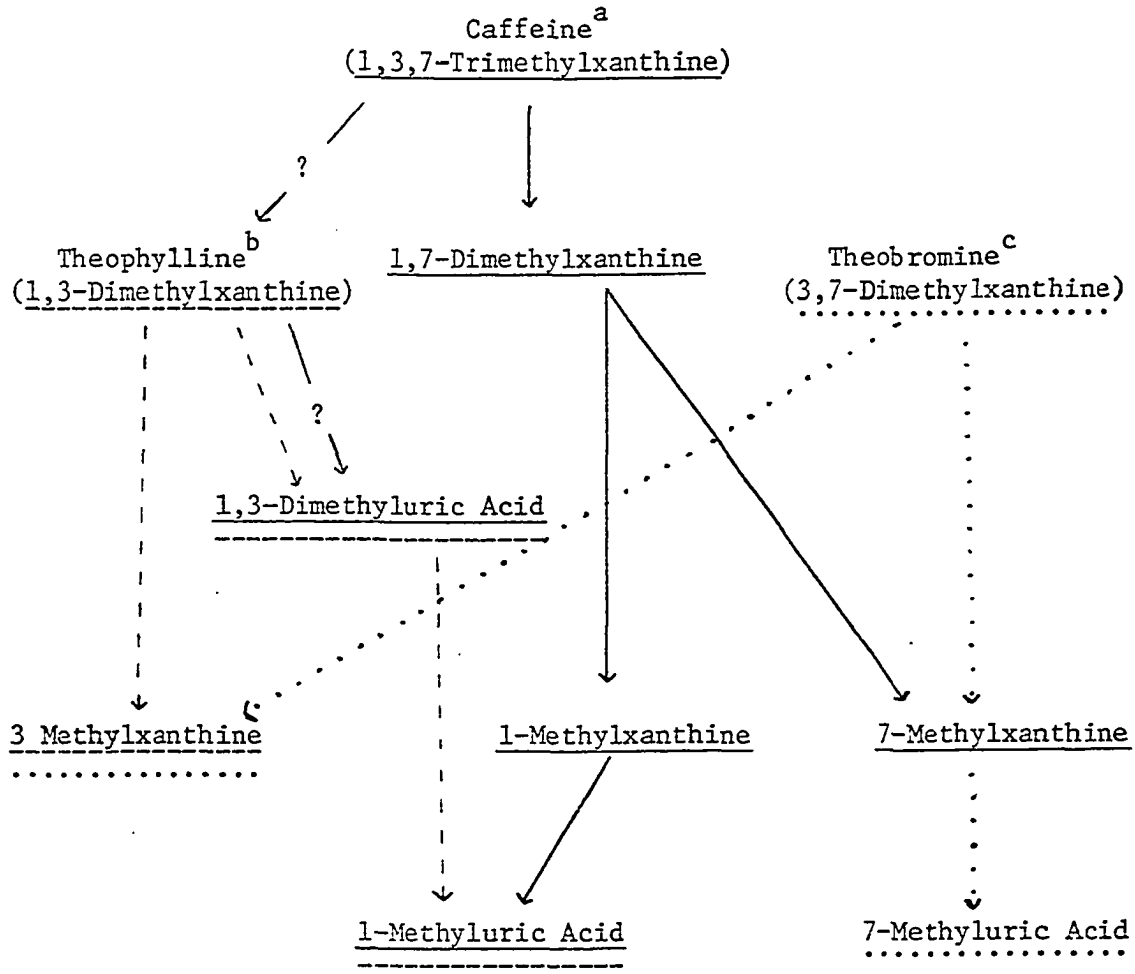
Investigations into the fate of caffeine in the body followed the observation of marked physiological effects upon drinking tea and coffee. Prior to 1916-1917 methods of detection were too inexact to be of value but with the development of colorimetric methods both Benedict[33] and Mendel and Wardell[34] reported that caffeine ingestion by humans increases uric acid excretion. A series of studies then followed. Myers and Wardell[35] reported in 1928 that caffeine and theophylline ingestion by humans causes increased uric acid excretion. However, they noted that such was not the case with theobromine and suggested that perhaps methylated uric acids responded to the colorimetric method in varying degrees, and were responsible for the observed results. Sixteen years later with the advancement of enzymatic procedures for distinguishing between uric

acid and its methylated derivatives, Buchanan, Block and Christman[36] reported that true uric acid is not excreted as a result of the conversion of caffeine and theophylline to uric acid. Evidence for the presence of 1-methyluric acid, 3-methyluric acid and 1,3-dimethyluric acid in human urine was presented. In 1951, Brodie, Axelrod and Reichenthal[37] studied the metabolism of theophylline in man and concluded that only a small amount of the drug appears unchanged in the urine. The major pathway of metabolic transformation is the oxidation to 1,3-dimethyluric acid, which was isolated. The following year, Weinfeld and Christman[38] succeeded in isolating 1-methyluric acid in human urine after ingestion of caffeine and both 1-methyl-and 1,3-dimethyluric acid after ingestion of theophylline. The work was completed with the report of Cornish and Christman[17] in 1957. The probable metabolic pathways of caffeine, theophylline and theobromine presented in their report is reproduced in Figure 1.

Following 1 gm doses of each of the compounds 62 per cent of the theobromine, 77 per cent of the theophylline and 66 per cent of the caffeine was excreted in the form of methylxanthines and methyluric acids within 48 hr. The major part of the theobromine was excreted as methylxanthine. Methyluric acids predominated as excretory products of theophylline. After caffeine administration, approximately equal amounts of methylxanthines and methyluric acids were present in the urine.

The order of demethylation in man, N_3, N_7 and N_1 is consistent with the results of Cavalieri and co-workers.[24] Total demethylation was excluded as a result of evidence which indicated that there was no

Figure 1



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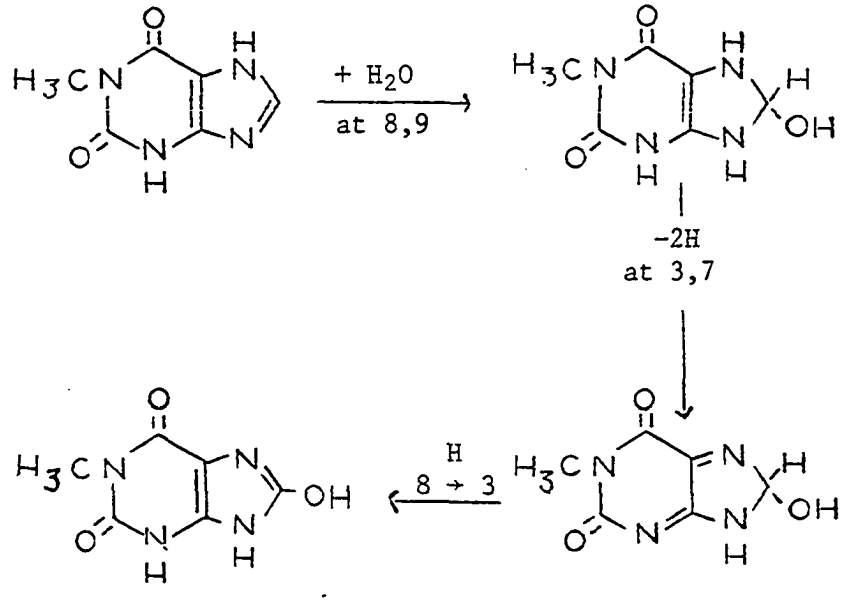
[a] Solid line = caffeine, [b] Dashed line = theophylline, [c] Dotted line = theobromine.

Compounds Excreted by Man after

Ingestion of Caffeine, Theobromine and Theophylline

accumulation of xanthine and that uric acid excretion was not significantly increased.

Milk xanthine oxidase, a flavoprotein enzyme, catalyzes the oxidation of certain purines, hypoxanthines and xanthine to uric acid. It was early characterized as a very specific enzyme; the introduction of amine and methyl groups into the xanthine molecule inhibits the activity of the enzyme.[39] Bergmann and Dikstein[40] observed that xanthine oxidase from cow's milk or human liver show identical substrate specificity. Among the N-methylated xanthines, only 1-methylxanthine is attacked by the enzyme and converted to 1-methyluric acid. The proposed hydration-dehydrogenation is illustrated in Equation 8. The



(8)

8-Lactim of

1-Methyluric Acid

decisive step is therefore the dehydrogenation of the grouping $HN_3-C_4=C_5-N_7H$ to the corresponding dienic system. Therefore, the other

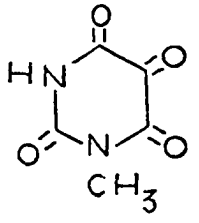
methylated xanthines are excluded from oxidation by this enzyme.

The occurrence of N-methylated uric acids in human urine led Dikstein, Bergmann and Henis[41] to seek another enzyme source. The presence of xanthine oxidase in the bacteria Pseudomonas aeruginosa and the positive results of Franke and Hahn[42] as regards its oxidation of N-methylated xanthines led to the choice of bacterial xanthine oxidases. The greater solubility of the N-methylated uric acids made it plausible for them to be produced by intestinal flora and absorbed into the general circulation.

Their studies indicate that contrary to the reports of Franke and Hahn, only 3-methylxanthine is oxidized to 3-methyluric acid by bacterial oxidase present in various strains of Pseudomonas and Vibrio. Since methylation at N_3 does not prevent oxidation, the enzymatic mechanism cannot be represented by dehydrogenation of $HN_3-C=C_5-N_7H$ as with the other xanthine oxidase. Instead the bacterial enzyme follows the manner of activity common to "aldehyde oxidase" with attack requiring an isolated C=N grouping. However, 1-methylxanthine also has an isolated C=N group but is not oxidized by the bacterial oxidase. Perhaps the secret to this specificity resides in the structure of the bacterial xanthine oxidase. A flavin group has been definitely established in the prosethetic group (the non-amino acid portion of the protein which lends stability to the protein) of mammalian xanthine oxidase.[43] The dienic system, $HC_3-C_4=C_5-N_7H$, in the dehydrogenated xanthine is analogous to the reactive portion of the riboflavin in the prosthetic group. Whether the bacterial xanthine oxidase contains flavin as its prosethetic group is not yet known.

Previous Electrochemical Studies

Only one study of the electrochemical oxidation of theobromine and caffeine is reported. Fichter and Kern[44] reported the oxidation of theobromine at a PbO_2 anode in 4 N H_2SO_4 and identified the products as methyl alloxan (16), 3,7-dimethyluric acid, methyl parabanic acid



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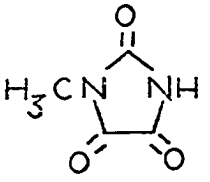
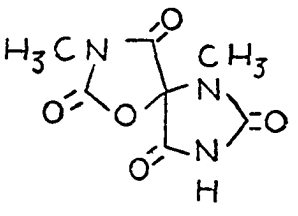


17

(17), ammonia, methyl amine and carbon dioxide. Caffeine oxidized in a similar manner yielded dimethylalloxan and apocaffeine (18) as identi-



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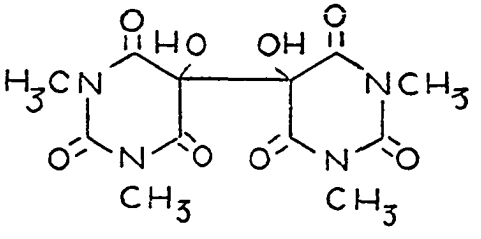
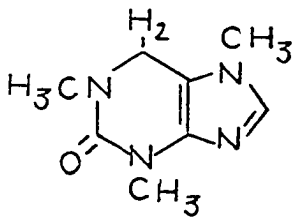


19

fied products. The same compound, oxidized without a diaphragm separating the anode and cathode compartments produced 1,1', 3,3'-tetramethyl alloxantin (19) in a 68.1% yield.

Although no reduction of the N-methylated xanthines was observed at the dropping mercury electrode in this present study, desoxycaffeine (20) and desoxytheophylline (21) have been reported in electrolytic-reductions of the parent compounds.[45,46]



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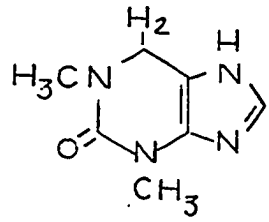


21

RESULTS AND DISCUSSION

Voltammetry of the N-Methylated Derivatives of Xanthine

Oxidation Potentials

Solutions of all the naturally occurring N-methylated xanthines were prepared in 1 M HOAc and voltammograms were obtained at sweep rates of 20 and 200 mv sec^{-1} respectively at the stationary pyrolytic graphite electrode (p.g.e.). The results are summarized in Table 4.

The most easily oxidized derivative is 1-methylxanthine (E_p = 1.10 v, 20 mv sec^{-1}; E_p = 1.15 v, 200 mv sec^{-1}). Xanthine methylated at the 7- and 3- position, respectively, are oxidized at potentials 160 to 200 mv more positive. The dimethylated derivative, 1,7-dimethylxanthine is also oxidized within the potential range of the latter two monomethylated derivatives (E_p = 1.25 v, 20 mv sec^{-1}; E_p = 1.31 v, 200 mv sec^{-1}) and is more easily oxidized than 1,3-dimethylxanthine (theophylline) by 50 to 60 mv. 3,7-Dimethylxanthine (theobromine) and 1,3,7-trimethylxanthine (caffeine) are the most difficultly oxidized (E_p = 1.5 v, 20 mv sec^{-1}; E_p = 1.6 v; 200 mv sec^{-1}). These latter peaks are very close to background discharge potentials, occurring as shoulders on the rise of the background discharge, and are therefore difficult to characterize with an accuracy of better than about 25 mv.

Table 4

Oxidation Potentials of N-Methylated Xanthines

at Pyrolytic Graphite Electrode in 1 M HOAc

Compound	Concn. mM	Sweep Rate $mv\ sec^{-1}$	Peak Potential v vs. S.C.E.	i_p/C[a] μa/mM	i_p/AC[b] $\mu a/mm^2 mM$
1-Methylxanthine	1.06	200	1.15	98.7	7.88
"	"	20	1.10	33.5	2.68
7-Methylxanthine	1.23	200	1.33	61.5	4.93
"	"	20	1.26	21.2	1.69
3-Methylxanthine	0.33	200	1.35	115.0	9.19
"	"	20	1.27	43.0	3.44
1,7-Dimethylxanthine	0.50	200	1.31	68.6	5.49
"	"	20	1.25	37.8	3.02
1,3-Dimethylxanthine	0.50	200	1.37	55.2	4.42
"	"	20	1.30	23.2	1.86
3,7-Dimethylxanthine	0.50	200	1.60	96.4	7.71
"	"	20	1.50	32.4	2.59
1,3,7-Trimethylxanthine	0.50	200	1.60	104.4	8.35
"	"	20	1.50	55.2	4.41

[a] i_p = Average peak current of triplicate scans

[b] A = Area of electrode; 12.5 mm^2

pH Studies

Solutions of xanthine and its N-methylated derivatives were prepared in background electrolyte solutions over the pH range 0 to 12.5. All solutions were 0.5 mM in concentration except for 3-methylxanthine (0.38 mM) and xanthine (which was very difficultly soluble and therefore used as a saturated solution). Voltammograms were obtained on a Sargent Model XV Polarograph at a sweep rate of 3.3 mv sec^{-1}. The results are summarized in Table 5.

The equations of the variation of peak potential (E_p) with pH are as follows:

xanthine (Figure 2)

$$E_p = 1.07 - 0.060\ pH; \qquad (9)$$

1-methlyxanthine (Figure 3)

$$E_p = 1.05 - 0.049\ pH; \qquad (10)$$

7-methylxanthine (Figure 4)

$$E_p^I = 1.19 - 0.049\ pH \qquad (11a)$$

and

$$E_p^{II} = 1.22 - 0.042\ pH; \qquad (11b)$$

3-methylxanthine (Figure 5)

$$E_p^I = 1.20 - 0.056\ pH \qquad (12a)$$

and

$$E_p^{II} = 1.27 - 0.050\ pH; \qquad (12b)$$

1,7-dimethylxanthine (Figure 6)

$$E_p = 1.31 - 0.059\ pH; \qquad (13)$$

1,3-dimethylxanthine, theophylline (Figure 7)

$$E_p^I = 1.35 - 0.069\ pH \qquad (14a)$$

Table 5

Effects of pH on Oxidation Potential of the Xanthines at

Background	pH	Xanthine v vs.S.C.E.	1-Methyl-xanthine v vs.S.C.E.	3-Methylxanthine v vs. S. C. E.			7-Methyl-xanthine v vs.S.C.E.		1,7-Di-xanth v vs.
2 M H_2SO_4	0	1.08	1.12		1.26			1.23	1.2
McIlvaine	2.0	0.91	0.93		1.18			1.13	1.1
1 M HOAc	2.3	0.98	1.01		1.18			1.21	1.2
McIlvaine	2.8	0.86	0.87		1.14			1.09	1.1
Acetate	3.6	0.87	0.87		1.09			1.11	1.1
McIlvaine	4.0	0.81	0.82		1.07			1.07	1.0
Acetate	4.6	0.78	0.79		1.04			1.04	1.0
McIlvaine	4.8	0.75	0.78			1.11		1.02	1.0
Acetate	5.5	0.71	0.74	0.90*	0.97	1.09		0.98	1.0
McIlvaine	6.1	0.68	0.73	0.87	0.97			0.96	0.9
McIlvaine	7.0	0.64	0.69	0.80	0.93		0.86	0.92	0.8
McIlvaine	8.1	0.64	0.63	0.73	0.87	1.09		0.88	0.8
Ammonia	8.5	0.54	0.60	0.73	0.87		0.76	0.89	0.7
Ammonia	9.0	0.50	0.56	0.69			0.73	0.87	0.7
Hydroxide	11.9	0.37	0.51[a]	0.52	0.63		0.62	0.75	0.7
Hydroxide	12.5	0.31	0.49[a]	0.52			0.57	0.70	0.7

* Potentials underlined appear as shoulders, not peaks; [a] Potentials very diffi

[b] pH = 5.7; [c] pH = 7.2; [d] Values not used in least square analysis.

Table 5

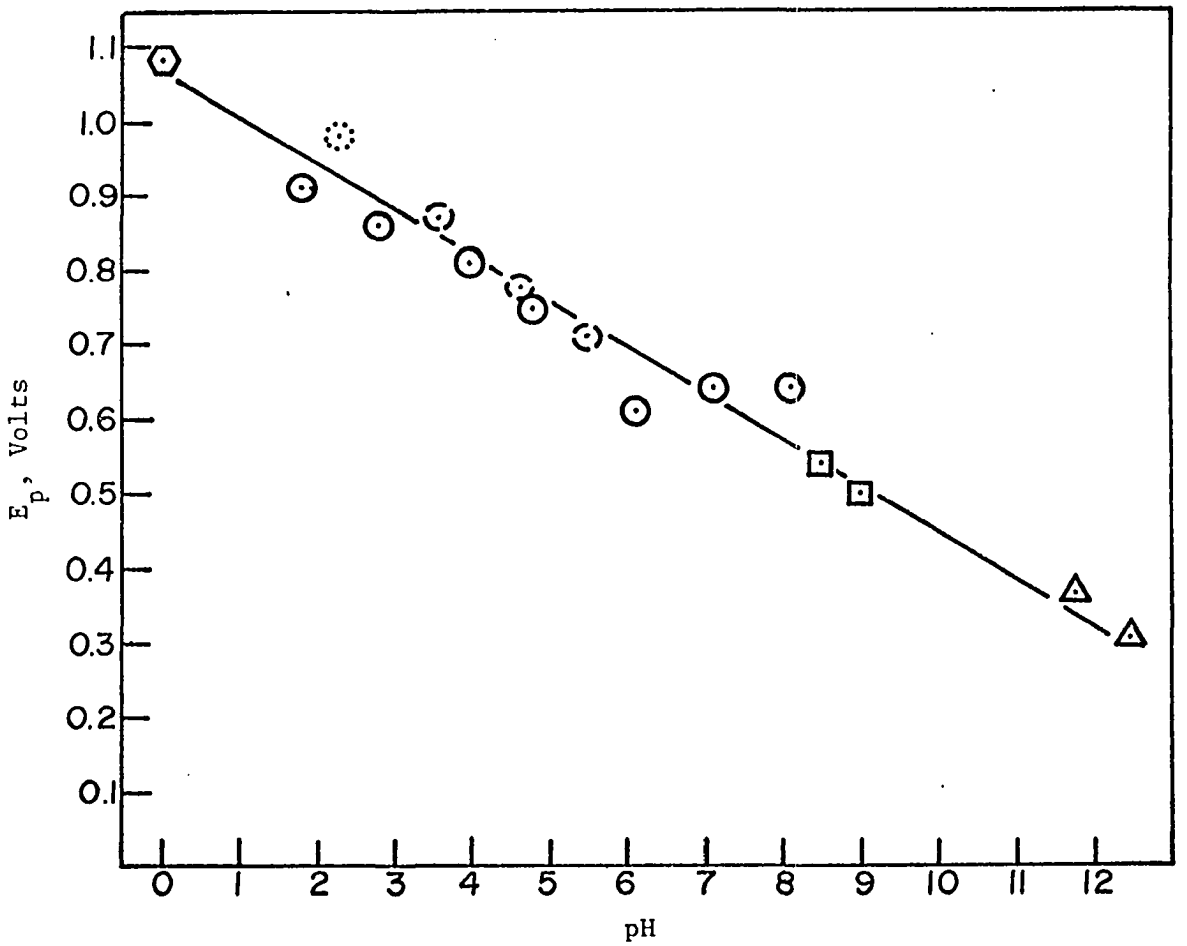
n Oxidation Potential of the Xanthines at Pyrolytic Graphite Electrode

yl-ne C.E.	3-Methylxanthine v vs. S. C. E.		7-Methyl-xanthine v vs.S.C.E.	1,7-Dimethyl-xanthine v vs.S.C.E.	1,3-Dimethyl-xanthine v vs.S.C.E.		3,7-Dimethyl-xanthine v vs.S.C.E.	1,3,7-Tri-methyl-xanthine v vs.S.C.E.
	1.26			1.23	1.24			
	1.18			1.13	1.17			
	1.18			1.21	1.20	1.32*	1.52	1.50
	1.14			1.09	1.15			
	1.09			1.11	1.15	1.23	1.43	1.43
	1.07			1.07	1.08	1.10		
	1.04			1.04	1.08	1.08 1.18	1.36	1.38
		1.11		1.02	1.05	1.00		
0.90*	0.97	1.09		0.98	1.02	0.98 1.14[b]	1.32	1.37
0.87	0.97			0.96	0.96	0.95 1.09		
0.80	0.93		0.86	0.92	0.87	0.85[c] 1.08[c]		
0.73	0.87	1.09		0.88	0.82	0.79 0.98		
0.73	0.87		0.76	0.89	0.79	0.77 0.98		
0.69			0.73	0.87	0.74[d]	0.77		
0.52	0.63		0.62	0.75	0.75[d]			
0.52			0.57	0.70	0.75[d]			

lders, not peaks; [a] Potentials very difficult to determine vs. M.S.E. reference electrode;

used in least square analysis.

Figure 2

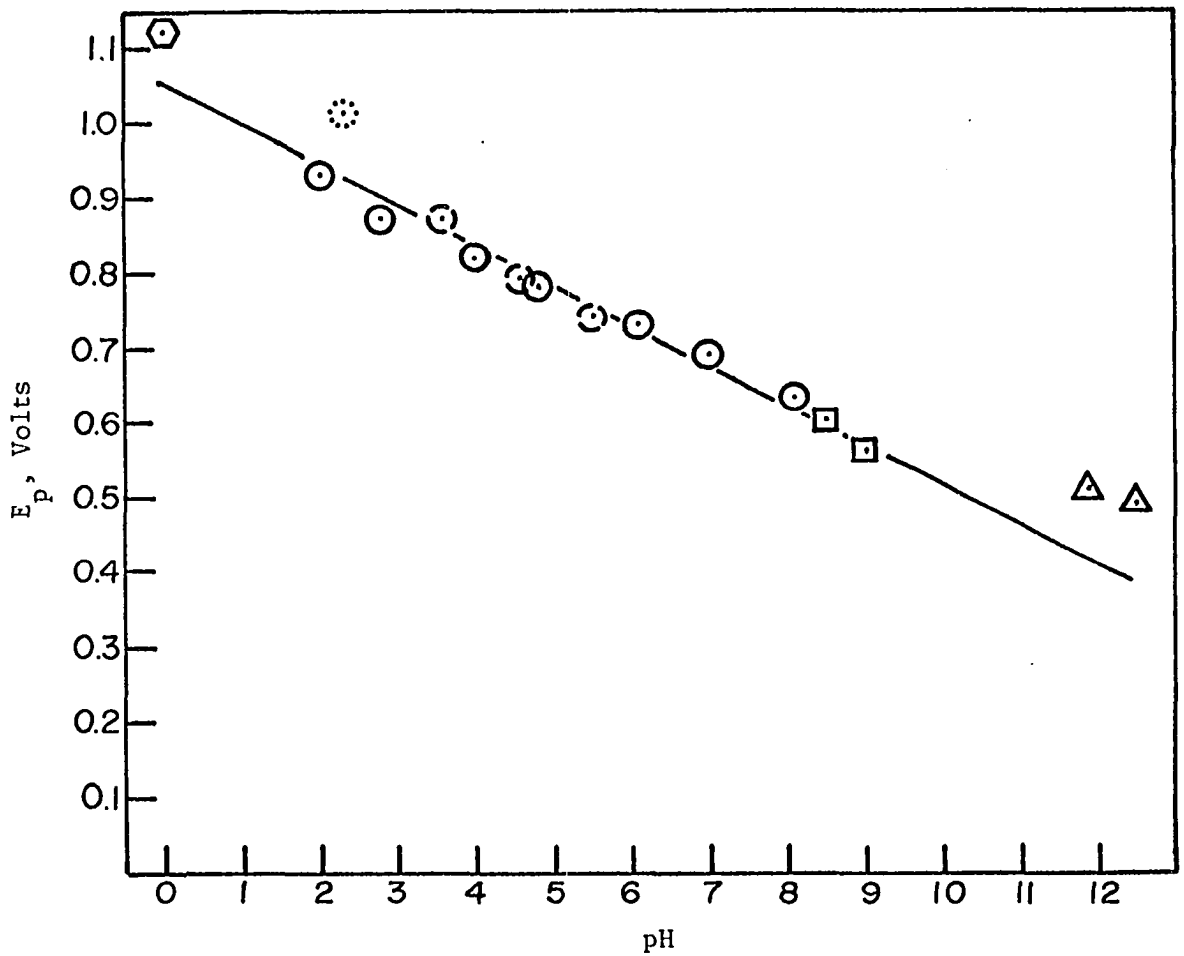


Notation for Figures 2 - 9

- = 2 M H_2SO_4
- = McIlvaine Buffer
- = 1 M HOAc
- = Acetate Buffer
- = Ammonia Buffer
- = Hydroxide Buffer

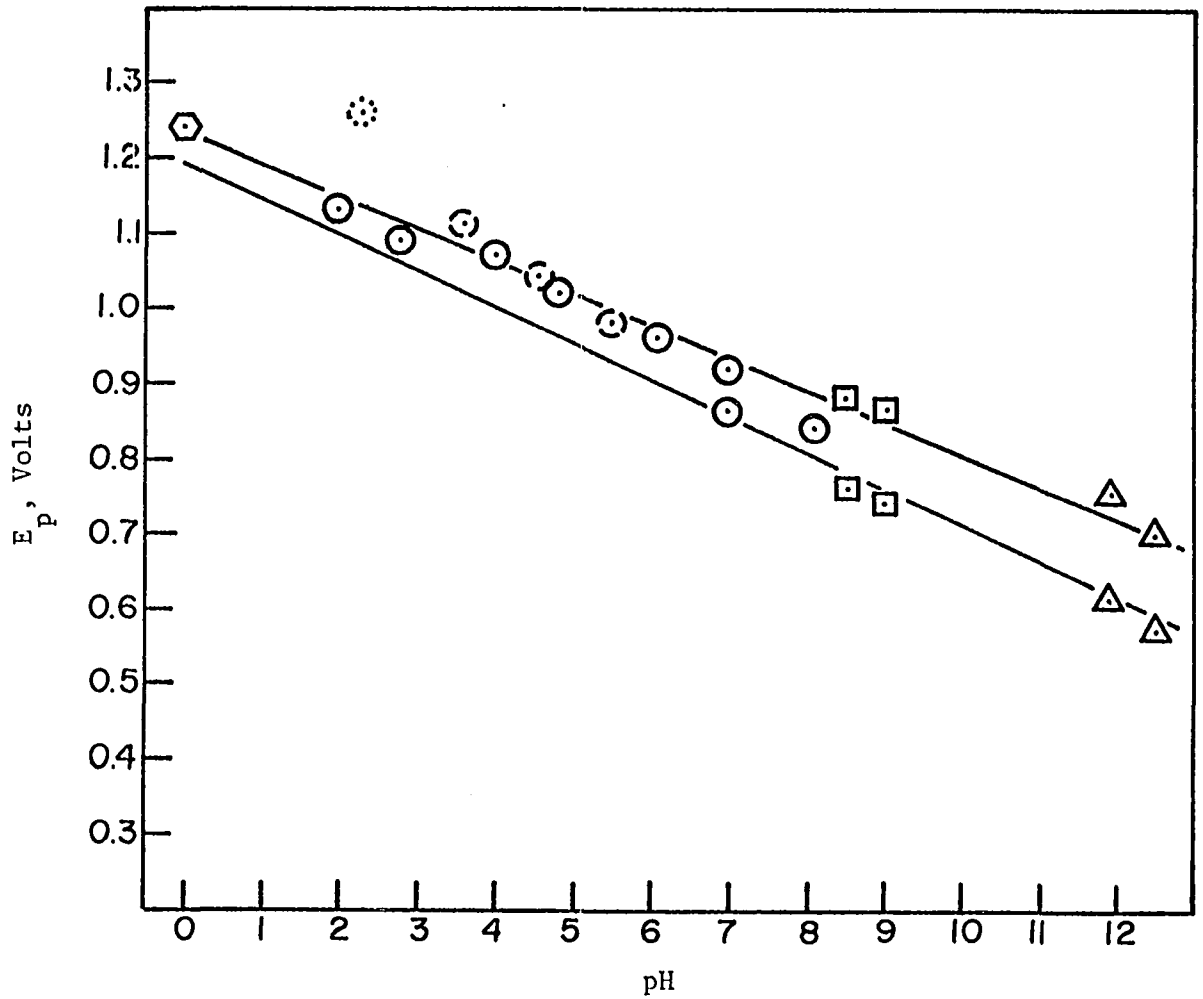
Variation of E_p with pH for Xanthine Oxidation

Figure 3



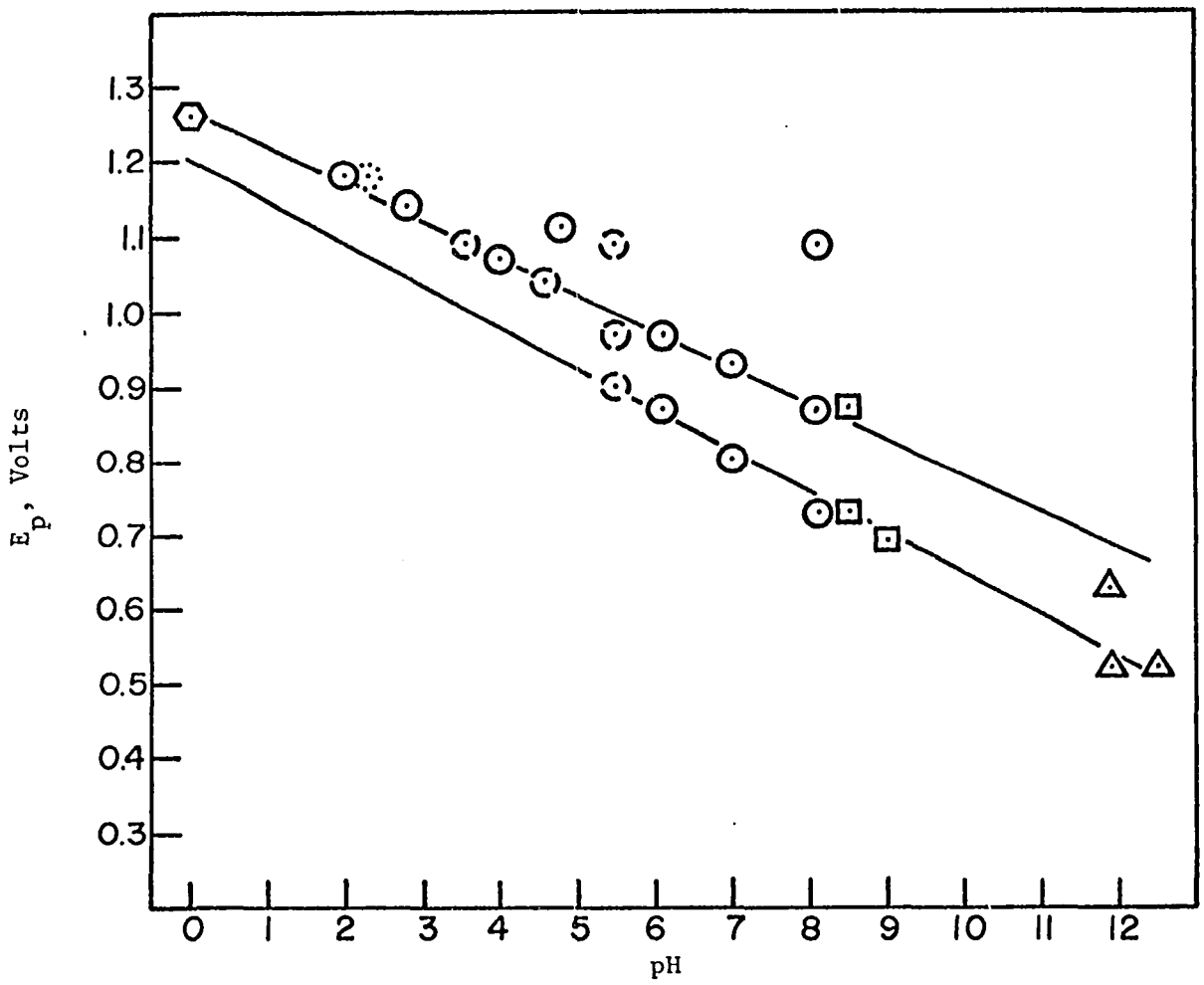
Variation of E_p with pH for 1-Methylxanthine Oxidation

Figure 4



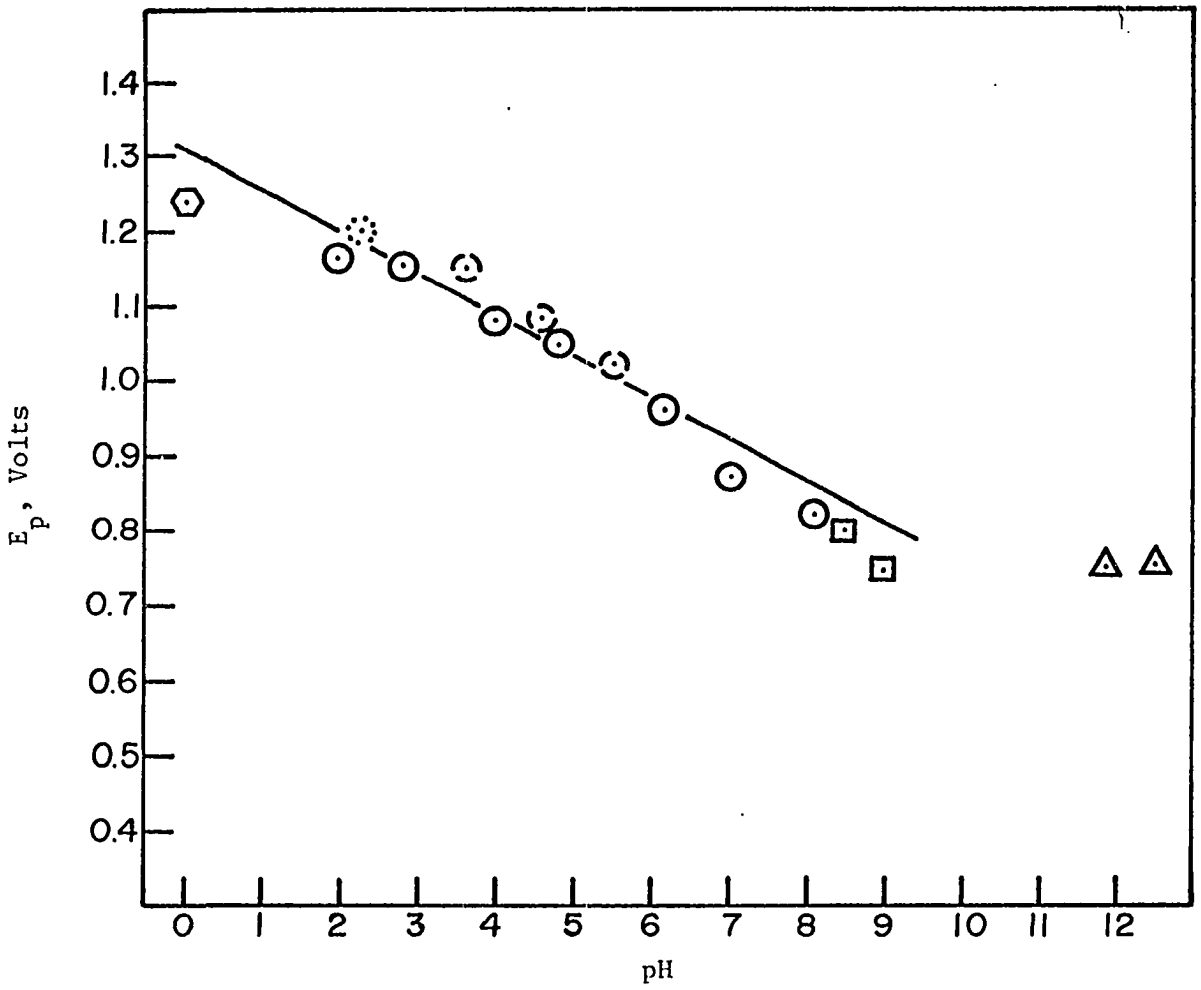
Variation of E_p with pH for 7-Methylxanthine Oxidation

Figure 5



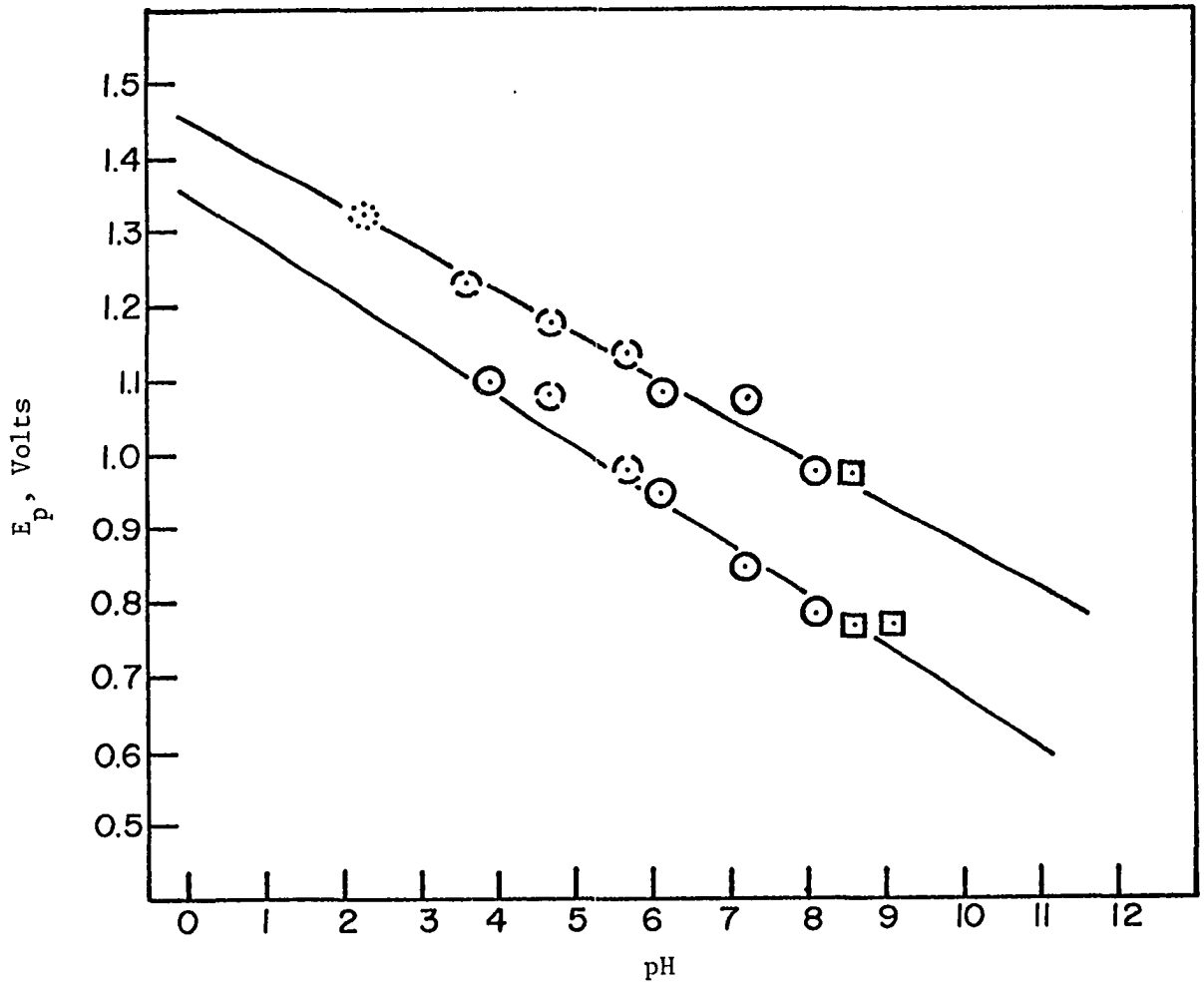
Variation of E_p with pH for 3-Methylxanthine Oxidation

Figure 6



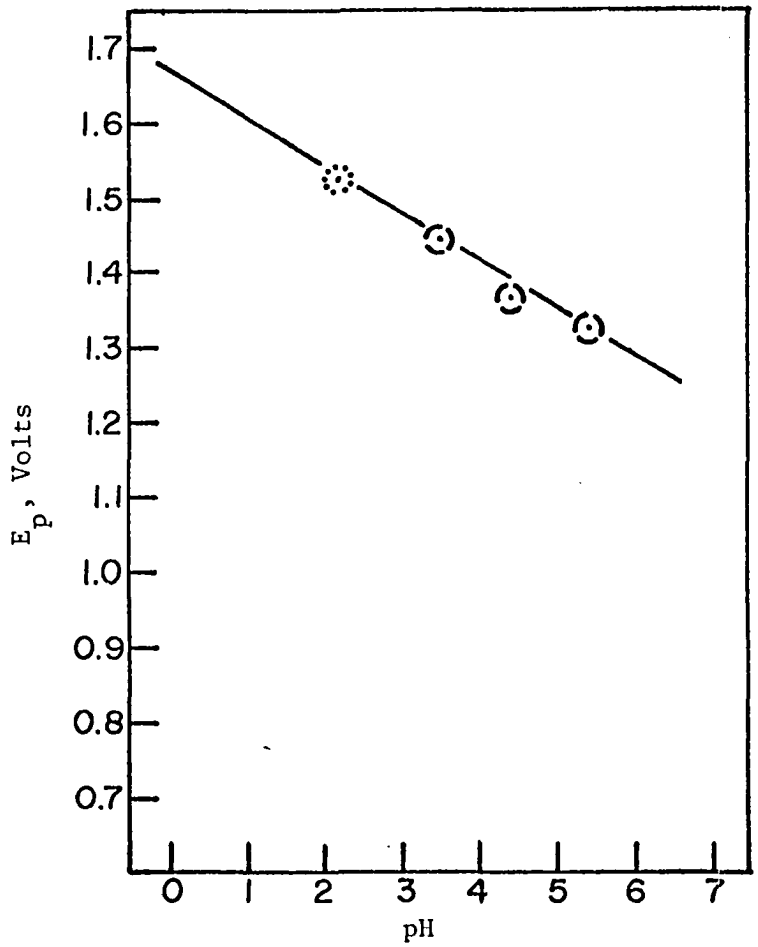
Variation of E_p with pH for 1,7-Dimethylxanthine

Figure 7



Variation of E_p with pH for 1,3-Dimethylxanthine (Theophylline) Oxidation

Figure 8

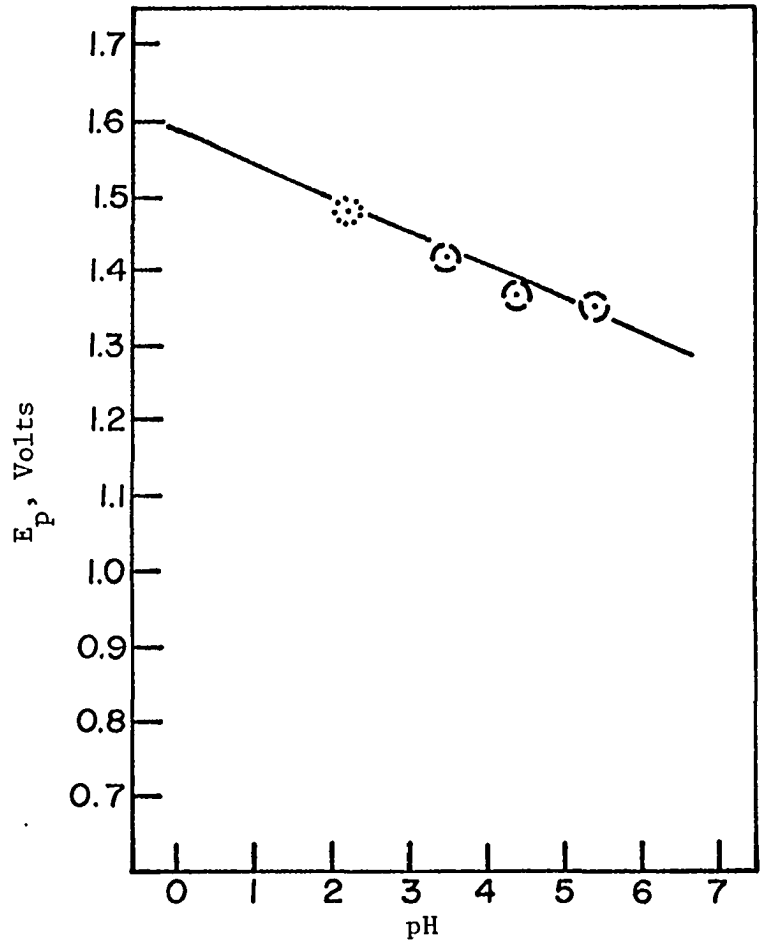


Variation of E_p with pH for

3,7-Dimethylxanthine

(Theobromine) Oxidation

Figure 9



Variation of E_p with pH for

1,3,7-Trimethylxanthine

(Caffeine) Oxidation

and

$$E_p^{II} = 1.45 - 0.056 \text{ pH} \qquad (14b)$$

3,7-dimethylxanthine, theobromine (Figure 8)

$$E_p = 1.67 - 0.064 \text{ pH}; \qquad (15)$$

1,3,7-trimethylxanthine, caffeine (Figure 9)

$$E_p = 1.59 - 0.042 \text{ pH}. \qquad (16)$$

The oxidation peaks of theophylline, theobromine and caffeine are masked by the background discharge in many of the McIlvaine buffers. Oxidation of the latter two can only be observed in 1 M HOAc and in acetate buffers, and even in these, oxidation occurs on the rise of the background discharge.

It is observed that 7-methylxanthine, 3-methylxanthine and 1,3-dimethylxanthine are oxidized by way of two pH dependent processes. A second oxidation peak at less positive potentials becomes apparent at pH 5.5 for 3-methylxanthine, pH 7.0 for 7-methylxanthine and pH 3.9 for 1,3-dimethylxanthine. The pH range involved and the presence of 7-methylxanthine in this phenomena rules out the possibility of linking this second observed process with the anion equibria expected for 3-methylxanthine, 1,3- and 3,7-dimethylxanthine and 1,3,7-trimethylxanthine in basic solutions as cited by Cavalieri and co-workers (Introduction).

Study of 1,3-dimethylxanthine at various sweep rates. Because theophylline is one of the compounds chosen for a thorough electrochemical study, a closer examination of the double oxidation process was undertaken for theophylline solutions at sweep rates of 5, 20, 200 and 500 mv sec^{-1}. Variations of E_p with pH for solutions 2.5 mM in theophylline are illustrated in Figures 10 - 13. Three pH dependent

electrode processes are evident. The oxidation occurring most easily, i.e., at the lowest positive potentials, first appears in the pH region 3.9 to 5.0 and is observed through pH 9.05. The variation of peak potential with pH for this reaction is stated as follows:

$$500 \text{ mv sec}^{-1};\ E_p = 1.42 - 0.070 \text{ pH} \qquad (17a)$$

$$200 \text{ mv sec}^{-1};\ E_p = 1.39 - 0.069 \text{ pH} \qquad (17b)$$

$$20 \text{ mv sec}^{-1};\ E_p = 1.26 - 0.056 \text{ pH} \qquad (17c)$$

$$5 \text{ mv sec}^{-1};\ E_p = 1.24 - 0.056 \text{ pH}. \qquad (17d)$$

A second process is observed occurring over the entire pH range at sweep rates of 5 and 20 mv sec^{-1}, but is not observed in every buffer used. It appears over the pH range 3.6 to 6.1 at sweep rates of 200 and 500 mv sec^{-1}. The variation of peak potential with pH is stated below:

$$500 \text{ mv sec}^{-1};\ E_p = 1.56 - 0.075 \text{ pH} \qquad (18a)$$

$$200 \text{ mv sec}^{-1};\ E_p = 1.52 - 0.073 \text{ pH} \qquad (18b)$$

$$20 \text{ mv sec}^{-1};\ E_p = 1.37 - 0.063 \text{ pH} \qquad (18c)$$

$$5 \text{ mv sec}^{-1};\ E_p = 1.32 - 0.059 \text{ pH}. \qquad (18d)$$

A third process is observed over the entire pH range 0 to 9.05 for the 5 and 20 mv sec^{-1} sweep rates. It is the only process occurring in 1 M HOAc at the fastest sweep rates. Equations for variation of peak potential with pH at the slower scan rates are as follows:

$$20 \text{ mv sec}^{-1};\ E_p = 1.44 - 0.061 \text{ pH} \qquad (19a)$$

$$5 \text{ mv sec}^{-1};\ E_p = 1.42 - 0.061 \text{ pH}. \qquad (19b)$$

It is noted that equations describing the pH dependence for the 5 and 20 mv sec^{-1} sweep rates are very similar in each case. Comparison of Equations 18c, 18d, 19a and 19b with 14a and 14b reveals that at the

Figure 10

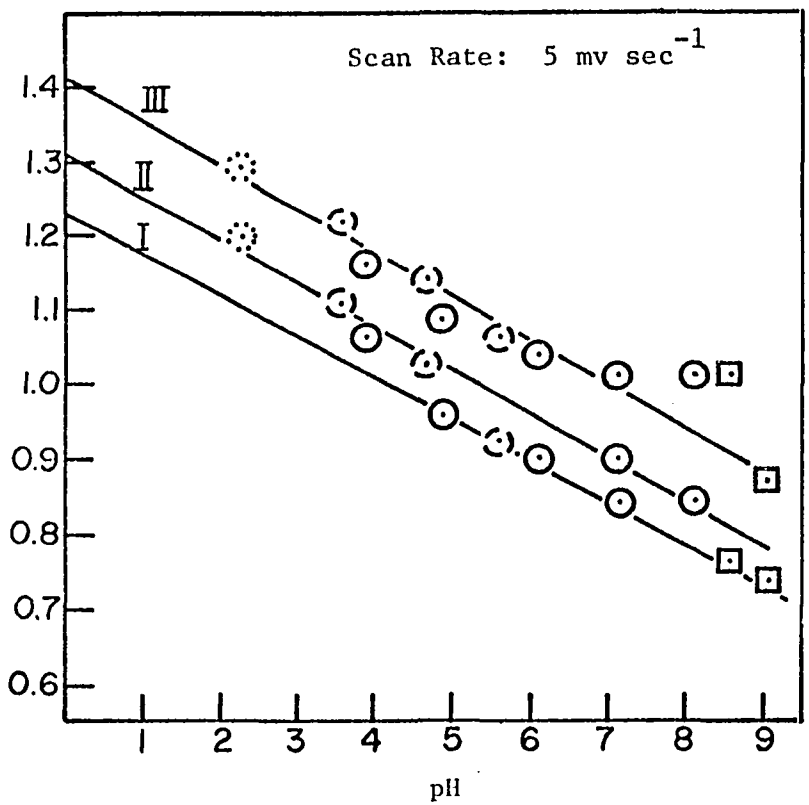
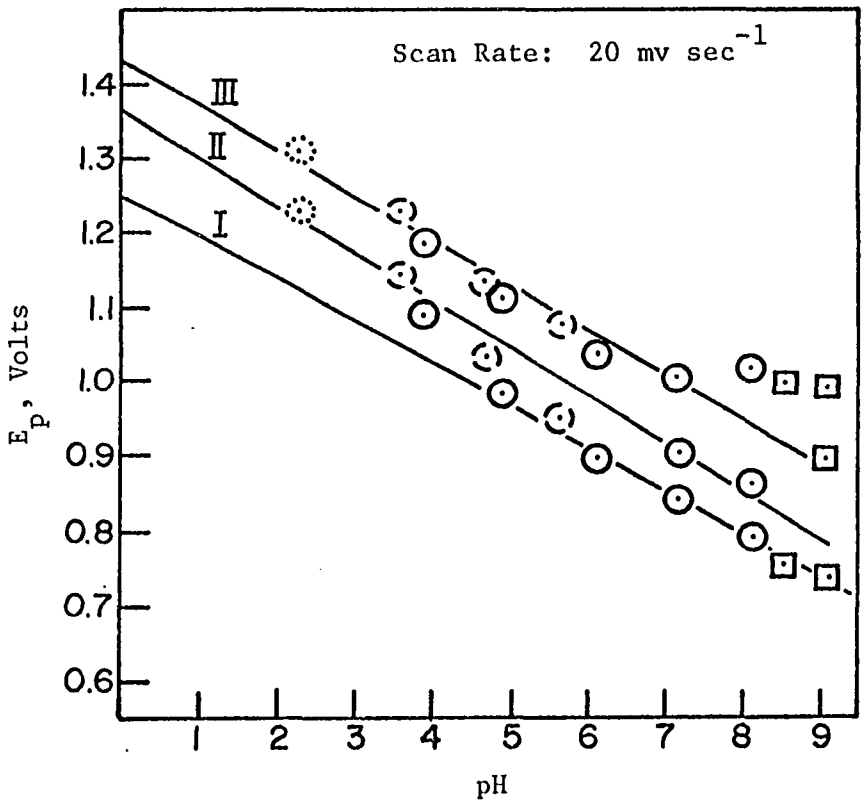


Figure 11



Figures 10 - 13:

⊙=1 M HOAc; ⊙= McIlvaine Buffer ⊙= Acetate Buffer ⊡ = Ammonia Buffer

Variation of E_p with pH for Theophylline Oxidation

Figure 12

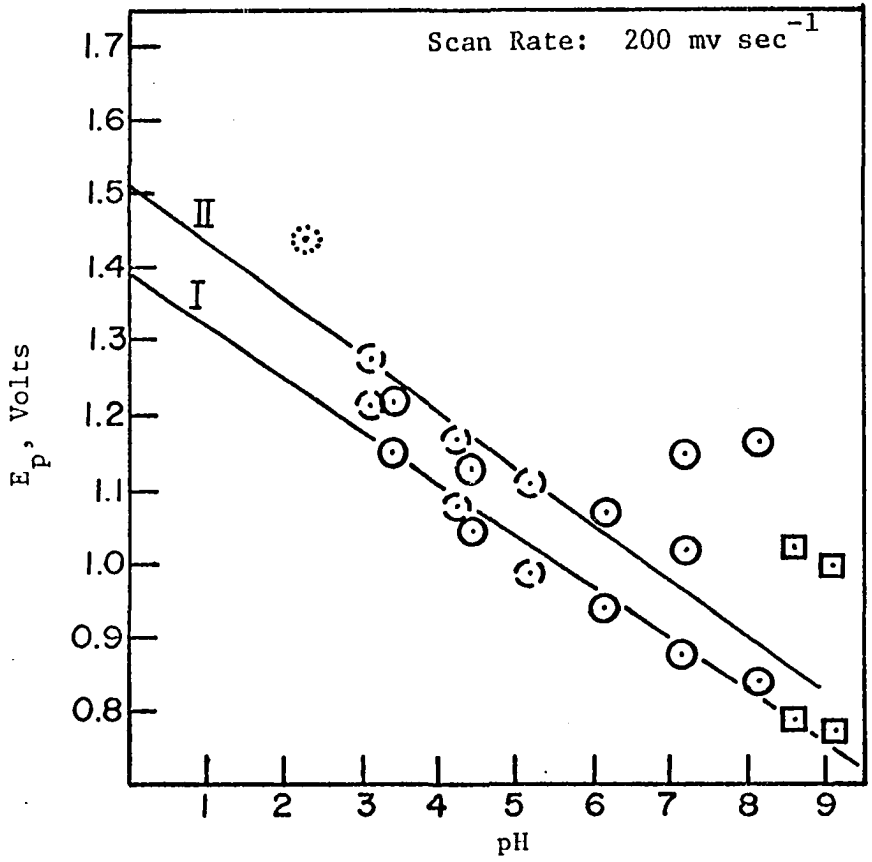
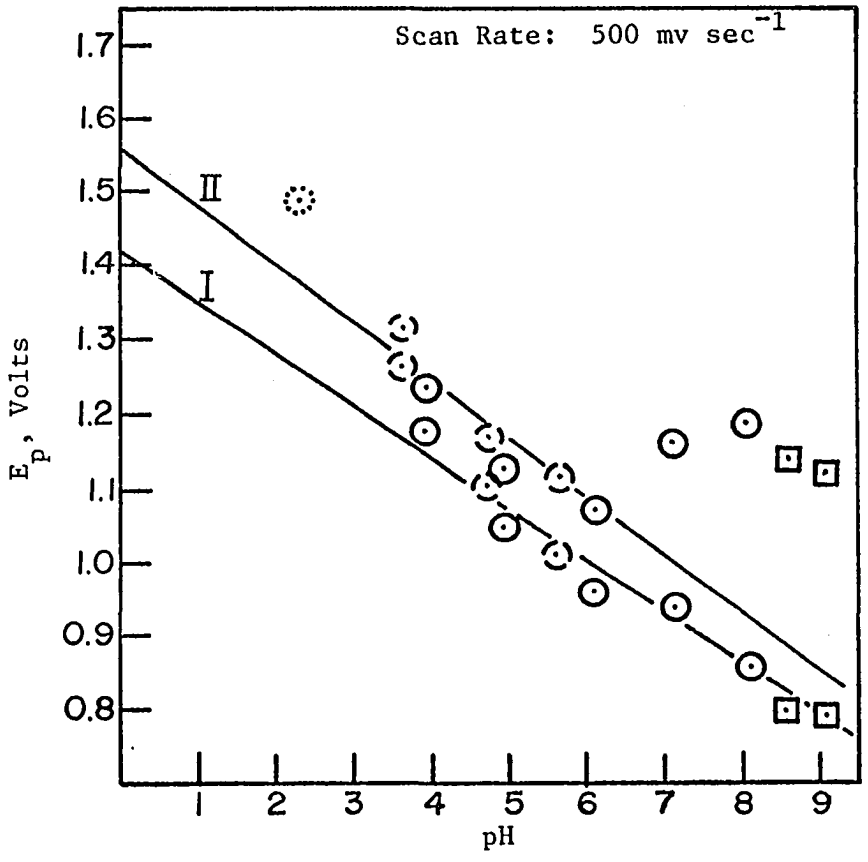


Figure 13



Variation of E_p with pH for Theophylline Oxidation

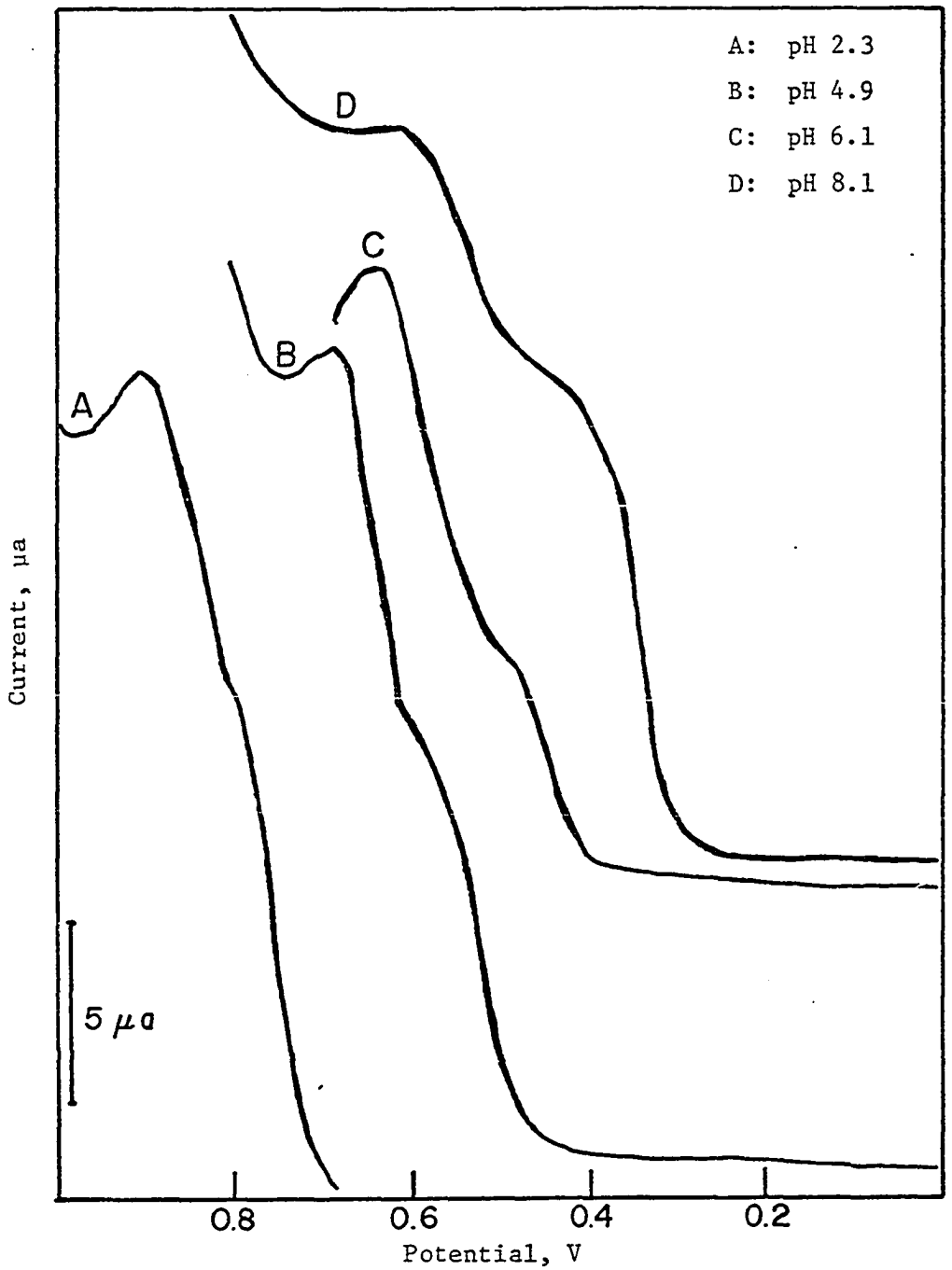
slow scan rate of 3.3 mv sec^{-1} and in a five-fold more dilute solution, only the second and third electrode processes are observed. In fact, in 1 M HOAc and pH 3.6 acetate buffers only the third process is observed.

Voltammograms of 2.5 mM theophylline in solutions of 1 M HOAc (pH = 2.3) and McIlvaine buffer (pH = 4.9, 6.1 and 8.05, respectively) are reproduced in Figure 14 (sweep rate = 5 mv sec^{-1}) and Figure 15 (sweep rate = 500 mv sec^{-1}). It is observed that as the scan rate increases the first peak shifts toward more positive potentials (at pH 4.9, 5 mv sec^{-1}, E_p^I = 0.560 v and E_p^{II} = 0.690 v; at 500 mv sec^{-1}, E_p^I = 0.655 v and E_p^{II} = 0.730 v) and the height of the first wave increases (at pH 4.9 the ratio of current height of peak I to total current is 0.44 at 5 mv sec^{-1} and 0.97 at 500 mv sec^{-1}). Similar effects were noted by Dryhurst[47] in the oxidation of 6-thiopurine at the p.g.e. and are characteristic of an adsorption wave where the product of the electrochemical reaction is strongly adsorbed.[48] It will be noted later that a solid product forms on the graphite electrode in the course of a macroscale electrolysis. This phenomena of coating the surface of the electrode, rather than adsorption in the strict sense, may be what is observed as the sweep rate is varied.

Cyclic Voltammetry at Rapid Sweep Rates

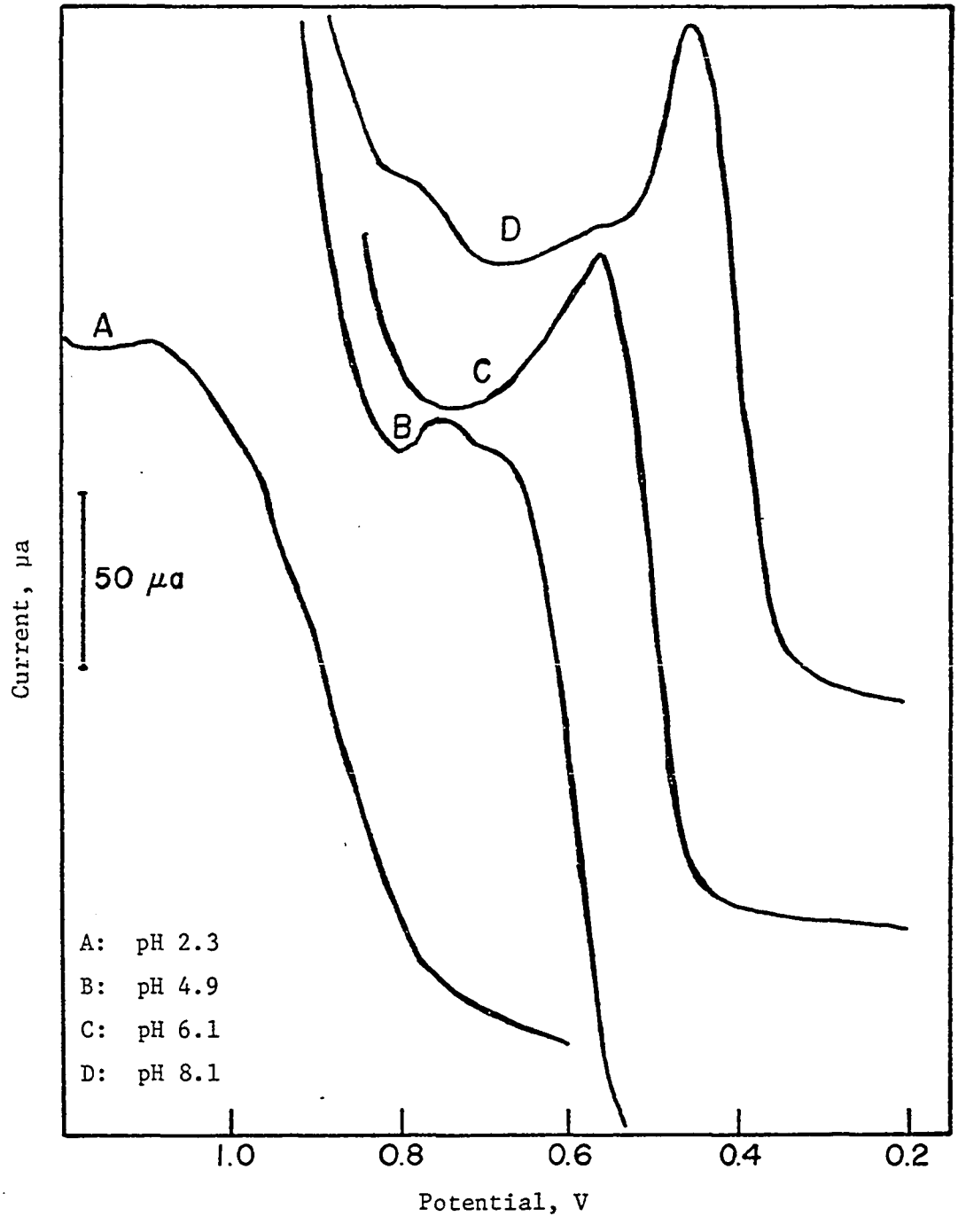
Saturated solutions of the xanthines under investigation and of uric acid and 1,3-dimethyluric acid were prepared in acetate buffer pH 4.7. Voltammograms at the p.g.e. obtained at 8 v sec^{-1} and recorded on a dual beam oscilloscope equipped with a polaroid camera are repro-

Figure 14



Voltammograms of Theophylline at Various pH Values (5 mv sec^{-1})

Figure 15



Voltammograms of Theophylline

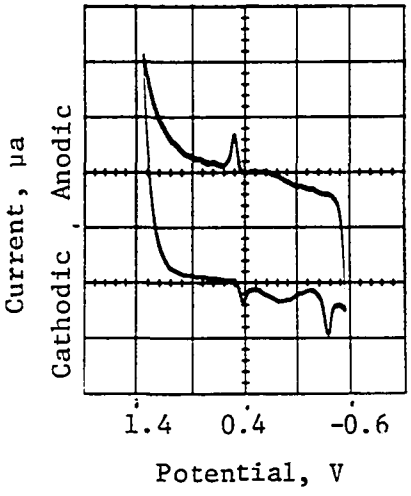
at Various pH Values (500 mv sec^{-1})

duced in Figures 16 - 25. The voltammograms represent cylic scans obtained after several cycles when a steady state had been achieved. Figure 18B represents early traces of xanthine electroactivity. The first scan, however, is not represented. By comparison with Figure 18A it is clear that a certain degree of clarity is sacrificed in achieving the steady state.

Uric acid is oxidized at a potential of 0.50 v to a species (the 4,5-diol) which is reduced at 0.40 v. (The peak potentials, under the conditions of sweep cyclic voltammetry with oscilloscopic trace recording, can at best be determined within an accuracy of ± 60 mv.) These results can be interpreted as formation of an almost reversible couple since peaks separate somewhat with increasing scan rate and the results are comparable to those reported by Dryhurst.[7] A secondary reduction, i.e., one which occurs only after the primary oxidation peak has been scanned, appears at -0.40 v. The peak, reported by Dryhurst, appearing at -0.80 v is not within the potential span of the voltammogram. A peak corresponding to the -0.40 v peak is not reported in that work, however. The explanation for this probably resides in the limits of potentials scanned. In the present study all voltammograms extend to 1.0 v or beyond; presumedly the species reduced at -0.40 v is formed between the 0.6 v limit scanned by Dryhurst and 1.0 v.

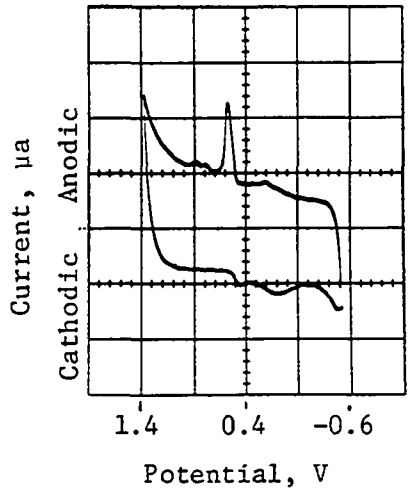
1,3-Dimethyluric acid is oxidized at 0.55 v and on the reverse scan a reduction peak is observed at 0.45 v. Noting that the oxidation potential shifts to less positive values as the pH increases, that the potentials of the oxidation and reduction are very similar to those observed for uric acid itself and that the nature of the couple is analogous to the almost reversible couple observed by Dryhurst[7] in

Figure 16



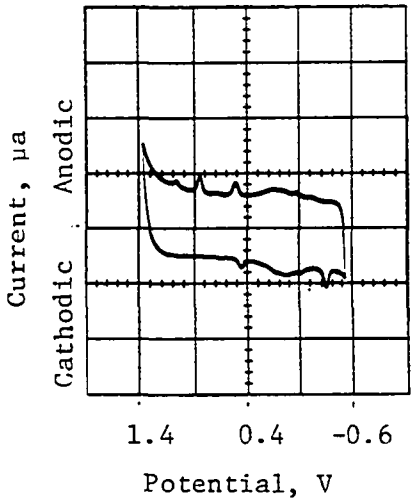
Uric Acid

Figure 17



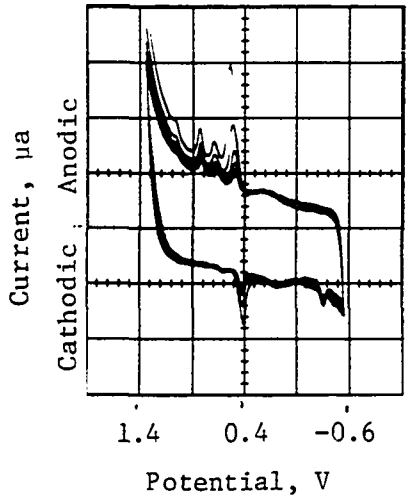
1,3-Dimethyluric Acid

Figure 18A



Xanthine

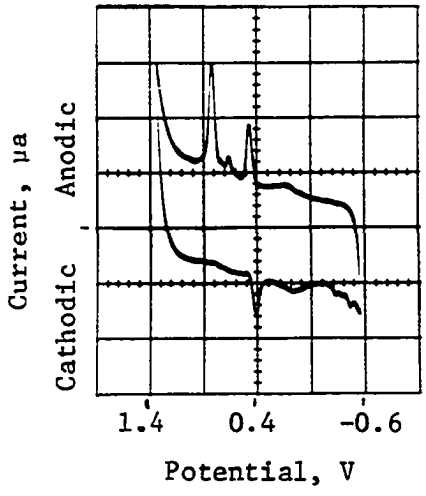
Figure 18B



Xanthine

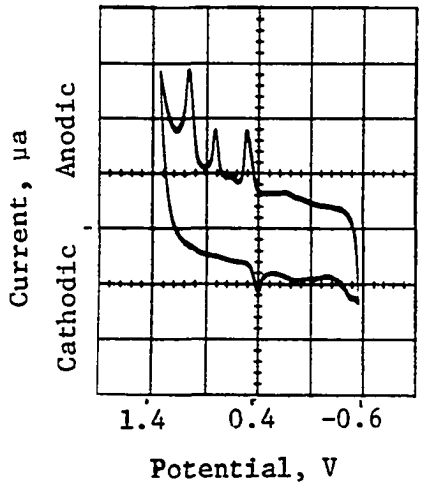
Background: pH 4.7 Acetate buffer
Current scale: 2µa per division

Figure 19



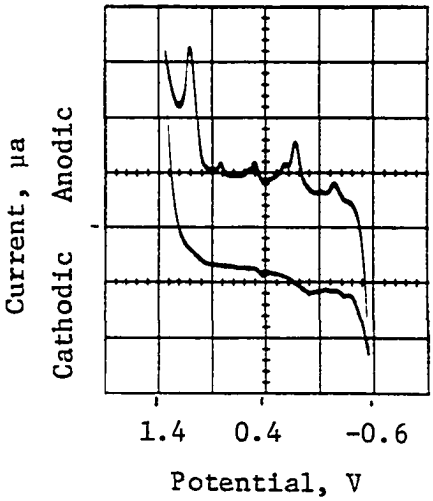
1-Methylxanthine

Figure 20



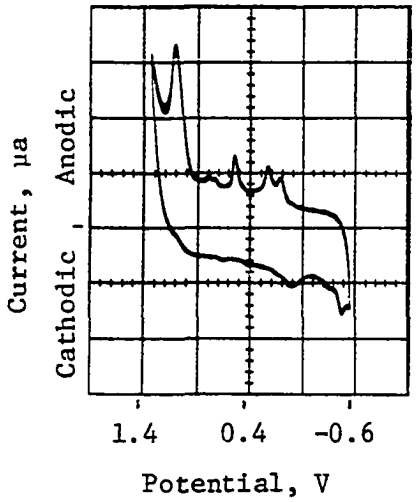
3-Methylxanthine

Figure 21



7-Methylxanthine

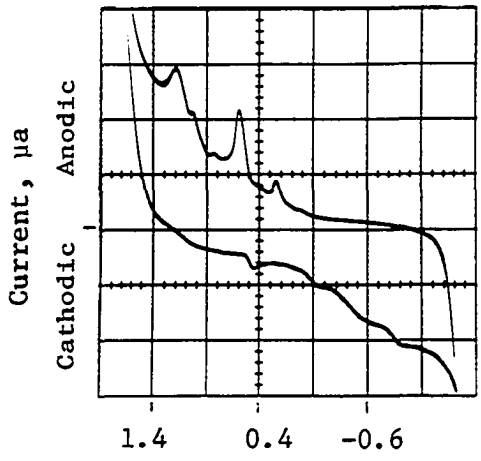
Figure 22



1,7-Dimethylxanthine

Background: pH 4.7 Acetate buffer
Current scale: 2µa per division

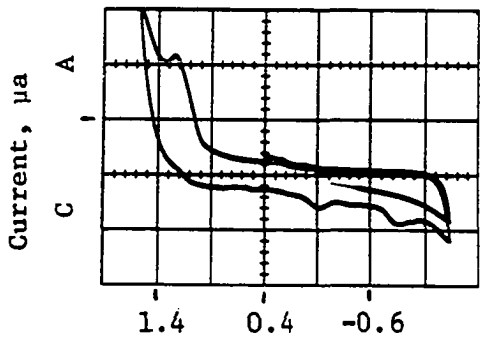
Figure 23A



1,3-Dimethylxanthine

(Theophylline)

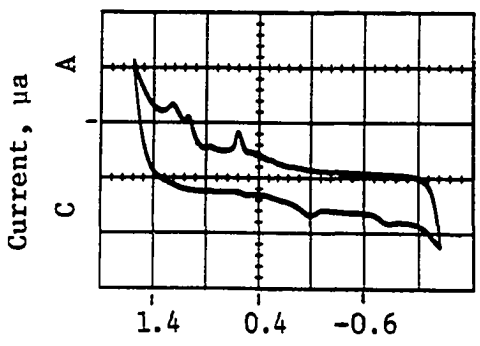
Figure 23B



Theophylline

First Scan

Figure 23C

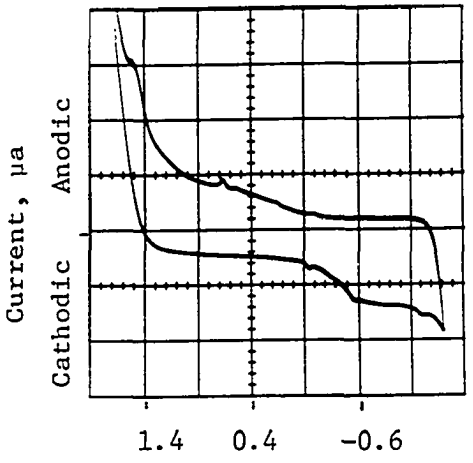


Theophylline

Steady State

Background: pH 4.7 Acetate buffer
Current scale: 23A, 5µa per division; 23B-C, 500µa per division

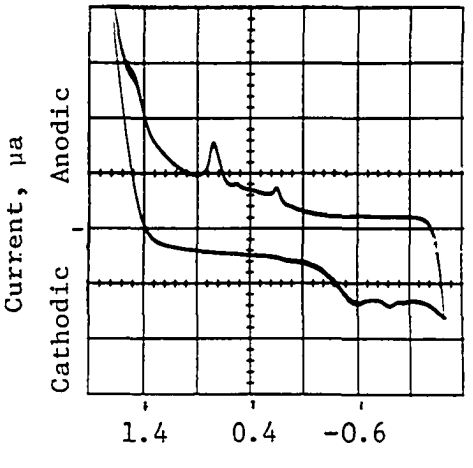
Figure 24



3,7-Dimethylxanthine

(Theobromine)

Figure 25



1,3,7-Trimethylxanthine

(Caffeine)

Background: pH 4.7 Acetate buffer
Current scale: 5μa per division

similar rapid sweep voltammetric studies, the 4,5-diol is also postulated to be the primary oxidation intermediate of electrochemical oxidation of 1,3-dimethyluric acid at the p.g.e.

Oxidation of the xanthines revealed that on the first scan a single anodic peak is observed, i.e., the primary oxidation peak. On the return scan and subsequent sweeps secondary cathodic and anodic peaks occur. This is illustrated in Figure 23B (the primary oxidation peak of theophylline appears followed by the secondary cathodic peaks) and Figure 23C (subsequent scans reveal the primary and secondary peaks). Peak potentials arranged in comparative columns are tabulated in Table 6.

Primary oxidation peak potentials of the methylated xanthines are in the same order as previously noted: 1-methylxanthine is the most easily oxidized, theobromine and caffeine are the most difficult to oxidize.

The reversible anodic-cathodic couple related to the 4,5-diol species can be observed in varying degrees for all the xanthines except theobromine and caffeine. A distinct anodic peak considerably more positive than the anodic peak of the couple appears in these latter compounds, $(E_p)_a$ = 0.68 v for theobromine and $(E_p)_a$ = 0.75 v for caffeine. No related cathodic peak is observed. In the case of 7-methylxanthine and 1,7-dimethylxanthine, anodic peaks $(E_p)_a$ = 0.50 v and $(E_p)_a$ = 0.55 v, respectively, are easily discerned. A very small cathodic peak, $(E_p)_c$ = 0.45 v, is detected for 7-methylxanthine. An indentation in the cathodic range of 0.40 v is the best related peak observed for 1,7-dimethyl-xanthine. Thus, methylation at the 7-position appears to affect the appearance of the reversible couple related to the presence of the

Table 6

Correlation of Peaks Observed in Uric Acid and Xanthine C

Compound	Peak Potential of Primary Oxidation v vs. S. C. E.	Peak Potentials of Secondary Reduction v vs. S. C. E.				
		A	B	C	D	E
Uric Acid		0.40		-0.40		[-0.80]*
1,3-Dimethyl-uric Acid		0.45		-0.45		
Xanthine	0.85	0.45		-0.35		[-0.85]
1-Methyl-xanthine	0.95	0.40		-0.35[b]	-0.45	[-0.85]
3-Methyl-xanthine	1.05	0.40		-0.40		[-0.85] [
7-Methyl-xanthine	1.10	0.45[b]	0.00[e]	-0.40[b]		[-0.85] [
1,7-Dimethyl-xanthine	1.10	0.40[b]	0.00[e]	-0.45		-0.90
1,3-Dimethyl-xanthine (Theophylline)	1.20	0.45	-0.10[e]	-0.45[b]		[-0.90] [
3,7-Dimethyl-xanthine (Theobromine)	1.50		-0.15[e]	-0.55		-0.90[d]
1,3,7-Trimethyl-xanthine (Caffeine)	1.50			-0.55		-0.90[d]

[a] This is the primary oxidation peak for the uric acids; [b] Very small peaks; [c] scan; [d] Relates to reduction potential of the parabanic acids at same scan rate

* Peaks appearing in brackets observed in photographs not reproduced in this text

Table 6

bserved in Uric Acid and Xanthine Cyclic Voltammograms (8 v sec^{-1})

Potentials of Secondary Reductions v vs. S. C. E.				Peak Potentials of Secondary Oxidations v vs. S. C. E.					
C	D	E	F	G	H	I	J	K	L
-0.40		[-0.80]*				0.50[a]			
-0.45						0.55[a]			
-0.35		[-0.85]				0.50			
-0.35[b]	-0.45	[-0.85]			0.65	0.45			
-0.40		[-0.85]	[-1.10]		0.80	0.50			
00[e] -0.40[b]		[-0.85]	[-1.10]		0.80[b]	0.50	0.25[e]	0.15[e]	-0.25
00[e] -0.45		-0.90			0.90	0.55	0.25[e]	0.15[e]	
10[e] -0.45[b]		[-0.90]	[-1.10]	1.05	0.90	0.55	0.25[e]		
15[e] -0.55		-0.90[d]	-1.20			0.68[a]			
-0.55		-0.90[d]				0.75[c]	0.15[e]		

uric acids; [b] Very small peaks; [c] Must scan beyond -1.0 v to see this peak on return
e parabanic acids at same scan rate; [e] Believed to be related to the alloxans;
tographs not reproduced in this text.

4,5-diol. Such a couple is not observed at all in 3,7-dimethyl and 1,3,7-trimethylxanthine. The cathodic peak is almost indiscernible in 1,7-dimethylxanthine and very small in 7-methylxanthine.

The secondary anodic peaks listed in colums G and H, and the peaks, $(E_p)_a$ = 0.68 v and $(E_p)_a$ = 0.75 v for theobromine and caffeine are believed to be related to the double wave character observed for some xanthines in the pH studies. They do not appear on the first scan at these extremely rapid sweep rates but are detected on subsequent traces.

Comparison of the potentials of the peaks listed in columns B, H and J with those observed for the alloxans under similar conditions links the cathodic peaks to the reduction of the respective alloxans and the anodic peak to the oxidation of the alloxan reduction product, dialuric acid. Alloxans are formed as secondary products of the electrochemical oxidation of these purines arising from total oxidation of the 4,5-position of the six-membered ring.

The cathodic peaks in column E compare with those observed for the reduction of the respective parabanic acids under similar conditions. The parabanic acids are also secondary products of purine electrochemical oxidation.

On the basis of the voltammogram data, it can be stated that all the xanthines studied, with the possible exception of theobromine and caffeine, are oxidized in a very similar manner. In those cases where the uric acid type diol is observed, a four-electron process is probably operative with oxidation of C_8 as the potential determining step since it is noted that oxidation of the $-C_4=C_5-$ double bond occurs at less positive potentials than that of the primary oxidation when viewed on

subsequent sweeps. Two electrons are removed from the $-N_9=C_8-$ double bond to give a C_8 carbonyl group and simultaneously two electrons are then removed from the $-C_4=C_5-$ double bond. On the reduction scan two electrons are replaced in the $-C_4-C_5-$ site and consequently, oxidation occurs on the anodic scan, but at a potential less than that of the primary oxidation and very similar to that observed for uric acid oxidation. It should be noted that the HC=N- double bond has been observed as the preferential position for oxidation by enzymes such as xanthine oxidase.[40,42] The p.g.e. oxidation of the xanthines studied exhibit the same preference as to the primary oxidation site.

Further investigations of the electrochemical oxidation of theobromine and caffeine, especially faradaic n value determinations are required before it can be affirmed that these compounds are oxidized in the above manner. It is possible that the related 4,5-diol compound is formed but is too unstable to be detected even by rapid sweep voltammetry.

The trend in oxidation potentials at the p.g.e. validates the following statements: (1) methylation of N_1 has little effect on the oxidation potential, (2) the effects of methylation of N_7 and N_3 are almost equal when occurring in monomethylated xanthines, (3) the effect of methylation at N_3 has a greater effect, i.e., makes oxidation more difficult than methylation at N_7 when dimethylated xanthines are being considered and (4) the combination of N_7 and N_3 methylation shifts the oxidation potential to the most positive values observed in the study and is unaffected by further methylation at N_1, as can be noted by comparing E_p for 3,7-dimethylxanthine and 1,3,7-trimethylxanthine.

These trends are counter to those predicted by quantum mechanical calculations for the effect of N-methylation on the electron-donor properties of the xanthines.[49] From the calculated energies of the highest occupied molecular orbitals it is predicted that methylation at N_1 should have little effect on the electron-donor properties of xanthine but that methylation at N_3 should increase these donor properties to a very large extent. Intermediate behavior is predicted for N_7 methylation. A similar anomaly has been noted for the reduction potentials of cytosine and adenine which are in the reverse order to that predicted from quantum mechanical calculations.[50] These disagreements are probably due to differences in the electrochemical kinetic process. Thus further study of the electrode kinetics will be needed to fully explain the differences.

Electrochemical Oxidation of Caffeine

Determination of Faradaic n Values

The results of coulometry at the controlled potential of 1.40 v in 2 M H_2SO_4, 1.40 v in 1 M HOAc and 1.30 v in pH 4.7 acetate buffer are summarized in Table 7. An average n value of 3.7 is obtained.

Identification of Oxidation Products

The oxidation products of caffeine were determined by polarography, thin layer chromatography and spectrophotometric analytical methods. The "possible" oxidation products were first determined by considering the methylated nature of the pyrimidine and imidazole ring and were based on the assumption, validated in general by cyclic voltammetry, and in particular by the four electron determination that xanthine oxidation resembles that of uric acid.[1] It was noted that upon

Table 7

Faradaic n Values for Electrochemical Oxidat

Number of Experiment	Background Electrolyte	pH	Controlled Potential v vs. S.C.E.	Electrolysis Time min
1	2 M H_2SO_4	0.0	1.40[a]	270
2	2 M H_2SO_4	0.0	1.40	270
3	2 M H_2SO_4	0.0	1.40	300
4	2 M H_2SO_4	0.0	1.40	240
5	2 M H_2SO_4	0.0	1.40	360
6	1 M HOAc	2.3	1.40	36
7	1 M HOAc	2.3	1.40	30
8	1 M HOAc	2.3	1.40	40
9	1 M HOAc	2.3	1.40	30
10	1 M HOAC	2.3	1.40	33
11	1 M HOAc	2.3	1.40	360
12	1 M HOAc	2.3	1.40	260
13	1 M HOAc	2.3	1.40	210
14	1 M HOAc	2.3	1.40	50
15	1 M HOAc	2.3	1.40	75
16	Acetate	4.7	1.30	370
17	Acetate	4.7	1.30	480

[a] Potentials controlled within ± 60 mv.

Table 7

n Values for Electrochemical Oxidation of Caffeine

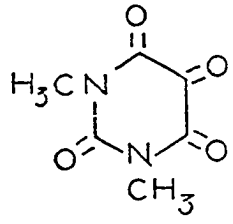
Controlled Potential vs. S.C.E.	Electrolysis Time min	Faradaic n Value	Comments on Conditions
1.40[a]	270	3.41	1-15: 1 mM initial concn.
1.40	270	4.09	1-5: not deoxygenated, complete
1.40	300	3.88	electrolysis.
1.40	240	3.43	6-10: working compartment de-
1.40	360	3.98	oxygenated, spectrophometric
1.40	36	3.18	determination of final caffeine
1.40	30	4.86	concn.
1.40	40	3.27	10-14: all compartments deoxygenated.
1.40	30	4.78	10-12: complete electrolysis.
1.40	33	3.60	13-14: oxidized until 10 ml
1.40	360	4.15	acid titrated.
1.40	260	3.02	15-16: complete electrolysis
1.40	210	3.40	of 0.5 mM solution.
1.40	50	3.12	
1.40	75	3.12	
1.30	370	3.89	
1.30	480	3.78	
		Av. = 3.68	

complete oxidation the UV spectrum characteristic of caffeine (λ_{max} = 272 mμ, pH 2.3) due to the $N\text{-}C_4\text{=}C_5\text{-}C_6\text{=}O$[25] was no longer detected, further indicating that oxidation did, indeed, occur at the $-C_4\text{=}C_5-$ double bond. The "possible" products, therefore, are dimethyl alloxan, dimethyl parabanic acid (from the six-membered ring), methyl parabanic acid (from the imidazole ring), trimethyl allantoin, dimethyl urea, methyl urea, ammonia methyl amine and carbon dioxide.

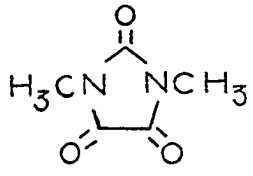
Solutions used for thin-layer chromatography were obtained by complete electrolysis of ca. 10 mM caffeine in 1 M HOAc; the other test solutions were from 1.0 mM caffeine oxidation in 1 M HOAc. The characteristics of the caffeine electrolysis product solution and of the "possible" oxidation products, also in 1 M HOAc are summarized in Table 8.

Dimethyl alloxan (22) was identified as responsible for the cathodic wave, $E_{\frac{1}{2}}$ = 0.04 v (potentials observed vs. M.S.E. and corrected as vs. S.C.E.), observed in d.m.e. studies of the electrolysis product solution. An UV-active spot, developed from the electrolysis product solution on a cellulose coated thin-layer sheet developed in butanol-acetic acid-water (120:30:50) with an r_f value of 0.88 was identical to that for the standard DMA solution.

In the same manner, dimethyl parabanic acid (23) was identified



22



23

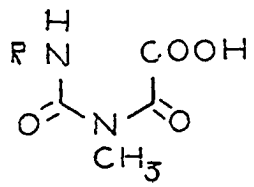
Table 8

Characteristic Properties and Identification of Caffeine Oxidation Products in 1 M HOAc[a]

Compound	$E_{1/2}$[b] v vs. S.C.E.	r_f[c]	λ_{max} mμ
Caffeine Oxidation Product Solution			
Oxidation Product Solution	0.04	0.88[d]	515[f]
	-0.63	0.76[e]	487[g]
	-0.91		380[h]
"Possible" Oxidation Products			
Dimethyl Alloxan	0.03	0.88[d]	
Dimethyl Parabanic Acid	-0.63, -0.93	0.87[d]	
Methyl Parabanic Acid	-0.64	0.78[d]	
Trimethyl Allantoin			515[f]
Methyl Urea		0.72[e]	487[g]
Dimethyl Urea		(-)[e]	(-)[g]
Ammonia			380[h]

[a] All tests were performed directly on the 1 M HOAc solution, pH = 2.3; [b] Average deviation ± 0.03 v; [c] Spots developed in butanol-acetic acid water (120:30:50) on Brinkman MN polygram cellulose UV_{254} coated plastic sheets; R_f = ratio of distance solvent front traveled to distance of spot from origin; [d] UV-active; [e] Ehrlich reagent; [f] glyoxylic acid chromophore; [g] diacetyl monoxime complex; [h] Nessler reagent; (-) = no response to test.

as a product being polarographically reduced with an $E_{1/2}$ = -0.65 v at the d.m.e. since the results of thin-layer chromatography spotted with the electrolysis product solution gives an UV-active spot with an r_f = 0.88. This when compared with the values from spots of standard solutions of dimethyl parabanic acid (r_f = 0.87) and methyl parabanic acid (r_f = 0.78) eliminates the methyl parabanic acid. Closer studies of dilute dimethyl and methyl parabanic acid solutions in 1 M HOAc revealed that a second cathodic wave appeared as the solution aged (30 min to 2 hr). This wave, $E_{1/2}$ = -0.93 v, accounts for the third cathodic wave, $E_{1/2}$ = -0.91 v, observed in d.m.e. studies of the electrolysis product solution and probably is due to decomposition of the parabanic acids to the respective oxaluric acids (24).[51]



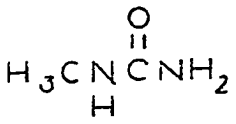
R = CH_3 or H

24

Methyl urea (25) was identified on the basis of the color and visible absorption peak exhibited when solutions of caffeine electrolysis product and standard solutions of methyl urea were carried through a modified Rosenthal test (Experimental). Both solutions appeared rose-yellow in color and exhibited an absorption with λ_{max} = 487 mμ. A yellow spot was observed upon thin-layer chromatography (as described above) when sprayed with Ehrlich reagent, r_f = 0.72 for standard methyl urea solution and r_f = 0.76 for the electrolysis solution. No spot

developed for standard dimethyl urea because the standard solution was applied as an aqueous solution. Only acidic (1 M HOAc) solutions give a positive test which is probably due to the rate of hydrolysis of dimethyl urea.

The electrolysis product solution was found to contain a species which gave a glyoxylic acid-phenylhydrazine-ferricyanide chromophore, λ_{max} = 515 mμ. This behavior is characteristic of allantoin (26) upon identical treatment (Experimental). The allantoin formed is 3,6,8-



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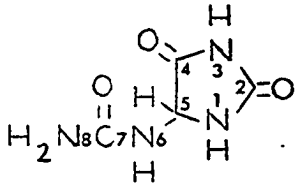


26

trimethyl allantoin since Brandenburger[52] has shown that in the formation of allantoin, bonds are never broken between C_4 and N_3 and N_9. Thus, 1,3,6- or 1,3,8-trimethyl allantoin is most unlikely.

Ammonia was identified by a positive reaction to Nessler reagent. A yellow colored solution exhibiting an absorbance in the 380 - 400 mμ region was obtained.

Quantitative Determination of Oxidation Products

The quantity of dimethyl alloxan and dimethyl parabanic acid present in an oxidation product solution (initial concentration of caffeine, 1.0 mM) was determined polarographically. The average limiting current was related to concentration by means of concentration calibration curves.

The concentrations of methyl urea and trimethyl allantoin were determined spectrophotometrically by comparison with standard calibration curves prepared with each determination using the same methods as used for identification.

Quantitative determination of ammonia spectrophotometrically could not be accomplished due to the interference by allantoin at the concentration levels present in the solutions. It was therefore determined by a difference technique.

The concentration of the individual products at stages in the electrolysis was also determined. The results are given in Table 9. Plots of millimoles of the respective products versus millimoles of caffeine electrolyzed (Figure 26) reveals that there is a linear increase in each concentration as the caffeine is oxidized.

Two solutions were electrolyzed until 0.054 and 0.051 millimoles of caffeine had been oxidized. Triple determinations were made for the concentration of each electrolysis product. The results are given in Table 10. The nitrogen content of the pyrimidine ring of caffeine should be accounted for by summing the millimoles of dimethyl alloxan, dimethyl parabanic acid and trimethyl allantoin if these are the only nitrogen-containing products formed from the six-membered ring. Similarly, the nitrogen of the imidazole ring should exist in trimethyl allantoin, methyl urea, ammonia and methyl amine. However due to the interference by allantoin, the ammonia concentration cannot be accurately determined. Therefore, the material balance, i.e., the products formed account for all the material oxidized, is based on the nitrogen content of the pyrimidine ring alone. The two experiments, accounting for 95

Table 9

Concentration of Oxidation Products at Stages in Caffeine Oxidation

Electrolysis Time min	Millimoles Caffeine Oxidized	Millimoles of Product Formed			
		Dimethyl Alloxan	Dimethyl Parabanic Acid	Methyl Urea	Trimethyl Allantoin
		Experiment 1			
60	0.054	0.033	0.002	0.016	-----[a]
120	0.105	0.053	0.004	-----[a]	-----[a]
380	0.148	0.086	0.009	0.003	0.037
		Experiment 2			
53	0.051	0.053[b]	0.003	0.011	-----[a]
90	0.099	0.054	0.005	0.024	0.024
360	0.152	0.084	0.008	0.030	0.039

[a] Concentration not determined; [b] High values due to presence of oxygen

Table 10

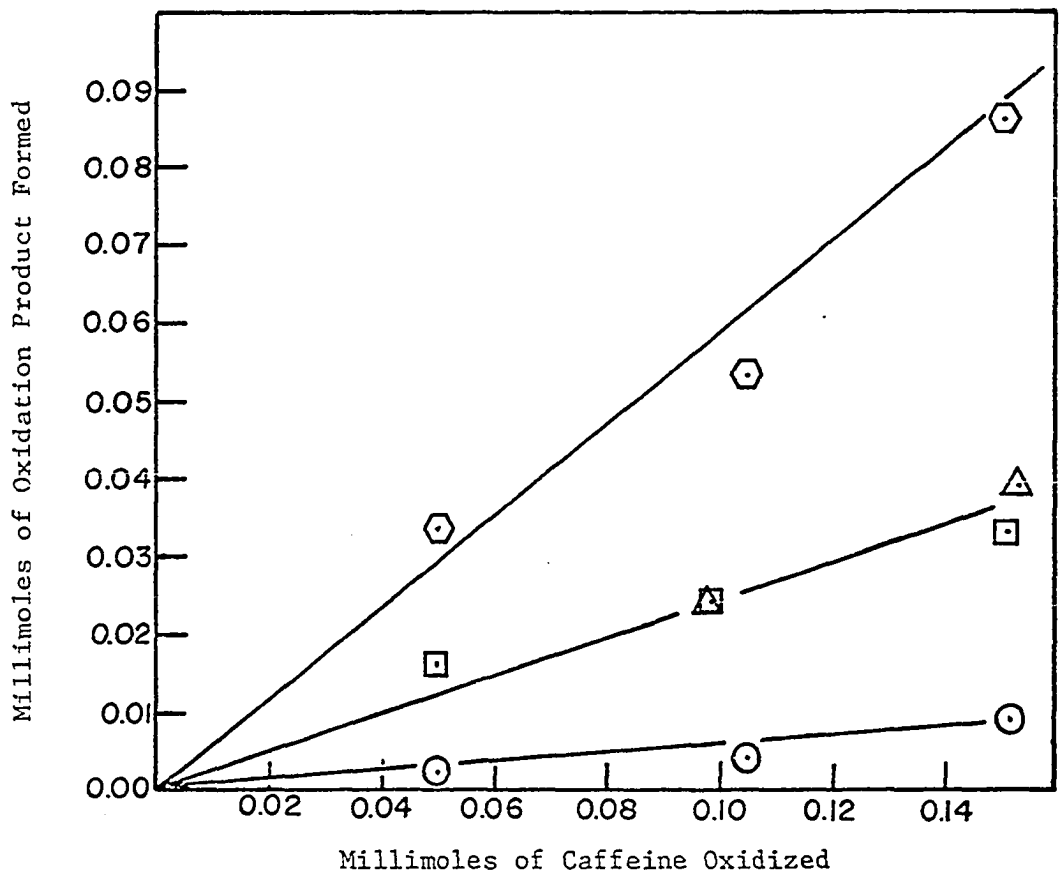
Determinations of Caffeine

Oxidation Product Concentrations

Millimoles Caffeine Oxidized	Millimoles[a] of Product Formed				Millimoles of Pyrimidine Ring Accounted for by Products	Percentage of Oxidized Caffeine Accounted for by Products
	Dimethyl Alloxan	Dimethyl Parabanic Acid	Methyl Urea	Trimethyl Allantoin		
0.054	0.030	0.002	0.013	0.019	0.051	95
0.051	0.030	0.002	0.012	0.019	0.051	100

[a] Average value from triplicate determinations

Figure 26



⬡ = Dimethyl Alloxan

⊡ = Methyl Urea

⊙ = Dimethyl Parabanic Acid

△ = Trimethyl Allantoin

Variation of Oxidation Product Concentrations with Caffeine Concentration

and 100 per cent of the caffeine oxidized, confirms that no other nitrogen-containing products are formed.

Mechanism of Electrochemical Oxidation of Caffeine

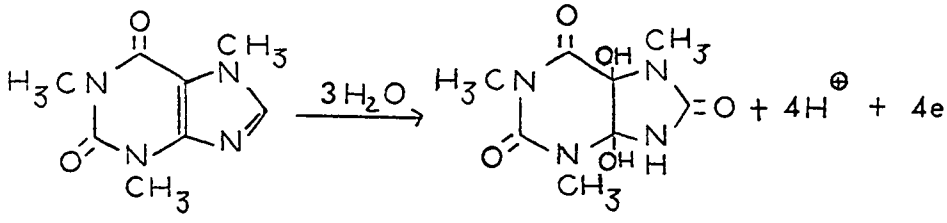
Cyclic voltammetry of the xanthines indicated that oxidation could be expected to be very similar to that of uric acid. Once oxidation occurs at the number eight position, the methylated xanthines exist as methylated uric acids. However, in the case of caffeine, the almost reversible couple previously identified as oxidation-reduction of the $-C_4=C_5-$ double bond was not distinguishable. The loss of the characteristic absorption of this bond (λ_{max} = 272 mμ for caffeine in 1 M HOAc) upon electrolysis, however, indicated that oxidation did occur at the $-C_4=C_5-$ position as well as at the number eight position and products similar to those of uric acid could be expected.

The determination of faradaic n values reveals that the oxidation involves close to four electrons. The millimoles of dimethyl alloxan, dimethyl parabanic acid and trimethyl allantoin formed upon oxidation balances the millimoles of caffeine oxidized and are the only nitrogen-containing products arising from the pyrimidine ring. The nitrogen of the imidazole ring not existing on methyl urea and trimethyl allantoin must then exist as ammonia and as a consequence of N_7 methylation, as methyl amine. Carbon dioxide is formed from both rings.

Equation 20 represents the primary electrochemical oxidation of caffeine to the 4,5-diol intermediate. Secondary reactions producing the identified products are illustrated in Equations 21 - 24. Dimethyl alloxan, trimethyl allantoin, dimethyl parabanic acid and methyl urea are fragmentation and oxidation products of the intermediate. Methyl

amine and ammonia are hydrolysis products of methyl urea. The further oxidation of the intermediate to dimethyl parabanic acid is negligible in the overall determination of the number of electrons involved in the reaction (cf. 0.08 parts in 4.0).

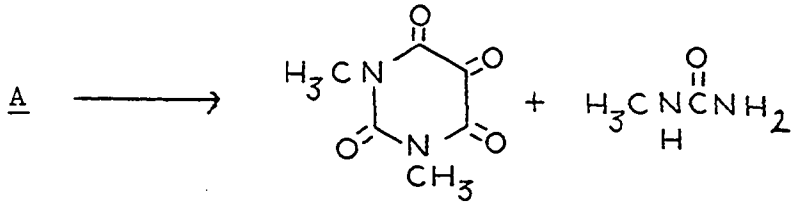
Primary Electrochemical Oxidation



(20)

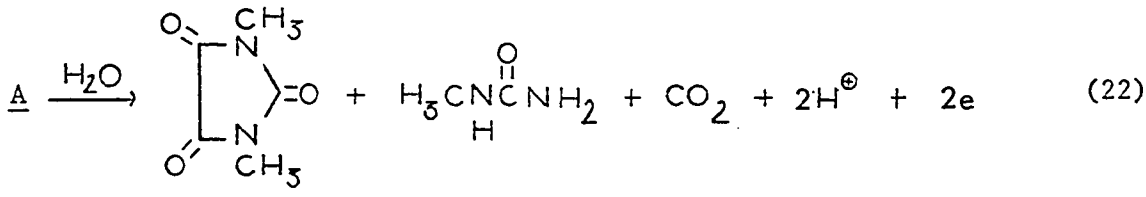
1,3,7-Trimethyl-xanthine (Caffeine)	1,3,7-Trimethyl-uric Acid-4,5-diol	
	A	
1.0 mole	1.0 mole	4.0 mole

Secondary Processes



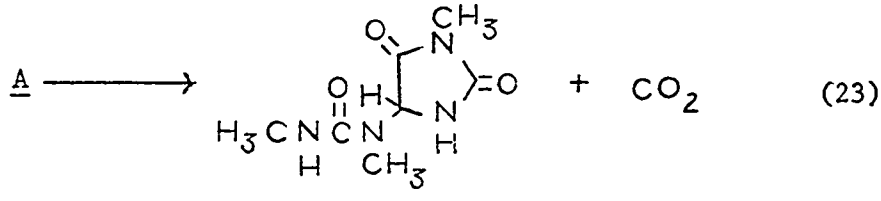
(21)

	Dimethyl	Methyl
0.59 mole	Alloxan	Urea
	0.59 mole	0.59 mole



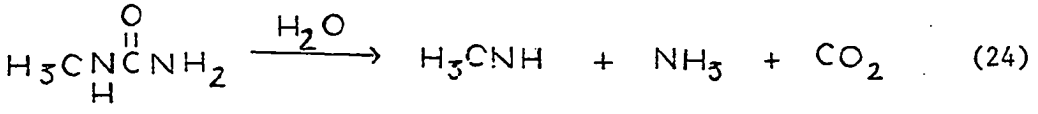
(22)

	Dimethyl Parabanic Acid	Methyl Urea	Carbon Dioxide	
0.04 mole	0.04 mole	0.04 mole	0.04 mole	0.08 mole



(23)

	3,6,8-Trimethyl Allantoin	Carbon Dioxide
0.37 mole	0.37 mole	0.37 mole



(24)

Methyl Urea	Methyl Amine	Ammonia	Carbon Dioxide
0.39 mole	0.39 mole	0.39 mole	0.39 mole

Electrochemical Oxidation of Theobromine

Determination of Faradaic n Values

The results of coulometry at a controlled potential of 1.44 v in 2 M H_2SO_4, 1.50 v and 1.40 v in 1 M HOAc and 1.30 v in pH 4.7 acetate buffer are summarized in Table 11. An average n value of 3.8 is obtained.

Identification of Oxidation Products

The identification process for theobromine oxidation products are the same as those used for caffeine. The "possible" oxidation products are methyl alloxan, methyl parabanic acid, dimethyl allantoin, methyl urea, ammonia, methyl amine and carbon dioxide.

The solutions used were obtained by complete electrolysis of 10 mM theobromine in 1 M HOAc. The standard solutions of the "possible" products were also made up in 1 M HOAc. The results are summarized in Table 12.

Table 11

Faradaic n Values for Electrochemical Oxidat

Number of Experiment	Background Electrolyte	pH	Controlled Potential v vs. S.C.E.	Electrolysis Time min
1	2 M H_2SO_4	0.0	1.44[a]	360
2	2 M H_2SO_4	0.0	1.44	480
3	1 M HOAc	2.3	1.50	30
4	1 M HOAc	2.3	1.50	30
5	1 M HOAc	2.3	1.50	330
6	1 M HOAc	2.3	1.50	395
7	1 M HOAc	2.3	1.50	135
8	1 M HOAc	2.3	1.50	150
9	1 M HOAc	2.3	1.50	165
10	1 M HOAc	2.3	1.50	120
11	1 M HOAc	2.3	1.40	40
12	1 M HOAc	2.3	1.40	40
13	1 M HOAc	2.3	1.40	40
14	1 M HOAc	2.3	1.40	40
15	Acetate	4.7	1.30	540
16	Acetate	4.7	1.30	455

[a] Potentials controlled within ± 60 mv.

Table 11

n Values for Electrochemical Oxidation of Theobromine

Controlled Potential vs. S.C.E.	Electrolysis Time min	Faradaic n Value	Comments on Conditions
1.44[a]	360	3.35	1-6: 1 mM initial concn.
1.44	480	3.37	1-8: Not deoxygenated.
1.50	30	4.65	1-2: Complete electrolysis.
1.50	30	4.00	3-4: Final concn. of theobromine
1.50	330	4.30	determined spectrophotometrically.
1.50	395	3.71	5-6: Complete electrolysis.
1.50	135	3.57	7-10: 0.2 mM initial concn.
1.50	150	3.26	7-10: Complete electrolysis.
1.50	165	3.94	9-14: Working compartment
1.50	120	3.67	deoxygenated.
1.40	40	4.18	11-14: 1 mM initial concn.,
1.40	40	3.78	Final concn. of theobromine
1.40	40	3.63	determined spectrophotometrically.
1.40	40	3.90	15-16: All cells deoxygenated,
1.30	540	3.56	complete electrolysis.
1.30	455	3.86	15: 1.0 mM initial concn.
			16: 0.5 mM initial concn.

Table 12

Characteristic Properties and

Identification of Theobromine Oxidation Products in 1 M HOAc[a]

Compound	$E_{1/2}$[b] v vs. S.C.E.	λ_{max} mμ
Theobromine Oxidation Product Solution		
Oxidation Product Solution	0.04 -0.65 -0.90	515[c] 487[d] 380[e]
"Possible" Oxidation Products		
Methyl Alloxan	0.02	
Methyl Parabanic Acid	-0.63, -0.93	
Dimethyl Allantoin		515[c]
Methyl Urea		487[d]
Ammonia		380[e]

[a] All tests performed on 1 M HOAC solution, pH = 2.3.

[b] Average deviation, ± 0.03 v.

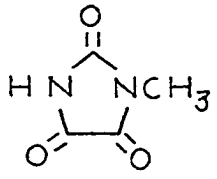
[c] Glyoxylic acid chromophore.

[d] Diacetyl monoxime complex.

[e] Nessler reagent.

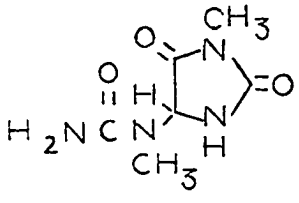
Methyl alloxan (27) and methyl parabanic acid (28) were identi-

27



28

fied by their polarographic half-wave potentials. Solutions of methyl urea and electrolysis product, identically treated, produced the diacetyl monoxime complex with characteristic absorption, λ_{max} = 487 mμ. The electrolysis product solution exhibited the behavior characteristic of allantoin, absorption with λ_{max} = 515 mμ, when treated to produce the glyoxylic acid-phenylhydrazine-ferricyanide chromophore. The allantoin present is 3,6-dimethyl allantoin (29). Ammonia was identified by a



29

positive reaction to Nessler reagent.

Quantitative Determination of Oxidation Products

The methods employed for quantitative determination of the oxidation products of theobromine are the same as those used for caffeine. Three solutions were electrolyzed until 0.060, 0.059 and 0.065 millimoles of theobromine had been oxidized and triple determinations were made for

each of the products. The results are shown in Table 13.

Table 13

Determination of Theobromine Oxidation Product Concentrations

Millimoles Theobromine Oxidized	Millimoles of Product Formed					
	Methyl Alloxan	Methyl Parabanic Acid	Methyl Urea	Dimethyl Allantoin	Millimoles of Pyrimidine Ring Accounted for by Products	Percentage of Oxidized Theobromine Accounted for by Products
0.060	0.036	0.002	0.025	0.016	0.054	90
0.059	0.032	0.002	0.028	0.016	0.050	85
0.065	0.049	0.004	0.031	0.015	0.068	103

The nitrogen content of the pyrimidine ring of theobromine is accounted for by summing the millimoles of methyl alloxan, methyl parabanic acid and dimethyl allantoin. An average of 93 per cent is obtained in the three experiments, which is a lower value than obtained for the caffeine analysis. However, theobromine is much less soluble than either caffeine or theophylline (Table 1) and it is very difficult to get a 1.0 m$\underline{M}$ equivalent of the solid into solution prior to the oxidation. Hence an error of ± 0.003 millimoles is possible in the concentration determinations.

Mechanism of Electrochemical Oxidation of Theobromine

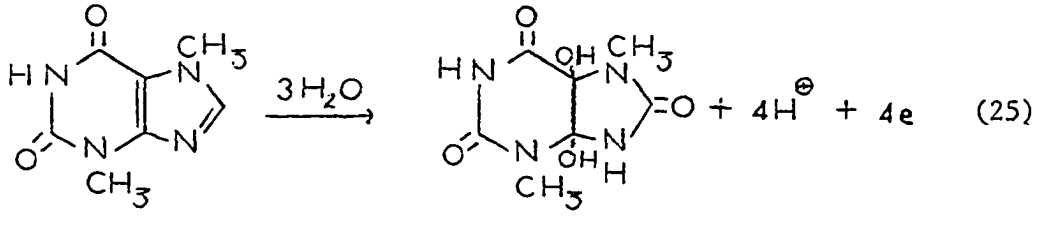
The electrochemical oxidation of theobromine proceeds in a way identical to that of caffeine. This similarity is governed by the methylation of N_3 and N_7 since it is observed in rapid scan voltammetry

that methylation at the seven position affects the presence of the almost reversible anodic-cathodic couple linked to the uric acid 4,5-diol species, and that dimethylation involving the number three position is more difficult to oxidize than the one-seven combination.

The determination of faradaic n values reveals that four electrons are transferred in the oxidation. The millimoles of methyl alloxan, methyl parabanic acid and dimethyl allantoin formed on oxidation balance the millimoles of theobromine oxidized and are, therefore, the only nitrogen-containing oxidation products of the pyrimidine ring. The nitrogen of the imidazole ring is present as methyl urea, dimethyl allantoin, ammonia and methyl amine. Carbon dioxide is formed from both rings.

Equation 25 represents the primary electrochemical oxidation to the 3,7-dimethyluric acid-4,5-diol intermediate. Secondary reactions producing the identified products are illustrated in Equations 26 - 29 and are completely analogous to those of caffeine.

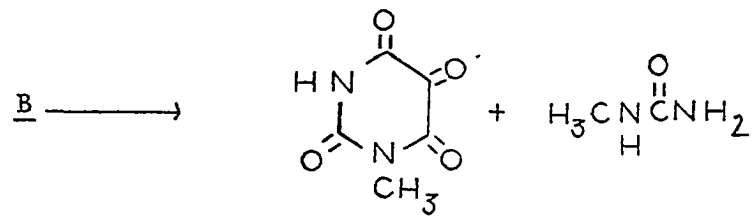
Primary Electrochemical Oxidation



(25)

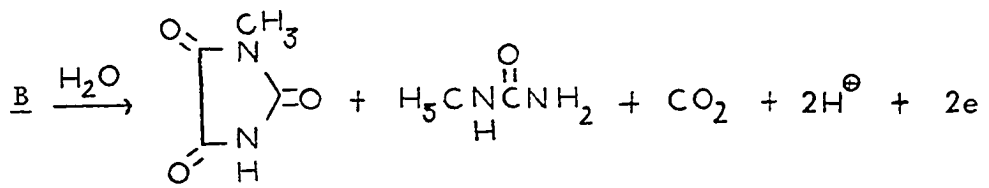
3,7-Dimethyl-xanthine (Theobromine)	3,7-Dimethyl-uric Acid-4,5-diol	
1.0 mole	1.0 mole	4.0 mole

Secondary Processes



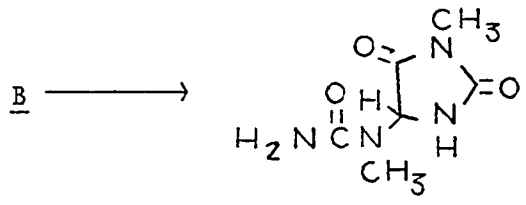
(26)

	Methyl Alloxan	Methyl Urea
0.75 mole	0.75 mole	0.75 mole



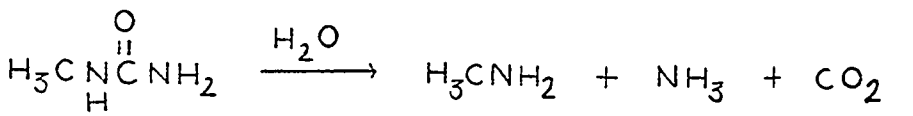
(27)

	Methyl Parabanic Acid	Methyl Urea	Carbon Dioxide	
0.03 mole	0.03 mole	0.03 mole	0.03 mole	0.06 mole



B		3,6-Dimethyl Allantoin	Carbon Dioxide
0.23 mole		0.23 mole	0.23 mole

(28)



Methyl Urea		Methyl Amine	Ammonia	Carbon Dioxide
0.27 mole		0.27 mole	0.27 mole	0.27 mole

(29)

Electrochemical Oxidation of Theophylline

Determination of Faradaic n Values

The results of coulometry at the controlled potential of 1.30 v in 2 M H_2SO_4 and ca. 1.25 v in 1 M HOAc are summarized in Table 14. An average n value of 3.3 with an average deviation of ± 0.04 is obtained.

Identification of Oxidation Products

Initial attempts to identify the products of theophylline oxidation in a manner analogous to that used for caffeine and theobromine revealed that major differences existed. The following differences were noted: the solutions obtained upon oxidation of 1.0 mM theophylline in 1 M HOAc were somewhat opaque, the limiting current of the cathodic wave normally related to the alloxan concentration was much less than in the previous cases, the limiting current of the cathodic wave related to the parabanic acid concentration was greater than observed in the other oxidation product solutions, allantoin tests at early stages in the electrolysis were negative, the electrolysis product solution became yellow when treated with base in the preliminary procedures of the allantoin test and a yellow solid was observed to form on the electrodes and in the solution when 10.0 mM solutions of theophylline in 1 M HOAc were oxidized.

Thin-layer chromatography. A 10.0 mM solution of theophylline in 1 M HOAc was oxidized at 1.25 v for six days and the solid product separated by filtration. A cellulose coated thin-layer sheet was spotted with 1 M HOAc solutions of dimethyl alloxan, dimethyl parabanic acid and parabanic acid and with the oxidation products filtrate, then treated

Table 14

Faradaic n Values for Electrochemical Oxidation of Theophylline

Number of Experiment	Background Electrolyte	pH	Controlled Potential v vs. S.C.E.	Electrolysis Time min	Faradaic n Value	Comments on Conditions
1	2 M H_2SO_4	0.0	1.30	210	3.48	1-7: 1.0 mM initial concn.; complete electrolysis. 1-3: not de-oxygenated. 4-7: all com-partments de-oxygenated.
2	2 M H_2SO_4	0.0	1.30	240	3.67	
3	2 M H_2SO_4	0.0	1.30	300	3.76	
4	1 M HOAc	2.3	1.20	90	2.40	
5	1 M HOAc	2.3	1.25	110	2.98	
6	1 M HOAc	2.3	1.25	120	2.98	
7	1 M HOAc	2.3	1.30	1980	3.68	
					Av = 3.28	

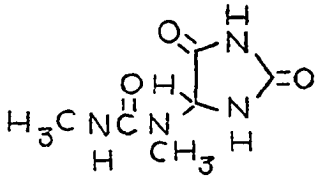
as described earlier. Two UV active spots developed from the latter with $r_f = 0.83$ and $r_f = 0.58$ corresponded to the reference spots of dimethyl alloxan and parabanic acid. Dimethyl parabanic acid may also exist in the filtrate since the standard solution of dimethyl parabanic acid yields an $r_f = 0.81$ which does not experimentally eliminate the possibility that some dimethyl parabanic acid exists in the filtrate spot developed with $r_f = 0.83$. However, the presence of parabanic acid itself indicates that, contrary to the results of caffeine and theobromine oxidation, a portion of the imidazole ring of theophylline exists as parabanic acid in the oxidation mixture.

The existence of dimethyl urea in the oxidation product solution was established by comparison of the r_f value of standard dimethyl urea in 1 M HOAc and the test solution. Two spots reactive to Ehrlich reagent were identified in the test solution with $r_f = 0.85$ and $r_f = 0.58$. These corresponded to the spots of standard dimethyl urea and urea respectively.

Spectrophotometric analytical methods. Urea was also identified as an oxidation product by use of the modified Rosenthal test on the electrolysis product solution and on standard urea solutions. The test solutions developed a yellow color and exhibited an absorption with $\lambda_{max} = 480$ mμ. Standard dimethyl urea solutions were observed to resist the Rosenthal test under the conditions used.

A positive test for the allantoin species was observed when treated as described previously. The compound is 6,8-dimethyl allantoin (30). Ammonia was identified by a positive reaction to Nessler reagent.

Identification of the solid product. Solid product collected from the oxidation described in the chromatography section above did not



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melt at the maximum temperature (310 °C) available on the melting-point block. The IR spectrum (KBr pellet) exhibited a distinct 1600 cm^{-1} peak not observed in a theophylline spectrum. Comparison with the IR spectrum of 1,3-dimethyluric acid[37] revealed that the 1600 cm^{-1} peak did exist in the spectrum of the dimethylated uric acid but other absorption peaks differed. The solid exhibited no absorption of UV light when in 1 M HOAc, due perhaps to its very low solubility in that media, but spectra of the solid product in H_2O and in pH 12 hydroxide buffer exhibited a strong UV absorption in the 360 - 330 mμ region. A dimeric structure was suspected based on the low solubility and high melting point as well as the fact that the coulometric determinations indicated that a low number of electrons were transferred in the oxidation. A dimer of 1,3-dimethyluric acid was tentatively postulated. The results of the elemental analysis for percentage composition of nitrogen, hydrogen and carbon are given in Table 15, sample H - I. The sample data does not match the theoretical calculation of the composition of the proposed dimeric structure.

Repeated macroscale electrolysis in 1 M HOAc were carried out with careful collection and washing of solid product. The IR and UV spectra of the various samples were consistent. The results of an elemental analysis on a homogeneous mixture of the collected solid are

given in Table 15, H - II.

Table 15

Percentage Composition Data for Solid Product

Sample Designation	Percentage		
	Carbon	Hydrogen	Nitrogen
	Experimental		
H - I[a]	45.10	3.91	28.87
H - II[a]	44.72	3.80	30.26
H - III	46.70	4.09	31.77
	Theoretical		
1,3-Dimethyl Uric Acid Dimer	39.4	4.0	26.4
Theophylline Dimer	47.0	3.94	31.2

[a] These samples contaminated with theophylline later removed *via* sublimation.

Observation of the sample heated in an open flame suggested the possibility of sublimation as a means of purification. Preliminary studies revealed that the solid sublimed at temperatures approaching 350 °C and the sublimate exhibited an IR spectrum possessing greater clarity in the 3100 - 3200 cm^{-1} region but in all other respects identical. A sublimation chamber was therefore designed (Experimental) and the

solid electrolysis product sublimed at ca. 330 °C. The sublimate formed in three overlapping zones. The front zone was white in appearance, the second appeared creme-colored and the final and thickest was very yellow. This latter, closest to the oven, was carefully separated and resublimed.

Material from the front and middle sublimate zones possessed IR characteristics identical to those of theophylline. The IR spectra of theophylline and of the resublimed unknown product are reproduced in Figures 27 and 28 respectively. The spectrum of the unknown solid possesses the same general characteristics observed in the spectra of the crude electrolysis product. However, a sharper peak of medium intensity is observed at 3160 cm^{-1} and the peaks at 1650 and 1690 cm^{-1} are of equal intensity. The UV spectra of the sublimed product dissolved in pH 12 hydroxide buffer and in water are reproduced in Figure 30. Three regions of UV absorption, ca. 340 mμ, 275 mμ and 232 mμ are observed.

In pH 12 hydroxide buffer the most intense UV absorption peaks at the longest wavelength values appear at λ_{max} = 339 mμ, with shoulders at 355 and 327.5 mμ. A weak absorption, λ_{max} = 275 mμ and a moderately strong absorption, λ_{max} = 232 mμ, are also observed. In water the absorbance is less owing to the decreased solubility. Absorption in the longest wavelength region appears to shift towards longer wavelengths as compared with pH 12 solutions; a peak appears with λ_{max} = 347.5 mμ and a shoulder at 362.5 mμ. Absorptions at λ_{max} = 274 mμ and λ_{max} = 233 mμ are also observed, the relative intensities comparable to those for pH 12 solutions. These measured wavelengths, determined on a Perkin Elmer-Hitachi spectrophotometer have a deviation of ± 2 mμ between duplicate scans.

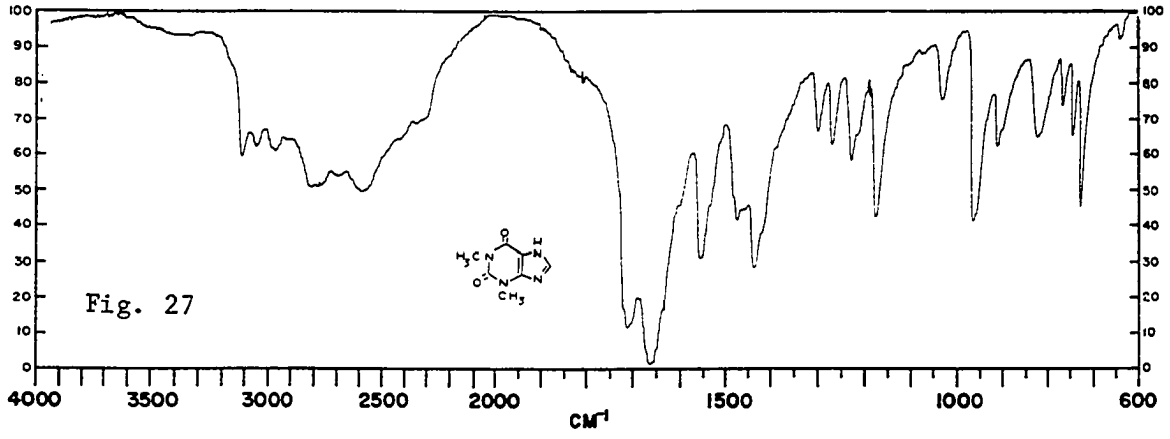


Fig. 27

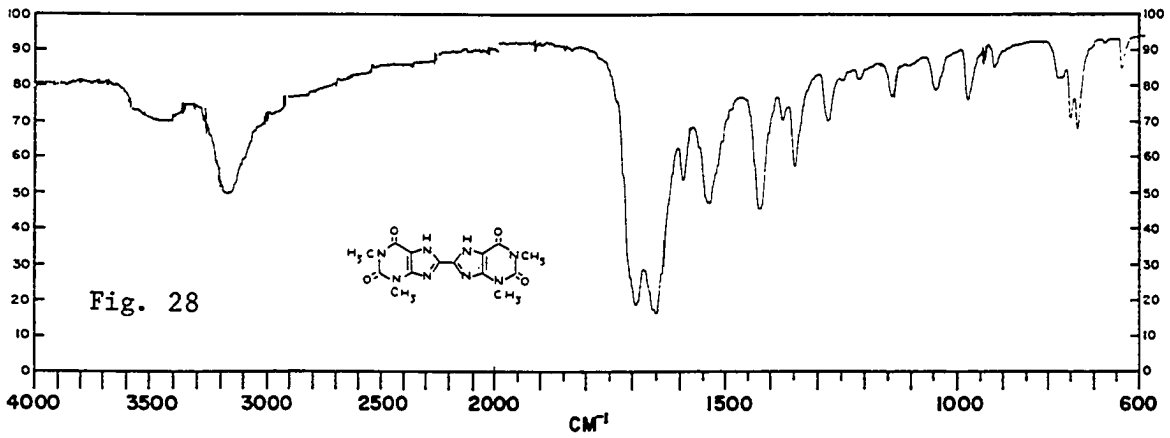


Fig. 28

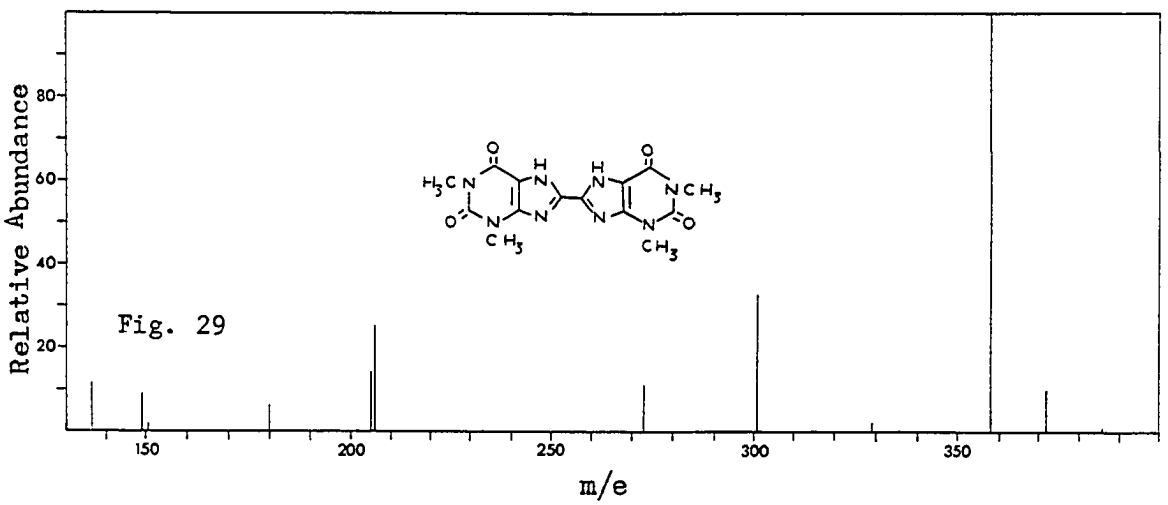
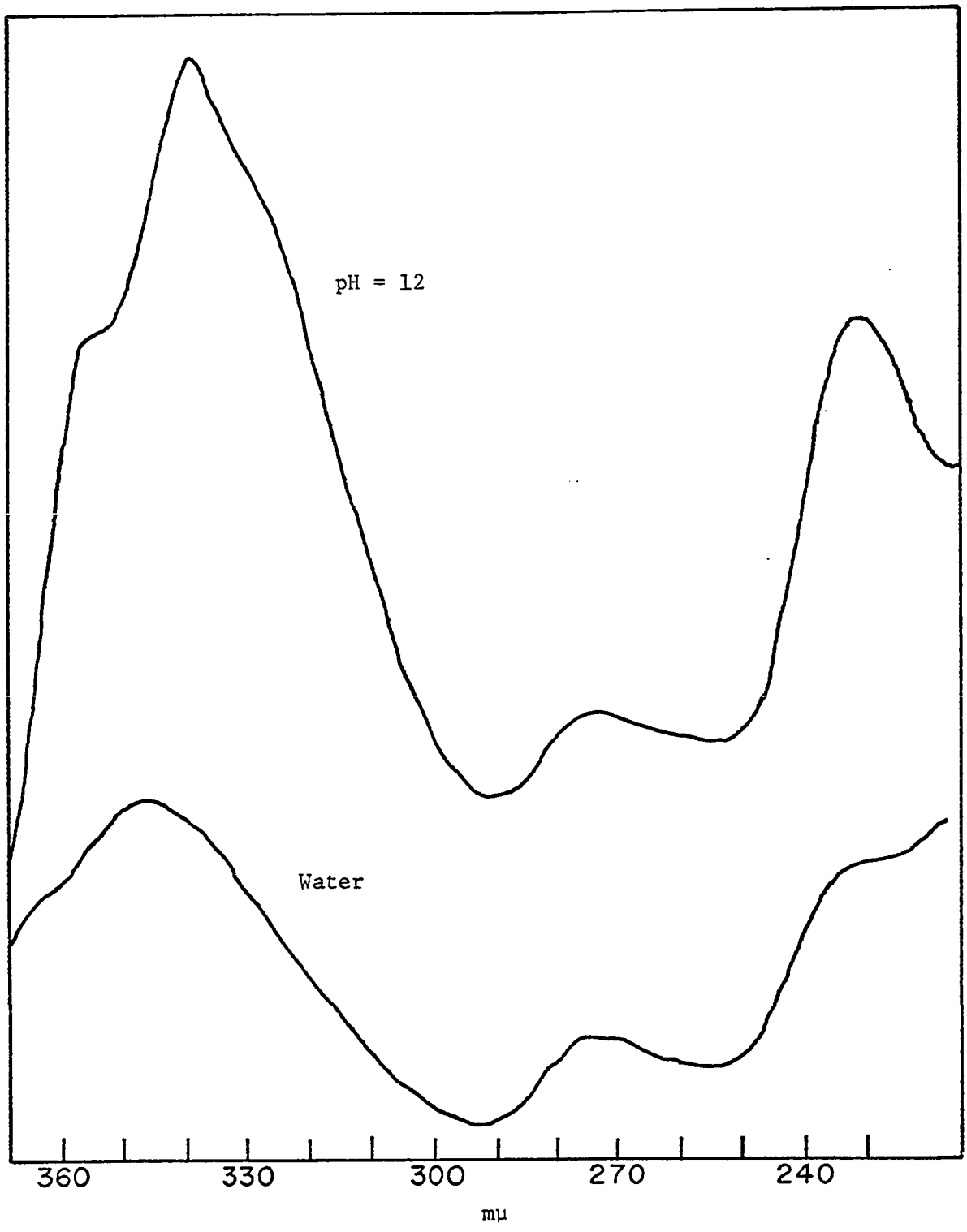


Fig. 29

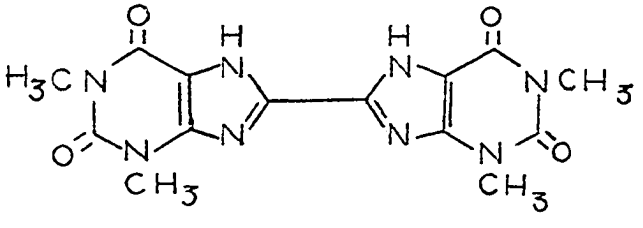
Figure 30



Ultraviolet Spectra of 8-(1,3-Dimethylxanthyl-8)-1,3-dimethylxanthine

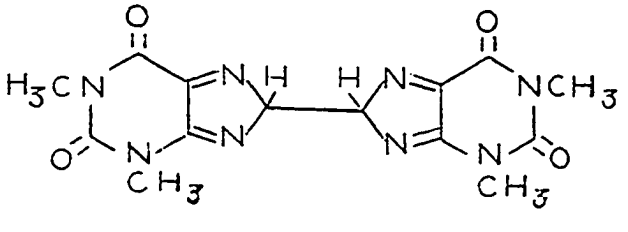
The mass spectrum of the sublimed product is reproduced in Figure 29. Elemental analysis data for the sample is given as H - III in Table 15. The mass spectroscopic molecular weight determination (358 parent ion) and the elemental analysis establish the composition as $C_{14}H_{16}N_8O_4$ which is the composition of a 1,3-dimethylxanthine (theophylline) dimer.

Structure of (1,3-Dimethylxanthyl-8)-1,3-Dimethylxanthine. The chemical composition and molecular weight of the molecule indicates that C_8 has not been totally oxidized to C=O and that therefore a one-electron oxidation has occurred followed by dimerization. Allowing that C_8 is indeed the position of primary attack in all the xanthines studied, and observing the elemental analysis and mass spectrograph fragmentation pattern, two structures are possible [(31) or (32)]:



31

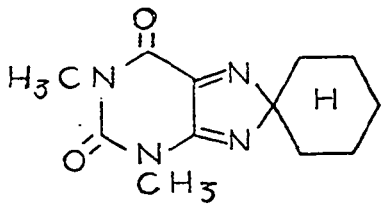
or



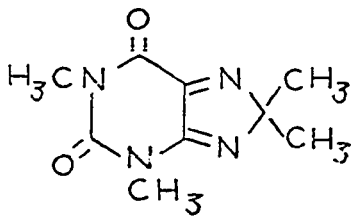
32

The fragmentation pattern does not distinguish between these two structures but consideration of analogous IR and UV spectra lend support to Structure 31 as the actual dimer molecule.

Published IR spectra of two compounds having the $-C_4=N_9-C_8-N_7=C_5-$ grouping of Structure 32, namely: 1,3-dimethyl-8,8-pentamethylene-8H-xanthine (33)[53] and 1,3,8,8-tetramethyl-8H-xanthine (34)[54] reveal that a



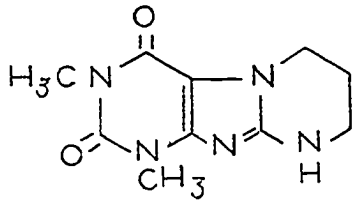
33



34

distinct carbonyl absorption due to C_6 is observed at ca. 1750 cm^{-1} in each case. The C_2 amide carbonyl absorption is closer to 1700 cm^{-1}. However, in compounds containing the $C_8=N_9-C_4=C_5-C_6=O$ grouping, in which both C_6 and C_2 can exist as amide carbonyls (C_6 exists in conjugation with $-N_9=C_8-$ through $-C_4=C_5-$) only one carbonyl absorption is observed, again in the 1700 cm^{-1} region. Such is true of theophylline (Figure 27), of the other methylated xanthines[55] and also of a recently prepared

theophylline derivative, 1,3-dimethyl-6,7,8,9-tetrahydropyriminido (2,1-f) purine-2,4 (1H, 3H) dione (35) which exhibits five absorptions



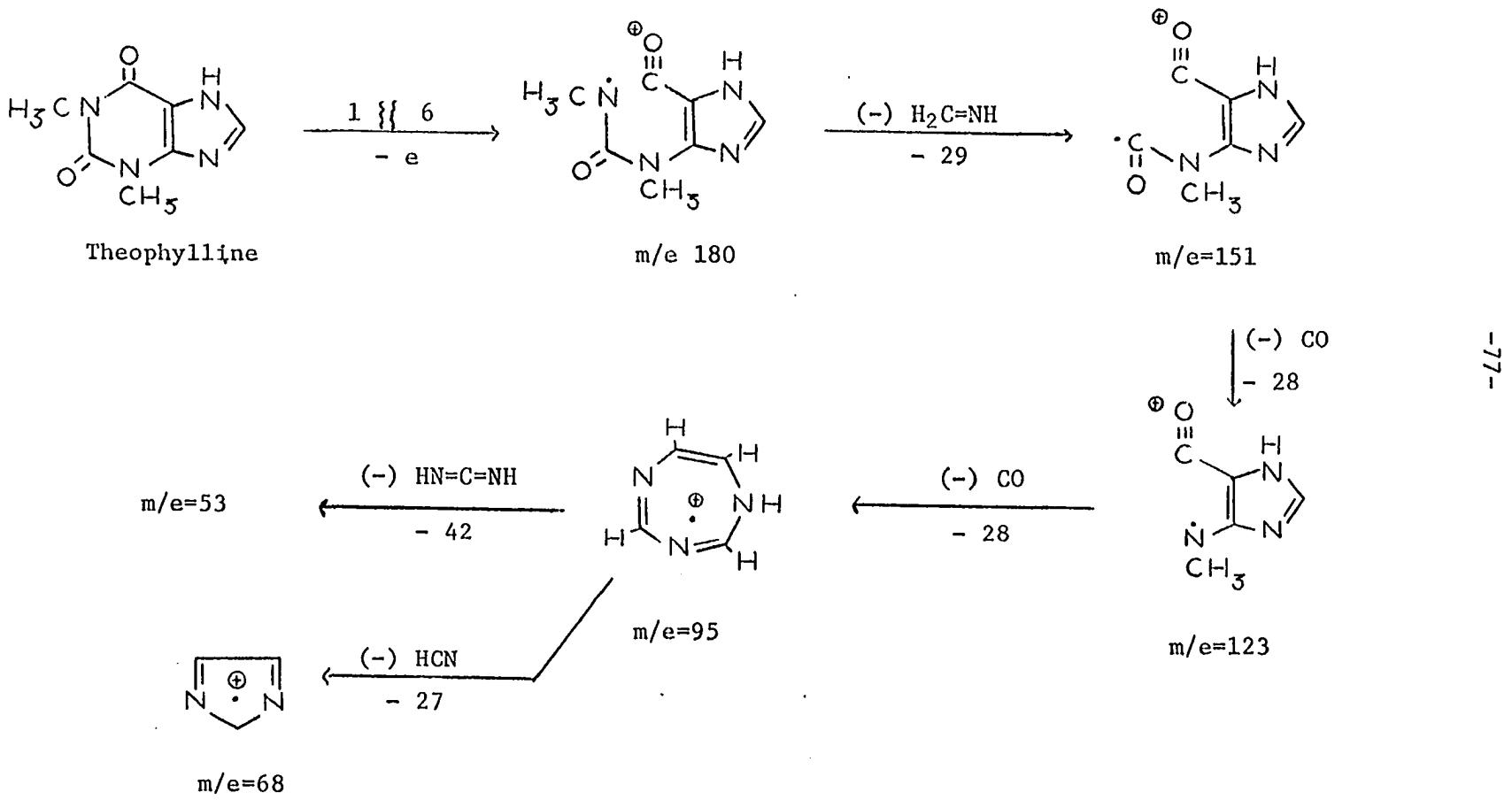
35

in the 1750 - 1500 cm^{-1} region, reported to be: 1715 cm^{-1} 1667 cm^{-1}, 1645 cm^{-1}, 1600 cm^{-1} and 1555 cm^{-1}.[56]

The UV absorption of theophylline due to the $-C_8=N_9-C_4=C_5-C_6=O$ grouping exhibits a λ_{max} = 278 mμ in alkaline solutions.[24] Noting that Bowden and co-workers[57] have shown that extension of an ethylenic bond by a tertiary nitrogen produces a 20 mμ auxochromic shift and that the increment for a double bond extending conjugation for enone absorption is given as 30 mμ,[58] it is not unlikely that a λ_{max} = 339 mμ in pH 12 hydroxide buffer is observed for the grouping in Structure 31 which is $O=C-C_5=C_4-N=C_8-C_8'=N-C_4'=C_5'-C=O$

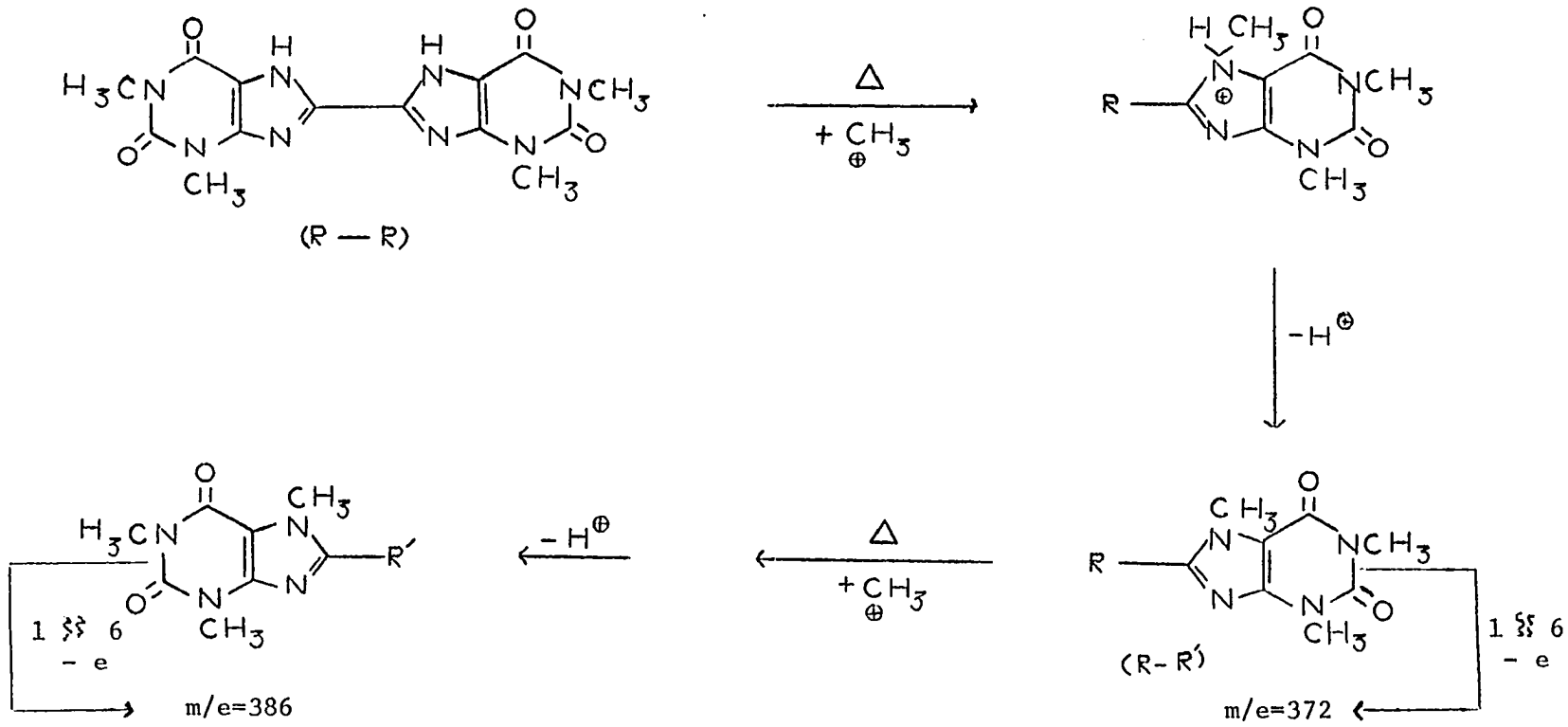
Discussion of mass spectra. The fragmentation pattern for theophylline as interpreted from mass spectrographs by Spiteller and Spiteller-Friedmann[59] is reproduced in Figure 31 for sake of comparison with that of the dimer fragmentation pattern (Figure 32B). The parent ion m/e = 180 is formed by cleavage of the N_1-C_6 bond. Successive loss of the methylated N_1 and of the C_2 carbonyl produce ions with m/e values of 151 and 123 respectively. Loss of the C_6 carbonyl and formation of a seven-membered ring follows, m/e = 95. Loss of HC≡N and HN=C=NH produces

Figure 31



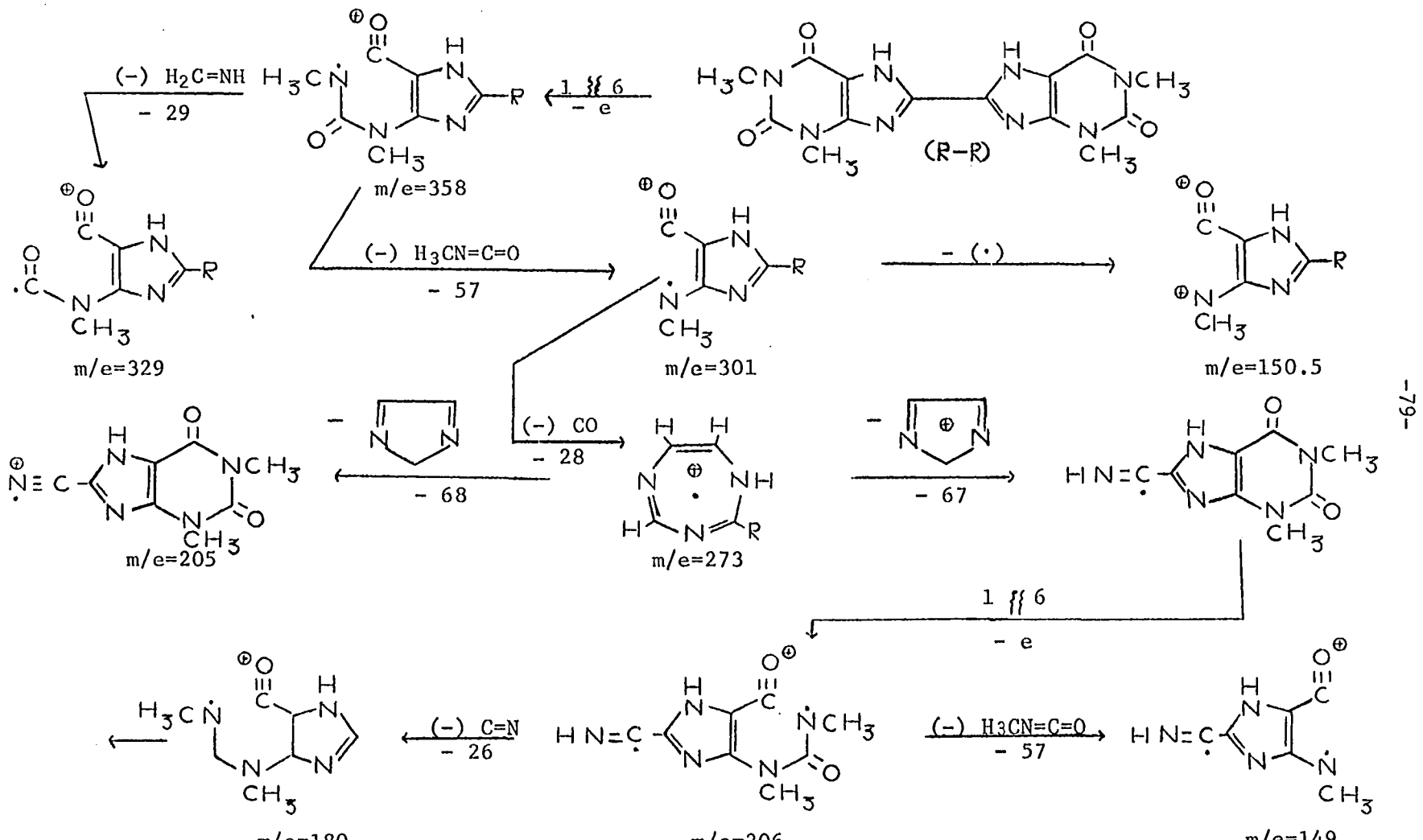
Mass Spectroscopic Fragmentation Pattern for Theophylline

Figure 32A



Proposed Scheme for Formation of Mass Spectrum Peaks at m/e=372 and 386

Figure 32B



Proposed Mass Spectroscopic Fragmentation Pattern for 8-(1,3-Dimethylxanthyl-8)-1,3-Dimethylxanthine

the ions observed at mass units 68 and 53 respectively.

In the spectrum of the dimer two peaks, 14 and 28 mass units above the expected dimer molecular weight are observed at 372 and 386 mass units. Similar higher homologs (differing by one or two $-CH_2$) groups are reported for some indole alkaloids.[60,61] By means of deuterium labelling these have been explained to occur by the thermal decomposition of one molecule, thereby liberating a $-CH_3$ group which quaternizes the basic nitrogen of a second molecule, followed by a Hofmann elimination[62] as illustrated for the theophylline dimer in Figure 32A.

The parent dimer cleaves between the N_1-C_6 bond and upon loss of 29 and 57 mass units produces ions of mass 329 and 301 respectively. Further loss of CO produces the ion of 273 mass units. Metastable peaks at mass units $(301)^2/358$ and $(273)^2/301$ affirm the direct fragmentation of a 57 mass unit from the parent dimer and a 28 mass unit from the 301 ion species. The peak at 206 mass units arises from theophylline bonded at C_8 to a C=NH remnant of the second theophylline molecule. Upon loss of 57 mass units, similar to that observed earlier in both the dimer (358 → 301) and theophylline itself (180 → 123) an ion of 149 mass units is observed. The similarity existing between the monomeric and dimeric fragmentation patterns is thus well established.

Quantitative Determination of Oxidation Products

The quantity of dimethyl alloxan and parabanic acid present in an oxidation product solution was determined polarographically. The concentration of urea and dimethyl allantoin were determined by the usual spectrometric analytical methods. The major difference exhibited by the oxidation product solution was in the low level of these products formed

Table 16

Determinations of Theophylline Oxidation Product Concentrations

Millimoles Theophylline Oxidized	Electrolysis Time min	Millimoles of Product Formed			
		Dimethyl Alloxan	Parabanic Acid	Urea	Dimethyl Allantoin
0.076	90	------[a]	0.011	0.015	------[a]
0.052	107	------[a]	0.009	0.010	------[a]
0.054	117	------[a]	0.008	0.010	------[a]
		Determinations During Course of Electrolysis			
0.048	96	------[b]	-----[b]	0.011	------[a]
0.076	190	------[b]	-----[b]	0.016	------[a]
0.126	450	------[b]	-----[b]	0.029	0.013
0.151	1630	0.033	0.021	0.032	0.016
0.069	75	------[a]	0.009	0.012	------[a]
0.107	180	0.020	0.015		------[a]
0.138	600	0.039	0.025	0.027	0.016
0.151	1980	0.040	0.030	0.029	0.020

[a] Concentration level too low to be determined.

[b] Concentration not determined.

due to the formation of the solid dimer. For this reason short-term electrolysis were not particularly practical for determining the alloxan and allantoin levels. The results of five electrolysis, two exhaustive and three short-term, are summarized in Table 16.

A material balance of the products in the usual manner is difficult since the quantity of solid product, ammonia, methyl amine and dimethyl urea are not known. Any dimethyl parabanic acid formed is not polarographically distinguishable from parabanic acid. Also, the determination of unoxidized theophylline concentration depends on the spectral properties of the theophylline and may be complicated by the spectra of the solid product dissolved in 1 M HOAc as it forms. However the following calculations can be used as evidence that all the products have been identified. Using the data listed in Table 16 for the 600 min electrolysis as a sample oxidation product solution it is possible to account for the theophylline oxidized as shown in Table 17. In this

Table 17

Oxidation Products Accounting for Nitrogen-Content of Pyrimidine Ring, Moles per Mole of Theophylline Oxidized		Oxidation Products Accounting for Nitrogen-Content of Imidazole Ring, Moles per Mole of Theophylline Oxidized	
Dimethyl Alloxan	0.282	Parabanic Acid	0.181
Dimethyl Allantoin	0.116	Urea	0.195
Dimethyl Urea	0.181	Dimethyl Allantoin	0.116
	0.579		
Solid Product	0.421		

calculation the concentration of dimethyl urea is determined to be equal to that of parabanic acid since for every mole of the 4,5-diol oxidized

to parabanic acid, the remaining molecule fragment produces dimethyl urea and carbon dioxide. These calculations indicate that 0.579 moles of theophylline are involved in a four-electron oxidation with transfer of 2.31 electrons per mole of theophylline initially taken. The 0.42 moles of dimer formed involves transfer of a further 0.42 electrons per mole of initial theophylline. Oxidation of the 4,5-diol to parabanic acid involves two electrons and this contributes 0.36 electrons per mole of initial theophylline. A total therefore of 3.09 electrons are transferred per mole of theophylline oxidized. Considering the large deviation observed in the n determinations and the rough method of calculation used, this value of 3.1 well represents the reaction. It should be noted that the quantity of parabanic acid formed is some indication of the ease of oxidation of the 4,5-diol intermediate. In the case of both caffeine and theobromine, in which the intermediate was too unstable for detection under rapid sweep voltammetry, the major secondary products are the respective alloxan and allantoin. Also, in these compounds, the very small quantity of the respective parabanic acid detected arises from the pyrimidine ring. However, in theophylline and indeed also in guanine[6] in which the 4,5-diol intermediate is detected, the major secondary product arises from further oxidation of the intermediate producing parabanic acid from the imidazole ring. The effect of N_7 methylation coupled with the shift in oxidation potentials to greater positive values for N_3, N_7-methylated xanthines is undoubtedly responsible for this phenomena.

Mechanism of Electrochemical Oxidation of Theophylline

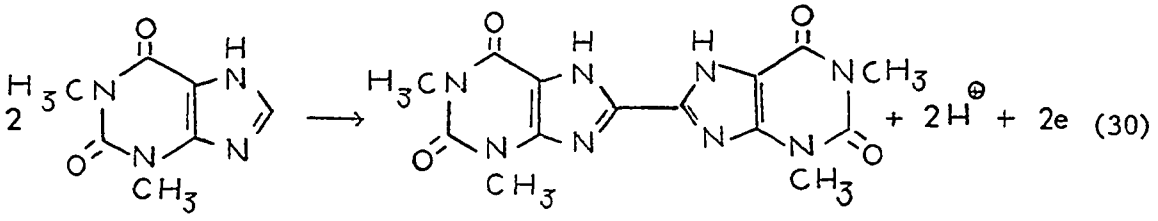
Cyclic voltammetry of theophylline indicates that the oxidation produces the 4,5-diol related to that observed in uric acid oxidation.

Faradaic n value determinations exhibited a rather large deviation between replicate experiments but averaged 3.3 electrons. To account for the low electron number, a mechanism involving a free radical and dimerization is followed, as is evidenced by formation of a solid dimeric product on the electrode and in solution. The purified solid product submitted to elemental analysis and a mass spectroscopic molecular weight determination identifies a compound with the composition $C_{14}H_{16}N_8O_4$. Mass spectrum fragmentation patterns and comparative IR studies support a structure bonded through the two C_8 positions with the remainder of the theophylline structure as in the parent molecule. This product is formed in a one electron per molecule oxidation and is illustrated in Equation 30.

The other identified solution products arise from the primary electrochemical oxidation intermediate, a 4,5-diol or similar structure of 1,3-dimethyluric acid. The potential determining step appears to involve the $-N_9=C_8-$ double bond of theophylline to give 1,3-dimethyluric acid (Equation 31). This oxidation is followed immediately by the two-electron, two-proton oxidation of the dimethylated uric acid to the related 4,5-diol (Equation 32a). Together Equations 32a - 32b represent the almost reversible oxidation-reduction observed in cyclic voltammetry.

Parabanic acid and dimethyl urea arise from a two-electron, two-proton oxidation of the 4,5-diol (Equation 33). Dimethyl alloxan and dimethyl allantoin are fragmentation products of this intermediate (Equations 34 - 35). Methyl amine and ammonia are hydrolysis products of the ureas (Equations 36 and 37). Carbon dioxide is also formed in the reactions. Dimethyl parabanic acid, if it exists, results from a two-electron oxidation of the 4,5-diol as illustrated for caffeine.

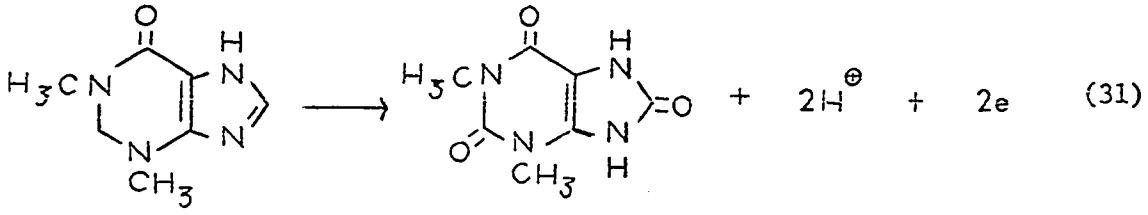
Oxidation to Theophylline Dimer



(30)

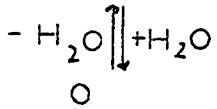
1,3-Dimethyl-xanthine (Theophylline)	8-(1,3-Dimethylxanthyl-8)-1,3-Dimethylxanthine	
0.42 mole	0.21 mole	0.42 mole

Primary Four-Electron Electrochemical Oxidation

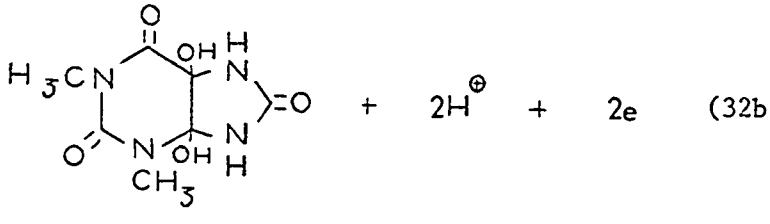


(31)

Theophylline	1,3-Dimethyl-uric Acid	
0.58 mole	0.58 mole	1.16 mole



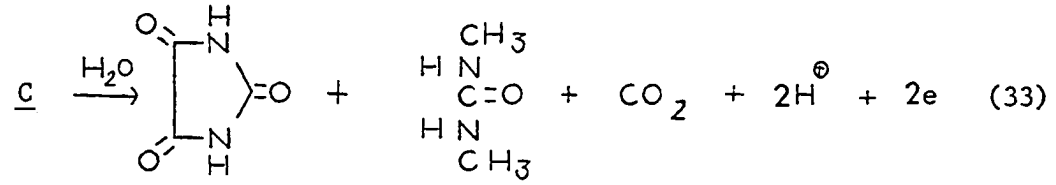
(32a)



(32b)

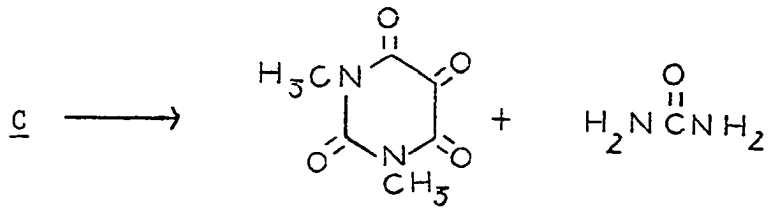
1,3-Dimethyl-uric Acid-4,5-diol	
0.58 mole	1.16 mole

Secondary Processes



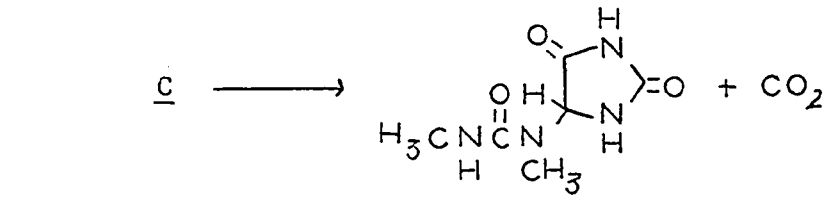
	Parabanic Acid	Dimethyl Urea	Carbon Dioxide	
0.18 mole	0.18 mole	0.18 mole	0.18 mole	0.36 mole

(33)



	Dimethyl Alloxan	Urea
0.28 mole	0.28 mole	0.28 mole

(34)

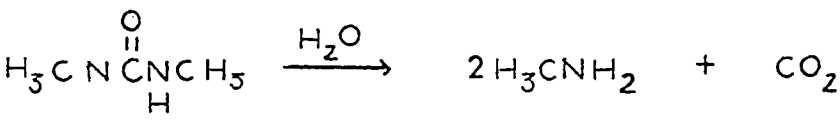


(35)

	6,8-Dimethyl Allantoin	Carbon Dioxide
0.12 mole	0.12 mole	0.12 mole

(36)

Urea	Ammonia	Carbon Dioxide
0.09 mole	0.18 mole	0.09 mole



(37)

Dimethyl Urea	Methyl Amine	Carbon Dioxide
?	?	?

EXPERIMENTAL

Chemicals

Dimethyl parabanic acid and methyl parabanic acid were prepared according to the method of Biltz and Topp.[63] Dimethyl urea (2.6 g) and oxalyl chloride (3.2 g) reacted vigorously upon mixing. The reaction product was dissolved in hot ethyl alcohol. Platelets resembling mother-of-pearl crystallized out and dimethyl parabanic acid was recrystallized from water (mp 150 - 151 °C). Methyl parabanic acid was obtained by heating a mixture of 4.45 g methyl urea, 7.5 g oxalyl chloride and 100 g anhydrous ether. The ether portion was separated from a yellow substance formed by the reaction and evaporated. The product was recrystallized from water (mp 151 - 154 °C).

Other chemicals were obtained from the sources listed: theobromine, caffeine, theophylline, xanthine and uric acid (Nutrional Biochemicals); 1-methyl and 7-methylxanthine (K and K Laboratories); 10 mg samples of 3-methyl and 1,7-dimethylxanthine (Burroughs Wellcome and Co.); 1,3-dimethyluric acid (Veb Arzneimittel, Dresden, compliments of Goldner, Dietz and Carstens);[54] diacetyl monoxime and phenylhydrazine (Fischer); urea (Merck); methyl urea, dimethyl urea and parabanic acid (Eastman); potassium ferricyanide (Mallinckrodt); bacto-agar (Difco Labs). Source of alloxan and the N-methylated alloxans is given in Experimental Part II. Any other chemicals and reagents used were of analytical re-

agent grade.

Buffer solutions were prepared with an ionic strength of 1.0 M, giving a 0.5 M ionic strength upon 1:1 dilution, and were constituted as follows: pH 0.5 - 3.0, HCl-KCl; pH 2.2 - 8.0, citric acid - NaH_2PO_4-KCl (McIlvaine); pH 3.5 - 5.8, NaOAc-HOAc; pH 8.5 - 9.0, NH_4Cl-NH_4OH; pH 11 - 13, NaOH-KCl. 2 M H_2SO_4 and 1 M HOAc were also used. Argon used for deoxygenation was equilibrated with water in a bubbling chamber. Nitrogen was used for deoxygenation of reference and working cells without purification. All mercury was triply distilled by Bethlehem Apparatus.

Thin-layer chromotography was carried out on Brinkman MN Polygram pre-coated plastic sheets of Cellulose 300, UV_{254}. Butanol-acetic acid water (120:30:50) was the developing solvent. Ehrlich reagent (10% w/v p-dimethylaminobenzaldehyde in concentrated HCl) was used for urea detection.

Apparatus

Polarograms and 3.3 mv sec^{-1} voltammograms were recorded on a Sargent Model XV polarograph using a water-jacketed three compartment cell maintained at 25° ± 0.2 °C with each compartment isolated by a fine-porosity centered glass disc; salt bridges were inserted on the counter and reference sides of the frit. The salt bridges were prepared by dissolving 4 g agar in 90 ml water, 8.5 g potassium sulfate was then added. The salt bridge was prepared with sulfate rather than chloride to avoid interference by chloride in potential determinations. Similarly, the saturated mercurous sulfate electrode (M.S.E.) was used as the reference electrode. M.S.E. has a potential of 0.40 v vs. S.C.E.[64] The counter electrode was platinum gauze in saturated potassium sulfate

solution.

The triangular potential sweep voltammetry was performed with the apparatus described by Dryhurst, Rosen and Elving.[65] The cyclic voltammograms were recorded on a Mosely Model 7001A X-Y recorder and on a Tektronix Model 502A Dual Beam Oscilloscope to which was attached a Model C-27 Polaroid camera equipped with Polaroid 3000 Black and White Speed Type 47 film. An Exact Electronics Model 502 Function Generator was used.

The dropping mercury electrode was constructed in the usual manner. An m value of 1.375 mg sec^{-1} and t = 5.08 sec^{-1} were determined in an open circuit with a column height of 70 cm. The pyrolytic graphite electrodes were prepared from G.E. pyrolytic graphite; 4 mm graphite rods (area = 12.5 mm^2) sealed in 6 mm glass tubes with epoxy resin. A solid resin seal with no air pockets was required. The electrode was resurfaced before each voltammogram by polishing on silicon carbide paper, 600 grade (Buehler Ltd.), mounted on a rotating disc. The electrode was then sprayed with a strong stream of distilled water to remove the powder from the surface and softly touched to tissue to remove the excess water. For coulometry and macroscale electrolysis six 3 in by 0.75 in plates of pyrolytic graphite inserted in a large rubber stopper were used. Resurfacing was accomplished by hand using silicon carbide paper. The plates were sprayed with water for final cleaning.

The temperature of the cell and water-jacketed bubbling chamber was maintained by circulating water from a bath heated by an incandescent bulb in circuit with 25 °C or 40 °C temperature regulators and a mercury relay (H - B Instrument Co.).

The cell used for coulometric and macroscale electrolysis consisted of a 250 ml beaker as the working compartment, with two 100 ml beakers attached with glass arms containing medium porosity sintered glass discs at approximately 90° to the side of the large beaker. These were the counter and reference compartments. Each compartment was fitted with a rubber stopper containing holes for electrodes and bubblers. Salt bridges were on the counter and reference sides of the frits. The reference electrode was M.S.E. and the counter electrode was a large platinum gauze in saturated potassium sulfate solution. The graphite plates described above, immersed in the solutions in the working electrode compartment served as the anode for the oxidations. Solutions were magnetically stirred with a Teflon-covered iron bar or by the bubbling gas used for deoxygenation.

A Wenking Potentiostat, Model 7214-66TA1, was used for the constant potenial source for the electrolytic and coulometric work. A titration coulometer based on the reaction:

$$2\ Ag + 2\ Br^- + 2\ H_2O = 2\ AgBr + H_2 + 2\ OH^-$$

was placed in the circuit for electron number determinations.[64] The titration vessel contained a platinum gauze electrode, a coiled silver wire (Sargent, #16) electrode, bubbler, glass electrode and a solution of 0.03 M potassium bromide and 0.2 M potassium sulfate. One mole of hydroxide ion per faraday is produced at the cathode. The hydroxide ion is titrated with standardized HCl, ca. 0.01 M, the equivalence point at pH = 7.0 maintained with the glass electrode. White light is excluded from the coulometer since light decomposition of AgBr produces hydrogen ion.

The pH measurements were made with a Beckman Zeromatic pH meter. Infrared spectra were obtained with a Beckman IR-8 Spectrophotometer. KBr pellets were made with a hydraulic press. Ultraviolet spectra were recorded on a Beckman Model DB Spectrophotometer and a Perkin Elmer-Hitachi Model 124 Spectrophotometer using 1 cm glass stoppered cells. Mass spectra recorded at the University of Oklahoma were recorded on a Hitachi MS-18 Mass Spectrometer. Melting points were obtained with an Arthur Thomas Co. Uni-Melt and a Fischer-John melting point block. Lyophilization was accomplished using a Kinney Model KC-2 vacuum pump; cooling traps contained isopropanol-dry ice. The lyophilization vessel was a round-bottomed flask of appropriate size. The solutions were shell frozen on the flask walls.

Standard Solutions

Caffeine and theophylline solutions, 5 mM and theobromine, 2.5 mM, were prepared as standard solutions in water. Concentrations of the other xanthines were: 1,7-dimethylxanthine, 0.99 mM in water; 3-methylxanthine, 0.75 mM in water; 1-methylxanthine, 1.06 mM in 1 M HOAc, 7-methylxanthine, 1.23 mM in 1 M HOAc. Solutions prepared in pH 4.7 acetate buffer for rapid sweep voltammetry were prepared as saturated solutions except those of theobromine, theophylline and caffeine, concentrations ca. 3 mM.

Preparation of Solutions

All solutions were appropriately diluted (total volume 10 or 20 ml), the pH recorded and were placed in the polarographic cell. Deoxygenation was not required for the pH studies. In the case of cyclic voltammetry, a heavy stream of argon was bubbled through the solution

for five minutes and a stream passed over the solution during the scans. The electrode was resurfaced between scans as described above. Triplicate determinations were obtained for each solution. Triplicate scans of background solution, prepared by dilution with water, were also obtained. Peak heights were obtained by subtracting average background current from the average peak current of the xanthine voltammogram.

Concentration Calibration Curves

Polarographic concentration studies were performed on the parabanic acids and the alloxans in 1 M HOAc. Solutions 0.1 to 2.0 mM were prepared in 1 M HOAc, triplicate polarograms obtained and the average current plotted versus concentration. Beer's Law plots were obtained for theophylline, theobromine and caffeine by determining the absorbance of triplicate solutions of 0.01 to 1.0 mM concentration. Absorbance (average) was plotted versus concentration. Straight lines passing through the origin were obtained in both the polarographic and spectrophotometric calibration.

Procedure for Determination of Faradaic n Values

The cell described above for macroscale electrolysis was used with the coulometer in the circuit. The applied potential was determined from the peak oxidation potential observed for each xanthine. Generally a potential about half way up the rise toward the oxidation maximum was chosen.

A background solution, 150 ml in volume, was oxidized until a constant current reading was attained on the potentiostat and a constant volume of HCl was titrated per unit time. This constant value is the

background level, i.e., current per unit time which flows as a result of oxidation of background. The potentiostat was then turned off and a weighed quantity of the xanthine added producing a solution 0.2 or 1.0 mM in concentration. Upon dissolution the potential was again applied and the time required for oxidation carefully noted. In all cases the standard acid was titrated to bring the pH of the coulometer solution to pH 7 about every 15 min.

The reaction was determined to be completed when the quantity of standard acid titrated per unit time equaled that of the constant value attained by the background. Absence of spectrophotometric absorbance by the solution at the 272 mμ wavelength was also used to indicate exhaustive oxidation.

The n values are calculated as follows: (1) determine total volume HCl titrated minus volume required for oxidation of background during period of electrolysis (2) multiply this volume by the factor of coulombs ml^{-1} represented by standard HCl solution to determine coulombs of electricity involved in oxidation of the xanthine (3) multiply the fraction of mole of material oxidized by 96,500 coulombs $equivalent^{-1}$ and (4) divide results of step 2 by results of step 3 to determine $equivalents\ mole^{-1}$ or number of electrons involved in oxidation.

Spectrophotometric Analytical Methods

Determination of Ureas

Urea and methyl urea were determined by a modification of Rosenthal's method.[65] A standard solution of urea (1 mg ml^{-1}) was stable for a month. Fresh solutions of methyl urea (1 mg ml^{-1}) were prepared

each week. The urea solutions used in the test, 10 μg ml^{-1}, were prepared by dilution of the standard solutions. All were made up in water.

One to four ml of electrolysis test solution were diluted in a 70 ml test tube to exactly 6 ml with water; 3.0 ml As (V) solution (10 ml concentrated HCl saturated with As_2O_5, then diluted to 35 ml with HCl) and 1.0 ml diacetyl monoxime (2.5% w/v in 5% HOAc) were added. Three such samples were prepared for each determination if practical (in the case where aliquots were removed during the course of electrolysis, a single sample was removed). Six test tubes containing 0, 10, 20, 30, 40 and 50 μg ml^{-1} of urea or methyl urea and four test tubes containing 2, 3, 4 and 5 ml respectively of 0.15 m$\underline{M}$ allantoin in 1 $\underline{M}$ HOAc (prepared from 3.0 m$\underline{M}$ standard solution in water) were treated in the same manner. Final total volume in every case was 10 ml.

The tubes, fitted with vented stoppers, were placed in boiling water for 30 min, cooled in tap water for 2 min and samples placed in a spectrophotometric cell with the solution containing no urea, but carried through the entire process, used as the blank. The absorbance of urea and allantoin solutions were determined at 480 mμ, the methyl urea at 487 mμ.

Calibration curves were prepared from the urea solutions and allantoin solutions. The allantoin calibration curve was used to correct for allantoin interference in the urea determination. Some error is involved in this correction in the case of methyl urea and the methylated allantoins in the test solution since standard methylated allantoins were not available for the test and therefore the 480 mμ absorbance is used rather than a 487 mμ absorbance. The concentration of the re-

spective urea in the test solution was determined by comparison with the calibration curve.

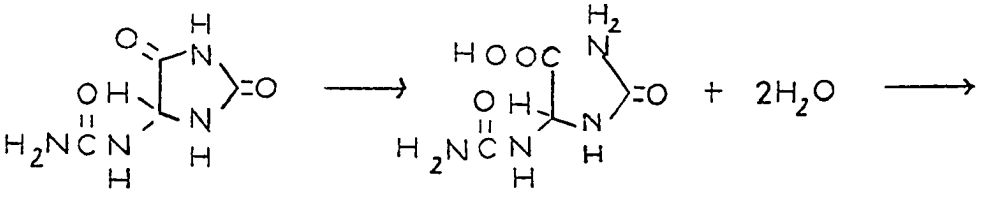
Bimethyl urea when treated as above did not result in a colored solution. A short-lived rose color developed in the first minutes of heating, then disappeared.

Allantoin Determination

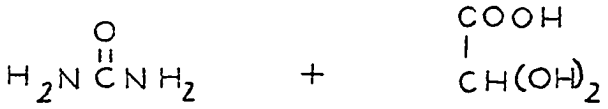
Allantoin species were determined by the Young and Conway adaptation of the Rimini-Schryver reaction.[67] This reaction is based on the glyoxylic acid-phenylhydrazine-potassium ferricyanide chromophore which proceeds as outlined in Equation 38.

Three test tubes are prepared with 1 - 4 ml electrolysis test solution (again only one sample is taken in those cases in which the concentrations are determined as the oxidation proceeds and the volume brought to 5 ml with 1 M HOAc. Similarly, five test tubes are prepared with 0, 2, 3, 4 and 5 ml 0.15 mM allantoin in 1 M HOAc and brought to 5 ml with 1 M HOAc. After adding 1.5 ml 5 M NaOH, the test tubes are placed in boiling water for 7 min, then immediately placed in ice water for 2 min. Two ml 5 M HCl and one ml phenylhyrazine (40 mg in 30 ml water) are added and the tubes returned to boiling water for 2 min. The tubes are then quickly cooled for 3 min in an ice water-salt mixture. One ml potassium ferricyanide (100 mg in 30 ml water) is added to each. The tubes are shaken to insure thorough mixing and allowed to stand for 80 min at room temperature. The absorbance of each solution is recorded at 515 mμ with the HOAc solution carried through the procedure serving as the blank.

A calibration curve is prepared from the standard allantoin



Allantoin Allantoic Acid



Urea Glyoxylic Acid (38)

$K_3Fe(CN)_6$ ⟶ Chromophore

solutions and the concentration of the test solutions determined from the curve.

Since the chromophore is based on glyoxylic acid derived from allantoin, the methylated character of the product allantoins was presumed to have no affect on the overall results. Colors observed in the test solutions well matched those of standard allantoin solutions.

Determination of Ammonia

Identification of ammonia was achieved by use of Nessler's reagent, prepared fresh each time solid was observed to have formed in the reagent bottle. The reagent was prepared as directed by King and Faulconer.[68] Potassium iodide (50 g) was dissolved in a minimum of cold distilled water (ca. 35 ml). A saturated solution of mercuric chloride was slowly added until a slight precipitate of red mercuric iodide persisted. A clarified 9 N solution of sodium hydroxide (400 ml) was added and the solution diluted to one liter with ammonia-free water. The clear supernatant liquid was decanted and used.

Standard solutions of ammonia were prepared by dissolving 0.3147 g NH_4Cl in 100 ml water thereby producing a solution 1000 μg ml^{-1} in NH_3. The solution used for testing was prepared by dilution and contained 10 μg ml^{-1} NH_3.

A calibration curve was prepared by adding 1, 2, 3, 4 and 5 ml NH_3 (10 μg ml^{-1}) to five test tubes and bringing the volume to 9 ml with water. A blank containing 9 ml water was also prepared. One ml Nessler reagent was added by a pipette to each tube with swirling and swirled for 30 sec. Each solution was allowed to stand for exactly 5 min, and the absorbance recorded at 380 mμ. About 0.5 ml test solution was the maximum used for a positive test. The allantoin concentration level present in the solutions was discovered to be responsible for a clouding effect noted. The test was therefore only of qualitative importance. Methyl and dimethyl alloxan were observed to give positive results. Preparations of methyl amine solutions produced some color but it was much less intense.

Preparation of Test Solutions for Analysis

Oxidations were carried out in the three-compartment cell described for macroscale electrolysis. One hundred and fifty ml of background solution, 1 M HOAc, was placed in the working compartment. In most cases, the coulometer was also in the circuit to follow the consumption of electrons. In those cases, the completely deoxygenated background solution was electrolyzed at the controlled-potential which corresponded to the oxidation potential of the xanthine until a constant current was maintained as determined by the constancy of the volume of standard HCl titrated per unit time. The potentiostat was then turned off and a weighed quantity of solid xanthine added to produce ca. a 1.0 mM solution. When dissolution had occurred, the potential was again applied.

Aliquots (10 ml) were taken at intervals usually gauged at 10, 20 and 30 ml standard acid additions to the coulometer. Single quantities were separated for urea and allantoin tests. Aliquots of 0.5 ml diluted to 10 ml with 1 M HOAc in the early stages and to 3 ml as the concentration of unoxidized xanthine decreased were used to determine concentration of unoxidized xanthine spectrophotometrically at 272 mμ. The remainder was used for polarographic analysis of electroactive electrolysis products and returned to the electrolysis cell.

Upon complete oxidation, determined spectrophotometrically and by observation that the current had returned to the level observed for background solutions, three samples were removed for urea tests, three for allantoin tests and triple undiluted samples subjected to spectrophotometric analysis. The entire electrolysis cell was placed under

the d.m.e. and polarographic analysis of the alloxan and parabanic acid concentration determined.

Short-term electrolysis were also performed. The constant used for timing was to continue oxidation until 10 ml standard HCl had been used in coulometer. In such, triplicate samples were removed for urea, allantoin and xanthine concentrations and the polarographic analysis was performed as above.

Thin-Layer Chromatography

Standard solutions used for thin-layer identification were prepared as follows: urea and methyl urea, 1000 μg ml^{-1} in water; dimethyl urea, 1000 μg ml^{-1} in 1 M HOAc; dimethyl parabanic acid, 2000 μg ml^{-1}; methyl parabanic acid, 1000 μg ml^{-1}; parabanic acid, 1000 μg ml^{-1}; alloxan, 1000 μg ml^{-1}; methyl alloxan 1500 μg ml^{-1} and dimethyl alloxan, 2000 μg ml^{-1}, all in 1 M HOAc.

Macroscale electrolysis of 10 mM solutions of the xanthines were performed and the volume reduced by lyophilization to about 50 ml. (Total lyophilization was not possible because the ammonia and alloxan products reacted together producing a red product presumed to be murexide.) The solution was then applied to the pre-coated cellulose sheets as were the standard solutions.

Spots were developed in butanol-acetic acid-water. The alloxans and parabanic acids were identified by UV light, the ureas with Ehrlich reagent. It was noted that alloxan in the presence of iron salts yields a purple color.[69] Several solutions containing iron were therefore tried and it was found that alloxan spots developed a purple color when sprayed with fresh aqueous solutions of ferrous ammonium sulfate.

Separation and Purification of Solid Theophylline Oxidation Product

Solid product was observed to form on the electrodes when the concentration of theophylline was greater than 1.0 mM. Quantities of the product were prepared by oxidation of very concentrated theophylline solutions (maintained lower than the point of saturation) at 1.20 to 1.30 v versus S.C.E. The solutions were magnetically stirred. Deoxygenation was not required. The electrodes were washed with streams of distilled water about every 12 hr, the washings collected in a large beaker and the solid was collected on sintered glass filters under vacuum. The electrodes were not scraped since graphite was thereby introduced in large quantities. The solution in the working compartment was filtered about every 24 hr and replaced with fresh 1 M HOAc. The counter electrode solution was also changed and frequently the salt bridge between the working and counter compartments also required changing.

A sublimation oven was designed as follows: a glass tube, open at both ends, 1 in diameter and ca.18 in long, was wrapped with nichrome wire, the coils were ca. 0.5 in apart and held firm by three pieces of asbestos string passing down the tube lengthwise, an outer tube, 1.5 in diameter, surrounded this wrapped tube which was held in place by asbestos wrappings at each end and the nichrome wire was soldered to heavier wire which served as lead posts. The oven was held in a horizontal position by clamps. Heating was accomplished electrically, using a simple rheostat and live current. A thermometer was held in a central position inside the inner tube at the far end by means of stoppers and clamps.

The sample tube consisted of a tube of such diameter as to fit inside the oven. It was 10 in long, sealed at one end and fitted with a 24-20 ground glass female joint at the opposite end. The sample, 30-50 mg, was deposited on the side of the tube very near the closed end with precaution taken so that sample was not spread along the wall of the tube. The sample tube was then inserted into the oven about 8.5 in. The tube was connected to a vacuum line and evacuated. Freezing traps of isopropanol-dry ice were included in the line.

The potential was gradually varied until temperatures approaching 330 °C were attained at which point sublimate was observed to form on the tube walls immediately outside the oven.

Three ringlets or sublimate zones were observed to form, that nearest the oven being the heaviest deposit and the most yellow in color. A sample of 30-50 mg size took about 36 hours to sublime at a regulated temperature nearest to the temperature at which sublimation was first observed to occur.

The sample tube was broken below the sublimate rings by etching and application of a heated rod (performed in glass blowing department). The sublimate zone nearest the oven was separated and resublimed for greatest purity.

Elemental analysis of nitrogen, hydrogen and carbon were performed by Chemalytics, Tempe, Arizona.

Mass spectra were obtained from the Massachusetts Institute of Technology and the University of Oklahoma laboratories.

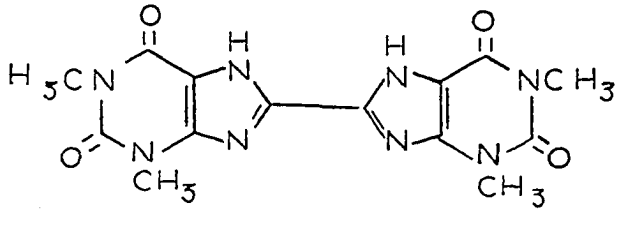
SUMMARY

Electrochemical oxidation of the naturally occurring N-methylated xanthines at the pyrolytic graphite electrode proceeds, in general, through the potential determining oxidation of the $-C_8=N_9-$ double bond producing the respective N-methylated uric acids, followed by further rapid oxidation of the $-C_4=C_5-$ double bond producing a 4,5-diol intermediate. A short-lived electrochemically reducible species related to this intermediate is detected by fast scan cyclic voltammetry for all but 1,3,7-trimethylxanthine (caffeine) and 3,7-dimethylxanthine (theobromine). The peak is due to regeneration of the $-C_4=C_5-$ double bond, which is subsequently oxidized to the 4,5-diol, detected as an anodic peak on the second and subsequent voltammetric sweeps. The anodic-cathodic peaks exist as an almost reversible couple. Reports of this intermediate formation under similar conditions for uric acid, adenine and guanine confirms that a large number of naturally occurring purines are oxidized by a mechanism involving a similar intermediate. It is also noted that the general mode of enzymic oxidation of these compounds in mammals other than man is mechanistically very similar, i.e., first oxidation at the -C=N- double bonds followed by oxidation of the $-C_4=C_5-$ double bond, and a very similar intermediate is postulated. However, since demethylation, observed in metabolism, does not occur in electrochemical oxidation, and also since the end metabolic product in human

metabolism of the purines is the related uric acids, the oxidation products identified upon electrochemical oxidation of caffeine, theobromine and theophylline are not those reported in human metabolic studies.

Macroscale electrolysis performed for faradaic n value determinations and product identification reveal that caffeine and theobromine are oxidized in a four-electron process suggesting a 4,5-diol intermediate which must be very rapidly fragmented or further oxidized since it is not detected electrochemically. Fragmentation into the respective alloxan and allantoin as the chief nitrogen-containing products of the pyrimidine ring is indeed observed. The end products of caffeine oxidation are dimethyl alloxan, 3,6,8-trimethyl allantoin, methyl urea, dimethyl parabanic acid, ammonia, methyl amine and carbon dioxide. Those of theobromine are methyl alloxan, 3,6-dimethyl allantoin, methyl urea, methyl parabanic acid, ammonia, methyl amine and carbon dioxide.

Theophylline is oxidized by two routes. About half is oxidized in a one-electron process at the C_8 position to the free radical with subsequent dimerization evidenced by formation of a yellow solid at the electrode and in solution. The compound, identified by a mass spectographic 358 parent mass unit peak and by elemental analysis to be $C_{14}H_{16}N_8O_4$ is believed by analogous IR and UV spectra to have the structure:



The remaining theophylline is oxidized in a four-electron process to the 4,5-diol intermediate which predominantly undergoes further oxidation to parabanic acid and dimethyl urea. Fragmentations of the intermediate, similar to those observed in caffeine and theobromine, produce dimethyl alloxan, 6,8-dimethyl allantoin and methyl urea. Hydrolysis of the ureas produces ammonia, methyl amine and carbon dioxide.

Three phenomena, observed in oxidation of caffeine and theobromine but not in theophylline, may be linked to the N_3-N_7- methylation combination of these compounds, namely: high positive oxidation potentials, failure to detect the anodic-cathodic couple under conditions of rapid sweep voltammetry and major production of end product via fragmentation of the intermediate rather than further oxidation. The latter two are certainly indicative of the extreme instability of the 4,5-diol intermediate.

The order of oxidation potentials observed among the N-methylated xanthines are as follows: 1,3,7-trimethylxanthine = 3,7-dimethylxanthine > 1,3-dimethylxanthine > 1,7-dimethylxanthine $\leq$ 7-methylxanthine $\geq$ 3-methylxanthine $\geq$ 1-methylxanthine $\geq$ xanthine.

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PART II

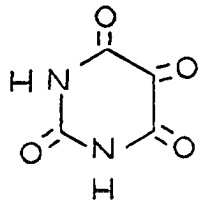
ELECTROCHEMICAL REDUCTION OF ALLOXAN, METHYL- AND DIMETHYL ALLOXAN IN AQUEOUS AND ACETONITRILE SOLUTIONS

INTRODUCTION

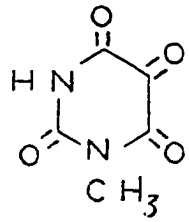
Statement of Problem

Alloxan and its N-methylated derivatives have previously been shown to be among the biological and electrochemical oxidation products of biologically important purines. For example, alloxan itself is produced upon electrochemical[1] and enzymatic[2,3] oxidation of uric acid in weakly acidic solutions. Similarly, electrochemical oxidation of theobromine, theophylline and caffeine gives rise to the appropriate methylated alloxans (Part I).

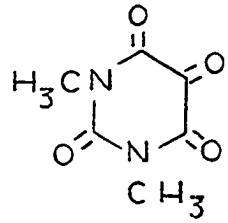
Initially it was proposed to study the polarography of alloxan (1a) methyl alloxan (1b) and dimethyl alloxan (1c) in the hope that



1a



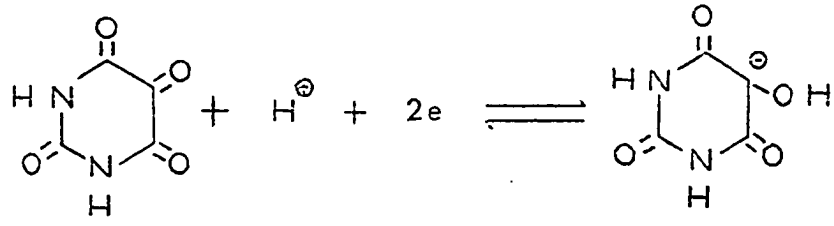
1b



1c

qualitative identification of each compound in a complex reaction mixture and quantitative determination of yield might be possible through electroanalytical methods.

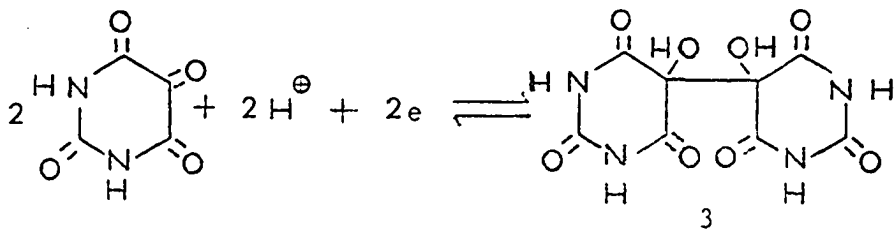
However, in view of the fact that the electrochemistry of the methylated alloxans had never previously been studied, it was decided to investigate both the mechanistic and analytical aspects of the entire system. Although alloxan itself has been studied electrochemically,[4,5] and behaves polarographically in a manner similar to the methylated alloxans, there appeared to be a need to clarify both the electrode processes and the nature of the electrochemical products. It is agreed[5] that alloxan exhibits two cathodic waves at the dropping mercury electrode and that prolonged reduction at the potential of the most positive wave produces dialuric acid (2) in a two-electron transfer process:



(1)

2

However, it would be expected on the basis of chemical evidence that the first product of the reduction of alloxan would be alloxantin (3):



(2)

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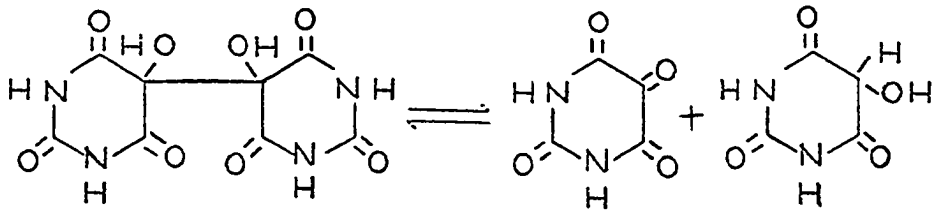
If in aqueous solutions and during prolonged electrolysis, the alloxantin disproportionates to alloxan and dialuric acid:



(3)

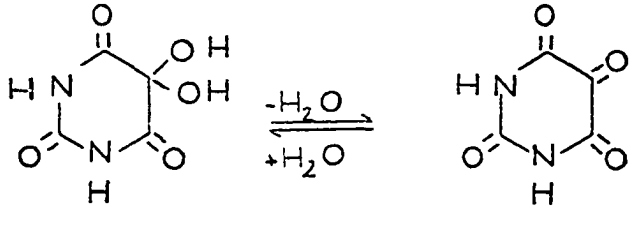
it is obvious that the ultimate product is dialuric acid. The literature contains several conflicting reports on the stability of alloxantin in aqueous solution. Biilman and Berg[6] indicate that given 100 g. of solution containing initially 0.2886 g. alloxantin seventy-eight percent would exist in the dissociated form. Similarly, Hill and Michaelis[7] in a potentiometric study report that alloxantin does not exist to any appreciable extent in aqueous solutions. However, Richardson and Cannon[8] through potentiometric titration experiments conclude that the existence of alloxantin is favored and by extrapolation of titration curves calculate an association constant (Ka) of 21 where:

$$K_a = \frac{[\text{alloxantin}]}{[\text{alloxan}][\text{dialuric acid}]} \qquad (4)$$

The results of Struck and Elving[5] suggest an even higher value. These latter workers also state that alloxantin is polarographically active and gives rise to a composite wave composed of a large anodic section and a small cathodic section at a potential corresponding to the first

cathodic wave of alloxan. This latter study, however, did not confirm whether the cathodic portion was due to the reduction of alloxan and the anodic portion due to oxidation of dialuric acid, these having formed by the disproportionation of alloxantin, or whether the composite wave is due to the oxidation-reduction behavior of alloxantin itself. Thus, available data does not indicate whether the reduction of alloxan at its first wave is a two-electron process to give dialuric acid or whether the primary electrode process is a one-electron reduction to alloxantin, which subsequently disproportionates in aqueous solution to alloxan and dialuric acid.

In order to resolve this rather subtle mechanistic problem, the electrochemistry of the alloxans was studied both in aqueous solutions and in an aprotic solvent, acetonitrile, in which the purported disproportionation of alloxantin would not be expected to occur. An additional advantage of such a non-aqueous solvent arises from the fact that the first polarographic wave of the alloxans in aqueous solution is kinetically controlled[4] by the well-known carbonyl dehydration process:



(5)

The hydrated form is favored in aqueous solutions, but only the dehydrated molecule is electroactive at the potential of the first wave.

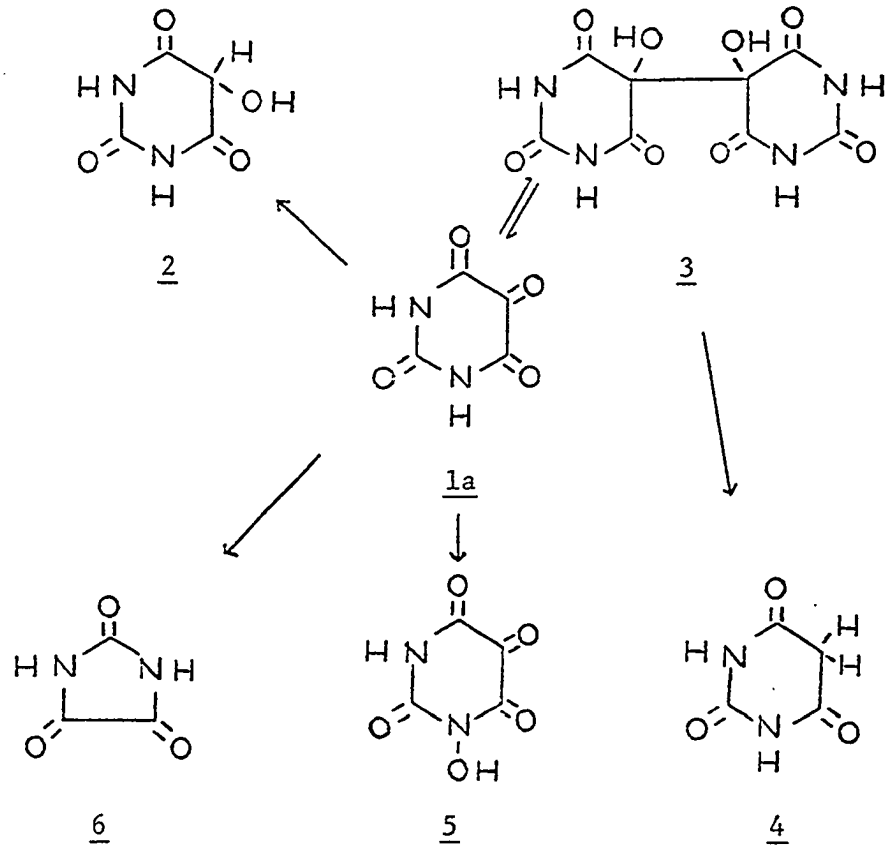
Accordingly, it was anticipated and later confirmed that in non-aqueous media the kinetic nature of this process was eliminated.

Struck and Elving[5] only briefly examined the diffusion controlled second wave of alloxan observed in aqueous solutions. The product of this reduction was postulated to be barbituric acid although determination of faradaic electron values for the wave and product isolation and identification were not carried out. Barbituric acid was postulated since if alloxan was reduced to dialuric acid in the first process it was reasonable to assume that the subsequent reduction was that of dialuric acid to the next stable species, i.e., barbituric acid. In the light of the uncertainties connected with the lack of definitive data on the second wave, a more detailed examination of the second wave was undertaken.

Alloxan and various of its derivatives are biologically active (vide infra). Accordingly, it was felt that a detailed investigation of the solution chemistry and of the electron-transfer properties of these compounds by electrochemical techniques might aid in understanding some of their biologically significant reactions at the molecular level.

Chemistry of Alloxan

The relationship of alloxan to related compounds is shown in the following scheme:[9]

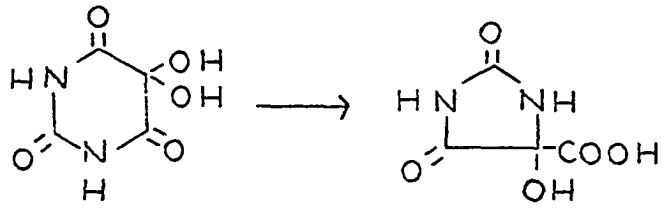


(6)

Reducing agents such as hydroiodic acid, stannous chloride, hydrogen sulfide or zinc and hydrochloric acid in the cold convert alloxan (1a) into alloxantin (3). On warming, alloxantin decomposes into dialuric acid (2) and alloxan. Solutions of dialuric acid and alloxantin are rapidly air oxidized to alloxan. Alloxantin digested with concentrated sulfuric acid gives barbituric acid (4). Alloxan oxime is known as violuric acid (5) and is the nitroso derivative of barbituric acid. On boiling alloxan with dilute nitric acid, parabanic acid (6) and carbon dioxide are formed. Alloxan in 1M HOAc partially and very

slowly decomposes to parabanic acid.[10]

Alloxan rearranges to alloxanic acid (7) at about pH 4.5;[11] the hydrolysis is very rapid in alkaline solutions.[12]



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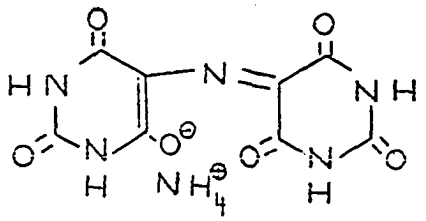
Several reactions of alloxan yield murexide[13] (8) such as the direct condensation of uramil with alloxan, the action of ammonia or



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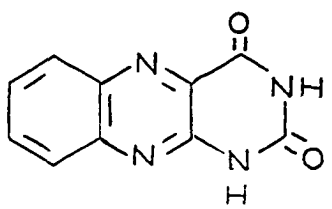
ammonium salts on alloxan or alloxantin, and the action of amino acids on alloxan and alloxantin.

Condensation of alloxan with amines occurs readily.[9] With o-phenylenediamine at pH < 5 alloxan yields alloxazine (9), the first



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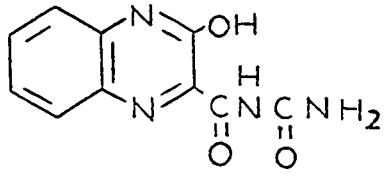
known pteridine derivative. Above pH 5, 3-hydroxy quinoxaline-2-carboxyureide (10) is formed.[2]



10

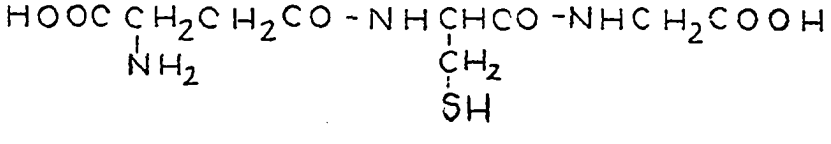
Biological and Biochemical Significance

Most interest in alloxan itself centers around its ability to induce experimental diabetes. Dunn and co-workers[14] first reported that intravenous injections of alloxan solutions into rabbits resulted in a deficient production of insulin (hypoglycemia). Selective death of the β-cells in the islets of Langerhans in the pancreas is observed. The mechanism suggested is that initially the insular tissue is excessively stimulated and death follows. The initial rise in insulin production (hyperglycemia) observed immediately upon injection supports this view. These workers note that alloxan is biologically derivable from uric acid and other purines and might conceivably be one of the components from which these are also formed *in vivo* and should therefore be studied more extensively for its relation to diabetes and other metabolic disorders. It has been known for some time[15] that an enzyme system which decomposes uric acid in the presence of oxygen, and which synthesizes uric acid from dialuric

acid and urea in the absence of oxygen is present in dog and calf liver. It has also been demonstrated[16] that alloxan introduced into the alimentary canal of rats is absorbed and passed through the portal circulation with retention of its ability to induce diabetes.

Tipson and Ruben[17] found alloxan or its reduction products in trichloracetic acid extracts of a number of animal tissues suggesting that alloxan is normally present in mammals. Indeed, it is known[17] that a certain level of alloxan can be maintained in the body without ill effects. This suggests that disorders which either cause over-production of alloxan or fail to destroy alloxan lead to the diabetic condition.

Diabetogenic dosages of alloxan lower blood reduced glutathione levels in rats.[18] Glutathione, a sulfhydryl compound, is a tripeptide containing cysteine, glutamic acid and glycine. It exists chiefly in the reduced form (11). The -SH, sulfhydryl radical, is

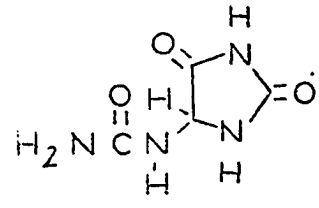


11

the portion of the molecule which acts as reductant. The oxidized form contains the -S-S- linkage. It appears that excess alloxan is oxidized by glutathione thus reducing the level of the latter in the blood. If as is assumed, alloxan functions in intermediary metabolism and biosynthesis, it has been postulated that uric acid and nucleic

acids might produce diabetes providing the blood reduced glutathione level is lowered before injection of these substances. It was confirmed that uric acid[18] and ribonucleic acid[19] injections in rabbits with lowered blood reduced glutathione caused a condition of excess blood sugar.

Studies of a comparative nature[20] on the end products of peroxidative destruction of uric acid with milk peroxidase and oxidation of uric acid with uricase have been reported. Ninety percent of the uric acid in the uricase reaction was accounted for as allantoin (12), whereas only one-third of the peroxidase end product was allantoin.



12

When aliquots of a reacting peroxidase system were added to a precipitating mixture of o-phenylenediamine and carrier alloxan, the radioactive derivative isolated suggested that alloxan formed as an intermediate in the peroxidation of uric acid.

Canellakis and Cohen[21] reported that uric acid oxidation by uricase in borate pH 7.2 produced urea and alloxanic acid.

Soberon and Cohen[2] observed that five to twenty percent of the uric acid broken down by the peroxidatic activity of leucocytes could be accounted for by the formation of alloxan upon acidification of the reaction mixture. The alloxan was trapped in the incubation

mixture at pH 6 with o-phenylenediamine and then acidified to determine the alloxan content by a fluorescence method.

Paul and Avi-Dor[3] studied the oxidation of 1-methyl uric acid with horse radish peroxidase and hydrogen peroxide. They reported a primary oxidation product obtained at pH 3-5 which could reversibly be transformed into two other forms. The primary oxidation product undergoes non-enzymatic decomposition to allantoin (pH 3-6) or to alloxan (pH $\leq$ 1).

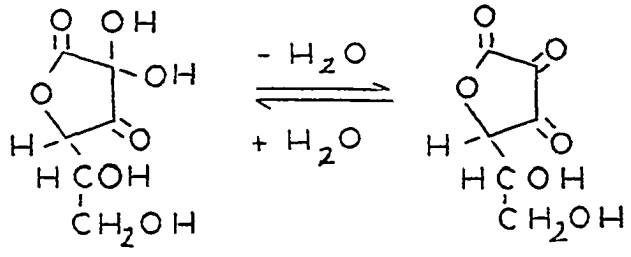
These results indicate that while it is unlikely that alloxan is formed in living organisms by peroxidase or uricase action, both primary oxidation products would yield alloxan given a pH $\leq$ 1. It is perhaps the parent substance which is responsible for the biological effects.

Reduction of the number five carbonyl group of alloxan to dialuric acid or molecular alteration to yield alloxantin does not affect the diabetic-producing activity.[22] Methylation of one of the pyrimidine nitrogens similarly does not diminish the activity. However, dimethyl alloxan and, therefore, tetramethyl alloxantin and dimethyl dialuric acids are not diabetogens.[23] Alterations to barbituric acid or violuric acid also destroys the activity.[22]

Alloxan inactivates both carboxylase[24] and urease[25] due to its ability to oxidize -SH containing enzymes to the -S-S- form. The oxidation of primary amines, notably amino acids, by the well-known Stricker Reaction[26] accounts for the amounts of murexide found in the blood and urine in cases of induced diabetes.

Previous Electrochemical Studies

Ono, Takagi and Wasa[4] reported that alloxan was reversibly reduced in a kinetically controlled step similar to that of dehydroascorbic acid (13) in which the dehydration kinetics controls the



(8)

13 13

reduction. The hydrated form is not electroactive. Alloxan also proved to be reducible in a polarographically reversible process since after controlled potential electrolysis the half-wave potential of the reduction product coincided with the oxidation wave of dialuric acid and the reduction wave of alloxan itself.

Struck and Elving[5] in a polarographic study of the alloxan-alloxantin-dialuric acid system reported that alloxan exhibited two well-defined cathodic waves at the dropping mercury electrode. They observed that the half-wave potential for Wave I (the most positive wave) shifted 0.031 volt more negative for each unit increase in pH; it was also found that 1.9 electrons were transferred in the reduction of a molecule of alloxan at the potential of Wave I. The wave was found to be kinetically controlled and the dehydration of the carbonyl

group in the number five position was postulated as the rate-controlling step, as suggested by Ono, Takagi and Wasa. An infrared spectrum of the reduction product was shown to be the sodium salt of dialuric acid. The second alloxan reduction (Wave II), observed only in the narrow pH range of 4.6 to 6.6, resulted from a diffusion controlled process. The diffusion current constant (I) ranged from 3 to 4 and, hence, the process involved was assumed to be the reduction of dialuric acid to barbituric acid and perhaps beyond.

Alloxantin was reported to show a combined cathodic-anodic wave at potentials corresponding to Wave I for alloxan and a second cathodic wave corresponding to Wave II of alloxan but with a considerably lower current. These workers concluded that alloxantin was only slightly dissociated in aqueous solutions.

Dialuric acid was shown to have an anodic wave at potentials corresponding to both cathodic Wave I of alloxan and the combined cathodic-anodic alloxantin wave.

Agreement of the half-wave potentials with potentiometric formal potentials[8] (E°) proved that the proposed reduction of alloxan and alloxantin to dialuric acid form a reversible couple.

Biilmann and Lund[27] determined the hydrogenation potential of quinhydrone and suggested quinhydrone could replace the usual H-electrode. Noting the similarity of alloxantin and quinhydrone, the hydrogenation potential of the former was also determined. Biilmann and Berg[6] used the cells of the type:

Pt/quinhydrone, 0.1$\underline{N}H_2SO_4$//0.1$\underline{N}H_2SO_4$//0.1$\underline{N}H_2SO_4$, alloxantin/Pt

and

Pt/quinhydrone, 0.1$\underline{N}H_2SO_4$//0.1$\underline{N}H_2SO_4$//0.1$\underline{N}H_2SO_4$,H_2(760mm.)/Pt.

These workers report the following emf values:

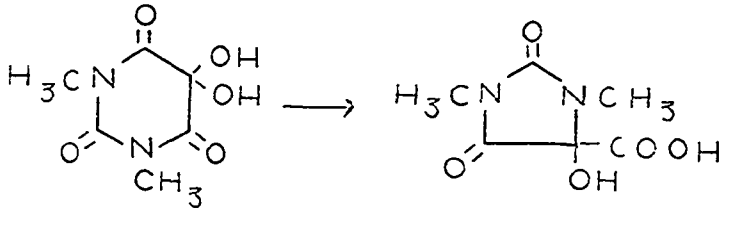
alloxantin, 0.3672v.; dimethyl alloxantin, 0.3648v.;

and tetramethyl alloxantin, 0.3633v.

RESULTS AND DISCUSSION

Polarography of Aqueous Solutions of Dimethyl Alloxan

The half-wave potentials ($E_{1/2}$) and diffusion current constants ($I = \bar{i}_l/Cm^{2/3}t^{1/6}$ where $\bar{i}_l$ is the average limiting current, μa; C is concentration, mM; m is the mass of the mercury drop, mg sec^{-1}; and t is drop time, sec) obtained for the reduction of dimethyl alloxan (DMA) in solutions of various pH are listed in Table 1. The decomposition of DMA into a non-electroactive compound begins near pH 5 and becomes very rapid as pH 7 is approached. By comparison with alloxan[11] this is the rearrangement of DMA to dimethyl alloxanic acid (14):



(9)

14

Three cathodic waves designated Ia, Ib and II are exhibited at the dropping mercury electrode (d.m.e.). Typical polarograms are illustrated in Figures 1 - 4. Variation of $E_{1/2}$ with pH is illustrated in Figure 5.

Waves Ia and Ib

Waves Ia and Ib both appear in 2M H_2SO_4, HCl-KCl solutions of

Table 1

Polarographic Behavior of Dimethyl Alloxan

pH	$E_{1/2}$ v vs. S.C.E.	i/C $\mu a(mM)^{-1}$	I[a]
	Wave Ia		
0.0 2M H_2SO_4	0.11	0.26	0.183
2.2 (M)[b]	0.03	0.28	0.191
2.3 1M HOAc	0.04	0.34	0.253
2.9 (M)	0.00	0.36	
3.4 (OAc)[c]	-0.03	0.55	0.362
3.7 (M)	-0.06	0.48	0.325
4.2 (OAc)	-0.05	0.40	0.288
4.8 (M)	-0.09	0.61	0.406
5.3 (OAc)	-0.09	0.55	0.368
5.8 (M)	-0.11	0.50[d]	0.352
6.9 (M)	-0.14	——[d]	
8.0 (M)	-0.18	——	
	Wave Ib		
0.0 2M H_2SO_4	-0.41	0.29	0.190
2.2 (M)	-0.29	0.21	0.140
0.5 (Cl)[e]	-0.34	0.31	0.191
1.0 (Cl)	-0.33	0.27	0.164
1.9 (Cl)	-0.33	0.29	0.181
3.0 (Cl)	-0.36	0.15	0.095
	Wave II		
4.8 (M)	-1.40	3.87	2.79
5.3 (OAc)	-1.42	3.15	2.19
5.8 (M)	-1.42	2.02	1.59

[a] $I = \bar{i}_l/Cm^{2/3}t^{1/6}$; [b] (M) = McIllvaine buffer; [c] (OAc) = acetate buffer; [d] Rearrangement to non-electroactive species too rapid for current measurements. [e] (Cl) = Hcl-KCl buffer.

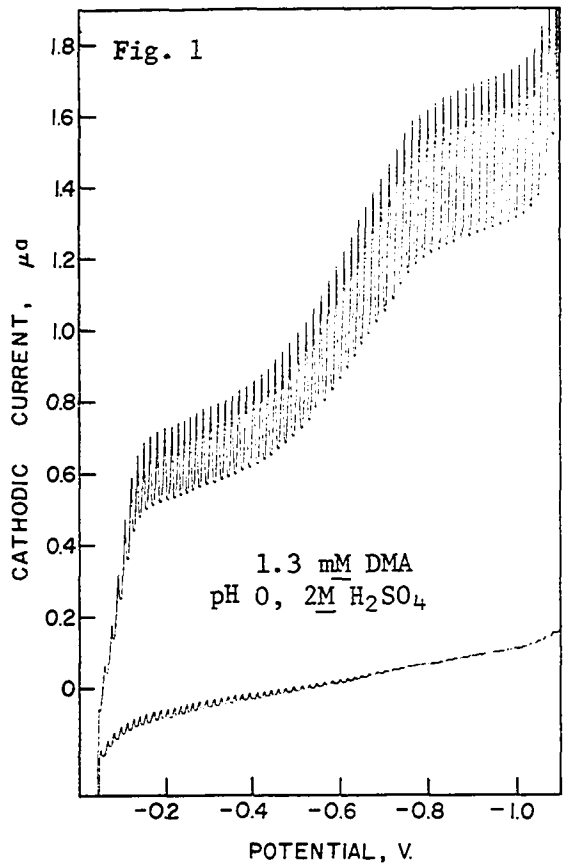


Fig. 1
CATHODIC CURRENT, μa
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1.3 mM DMA
pH 0, 2M H_2SO_4
-0.2
-0.4
-0.6
-0.8
-1.0
POTENTIAL, V.

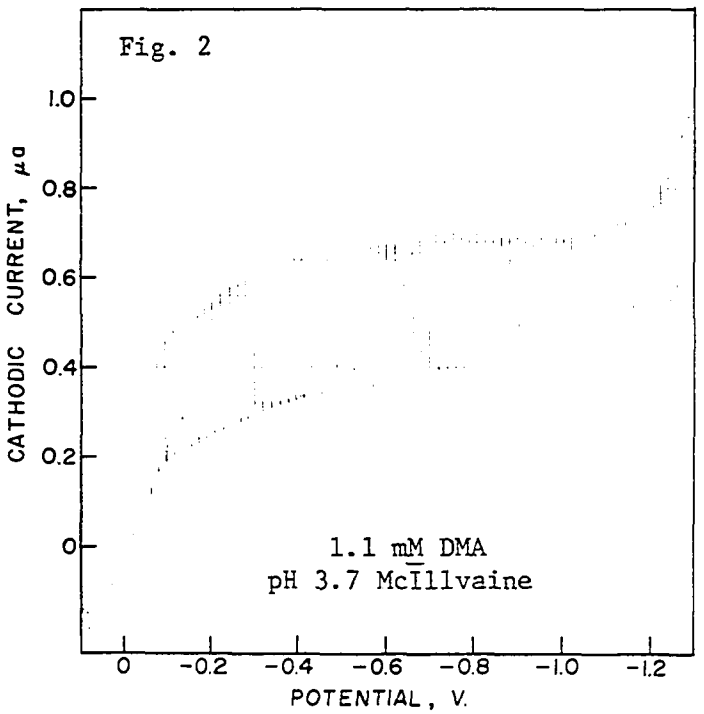


Fig. 2
CATHODIC CURRENT, μa
1.0
0.8
0.6
0.4
0.2
0
1.1 mM DMA
pH 3.7 McIlvaine
0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
POTENTIAL, V.

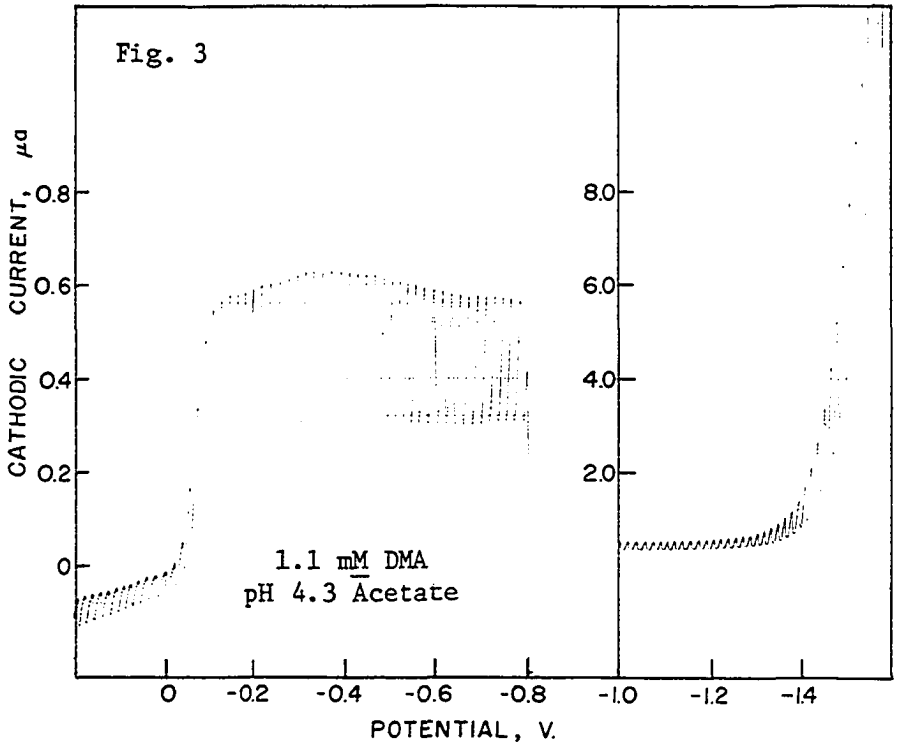


Fig. 3
CATHODIC CURRENT, μa
0
0.2
0.4
0.6
0.8
2.0
4.0
6.0
8.0
1.1 mM DMA
pH 4.3 Acetate
0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
POTENTIAL, V.

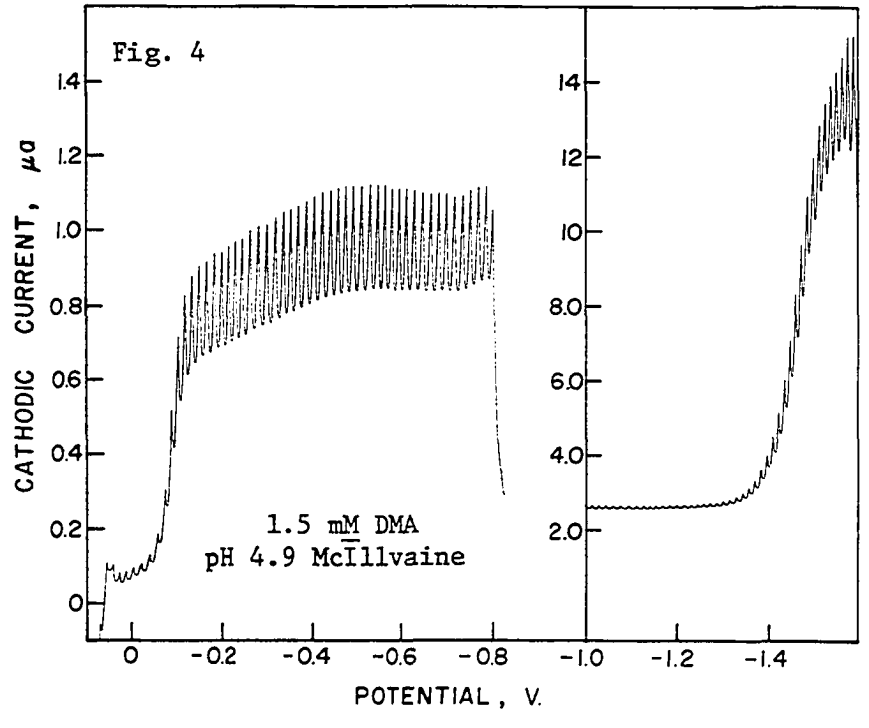
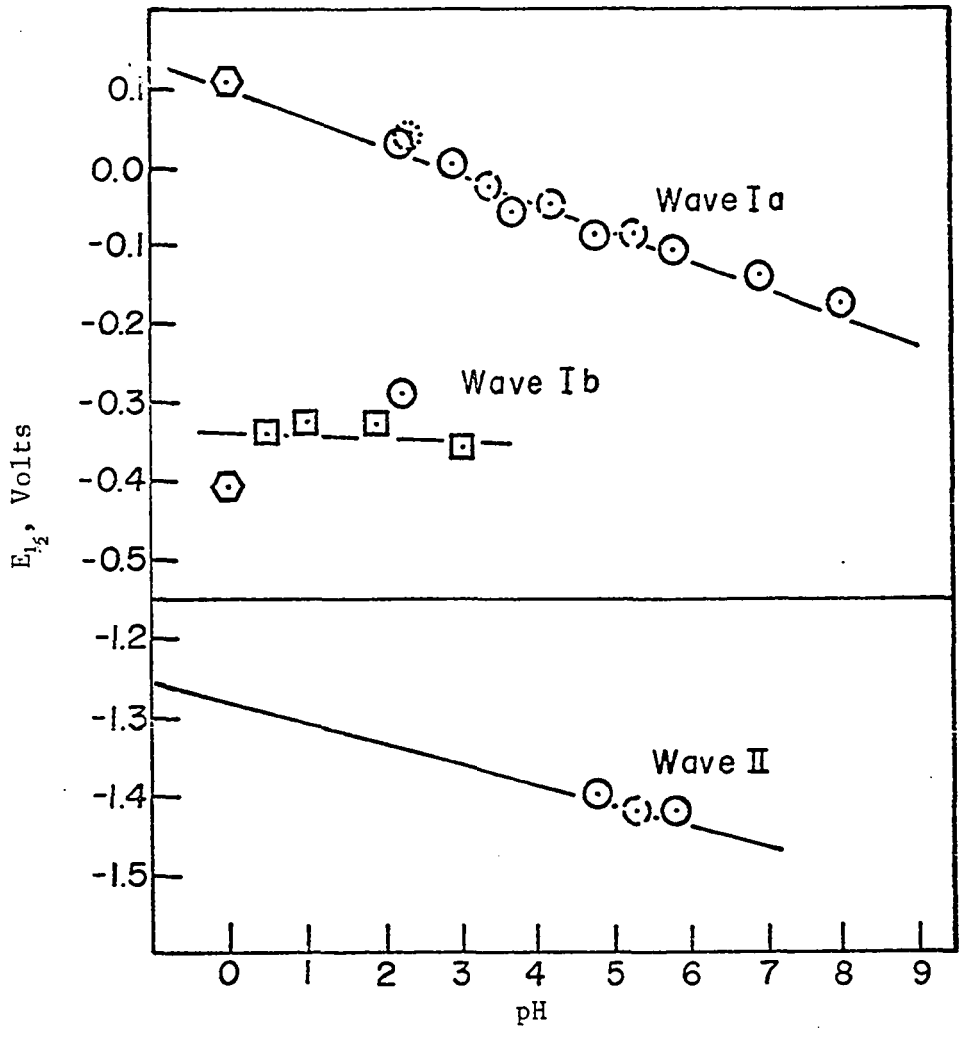


Fig. 4
CATHODIC CURRENT, μa
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
2.0
4.0
6.0
8.0
10
12
14
1.5 mM DMA
pH 4.9 McIlvaine
0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
POTENTIAL, V.

Figure 5



⊙ 2M H_2SO_4 ⊙ 1M HOAc ⊡ Chloride Buffer

⊙ McIlvaine Buffer ⊙ Acetate Buffer

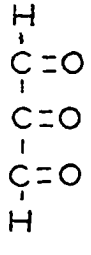
Variation of $E_{1/2}$ with pH for DMA Reduction Waves

pH 0.5 to 3.0 and in pH 2.2 McIllvaine buffer. Above pH 3 Wave Ib no longer appears.

The diffusion current constants for Waves Ia and Ib are very small. Table 2 A-C illustrates that the limiting currents are independent of the height of the mercury column indicating that a kinetic-controlled process is involved.[28] Kinetic currents arise when the height of a wave is partly or wholly determined by the rate of a chemical reaction that produces an electroactive substance in a thin layer of solution around the mercury drop.[28] A temperature coefficient (T.C.) of 5.2% / °C for Wave Ia was calculated by use of the equation:

$$T.C. = \frac{2.303}{\Delta T} \log \frac{i_2}{i_1} \qquad (10)$$

This T.C. is larger than the 1.3 to 1.6% / °C expected for a diffusion-controlled process and further substantiates that a kinetic-controlled process is involved. Dehydration of the hydrated carbonyl group in the number five position is, by analogy to alloxan,[5] undoubtedly the rate-controlling step involved. In addition to dehydroascorbic acid already cited, mesoxalic aldehyde (15) and dehydrodoreductic acid (16)[29] (all of which possess similar carbonyl groups) when observed polarographically



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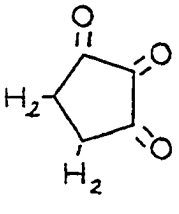


16

produce kinetic waves limited in height by the rate of the dehydration

Table 2

Effect of Mercury Column on Size of

Limiting Current for Reduction of Dimethyl Alloxan

(A) Wave Ia in 1M HOAc; DMA=1mM

Height cm	i μa
90	0.55
70	0.52
50	0.53
30	0.55

(B) Waves Ia and Ib in 2M H_2SO_4; DMA=1mM

Height cm	(Ia)i μa	(Ib)i μa
90	0.42	0.45
80	0.40	0.38
70	0.44	0.39
60	0.52	0.49

(C) Waves Ia and Ib in 2M H_2SO_4; DMA=10mM

Height cm	(Ia)i μa	(Ib)i μa
90	3.7	5.1
80	3.8	5.6
70	3.8	5.9
50	4.0	6.4

Table 2 (continued)

(D) Wave II in pH 5.3[a] (OAc); DMA=1mM

Height (h) cm	i μa	$ih^{-1/2}$ μa $cm^{-1/2}$
100	4.35	4.4
70	3.15	3.8
50	2.49	3.5

[a] Rearrangement to non-electroactive species is occurring at this pH.

of the carbonyl group. This effect of hydration on polarographic reduction of carbonyl compounds was first observed in formaldehyde and extensively studied by Brdicka.[30]

Wave Ia. Wave Ia, over the entire pH 0-8 region, linearly shifts 0.036 v. more negative for each unit increase in pH according to the equation:

$$E_{\frac{1}{2}} = 0.10 - 0.036\ pH \qquad (11)$$

This compares favorably with the equation of Struck and Elving[5] for alloxan, which they interpreted as indicating that the reduction involves two electrons and one proton. Hydrogen ions are generally consumed when organic compounds are reduced.[31] The reaction can be represented as:

$$O + pH^{+} + ne = RH_p \qquad (12)$$

where O is the oxidized form of the organic compound; RH is the reduced form, and p and n are the number of protons and electrons respectively. The current-potential curve for such a reversible reaction at 25°C can be described by the equation:

$$E_{d.e.} = E_{\frac{1}{2}} - \frac{0.059}{n} \log \frac{i - (i_d)_a}{(i_d)_c - i} \qquad (13)$$

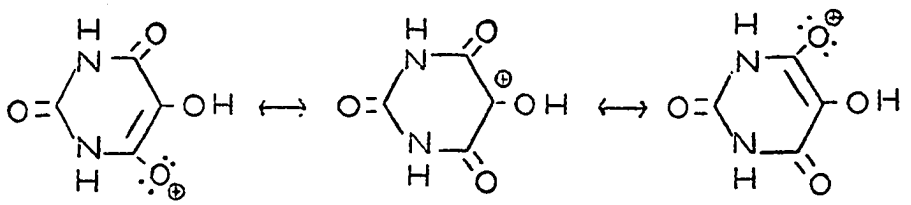
where $E_{d.e.}$ is the potential at the surface of the drop versus a suitable reference electrode, e.g., S.C.E.; i is the current and $(i_d)_a$ and $(i_d)_c$ are the diffusion current values at any point on the plateau of the anodic (a) or cathodic (c) wave. The half-wave potential, neglecting activity coefficients and liquid junction potentials is described by the equation:

$$E_{\frac{1}{2}} = E^{\circ} - \frac{0.5915}{n} \log \left(-\frac{k_o}{k_{RHp}}\right) - \frac{0.5915\ p}{n} pH \qquad (14)$$

where E° is the standard potential of the half-reaction, and k_o, k_{RHp} are the respective constants for the oxidized and reduced forms. In general, k is equal to $607nD^{\frac{1}{2}}m^{2/3}t^{1/6}$ with D being the diffusion coefficient for the respective compound. Therefore:

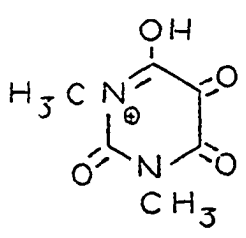
$$\frac{d\ E_{\frac{1}{2}}}{d_{(pH)}} = -\frac{0.05915}{n}\ p. \qquad (15)$$

Wave Ib. Wave Ib has a half-wave potential that is more dependent on the nature of the background material than on pH itself (see Figure 5). A pH independence is assumed since $E_{\frac{1}{2}}$ shifts only 0.03 v over the pH range 0.5 to 3.0 in chloride buffers. A deviation of ± 0.02 v between replica runs is within the acceptable range of instrumental accuracy. The half-wave potential in 2M H_2SO_4 is -0.41 v; in pH 2.2 McIllvaine buffer, $E_{\frac{1}{2}}$ = -0.29 v. The limiting current decreases significantly in chloride buffer as the pH approaches pH 3.0. At pH 1.9 the current is 0.29 μa; at pH 3.0 it becomes 0.15 μa. This evidence suggests a species which exists only in the pH region 0 - 3, which decreases in concentration with increasing pH and which is more difficult to reduce then the simple dehydrated form of DMA which undoubtedly gives rise to Wave Ia. Richardson and Cannan[8] reported a basic dissociation constant for alloxan with a pK < 1. Patterson, Lazarow and Levey[32] found that in a ten per cent sulfuric acid solution, alloxan exhibited an absorption peak, λ_{max} = 265 mμ, with an extinction coefficient of only 94. These workers concluded that a cation forms in acid solutions and suggested the structure (17):



17

However, a separate absorbing species with λ_{max} in the 265 mμ region could not be detected for DMA, if present it was masked by high end absorbance. But, in view of the second reduction wave, Wave Ib, and by analogy with alloxan, that such a cationic species exists in the limited pH range 0 - 3 is certainly not unlikely for DMA. A structure which is the same as that above for alloxan except that the positive charge resides on the nitrogen which is less electronegative than oxygen is shown as (18). However since other resonance forms are also possible the protonated structure will be designated in the general form, (19).



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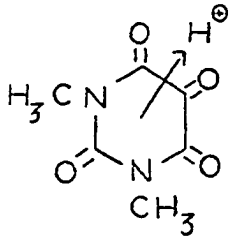


19

Since Waves Ia and Ib of DMA occur to the left of the electrocapillary maximum (mercury positively charged), it is more difficult for the polarized or positively charged material to approach the electrode (cf. Elving and Leone).[33] This accounts for the fact that the cation is

reduced at more negative potentials than the uncharged dehydrated molecule.

In pH 1.9 chloride buffer, the temperature coefficient of the limiting current of Wave Ib, 1.2%/°C, is smaller than expected for a kinetic-controlled process under usual conditions. However by analogy with cationic acids, acid dissociation constants increase with increase in temperature,[34] and accordingly, the concentration of the cationic species is much less at 40 °C than at 25 °C.

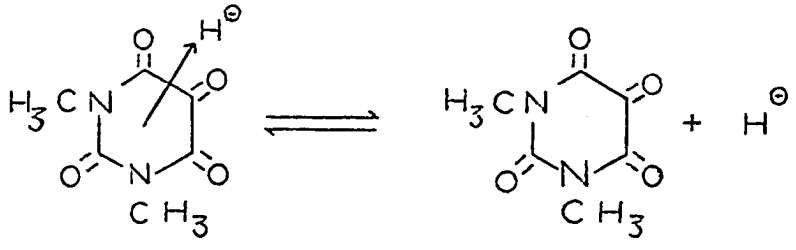
Consider the dissociation,

$$A\,H^{+} = A + H^{+}.$$

The number of charge centers remains constant. This is known as "isoelectric transference of a proton".[35] The variation of dissociation with temperature in such a case is described as follows:[34]

$$\log K = A - BT^{-1} \qquad (16)$$

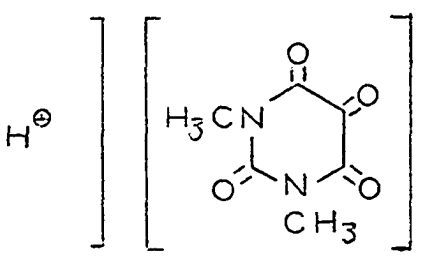
where $A = \Delta H^{\circ}_{o}/2.303R$ and $B = \Delta S^{\circ}_{o} - \Delta C_{p}/2.303R$ (ΔH°_{o} is the hypothetical standard entholpy change at absolute zero, R is the gas constant, ΔS°_{o} is the hypothetical standard entropy change at absolute zero, and ΔC_{p} is the difference in molal heat capacities of products and reactants). Hence the acid dissociation constant increases with increase in temperature. In the case of dimethyl alloxan the dissociation involved is:

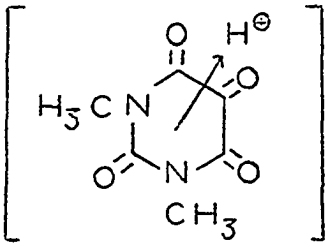


(17)

The dissociation constant can be calculated from the expression:

$$K_d = \frac{\left[H^{\oplus} \right] \left[\text{DMA} \right]}{\left[\text{DMA-H}^{\oplus} \right]} \qquad (18)$$





Thus at any pH as K_d increases with temperature increase, the concentration of the cationic species decreases and the concentration of the uncharged species increases. That this is indeed the case with DMA solutions in pH 1.9 chloride buffer can be observed in Table 3.

Table 3

Variation of Current with Temperature

Temperature °C	Current for Wave Ia μa	Current for Wave Ib μa	Ratio Wave Ia: Wave Ib
25°	0.262	0.352	0.745
40°	0.635	0.425	1.490

This decrease in concentration of the cationic species, if significant, would account for the low temperature dependence of Wave Ib.

To directly prove that the decrease in concentration of the

cationic form of DMA with temperature is of sufficient magnitude, thermodynamic data is needed. However, published values for the dissociation constant of the ammonium ion indicate that the change in concentration of a cationic species in an analogous situation is considerable; Everett and Wynne-Jones[36] report the values given in Table 4.

Table 4

Variation of Dissociation Constant with Temperature

Temperature °C	-log K	K
5°	9.898	1.26×10^{-10}
25°	9.248	5.65×10^{-10}
45°	8.678	2.10×10^{-9}

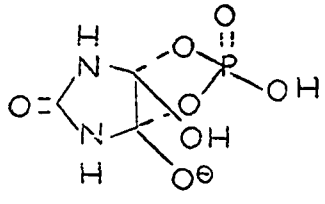
Interpolation of values for the dissociation constant versus temperature yields a value of $K = 1.45 \times 10^{-9}$ for T = 40 °C. Thus, the constant increases about three times over the 15 °C temperature variation under consideration. A three-fold increase in the dissociation constant at a constant pH represents a three-fold increase in the ratio of concentration of uncharged to cationic species and is therefore of sufficient magnitude to account for the low temperature coefficient of Wave Ib. Thus the 1.2%/ °C temperature coefficient of Wave Ib, although of the magnitude normally associated with a diffusion-controlled process, represents instead a kinetic-controlled process modified by appreciable changes in the solution concentration of the electroactive species as

a result of the temperature dependence of the dissociation equilibria. The kinetic step is basically the same as that for Wave Ia, i.e., the dehydration of the hydrated carbonyl in the number five position. The dehydrated species then becomes involved in the acid dissociation equilibrium.

Wave Ia in the absence of Wave Ib. Only Wave Ia appears in solutions of 1M HOAc, although at pH 2.3 the reduction of the cationic species to produce Wave Ib would be expected. The limiting current plateau exhibits of maximum of the first kind in the -0.1 to -0.4 v region. It was first thought that the maximum masked Wave Ib. Addition of Triton X, a typical maximum suppressor, resulted in the formation of a single normal-shaped wave (Wave Ia). The explanation probably resides in the fact that 1M HOAc does not constitute a buffered solution in the normal sense. Hydrogen ions are consumed in the reduction of most organic compounds. Therefore, in a poorly buffered solution the pH at the electrode surface increases as the cathodic current increases.[38] Pronounced changes in the characteristics of the polarogram often reflect this variation of pH. The cationic species reduced at the potential of Wave Ib does not appear to exist above pH 3 and, therefore, may not exist in the vicinity of the electrode in a poorly buffered solution such as 1M HOAc.

Wave Ib does not appear in McIllvaine buffers pH 2.9 to pH 8.0 and in acetate buffers pH 3.4 to pH 5.3. Comparison of Figures 2 and 4 with Figure 3 illustrates, however, that the shape of the current-potential curve for Wave Ia at potentials immediately more negative than the half-wave potential differs between the two buffers. In

McIllvaine buffers this region is more drawn out; a constant level of limiting current is reached at potentials approximately -0.3 v. more negative than with acetate buffers. However, this sloping effect does not constitute a second distinguishable reduction wave. A similar phenomena, i.e., a wave more drawn out than is generally expected, was observed in this laboratory for the second reduction wave ($E_{\frac{1}{2}}$ in the region of -1.4 v vs. M.S.E.) for parabanic acid and its N-methylated derivatives in solutions of McIllvaine buffers over a limited pH range. It was proposed[37] that the reduction of a phosphate complex of the respective parabanic acid is the process involved. The $H_2PO_4^-$ -parabanic acid (20) and the HPO_4^{-2} -parabanic acid (21) complexes are postulated to be:



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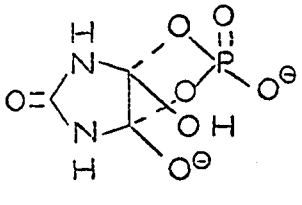


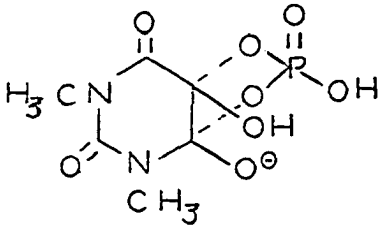
21

This very weak complex of parabanic acids with phosphate was reduced at a more negative potential than the free acid.

Since the nature of the carbonyl groups of DMA are similar to those of parabanic acid, complexes analogous to those above are plausible for a DMA-phosphate interaction. The anomaly observed in the reduction wave of DMA in McIllvaine buffers further suggests such a complex.

However, in the case of DMA, two well-formed reduction waves, i.e., one for the regular dehydrated molecule and one for the DMA-

phosphate complex are not observed. Furthermore, Wave Ia maintains a consistent linear variation of $E_{\frac{1}{2}}$ with pH over the entire pH range in all the solutions, i.e., the reduction of DMA whether in phosphate or acetate buffers involves a consistent process. The DMA-phosphate complex must, therefore, be reduced at the same potential as the free DMA and simultaneously with the free alloxan. Thus, the Wave Ia reduction in McIlvaine buffers ≥ pH 2.9 probably represents the reduction of both dehydrated DMA and the DMA-phosphate complex, the latter having the proposed structure (22)



22

Wave II

Wave II for DMA is evident in McIlvaine buffers pH 4.8 and 5.8 and acetate buffer pH 5.3. It was not possible to observe Wave II in pH < 4.7 due to the onset of hydrogen discharge; above pH 5.8 the rearrangement of DMA to dimethyl alloxanic acid (see Equation 9) is too rapid to permit the observation of Wave II. Accurate measurement of the limiting current is also complicated by the rate of DMA rearrangement even at pH values where Wave II is observed. The limiting current, however, appears to be proportional to the square root of the mercury height (Table 2). This proportionality and the mean value of 2.79 for

the diffusion current constant at pH 4.8 (Table 1) suggests that the Wave II process is diffusion-controlled. The half-wave potential shifts linearly 0.03 v more negative for each unit increase in pH according to the equation:

$$E_{\frac{1}{2}} = -1.26\ (\pm 0.04) - 0.030\ (\pm 0.01)\ pH$$

It should be noted that this dependence is based on only three data points with a deviation of ± 20 mv between replica runs at any pH.

Polarography of Aqueous Solutions of Methyl Alloxan

The half-wave potentials and diffusion current constants obtained for the reduction of methyl alloxan (MA) in solutions of increasing pH are presented in Table 5. Three cathodic waves designated Ia, Ib and II are exhibited at the dropping mercury electrode in a manner analogous to the observed polarographic behavior of DMA. The variations of $E_{\frac{1}{2}}$ with pH are shown in Figure 6.

Wave Ia and Ib

Wave Ia shifts linearly more negative with increasing pH according to the equation:

$$E_{\frac{1}{2}} = 0.08 - 0.027\ pH. \qquad (20)$$

The kinetic character of Wave Ia is obvious from the low value of diffusion current constants, the constancy of the limiting current with change in height of the mercury column (Table 6) and a temperature coefficient of 4.3%/ °C.

Wave Ib is observed in solutions pH 0 - 3 and is essentially

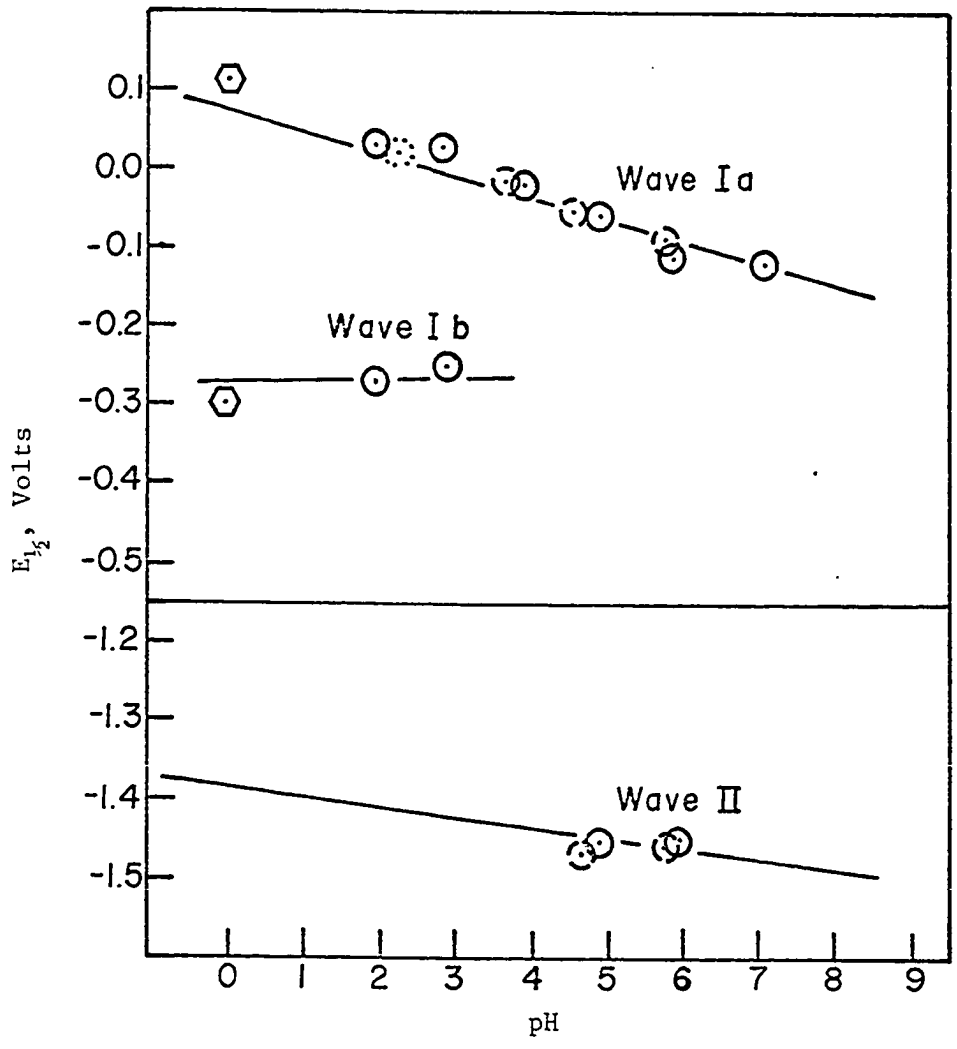
Table 5

Polarographic Behavior of Methyl Alloxan

pH	$E_{1/2}$ v vs. S.C.E.	i/C $\mu a(mM)^{-1}$	I[a]
	Wave Ia		
0.0 2M H_2SO_4	0.11	0.34	0.195
2.0 (M)[b]	0.03	0.24	0.135
2.3 1M HOAc	0.02	0.36	0.219
2.9 (M)	0.02	0.26	0.159
3.7 (OAc)[c]	-0.02	0.30	0.179
3.9 (M)	-0.02	0.34	0.209
4.6 (OAc)	-0.06	0.51	0.309
4.9 (M)	-0.06	0.42	0.257
5.8 (OAc)	-0.09	0.50	0.309
5.9 (M)	-0.11	0.59	0.362
7.1 (M)	-0.12	----[d]	
	Wave Ib		
0.0 2M H_2SO_4	-0.30	0.20	0.124
2.0 (M)	-0.27	0.13	0.080
2.9 (M)	-0.25	0.09	0.055
	Wave II		
4.7 (OAc)	-1.47	4.8	3.76
4.9 (M)	-1.46	4.4	2.79
5.8 (OAc)	-1.46	3.0	1.91
5.9 (M)	-1.46	2.6	1.66

[a] $I = \bar{i}_l/Cm^{2/3}t^{1/6}$; [b] (M) = McIllvaine buffer; [c] (OAc) = acetate buffer; [d] Rearrangement to non-electroactive species too rapid for current measurements.

Figure 6



2M H_2SO_4 1M HOAc McIlvaine Buffer

Acetate Buffer

Variation of $E_{1/2}$ with pH for MA Reduction Waves

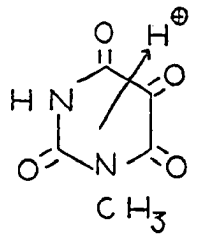
Table 6

Effect of Mercury Column Height on Size

of Limiting Current for Reduction of Methyl Alloxan

Wave I in 1M HOAc; MA=1mM	
Height cm	i μa
90	0.41
70	0.35
55	0.34
35	0.32

pH independent (see Figure 6). The reduction of the methyl alloxan cation (23) is undoubtedly the process involved, analogous to DMA Wave Ib.



23

The current is smaller than that of DMA Wave Ib: accordingly, the protonation of MA is presumedly less favored than the protonation of DMA. This effect is not entirely unexpected since Bergmann and Dikstein[39] have shown that the inductive effect of an electron-repelling substituent, e.g., $-CH_3$, is similar to a negative charge at nitrogen, though less pronounced. Thus, a methylated amide carbonyl is a more favored proton acceptor than a non-methylated amide carbonyl. Experiments in this laboratory did in fact reveal that Wave Ib is not observed in 1mM solutions of alloxan, which has no N-methyl groups, in 2M H_2SO_4. A 10 mM solution exhibits a height of Wave Ib comparable to a 2mM solution of DMA (Table 7).

Wave II

Wave II appears in the limited pH range 4.7 to 5.9. Below pH 4.7 the hydrogen discharge current masks the wave; above pH 5.9 the rate of rearrangement of methyl alloxan to non-electroactive methyl alloxanic acid (cf. Equation 9) is too rapid to permit observation of Wave II. The half-wave potential shifts 0.014 v more negative per unit increase

Table 7

Comparative Current Data for

Alloxan and Dimethyl Alloxan in 2M H_2SO_4

Concn. mM	Current for Wave Ia μa	Current for Wave Ib μa
	Alloxan	
1.0	0.38	
5.0	1.32	0.54
10.0	3.28	1.84
	Dimethyl Alloxan	
1.0	0.32	0.29
2.0	0.71	1.03

in pH as described by the equation:

$$E_{\frac{1}{2}} = -1.37\ (\pm\ 0.07) - 0.014\ (\pm\ 0.01)\ pH \qquad (21)$$

Again, as in DMA, the dependence is based on very few data points with an instrumentally inherent ± 20 mv deviation in potentials between replica runs.

Determination of Faradaic n Values of Alloxan and N-Methylated Derivatives

Data from coulometric determinations of the number of electrons involved in the reduction of alloxan, methyl alloxan and dimethyl alloxan at controlled potentials corresponding to Waves Ia and II respectively is summarized in Table 8.

Wave Ia

Exhaustive electrolysis of a 1mM DMA solution in 1M HOAc at a potential of -0.40 v required an average time of 14 hours. Methyl alloxan was similarly electrolyzed but for only 6 hours and the concentration of methyl alloxan not yet reduced determined from a polarographic concentration curve. In all cases about 2 electrons are transferred per molecule of the respective alloxan reduced.

Wave II

It has already been stated that Wave II is only observed over a limited pH range and that solutions of the respective alloxans are not stable over long periods at these pH values. Accordingly, complete electrolysis was not attempted, but rather, the electrolysis time was

Table 8

Coulometric Determination of the Number of Electrons

Involved in Reduction of Alloxan and N-Methylated Derivatives

Supporting Electrolyte	pH	Controlled Potential v	Millimoles Electrolyzed	n
		Dimethyl Alloxan		
		Wave Ia		
1M HOAc	2.3	-0.40	0.150	2.08
1M HOAc	2.3	-0.40	0.150	1.83
1M HOAc	2.3	-0.40	0.150	1.82
				Av. 1.91
		Wave II		
McIlvaine	4.9	-1.40	0.088[a]	1.84
McIlvaine	4.9	-1.40	0.074	1.85
McIlvaine	4.9	-1.40	0.074	1.93
				Av. 1.87
		Methyl Alloxan		
		Wave I		
1M HOAc	2.3	-0.40	0.095[b]	1.89
		Wave II		
McIlvaine	4.8	-1.50	0.083[a]	1.58
		Alloxan		
		Wave I[c]		
Acetate	4.0	-----	10.0	1.9
		Wave II		
McIlvaine	4.8	-1.50	0.068[a]	2.20
McIlvaine	4.8	-1.50	0.074	2.42
				Av. 2.31

Table 8 (continued)

[a] Final concn. determined spectrophotometrically; t = 30 min.

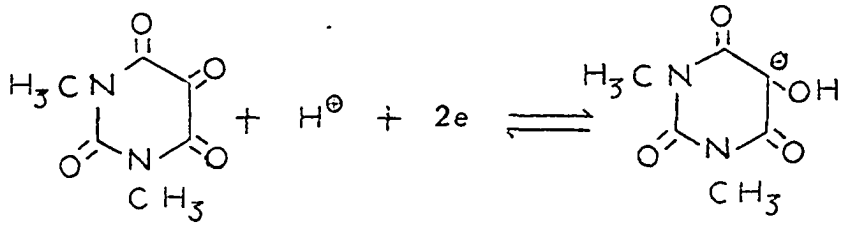
[b] Final concn. determined polarographically; t = 6 hr.

[c] Values from work of Struck and Elving.[5]

limited to 30 minutes. Polarographic concentration curves were prepared (Experimental) for solutions of each alloxan; the solutions were scanned 30 minutes after preparation in appropriate buffer (pH 4.7 to 4.9). The concentration of the alloxan remaining in the solution after a 30 min. reduction at -1.5 v was determined by comparison of the limiting current with the calibration curve. Average faradaic n values of 2.3, 1.6 and 1.9 were obtained for alloxan, MA and DMA, respectively. The reduction of the alloxans at the potential of Wave II is, therefore, a two-electron process.

Identification of the Wave I Reduction Products of DMA

The data presented in Table 8 is evidence that the alloxans undergo analogous electron-transfer processes. Hence, DMA was chosen for a more extensive study of the reduction products. DMA is reduced at the potential of Wave I (Wave I includes Ia and Ib) in a two-electron process. Two possibilities exist: either DMA is directly reduced to the dimethyl dialuric acid anion (the anion is proposed because the $E_{1/2}$-pH dependence supports the view that only one proton is involved) as shown in Equation 22, or DMA is reduced to tetramethyl alloxantin which disproportionates to DMA and dimethyl dialuric acid. In either case Equation



(22)

22 represents the overall process. Polarographic and spectrophotometric studies were carried out on the reduction product solutions with these two possibilities under consideration.

Polarographic Observations

Polarography of a 1M HOAc solution of DMA reduced at a constant controlled potential of -0.4 v revealed a single large anodic wave with an $E_{1/2}$ = 0.01 v (Figure 8).

Upon lypholization, the reduction product was faintly blue and turned red upon standing; it was hygroscopic. Polarograms of the product dissolved in 1M HOAc, revealed an anodic-cathodic couple at $E_{1/2}$ = -0.01 v and a very small cathodic wave at $E_{1/2}$ = -0.57 v.

Discussion of polarographic results. The sole anodic wave in the reduction product solution supports the assumption that dimethyl dialuric acid has been formed since dialuric acid shows analogous behavior.[5] The appearance of the cathodic wave in conjunction with the anodic wave in the lypholized reduction product can be readily explained. Reduction products of alloxan in aqueous solution, whether alloxantin or dialuric acid, are very susceptible to air oxidation back to alloxan.[5,32] Analogous behavior for the N-methylated derivatives is expected. Thus, it is reasonable to expect that some oxidation occurs while preparing the solutions for lypholization. The small cathodic wave ($E_{1/2}$ = -0.57 v) coincides with that observed for dilute solutions of dimethyl parabanic acid (24) in 1M HOAc. It has been cited (Introduction) that alloxan is observed to slowly decompose to parabanic acid. The analogous decomposition of DMA is represented as:

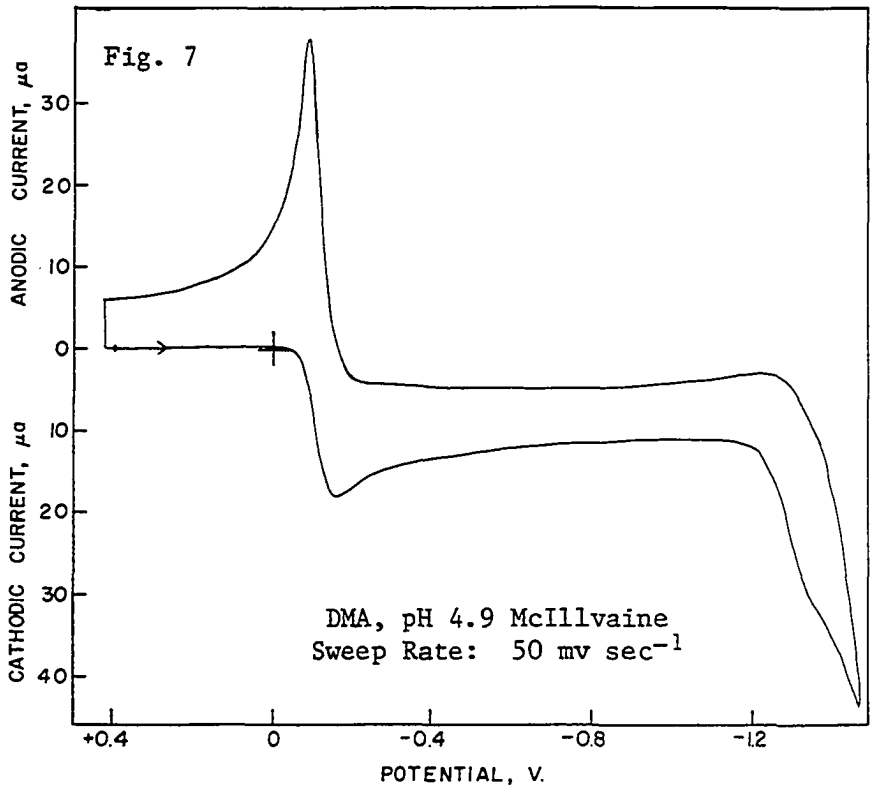


Fig. 7
ANODIC CURRENT, μa
CATHODIC CURRENT, μa
30
20
10
0
10
20
30
40
DMA, pH 4.9 McIllvaine
Sweep Rate: 50 mv sec⁻¹
+0.4
0
-0.4
-0.8
-1.2
POTENTIAL, V.

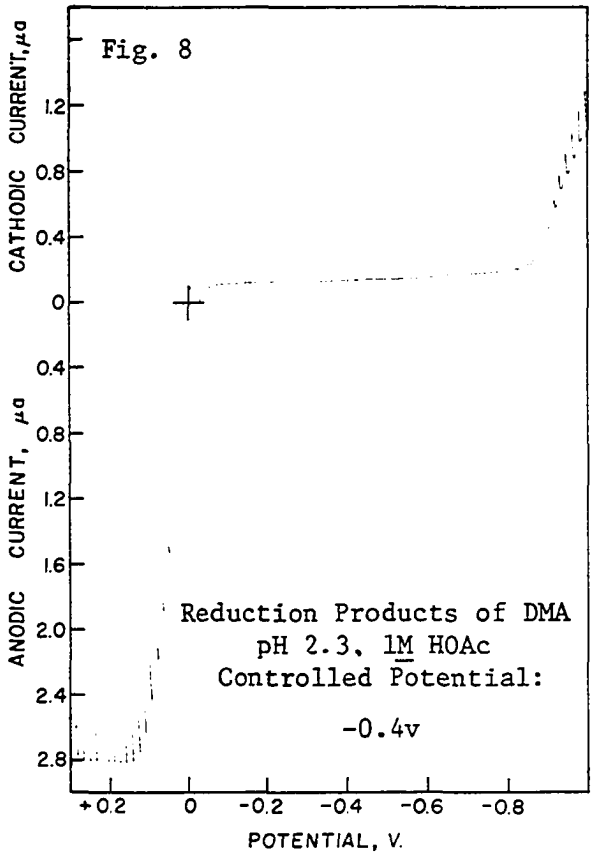
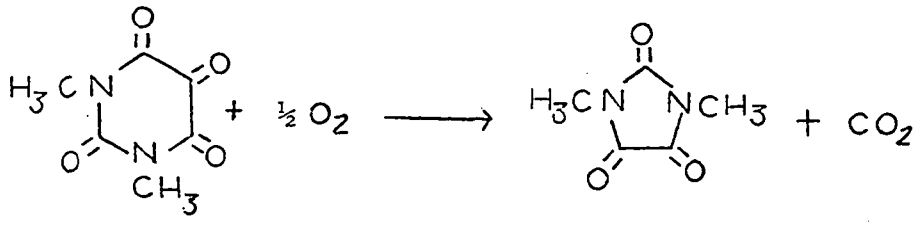


Fig. 8
CATHODIC CURRENT, μa
ANODIC CURRENT, μa
1.2
0.8
0.4
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
Reduction Products of DMA
pH 2.3, 1M HOAc
Controlled Potential:
-0.4v
+0.2
0
-0.2
-0.4
-0.6
-0.8
POTENTIAL, V.



(23)

14

Spectrophotometric Observations

In order to gain information regarding the nature of the products of the Wave I reduction throughout the course of the reduction, aliquots were taken at regular intervals and examined spectrophotometrically. Despite the difficulty of maintaining an oxygen-free atmosphere, the production of an oxygen-sensitive species absorbing at λ_{max} = 274 mμ is observed (Table 9). Upon exhaustive electrolysis, a 1:5 dilution of the reduction solution has an absorbance (A) of 0.408 at λ_{max} = 274 mμ. After exposure to air for 12 hours, the 274 mμ peak has disappeared.

Comparative study of alloxantin and dialuric acid. Attempts to synthesize tetramethyl alloxantin and dimethyl dialuric acid, which are the compounds involved in DMA reduction, were unsuccessful. Therefore, the spectrophotometric behavior of solutions of alloxantin and dialuric acid were examined under similar conditions for the sake of comparison. The results are summarized as follows:

Case 1

Alloxantin was dissolved in 1M HOAc with no precaution taken to exclude oxygen. The initial solution exhibited a peak with λ_{max} = 271

Table 9

Spectrophotometric Data[a] for Reduction Solution of DMA at Intervals throughout a Controlled - Potential Electrolysis at -0.4 v in 1M HOAc

Electrolysis Time hr	Dilution Factor	Absorbance λ_{max} 320–315 mμ	Absorbance λ_{max} 274 mμ
0	1:6	0.025	-----
2	1:6	0.040	0.100
5	1:5	0.075	0.132
9	1:5	0.128	0.340
13	1:5	0.145	0.408
--[b]	1:5	0.141	-----

[a] Obtained on Beckman DB Spectrophotometer

[b] Final solution after exposure to air for 12 hr.

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--[b]	1:5	0.141	-----

[a] Obtained on Beckman DB Spectrophotometer

[b] Final solution after exposure to air for 12 hr.

mμ. After 40 minutes, only a slight shoulder was observed in the 265 - 255 mμ region and there was a sharp end absorbance at 237 mμ.

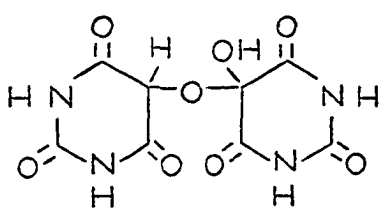
Case 2

An alloxantin solution was prepared in 1M HOAc with due precaution for excluding oxygen at all stages. The initial solution exhibited a peak at λ_{max} = 272 mμ, A = 0.318. After 7 hours a broad peak was observed in the region of 270 - 265 mμ, A = 0.138. A shoulder with A = 0.210 was observed at 237 mμ.

Case 3

A solution of dialuric acid was treated as described in Case 2. The initial solution exhibited a peak at λ_{max} = 269 mμ, A = 0.260. After 7 hours, a shoulder was observed at 237 mμ, A = 0.318. It is seen, therefore, that the behavior of the DMA reduction solution is quite comparable to that of alloxantin and dialuric acid.

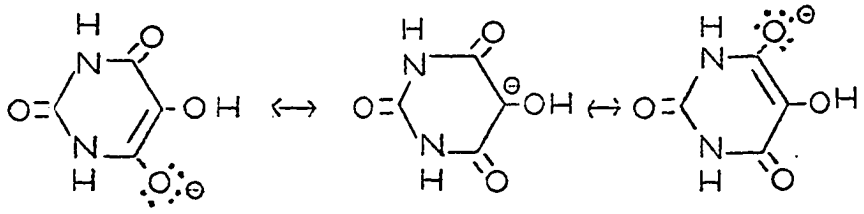
Very early work done on the absorption spectrum of the ureides,[40] led to the conclusion that simple ureides, i.e., alloxan or dialuric acid, exhibit spectra without absorption bands but that by linking together two simple ureides by one or more polyvalent atoms causes a powerful selective absorption. The structure then believed to represent alloxantin was (25) and it, therefore, was believed to exhibit an ab-



25

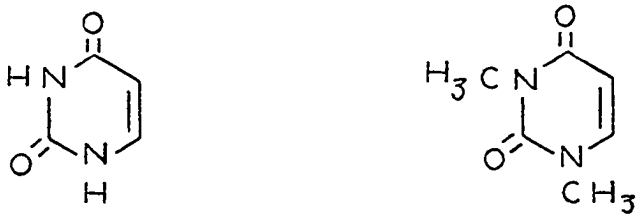
sorption band. It is now known that dialuric acid exhibits an absorption spectrum:[32] at pH 7.4, λ_{max} = 275 mμ; in 1% HCl, λ_{max} = 270 mμ. The absorption observed, therefore, in alloxantin solutions would appear to arise from the disproportionation of alloxantin into alloxan and dialuric acid.

The absorbance observed in dialuric acid is the result of the keto-enol equilibrium and ionization of the acidic hydrogen on carbon 5. In acid solutions this equilibrium (26) is represented as:



26

Discussion of spectrophotometric results. It is, therefore, evident that the behavior of the reduction product solution follows very closely that of alloxantin and dialuric acid solutions treated in a similar manner. The values of λ_{max}, however, differ slightly; the reduction product solution has a λ_{max} = 274 mμ, alloxantin and dialuric acid solutions have a λ_{max} = 270 mμ. Wave lengths can be determined within an accuracy of ± 2 mμ on the instrument used for these experiments (Beckman DB Spectrophotometer). Furthermore, the reduction product solution of DMA contains tetramethyl alloxantin and/or dimethyl dialuric acid. It can be shown by analogy with reported λ_{max} values[39] for uracil (27) and N-methylated uracils that methylation, in general, shifts the



27

absorption maximum of the non-ionized molecule toward the red, e.g., at pH 6.0, uracil exhibits an absorbance with λ_{max} = 260 mμ and 1,3 dimethyl uracil, λ_{max} = 266 mμ. Since it is $-N_3-C_4=C_5-C=O$ which is the chromophoric group in both sets of compounds, dimethyl dialuric acid is the absorbing species in the reduction product solution.

The species absorbing with λ_{max} in the 320 - 315 mμ region was not observed in short term electrolysis nor did it appear as a chemical decomposition product upon standing. Therefore, it is probably either a secondary electrochemical reduction product or a chemical decomposition product of such. It appears as a function of electrolysis time because of concentration.

Dissociation Study

The postulation of tetramethyl alloxantin as the primary electrode reduction product at the potential of Wave I requires that the degree of dissociation of alloxantin and the N-methylated derivatives in aqueous solution is indeed large so that the ultimate product is a dialuric acid.

A study of Struck and Elving[5] with alloxantin was therefore repeated. A 0.2 mM solution of alloxantin in pH 4.7 acetate buffer was observed to produce an anodic-cathodic couple with $E_{1/2}$ = 0.02 ± 0.01 v

and a second cathodic wave at $E_{\frac{1}{2}}$ = -1.51 v. The workers cited above observed that "if alloxantin were largely dissociated at the concentrations used, as indicated in earlier literature, the alloxan present as a result of such dissociation should give a Wave II for alloxantin completely comparable to Wave II of alloxan". The results summarized in Table 10 indicate that this is indeed observed and contradicts the findings of Struck and Elving. The second cathodic wave in alloxantin ($E_{\frac{1}{2}}$ = -1.51 v) is ten times that of the first cathodic wave as expected for an alloxan solution.

Macroscale Electrolysis

Struck and Elving[5] were able to isolate the sodium salt of dialuric acid as a solid product formed in the cell during a macroscale electrolysis (10 mM) of alloxan in pH 4.0 acetate buffer. Similar macroscale reductions of DMA were, therefore, carried out in 2M H_2SO_4 and in pH 3.7 acetate buffer. A solid product was not observed. Thus, dimethyl dialuric acid must be more soluble or less stable than dialuric acid.

Voltammetry of Aqueous Solutions of Dimethyl Alloxan

Cyclic voltammetry of DMA at the stationary pyrolytic graphite electrode (p.g.e.) revealed that a cathodic peak was produced. On the reverse sweep an anodic peak, with a current considerably larger than the cathodic current, appears in the same region. Figure 7 illustrates the nature of the scan in pH 4.9 McIllvaine buffer. Two cathodic peaks $(E_p)_c^I$ = -0.16 v and $(E_p)_c^{II}$ = 1.35 v and one anodic peak $(E_p)_a$ = -0.085 v are evident. Some general trends in the voltammetric peak current (i_p)

Table 10

Polarographic Date for Alloxantin in

pH 4.7 Acetate Buffer

No. of Experiment	$E_{1/2}$ v vs. S.C.E.	i μa
	0.03 v	11.25 anodic
1	0.03 v	0.51 cathodic
	-1.51 v	5.75 cathodic
	0.02 v	3.84 anodic
2	0.02 v	0.30 cathodic
	-1.51 v	3.75 cathodic

are observed. The peak current of cathodic Peak I is obviously small due to the kinetic nature of the process. Anodic Peak I is noticeably larger than the cathodic Peak I reflecting the diffusion-controlled nature of the process. Cathodic Peak II involves a smaller current than would be expected from previous d.m.e. results, i.e., a ten-fold increase.

Theoretically, a fundamental criterion of reversibility[41] is:

$$(E_{p/2})_c - (E_{p/2})_a = 56.0/n \text{ mv.} \qquad (24)$$

It can further be shown that:

$$(E_p)_c - (E_p)_a = -56.0/n \text{ mv.} \qquad (25)$$

The difference in peak potentials of cathodic Peak I and anodic Peak I for a solution of DMA in pH 4.9 McIlvaine buffer, observed at a scan rate of 50 mv sec^{-1} is -75 mv. This value suggests a slightly irreversible one-electron transfer or, in view of the relatively high scan rate, a rather irreversible two-electron process. The latter is more consonant with the polarographic data.

Proposed Mechanism for Wave I Reduction of DMA

Wave I, including Ia and Ib is clearly a kinetic-controlled process since (1) the limiting current is essentially independent of the height of the mercury column (2) the temperature coefficients are higher than those of a diffusion-controlled process (3) the diffusion current constants are very small and (4) the kinetic process of dehydration of a carbonyl in similar carbonyl structured compounds is well-substantiated polarographically. The kinetic-controlled process is the

dehydration of the hydrated form of DMA. Equation 26 represents the dehydration reaction.

The $E_{1/2}$ - pH variation (Equation 11) indicates that Wave Ia is a two-electron, one-proton process and determination of the faradaic n value verifies that two electrons are indeed involved. The reduction product solution in 1M HOAc at the d.m.e. exhibited a single anodic wave, $E_{1/2}$ = 0.04 v in 1M HOAc. Thus, the anodic-cathodic waves exist as an almost reversible couple. The UV absorption peak of the reduction product solution in 1M HOAc, λ_{max} = 274 mμ is consistent with that expected for dimethyl dialuric acid. Coulometric determinations of the two-electron reduction of DMA at the potentials of Wave I and II indicate that though the processes somehow differ, the end product is the same two-electron reduction product.

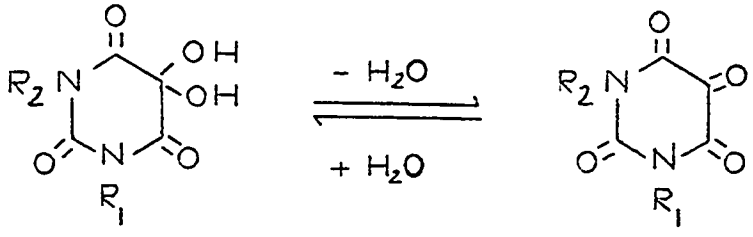
It is, therefore, proposed that at the potential of Wave Ia, dimethyl alloxan is reduced in a two-electron, one-proton process to the dimethyl dialuric acid anion. Equation 27 illustrates the proposed mechanism. This reaction may in part be occurring by way of dimerization of an alloxan free radical (a one-electron reduction product) to form the alloxantin, which rapidly dissociates in aqueous solutions to the respective alloxan and dialuric acid as represented by Equation 27b.

Wave Ib is the reduction of the cationic form of DMA in a pH independent process. The reduction is identical to Wave Ia in all other respects. Equation 28 illustrates the proposed mechanism.

A comparison of polarographic behavior, $E_{1/2}$ - pH variations and faradaic n values reveals that alloxan, methyl alloxan and dimethyl alloxan exhibit identical electrochemical characters and can be expected

to follow the same mechanistic routes during reduction. Thus, the equations which follow are for the alloxan molecule in general.

Dehydration of the Alloxans

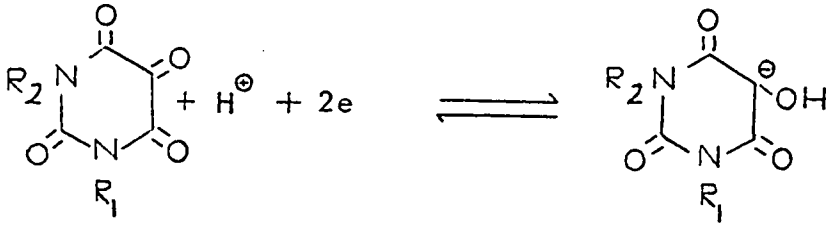


(26)

Hydrated Alloxan

Dehydrated Alloxan

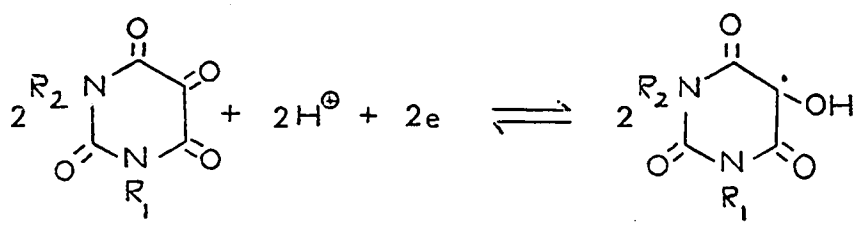
Electrochemical Reduction of the Alloxans at the Potential of Wave Ia



(27a)

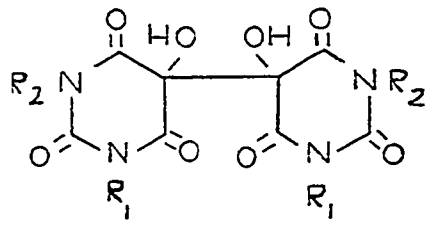
Alloxan

Dialuric Acid Anion



Alloxan + $2H^{\oplus}$ + 2e ⇌ 2 Alloxan Free Radical

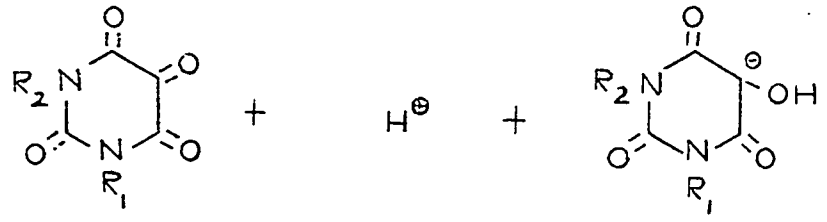
Rapidly Dimerizes



Alloxantin

(27b)

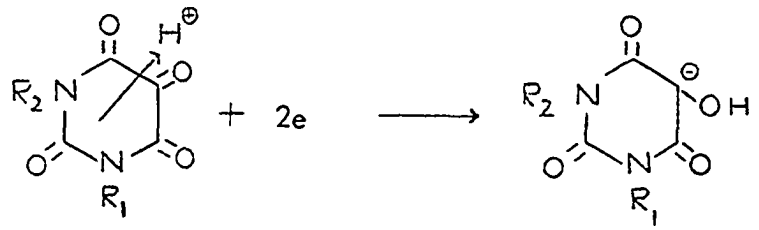
H_2O



Alloxan + $H^{\oplus}$ + Dialuric Acid Anion

Electrochemical Reduction of

the Alloxans at the Potential of Wave Ib



(28)

Alloxan Cation

Dialuric Acid Anion

Alloxan: $R_1 = R_2 = H$
Methyl Alloxan: $R_1 = CH_3$, $R_2 = H$
Dimethyl Alloxan: $R_1 = R_2 = CH_3$

Identification of Wave II Reduction Product of DMA

The mechanism proposed above allows for the assumption that the reduction product at the potential of Wave II for dimethyl alloxan is also dimethyl dialuric acid. Faradaic n values presented in Table 8 support this assumption. The reduction product solution was further studied at the d.m.e. and spectrophotometrically.

Polarographic and Spectrophotometric Observations

A small scale electrolysis of DMA in pH 5.1 McIllvaine buffer was performed in a regular polarographic cell. Polarograms obtained at intervals followed the course of the reduction. The anodic wave appeared and increased in height while the cathodic waves decreased. Wave II initially exhibited a limiting current about ten-fold that of Wave I.

Wave II consistently decreased in height as DMA was reduced.

Controlled-potential reduction of DMA in pH 5.1 McIllvaine buffer for 30 min. at -1.4 v was performed. Polarograms depicting the behavior of the initial and electrolyzed solutions at the d.m.e. are reproduced in Figure 9. An anodic wave with $E_{\frac{1}{2}}$ = -0.09 v is observed. DMA also exhibits a cathodic wave with $E_{\frac{1}{2}}$ = -0.09 v in pH 5.1 McIllvaine buffer. The solution contained a species which absorbed at λ_{max} = 276 mμ and disappeared upon exposure to air.

Discussion of d.m.e. and spectrophotometric results. The consistent decrease in the height of Wave II as the reduction proceeds, definitely disproves the assumption of previous workers[5] that, in the case of alloxan, Wave II is the reduction of dialuric acid to barbituric acid. (Wave II would increase as the anodic wave, i.e., the concentration of dialuric acid, increases.)

The half-wave potential of the anodic wave upon reduction at the potential of Wave II is identical to that of cathodic Wave Ia: the electroactive species forms a reversible couple. The reduction product exhibits a susceptibility to air-oxidation expected for dialuric acid and the N-methylated derivatives. The UV absorption peak with λ_{max} = 276 mμ is within the range expected of dimethyl dialuric acid by comparison with dialuric acid and by consideration of the effect of methylation on the uracils.

Proposed Mechanism for Wave II Reduction of DMA

Wave II has diffusion current constants compatible with a two-electron diffusion-controlled process. Thus the kinetic-controlled reaction of Wave I is unimportant at the more negative potentials. Deter-

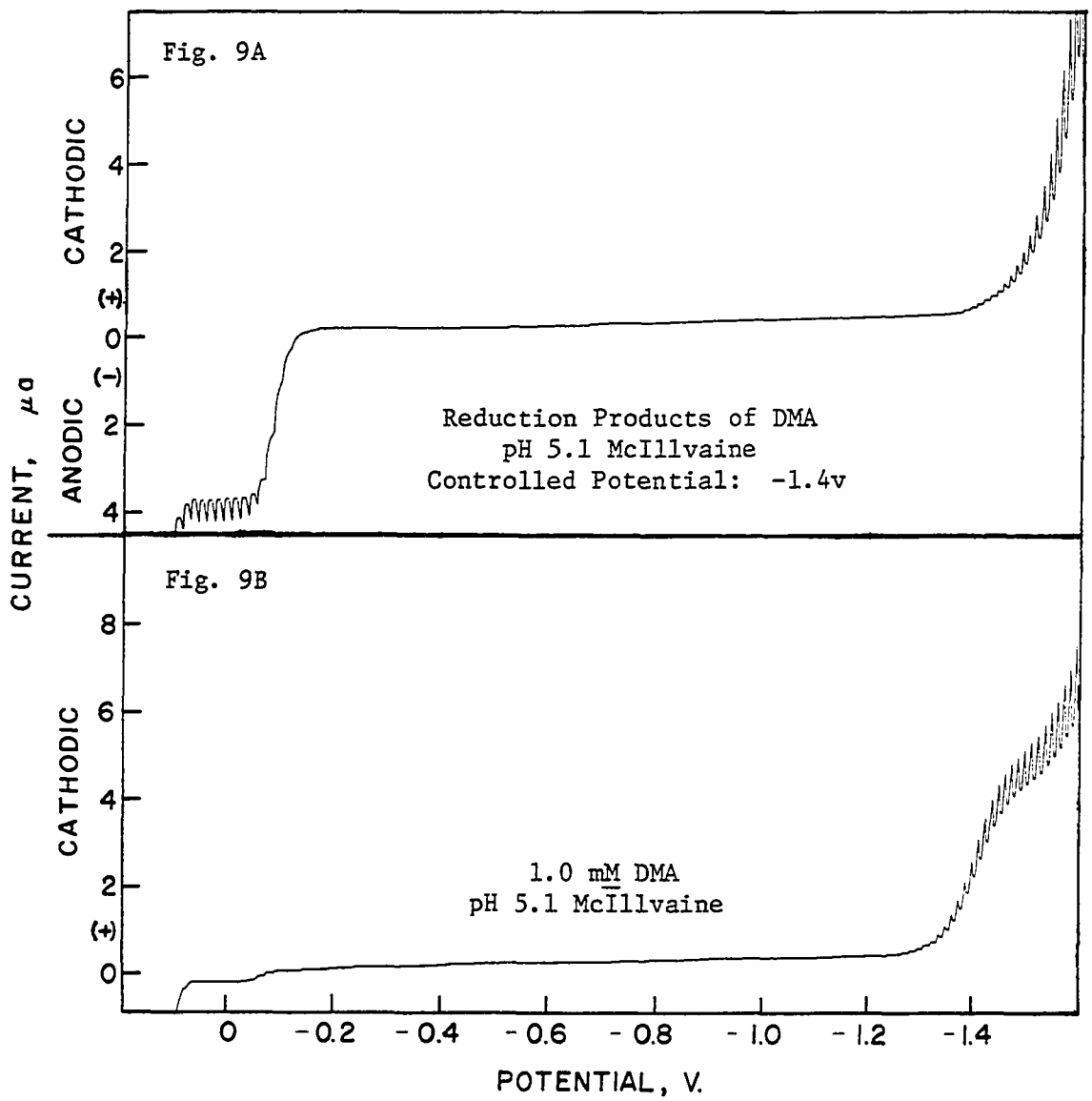


Fig. 9A
CATHODIC
ANODIC
(+)
(-)
6
4
2
0
2
4
Reduction Products of DMA
pH 5.1 McIllvaine
Controlled Potential: -1.4v
Fig. 9B
CATHODIC
(+)
8
6
4
2
0
1.0 mM DMA
pH 5.1 McIllvaine
0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
POTENTIAL, V.
CURRENT, μa

mination of faradaic n values substantiates that two electrons are involved in the reduction of DMA at the potential of Wave II.

The reduction product exhibits a single anodic wave at $E_{1/2}$ identical to that of DMA at the same pH. The UV absorption with λ_{max} = 276 mμ is within the expected range of dimethyl dialuric acid and the absorbing species exhibits a susceptibility to air-oxidation common to all the dialuric acids. It is, therefore, postulated that the hydrated DMA molecule, which is the predominant form in solution, must be directly reduced in a two-electron reduction to the corresponding dialuric acid.

In order to propose a general mechanism for the alloxans a comparison of the $E_{1/2}$ - pH dependence and αn_a values will be considered:

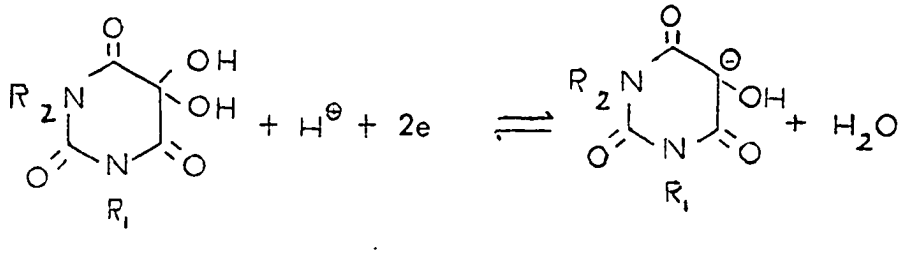
Alloxan[5], $E_{1/2}$ = -1.44 -0.015 pH, αn_a = 1.08

Methyl Alloxan, $E_{1/2}$ = -1.37 (± 0.08) -0.014 (± 0.01) pH, αn_a = 1.07

Dimethyl Alloxan, $E_{1/2}$ = -1.26 (± 0.04) -0.030 (± 0.01) pH, αn_a = 1.07

where $\alpha n_a = \frac{0.056}{E_{3/4} - E_{1/4}}$. Considering the deviations involved in the $E_{1/2}$ determinations, the decomposition rate of the electroactive species and the few data points a mechanism using two electrons and one proton is postulated in Equation 29.

Electrochemical Reduction of the Alloxans at the Potential of Wave II



(29)

Polarography of Alloxan in Acetonitrile Solutions

The mechanism proposed for the reduction of alloxan, methyl alloxan and dimethyl alloxan in aqueous solutions indicates that alloxantin and the N-methylated alloxantin derivatives are dissociated in aqueous solutions. Substantial evidence was presented but it was thought likely that the use of an aprotic solvent would eliminate both the dissociation process and the kinetic-controlled dehydration step, and therefore simplify the interpretation of the electrode mechanisms. An extensive study of alloxan (the behavior of the N-methylated derivatives was expected to be quite similar) in acetonitrile was therefore undertaken.

Alloxan in acetonitrile containing 0.1 M tetraethylammonium perchlorate (TEAP) as supporting electrolyte can give rise to a total of four cathodic waves at the d.m.e. (Figure 10). At very low concentrations, (0.1 to 0.3 mM) a single wave is observed, $E_{1/2}$ = -0.58 ± 0.02 v. (All potentials in the non-aqueous system are vs. Ag - 0.1 M $AgNO_3$ which is 0.337 v vs. S.C.E.)[42] At about the 0.4 mM concentration level a second wave appears, $E_{1/2}$ = -0.87 ± 0.04 v. Occasionally, a third wave is also evident at this concentration, $E_{1/2}$ = -1.36 ± 0.02 v. At 0.6 mM and above, a third wave always appears with $E_{1/2}$ = -1.27 ± 0.02 v and a fourth wave, $E_{1/2}$ = -1.55 ± 0.05 v, is also exhibited. Some variations of $E_{1/2}$ with concentration (Figure 11) was noted, the second wave exhibits the most noticeable shift (-0.91 to -0.72 v) over the 0.4 to 2.0 mM concentration range.

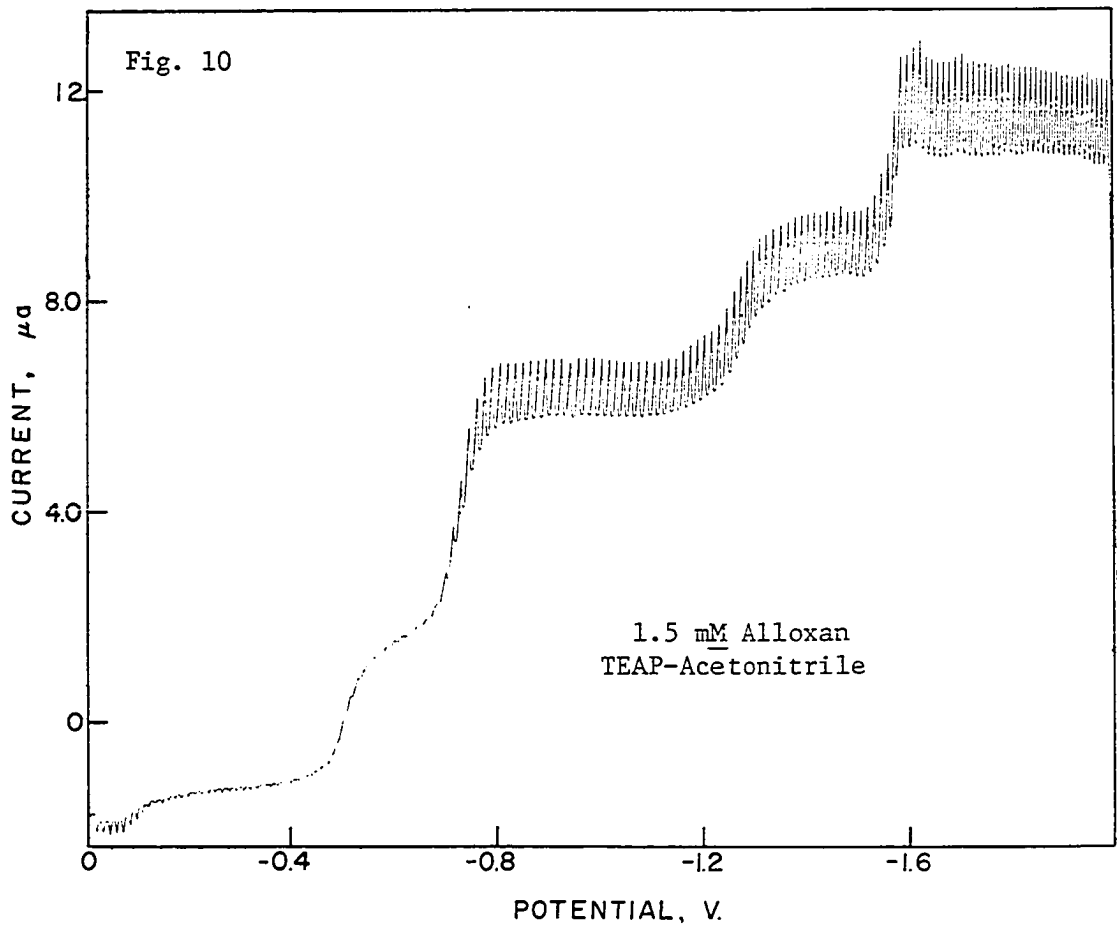
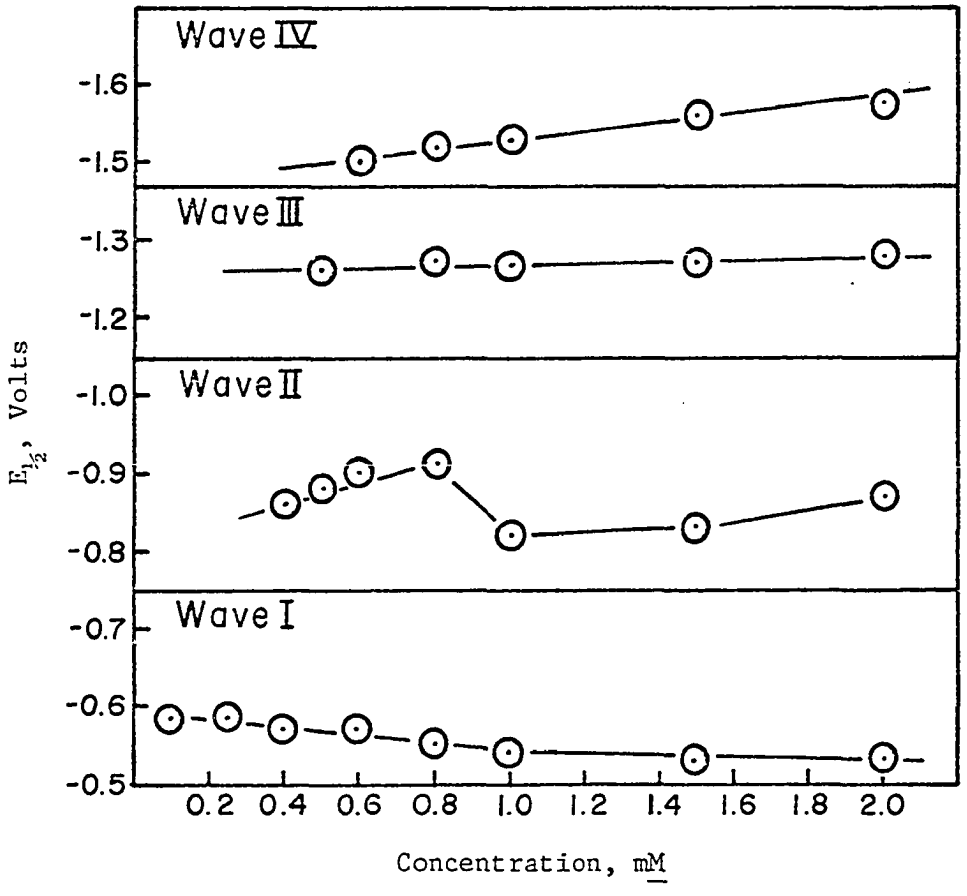


Fig. 10
12
8.0
4.0
0
CURRENT, μa
1.5 mM Alloxan
TEAP-Acetonitrile
0
-0.4
-0.8
-1.2
-1.6
POTENTIAL, V.

Figure 11



Variation of $E_{1/2}$ with Concentration for

Alloxan in TEAP-Acetonitrile

Wave I and Wave II

The occurrence of only Wave I at the lowest concentrations and the fact that this wave becomes concentration independent is indicative of an adsorption process.[43] If the reduction product of alloxan is adsorbed at the electrode surface, activity of the reduction product is lower in the adsorbed state than in solution and the reduction of alloxan is facilitated. Therefore, at very low alloxan concentrations a single wave is observed, representing the reduction of alloxan to the adsorbed reduction product. The height of such a wave would be expected to be diffusion-controlled, proportional to the concentration of alloxan and the square root of the corrected height of the mercury column[43] ($h^{\frac{1}{2}}_{corr}$) where $h_{corr} = h - \frac{3.1}{(mt)^{1/3}}$.

Wave I appears to be proportional to concentration up to the 0.4 mM - 0.5 mM level (Figure 12). Table 11 contains the polarographic data obtained with a 0.2 mM solution of alloxan. The diffusion current constant is 2.7 - 2.8 which is the predicted value for a one-electron diffusion-controlled process in acetonitrile.[44] A height study reveals a proportionality constant of 0.109 ± 0.006 for the value of $ih^{-\frac{1}{2}}_{corr}$.

Wave I becomes concentration independent above the 0.4 to 0.5 mM concentration level and Wave II appears. As the concentration of alloxan increases a point is reached at which the amount of reduction product formed completely covers the surface during the life of the drop. Further reduction of alloxan then requires that the excess reduction product remain in solution. However, since it is more difficult to reduce alloxan to the dissolved reduction product than to the adsorbed reduction product, the reduction of the excess alloxan produces a second

Table 11

Polarographic Data for 0.2 mM Solution

of Alloxan in 0.1M TEAP in Acetonitrile

Height cm	$E_{1/2}$ v vs. Ag-0.1M $AgNO_3$	$ih_{corr}^{-1/2}$ $\mu a\ cm^{-1/2}$	I[a]
90	-0.59	0.107	2.84
80	-0.59	0.100	
70	-0.59	0.115	2.89
60	-0.58	0.116	
50	-0.59	0.105	2.74
39	-0.59	0.102	

[a] I = diffusion current constant.

wave at a more negative potential.[43]

Wave I is therefore an adsorption wave, whose height remains constant and independent of any further increase in alloxan concentration. Wave II is termed a "normal wave"[43] and represents the reduction of alloxan to dissolved reduction product. Wave II is observed to be linearly dependent on concentration (Figure 13) but does not pass through the origin. However, the sum of the limiting currents for Waves I and II is linearly proportional to concentration giving a straight line relationship which passes through the origin (Figure 14). Height study data for a 1.0 mM alloxan solution is summarized in Table 12. The ratio $ih_{corr}^{-\frac{1}{2}}$ is constant with a mean value of 0.485 and a maximum deviation of ± 0.02 when the total current of Wave I + Wave II is employed. An average diffusion current constant of 2.71 is obtained for the sum of Waves I and II (Table 13); the two waves therefore would appear to represent a one-electron reduction.[44] Thus, the total height of the double wave, which corresponds to all of the alloxan diffusing to the surface of the drop, is diffusion-controlled and proportional to both concentration and $h_{corr}^{\frac{1}{2}}$, i.e., the theory of adsorption waves[43] is perfectly obeyed.

A diagnostic criteria of an adsorption wave is the proportionality of limiting current to h_{corr}.[43] Wave I gives a constant of 0.025 ± 0.002 for $i_l h_{corr}^{-1}$ in 1 mM solution (Table 12). Further confirmation of the adsorption character of Wave I is the appearance of Wave II in a 0.2 mM solution upon lowering the height of the mercury column to 25 cm (the normal height employed is 70 cm). The limiting adsorption current (i_{ad}), which represents the current that flows on reducing alloxan to the adsorbed product, is directly proportional to the height of the mercury

Figure 12

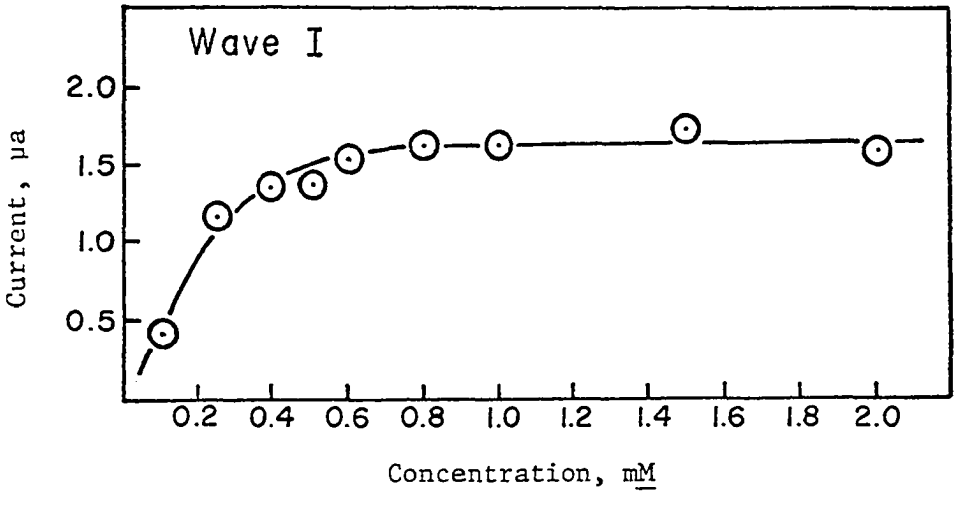
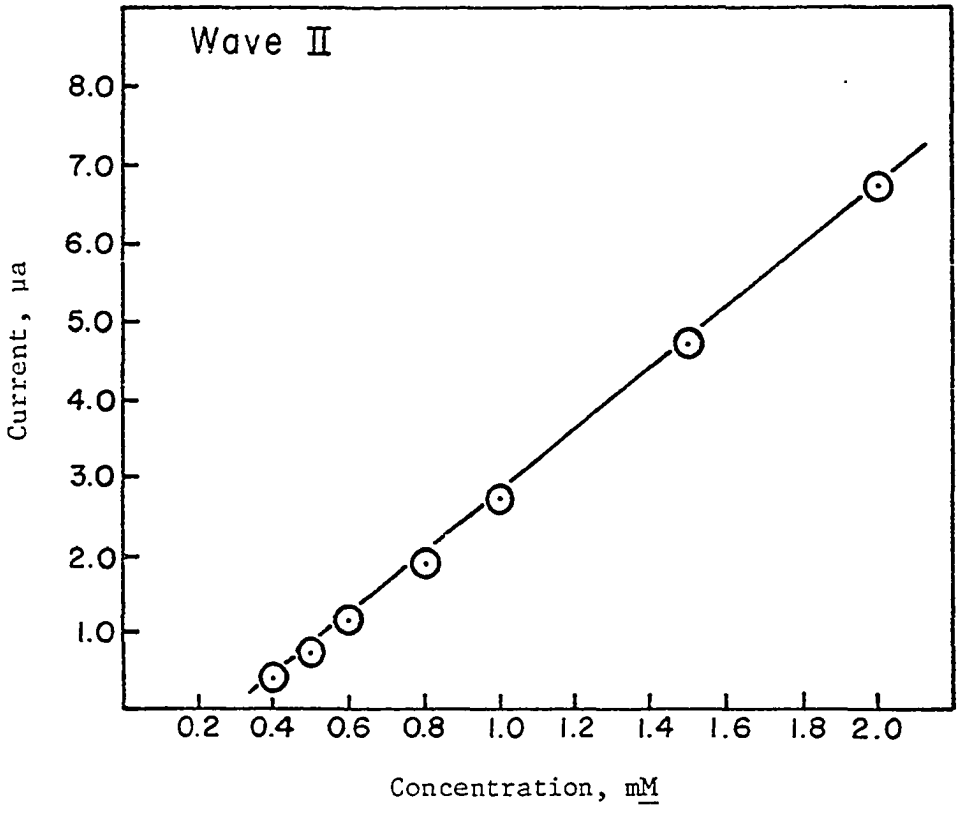
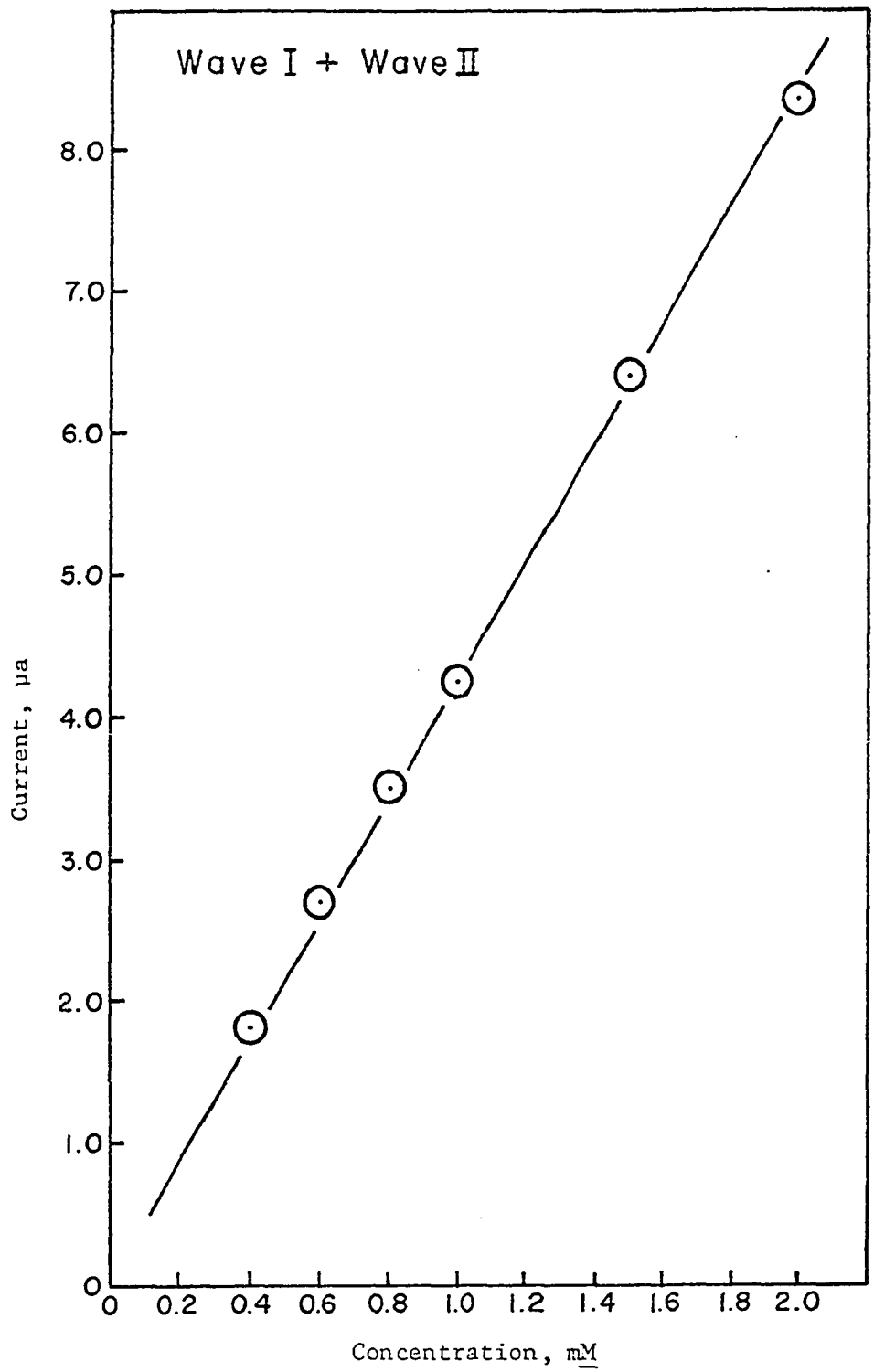


Figure 13



Variation of Current with Concentration for Wave I and Wave II of Alloxan in TEAP-Acetonitrile

Figure 14



Variation of Current with Concentration for Waves (I + II) of Alloxan in TEAP-Acetonitrile

Table 12

Effect of Mercury Column on Limiting Currents

for Reduction of 1.0 mM Alloxan in 0.1 M TEAP in Acetonitrile

Height cm	$i_l h_{corr}^{-\frac{1}{2}}$ μa $cm^{-\frac{1}{2}}$					ih_{corr}^{-1} μa cm^{-1}
	Wave					Wave
	I	II	I + II	III	IV	I
90	0.221	0.254	0.475	0.234	0.197	0.0233
80	0.239	0.259	0.498	0.242	0.179	0.0267
70	0.212	0.265	0.478	0.256	0.150	0.0254
60	0.201	0.279	0.480	0.222	0.165	0.0260
50	0.169	0.305	0.474	0.206	0.134	0.2239
40	0.168	0.342	0.510	0.201	0.146	0.0264

Table 13

Diffusion Current Constants for Reduction

Waves of Alloxan in 0.1 M TEAP in Acetonitrile

Concn.	Diffusion Current Constants					
	Wave					
mM	I	II	I + II	III	IV	III + IV
2.0	0.641	2.12	2.76	1.05	1.45	2.50
1.5	0.721	1.99	2.71	1.17	1.12	2.29
1.0	1.02	1.63	2.65	1.20	1.16	2.36
0.5	1.76	0.96	2.72			
0.1	2.77					

column (h); the diffusion current (i_d), which represents the current required to reduce all of the alloxan that reaches the surface of the drop, is proportional to $h^{\frac{1}{2}}$. Thus, as the height of the column is reduced, i_{ad} decreases by a greater factor than does i_d and at some level more alloxan will reach the drop surface than can be reduced and the "normal wave" appears.[43]

Wave III and Wave IV

Wave III and Wave Iv, which both appear at a concentration of about 0.6 mM alloxan, are both linearly dependent on concentration through 2.0 mM but do not pass through the origin (Figures 15 and 16, respectively). However, when the currents of the two waves are added a linear dependence passing through the origin is obtained (Figure 17). The value of $i_l h_{corr}^{-\frac{1}{2}}$ for Wave III is 0.23 ± 0.03 and 0.16 ± 0.03 for Wave IV (see Table 12). The diffusion current constant for the sum of the currents of Waves III and IV (Table 13) lies between 2.3 and 2.5, which is a reasonable value for a one-electron reduction in acetonitrile.[44]

The nature of Waves III and IV is difficult to interpret. The waves undoubtedly represent the further reduction of the products of Waves I and II but are complicated by the effect of the background electrolyte (vide infra).

Interpretation of the Reduction Waves

The diffusion current constants for the two sets of double waves, i.e., Waves I and II and Waves III and IV, compare favorably with those reported for the two-step reduction of quinones in acetonitrile. Some data of Wawzonek and co-workers[45] for various quinones in

Figure 15

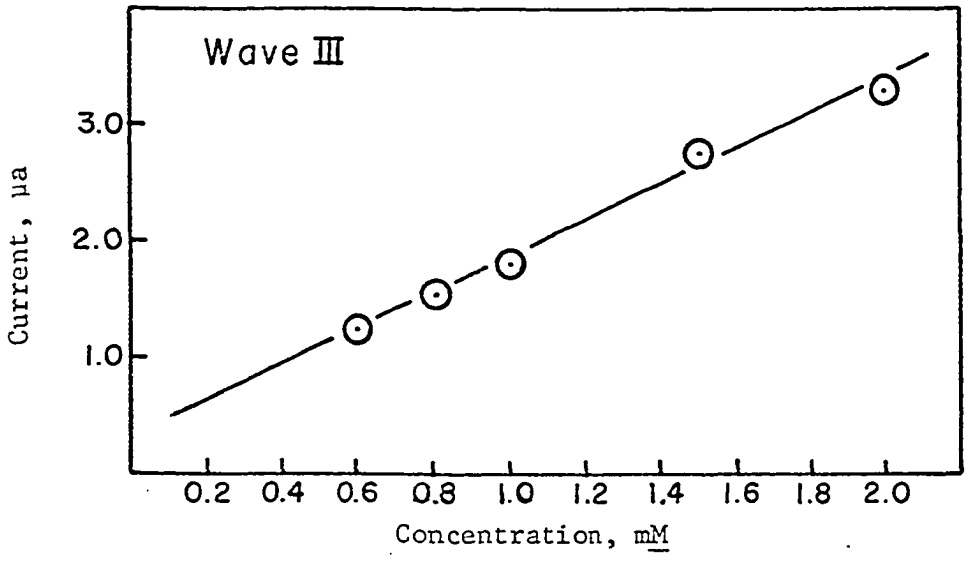
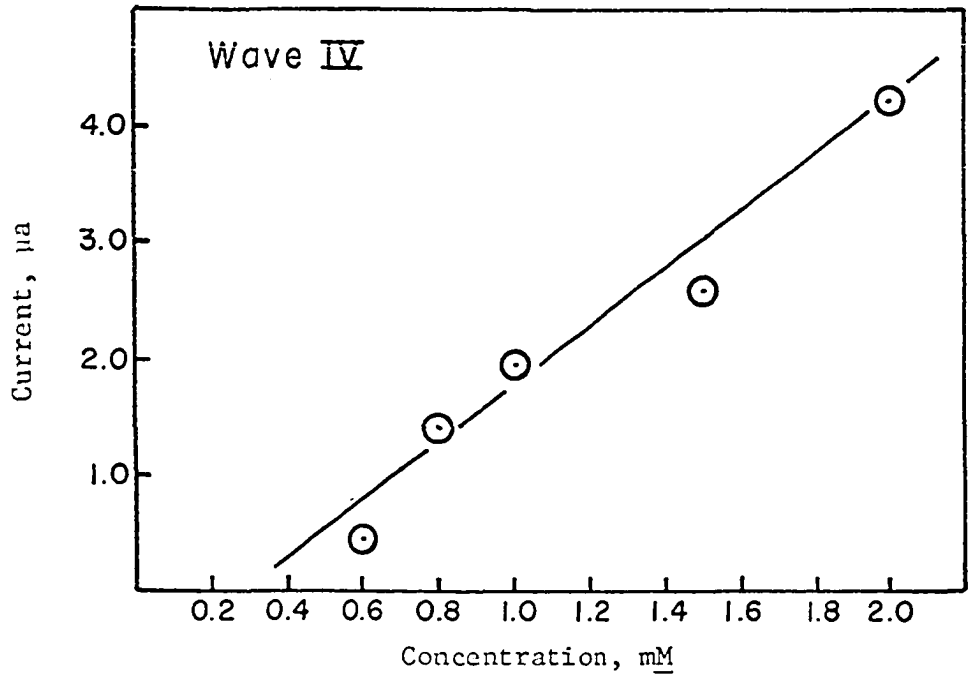
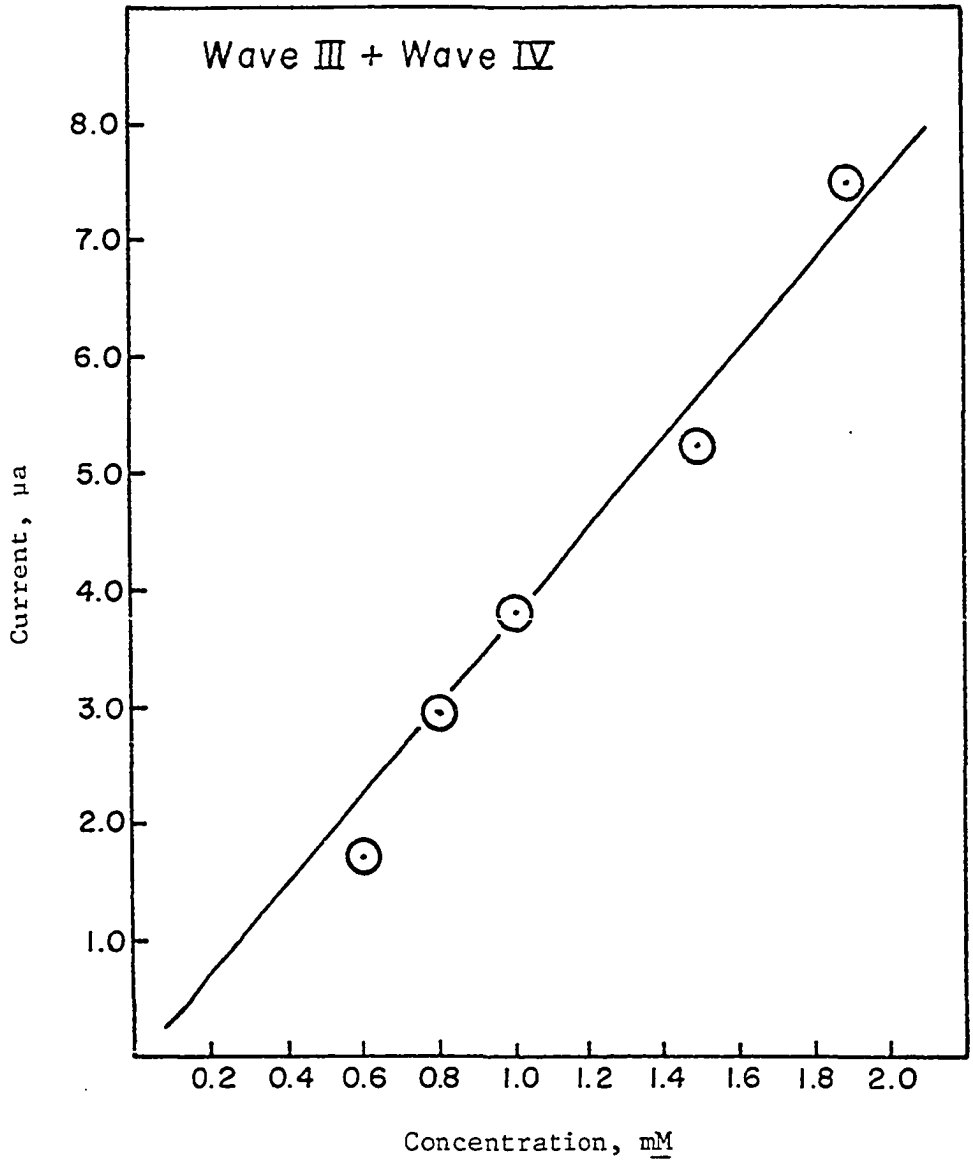


Figure 16



Variation of Current with Concentration for Wave III and Wave IV of Alloxan in TEAP-Acetonitrile

Figure 17



Variation of Current with Concentration for Wave (III + IV)

of Alloxan in TEAP-Acetonitrile

Table 14

Data of Wawzonek and Co-workers

Quinone	Concn. millimoles per liter	i_d μa		$I_d = i_d/Cm^{2/3}t^{1/6}$	
		1st wave	2nd wave	1st wave	2nd wave
Benzoquinone	1.00	4.67	3.58	3.07	2.35
Duroquinone[a]	1.00	5.37	3.98	3.53	2.62
Anthraquinone	1.00	4.97	3.78	3.27	2.49

[a] Tetramethyl benzoquinone

Table 15

Current and Diffusion Current

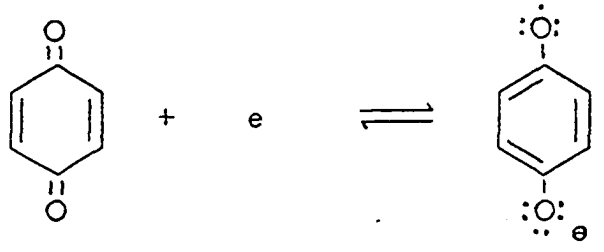
Constants for Alloxan in 0.1 M TEAP in Acetonitrile

Concn. mM	Wave I	Wave II	Waves I + II	Wave III	Wave IV	Waves III + IV
			Current			
1.0	1.68	2.90	4.58	1.80	2.04	3.84
1.5	1.72	4.72	6.44	2.74	2.52	5.26
2.0	1.62	6.72	8.34	3.28	4.36	7.64
			Diffusion Current Constant			
1.0	1.02	1.63	2.65	1.20	1.16	2.36
1.5	0.72	1.99	2.71	1.17	1.12	2.29
2.0	0.64	2.12	2.76	1.05	1.45	2.50

acetonitrile solutions containing 0.1 M tetrabutylammonium bromide is reproduced in Table 14. Current and diffusion current constants for solutions of alloxan in 0.1 M TEAP in acetonitrile is given in Table 15.

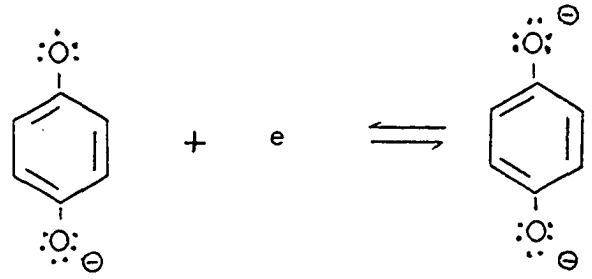
The height of the second wave for the quinone series (Table 14) is, in all cases, less than the height of the first wave by a factor of about eight-tenths. Similarly (Table 15), the height of the second double wave for alloxan (Wave III + IV) is less than the height of Wave I + II by a factor of about nine-tenths. It is clear, therefore, that at some concentration (in this case 0.4 to 0.6 mM) only the first double wave appears. Voriskova reports similar behavior for the polarographic reduction of pyocyanine and validates the application of the adsorption theory presented above to stepwise oxidations and reductions.

Wawzonek and co-workers interpreted the data obtained in the study of quinones at the d.m.e. in acetonitrile as a two-step reduction, first to the semiquinone and then to the hydroquinone dianion. The reactions involved are illustrated for benzoquinone as follows:



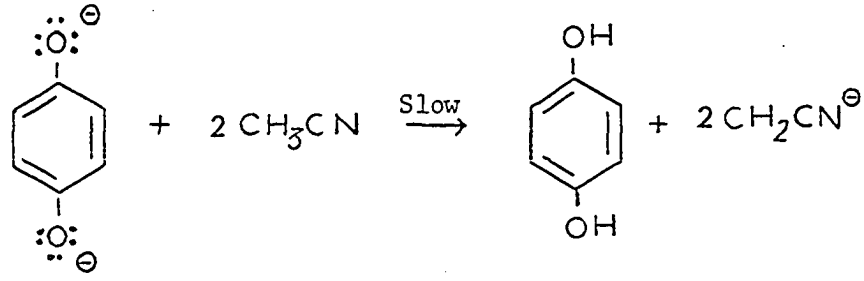
(30)

Quinone Semiquinone



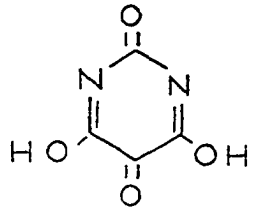
Semiquinone Hydroquinone Dianion (31)

the hydroquinone dianion then reacts slowly with the solvent producing hydroquinone:

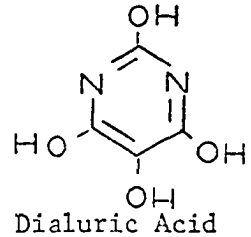


Hydroquinone Dianion Hydroquinone (32)

Alloxan bears the same relationship to dialuric acid that quinone bears to hydroquinone:



Alloxan
(Tautomerized Form)



Dialuric Acid

Furthermore, alloxantin is an intermediate product in the chemical reduction of alloxan as well as the oxidation of dialuric acid[47] and bears a relationship to dialuric acid and alloxan similar to that which quinhydrone bears to hydroquinone and quinone. The work of Biilmann and Lund[27] cited earlier (Introduction) is an example of the early recognition of the electrochemical utility of the alloxan system, i.e., as a replacement for the H-electrode, based on their similarity. Volke[48] cites alloxan as an example of the fully reversible quinone system,

$$Q + 2e = Q^{=}.$$

He observes that such systems are frequently complicated because of the possibility of formation of semiquinones and dimers and by adsorption, which is confirmed in the present study.

The report that two one-electron transfer steps are involved in the reduction of quinones in acetonitrile, the similarity of the alloxan-dialuric acid system to that of quinone and hydroquinone, and the data of Wave (I + II) and Wave (III + IV) permits the postulation that alloxan in acetonitrile is reduced in a one-electron step to the alloxan free radical, followed by another one-electron reduction to the dialuric acid dianion.

Effect of Protons on Polarography of Alloxan in Acetonitrile

Interpretation of the quinone-hydroquine study in acetonitrile[45] was clarified by observations of the effect of protons (benzoic acid and water, respectively, were added) on the half-wave potential and

current magnitude. Similar experiments were, therefore, designed for the present study.

Addition of Benzoic Acid

Benzoic acid varying in concentration from 0.1 to 22.0 m$\underline{M}$ was added to a 2.0 m$\underline{M}$ solution of alloxan in TEAP-acetonitrile. The polarographic data is presented in Table 16. (A comparison of $E_{\frac{1}{2}}$ and current for Wave IV in Table 16 with the data of Figures 11 and 16 reveals that the character of Wave IV has changed. This is probably due to a change in the TEAP used as supporting electrolyte. Solutions prepared with any one batch of TEAP were consistent; variations occurred with different batches.) Addition of protons does not affect the $E_{\frac{1}{2}}$ of Waves I, II or III. The $E_{\frac{1}{2}}$ of Wave IV shifts 0.01 v in a positive direction as the benzoic acid concentration reaches 0.2 m$\underline{M}$, and then the wave disappears entirely. Wave III disappears upon reaching a 1.0 m$\underline{M}$ level of benzoic acid. The height of Wave II increases with increasing proton concentration.

Addition of Water

The polarographic data obtained upon addition of water to the TEAP-acetonitrile system is summarized in Table 17. A 0.04 per cent by volume solution of water yields an essentially aqueous system, $\underline{i.e.}$, a very small cathodic wave, corresponding to the kinetic-controlled wave, and a larger second cathodic wave are observed. The wave comparable to the kinetic-controlled wave in normal aqueous solutions appears at $E_{\frac{1}{2}}$ = -0.56 v (-0.22 v $\underline{vs.}$ S.C.E.) and the second cathodic wave appears at $E_{\frac{1}{2}}$ = $\underline{ca.}$ 2.0 v ($\underline{ca.}$ -1.7 v $\underline{vs.}$ S.C.E.). The ratio of current sizes

Table 16

Effect of Benzoic Acid Addition on Reduction

Waves of Alloxan[a] in 0.1 M TEAP in Acetonitrile

Concn. of Benzoic Acid mM	Wave I		Wave II		Wave III		Wave IV	
	$E_{1/2}$ v	i μa	$E_{1/2}$ v	i μa	$E_{1/2}$ v	i μa	$E_{1/2}$ v	i μa
0.0	-0.48	0.88	-0.67	7.12	-1.28	3.28	-1.78	1.28
0.1	-0.47	0.72	-0.67	7.52	-1.28	3.60	-1.75	1.28
0.2	-0.47	0.80	-0.66	7.80	-1.28	3.48	-1.69	0.32
0.8	-0.46	0.80	-0.66	8.56	-1.30	1.52		
1.0	-0.47	0.80	-0.66	9.16				
2.0	-0.47	1.04	-0.66	9.25				
4.0	-0.45	0.96	-0.64	9.20				
22.0			-0.57[b]	10.48				

[a] Concentration of alloxan, 2.0 mM.

[b] Wave I could not be clearly distinguished.

Table 17

Effect of Water Addition on Reduction

of Alloxan[a] in 0.1 M TEAP in Acetonitrile

Concn. of Water Per cent by Volume	Wave I		Wave II		Wave III		Wave IV	
	$E_{1/2}$ v	i μa	$E_{1/2}$ v	i μa	$E_{1/2}$ v	i μa	$E_{1/2}$ v	i μa
0	-0.56	1.84	-0.78	2.72	-1.30	2.16	-1.57	1.60
0.0033	-0.56	1.76	-0.91	1.92	-1.28	2.40	-1.37	1.60
0.0067	-0.57	1.60	-0.87	0.83	-1.29	4.26		
0.0133	-0.58	1.68	-0.88	0.80	-1.26	5.52		
0.020	-0.59	1.48	[-1.14]*	[2.69]	[-1.26]	[3.24]		
0.027	-0.59	1.36	[-1.12]	[2.76]	[-1.31]	[3.45]		
0.033	-0.56	0.76	[-1.1][b]	----	[-1.55][c]			
0.040	-0.56	0.58					>2.0	5.92

[a] Concentration of alloxan, 1.0 mM.

[b] Appears in -1.0 to -1.2 v region.

[c] Appears in -1.5 to -1.6 v region.

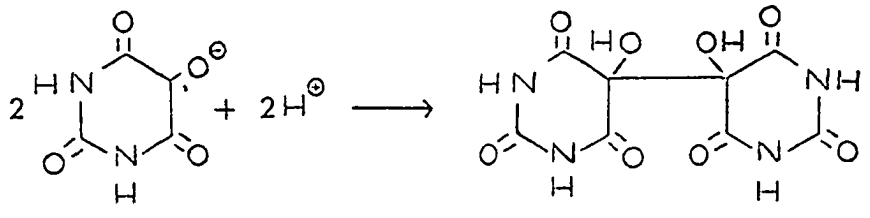
* Relationship of bracketed data to original Wave II and III uncertain.

is very comparable to that observed in normal aqueous solutions, i.e., a ratio of one to ten.

The half-wave potential for Wave I remains constant as water is added. Correspondingly, the height of the wave decreases. As an essentially aqueous system is approached, the kinetic character of Wave I becomes dominant. Wave II shifts 0.1 v more negative and the height decreases as small amounts of water are added. The $E_{1/2}$ of Wave III remains constant, the height increases with Wave III assuming the height of the additive III and IV currents when Wave IV disappears. Only Wave I is consistently observed. Two waves, whose relationship to the original Wave II and Wave III is difficult to assess, are discernible as the percentage by volume of water varies from 0.02 to 0.33.

Interpretation of Data

The date implies that the potential-determining steps do not involve protons since no shift of $E_{1/2}$ is observed as protons are added. The fact that Waves III and IV disappear as benzoic acid is added indicates that the reduction product of Waves I and II assumes a non-electroactive character in the presence of protons. Dimerization of an alloxan free radical (reduction product of Waves I and II, vide infra) in the presence of protons to produce alloxantin as proposed in Equation 33 fits the data very well. Alloxantin is non-electroactive in acetoni-



(33)

trile solution.

Determination of Faradaic n Values for Reduction of Alloxan in Acetonitrile

One-electron reductions have been assumed from the size of the diffusion current constants for the two sets of double waves, i.e., Wave I + II and Wave III + IV. The results of the reduction of 1.0 mM solutions of alloxan in 0.1 M TEAP in acetonitrile at controlled potentials of -1.0 v (corresponding to Wave I + II) and -1.8 v (corresponding to Wave III + IV) are summarized in Table 18.

Table 18

Determination of Faradaic n Values for Reduction of Alloxan in 0.1 M TEAP in Acetonitrile

Controlled-Potential v	n
-1.0	0.94
-1.0	1.16
-1.0	0.82
-1.0	0.97
-1.0	1.05
	Av. 0.99
-1.8 v	1.62
-1.8 v	1.65
-1.8 v	1.63
-1.8 v	1.77
	Av. 1.67

The reduction of alloxan at -1.0 v is clearly a one-electron process. An average of 1.7 electrons is involved in the reduction at -1.8 v. Thus, the total reduction process does indeed involve two electrons and proceeds by way of two one-electron transfer steps.

Physical Appearance of Electrolysis Solution

The electrolysis solution at either controlled potentials became cloudy within a few moments after the potential was applied. In almost every experiment, the solution also became pink in color. Solid product formation could be distinguished as the cause of the cloudiness within five to ten minutes. The quantity of solid formed varied but was generally most abundant within 25 to 30 minutes. As the reduction continued the quantity of solid product diminished until the solution returned to a clear state. A range of color from pink to deep purple to light pink was observed during the reduction. The two phenomena, i.e., the solid product formation and the color, are separate entities. In the few cases in which no color developed the solid product was white in appearance. However, solid product which was separated from a highly colored solution was pink to purple in appearance and the color was not diminished by washing.

Polarography of Electrolysis Solution

Typical polarograms of the electrolyzed solution at the completion of the -1.0 and -1.8 v electrolysis are shown in Figures 18 and 19 respectively. An anodic current is observed in the 0 to -1.0 v region. Two waves are perceptible in this region which will be referred to by the potential range of the limiting current rather than by half-

Fig. 18

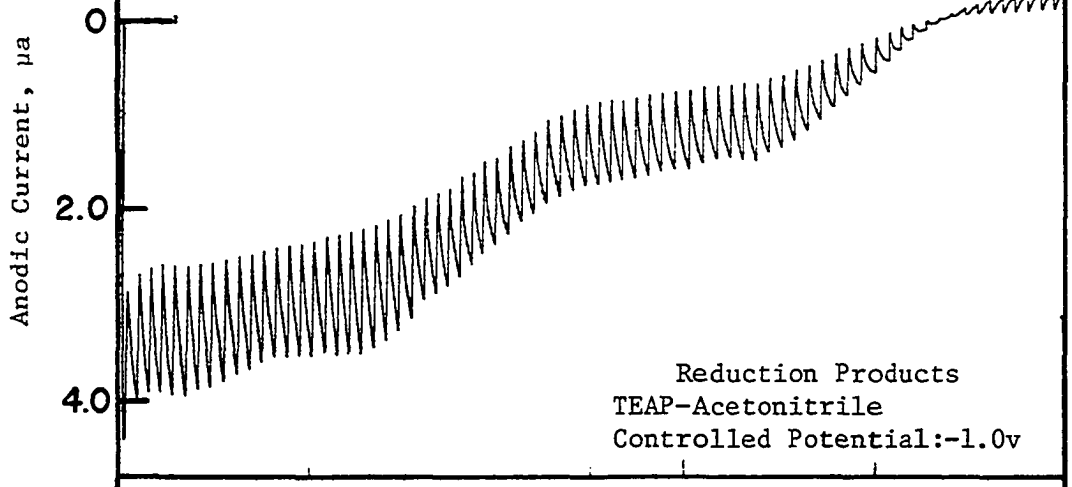
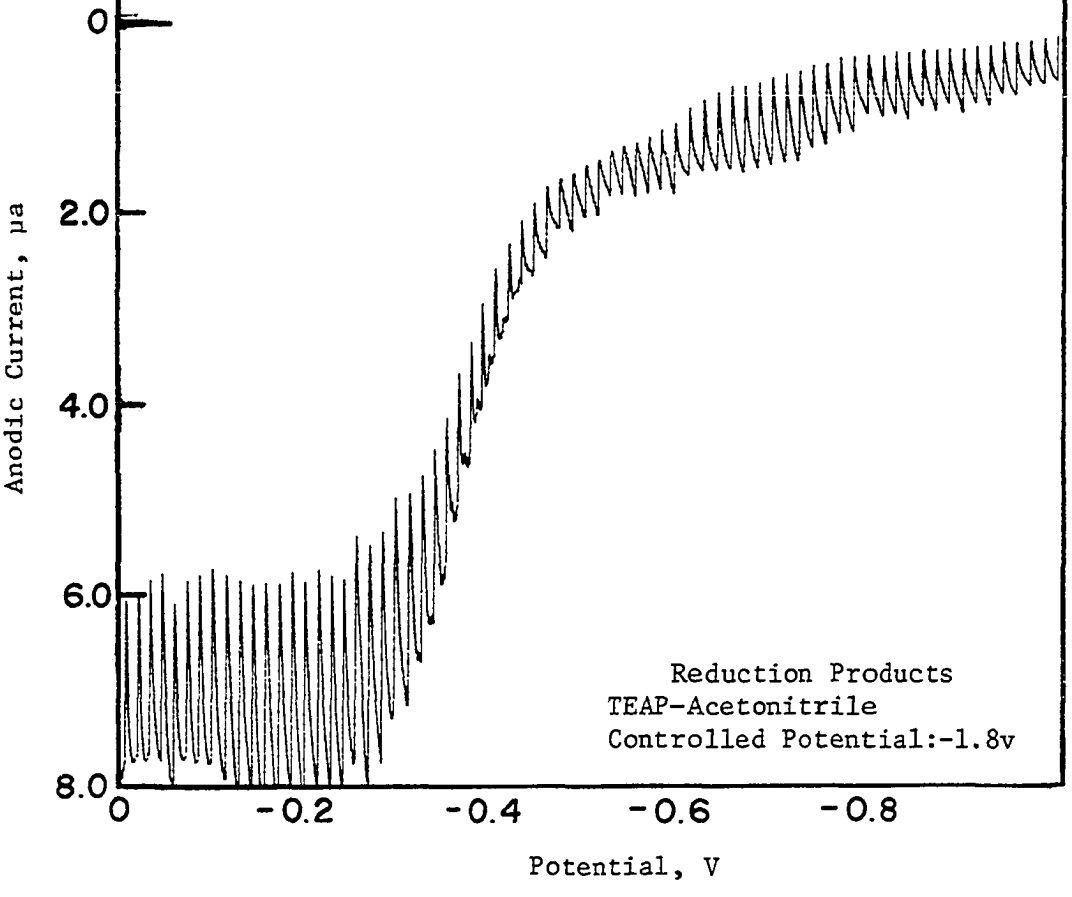

Anodic Current, μa
0
2.0
4.0
Reduction Products
TEAP-Acetonitrile
Controlled Potential:-1.0v

Fig. 19


Anodic Current, μa
0
2.0
4.0
6.0
8.0
Reduction Products
TEAP-Acetonitrile
Controlled Potential:-1.8v
0
-0.2
-0.4
-0.6
-0.8
Potential, V

wave potential, the latter being very difficult to define due to the erratic nature of the waves. Allowing for a ± 0.05 v deviation, the half-wave potentials appear at about -0.4 and -0.8 v. However, the plateau of the limiting current of the more positive wave is in the 0 to -0.3 v range; the plateau of the more negative wave in the -0.5 to -0.7 v range. The appearance of the waves is the same in solutions reduced at either potential, though the current height of the 0 to -0.3 v wave is greater in the -1.8 v reduction solution.

A diminution of current was observed for the -0.5 to -0.7 v wave when the reduction product solution was exposed to air. The height of the less negative anodic plateau (0 to -0.3 v) changed very little even after 48 hours.

Background solution electrolyzed under identical conditions showed the formation of a single distinct anodic wave, $E_{\frac{1}{2}}$ = ca.-0.85 v, but the height of the wave was considerably less than that of an electrolyzed alloxan solution.

Solutions of the expected products, alloxantin and dialuric acid, were prepared for comparative purposes. No anodic or cathodic activity was observed. Therefore, the anodic character of the reduction product solution is not due to the expected reduction products.

It should be noted that both the mechanism of the reduction of the alloxans in aqueous solution, and the proposed dimerization of the alloxan free radical in acetonitrile (Equation 33) are based on the assumption that alloxantin, in itself, is not electroactive. However, the resistance to oxidation of dialuric acid in acetonitrile is at first surprising. The best explanation for this resistance is probably that

offered by Wawzonek and co-workers[45] for the analogous behavior of hydroquinone. A prerequisite ionization of hydroquinone is assumed necessary for oxidation. Since acetonitrile has a dielectric constant of about 35, it is a poor ionizing solvent and therefore renders the hydroquinone electrochemically inert by inhibiting the ionization.

Spectrophotometric Behavior of Electrolysis Solution

The UV spectrum of the electrolyzed background solution exhibited strong absorbance in the UV region and precluded any direct identification of product absorbance. However, if an aliquot of the electrolysis solution was removed during the interval when cloudiness persisted in the solution and was greatly diluted with water under as anaerobic conditions as possible, an absorbing species, λ_{max} = 274 mμ, was observed. The absorbance rapidly diminished upon exposure to air. Both the λ_{max} and the behavior toward air favorably compare with dialuric acid as noted in the aqueous study.

Attempts at chromophore identification. The colored electrochemical reduction product solutions were not consistently produced nor was the duration of the presence of color constant. Colored solutions of electrolysis product (-1.0 v) could be stored for days and remain unchanged. At other times the color changed in intensity, or even disappeared, while the electrolysis was in progress.

The color was observed to closely resemble that of a murexide solution. Therefore, the colored solid product of a -1.0 v reduction was collected on a filter and dried under anaerobic conditions. Comparisons of the IR spectra of this crude, colored electrolysis product with those of commercial alloxantin and murexide indicates a possible

mixture. The 1900 to 1500 cm^{-1} region of the three spectra are reproduced in Figure 20. The electrolysis product exhibits both the 1610 cm^{-1} peak of alloxantin and the 1650 cm^{-1} peak of murexide.

Comparisons of the polarography of the solid electrolysis product, alloxantin and murexide in pH 4.7 acetate buffer are summarized as follows:

Electrolysis Product

$E_{1/2}$ = -0.01 v; anodic-cathodic couple
$E_{1/2}$ = -0.88 v; cathodic wave

Murexide

$E_{1/2}$ = 0.33; anodic wave
$E_{1/2}$ = -0.11; cathodic wave

Alloxantin

$E_{1/2}$ = -0.01; anodic cathodic couple

Electrolysis Product after Exposure to Air

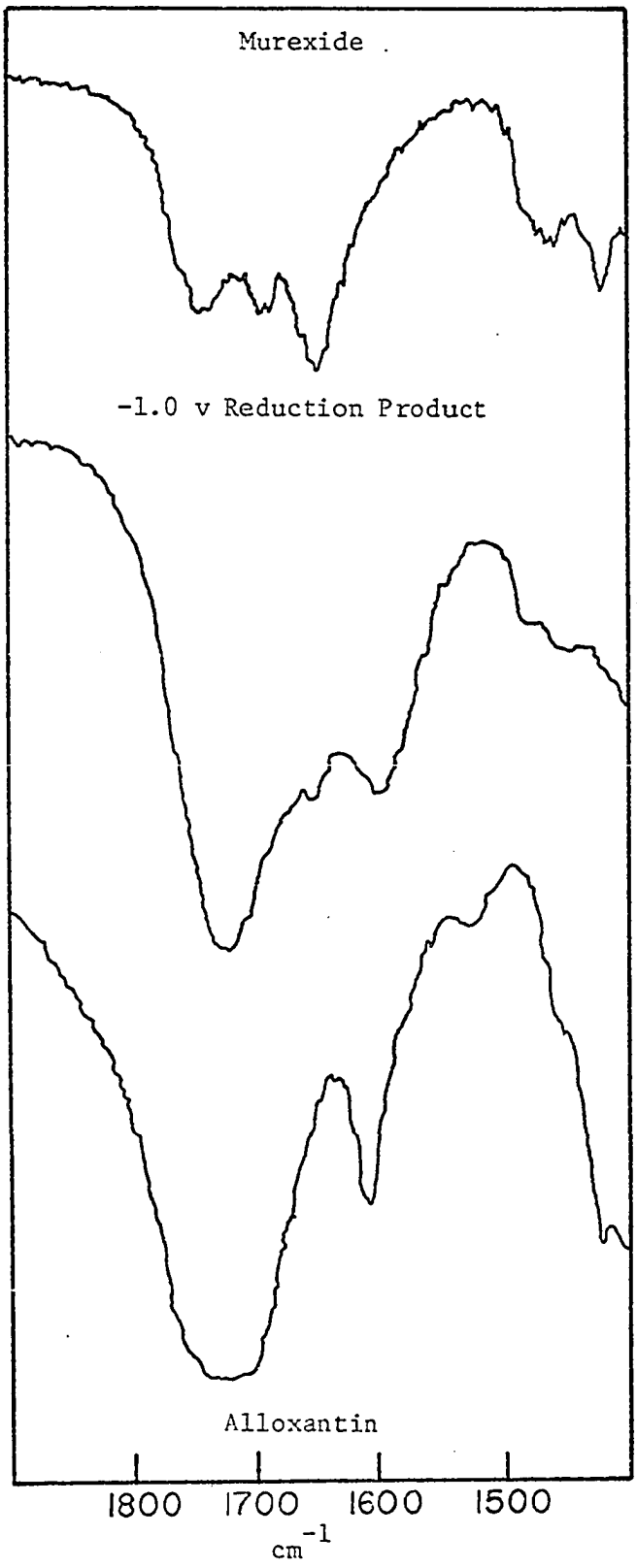
$E_{1/2}$ = -0.01; cathodic wave
$E_{1/2}$ = -0.50; cathodic wave
$E_{1/2}$ = -0.88; cathodic wave

Alloxantin + Murexide after Exposure to Air

$E_{1/2}$ = -0.01; cathodic wave
$E_{1/2}$ = -0.11; cathodic wave
$E_{1/2}$ = -0.44; cathodic wave

Since ammonia and/or urea are generally considered necessary for murexide formation from alloxan and alloxantin (Introduction) a 500 g batch of TEAP was recrystallized from acetonitrile in an attempt to eliminate these potential contaminants. Solutions of alloxan prepared with the recrystallized TEAP still developed the previously observed color upon electrolysis.

Figure 20


Murexide
-1.0 v Reduction Product
Alloxantin
1800
1700
1600
1500
cm-1

Interpretation of Results

The anodic activity exhibited in solutions of the reduction product in acetonitrile cannot be used to identify either alloxantin or dialuric acid. The behavior of aqueous solutions of the electrolysis product is more useful. The spectrophotometric characteristics are those of dialuric acid and the polarography is that expected of a dialuric acid or alloxantin solution which is partially oxidized to alloxan.

The color produced may be due to murexide or a similar compound. The determination of faradaic n values which are constant within experimental accuracy supports the view that the production of the colored compound is not part of the primary reduction mechanism, i.e., it does not interfere in the electron-transfer step to any appreciable extent, for if it did, a far greater variation in n values could be expected due to the variations in the color reaction already noted. Further identification of the compound was therefore not considered necessary.

Investigation of the Anodic Character of the Reduction Product Solution

A series of experiments were designed to clarify the nature of the species producing the anodic waves at the d.m.e. The various chemical parameters were eliminated one at a time to facilitate a systematic study of the effects of mercury, mercurous ion, silver ion and TEAP itself on the anodic character of the reduction product at the d.m.e. The reduction of alloxan at -1.0 v was then carried out in a regular polarographic cell.

Elimination of Mercury

Mercury was eliminated by using a graphite electrode as the cathode. The solution continued to become colored during the reduction. No clouding, i.e., no forming of a solid product, was noted. A polarogram was obtained on the partially electrolyzed solution with the graphite electrode disconnected but remaining in the deoxygenated solution. The anodic character was identical to that previously noted. The solution was then oxidized at -0.4 v with the graphite as the anode. A polarogram of this reversed electrolysis solution revealed that the height of the -0.5 to -0.7 plateau had diminished. An increase in the cathodic wave height (I + II) was also observed. This was proof of the somewhat reversible character of the -0.5 to -0.7 v region. It must be noted that the polarographic analysis of the solution did reintroduce mercury into the solution.

Elimination of Silver Ion

The presence of the silver cation was eliminated by using a mercury pool as the reference electrode during the reduction. Polarograms of the reduction product solution exhibited the typical anodic activity.

Elimination of TEAP

Alloxan in a 0.8 M $LiClO_4$ acetonitrile solution was reduced at -0.4 v. (This potential corresponds to the -1.0 v region on a TEAP polarogram.) No anodic activity beyond that of background was observed in the electrolysis product. This was evidence that TEAP itself must be involved in an interaction with the primary products of alloxan reduction thereby producing an electrochemically active product as

evidenced by the presence of two anodic waves upon polarographic analysis of the reduction product solution.

Polarography of Alloxan in Recrystallized TEAP in Acetonitrile

Polarograms of alloxan in a solution of 0.1 M TEAP which has been recrystallized from acetonitrile revealed a decreased anodic background activity. Shifts in $E_{\frac{1}{2}}$ and a modification of the character of the cathodic waves was also observed. A typical polarogram is reproduced in Figure 21. A comparative study of $E_{\frac{1}{2}}$ and i for solutions of alloxan of equal concentration in regular TEAP and in recrystallized TEAP is summarized in Table 19.

No changes were observable in the pattern or products of the electrolysis in the recrystallized background. Faradaic n values were compatible with those formerly obtained. Recrystallization probably altered the adsorbing properties of the product interactions but did not change the overall reaction process.

Voltammetry of Alloxan in Acetonitrile Solutions

Cyclic voltammograms of the acetonitrile-alloxan system at p.g.e. were obtained at various scan rates. A typical voltammogram, obtained at a rate of 200 mv sec^{-1} is reproduced in Figure 22. A general pattern of three major cathodic and four anodic peaks are observed.

The major cathodic peak, $(E_p)_c$ = -0.84 v, is coupled with an anodic peak, $(E_p)_a$ = -0.64 v. The 200 mv difference is obviously too great for a perfectly reversible system but does reflect the somewhat

Table 19

Effect of TEAP on Half-Wave Potential

and Current of Equal Concentration Alloxan in Acetonitrile

	Regular TEAP		Recrystallized TEAP	
	$E_{1/2}$ v	i μa	$E_{1/2}$ v	i μa
	0.6 mM			
Wave I	-0.56	1.63	-0.50	0.48
Wave II	-0.90	1.87	-0.71	2.50
Wave III	-1.27	1.24	-1.38	1.90
Wave IV	-1.50	0.42	-1.92	0.56
	1.0 mM			
Wave I	-0.53	1.68	-0.49	0.40
Wave II	-0.83	2.90	-0.73	4.00
Wave III	-1.25	1.80	-1.38	2.88
Wave IV	-1.55	2.04	-1.95	1.04

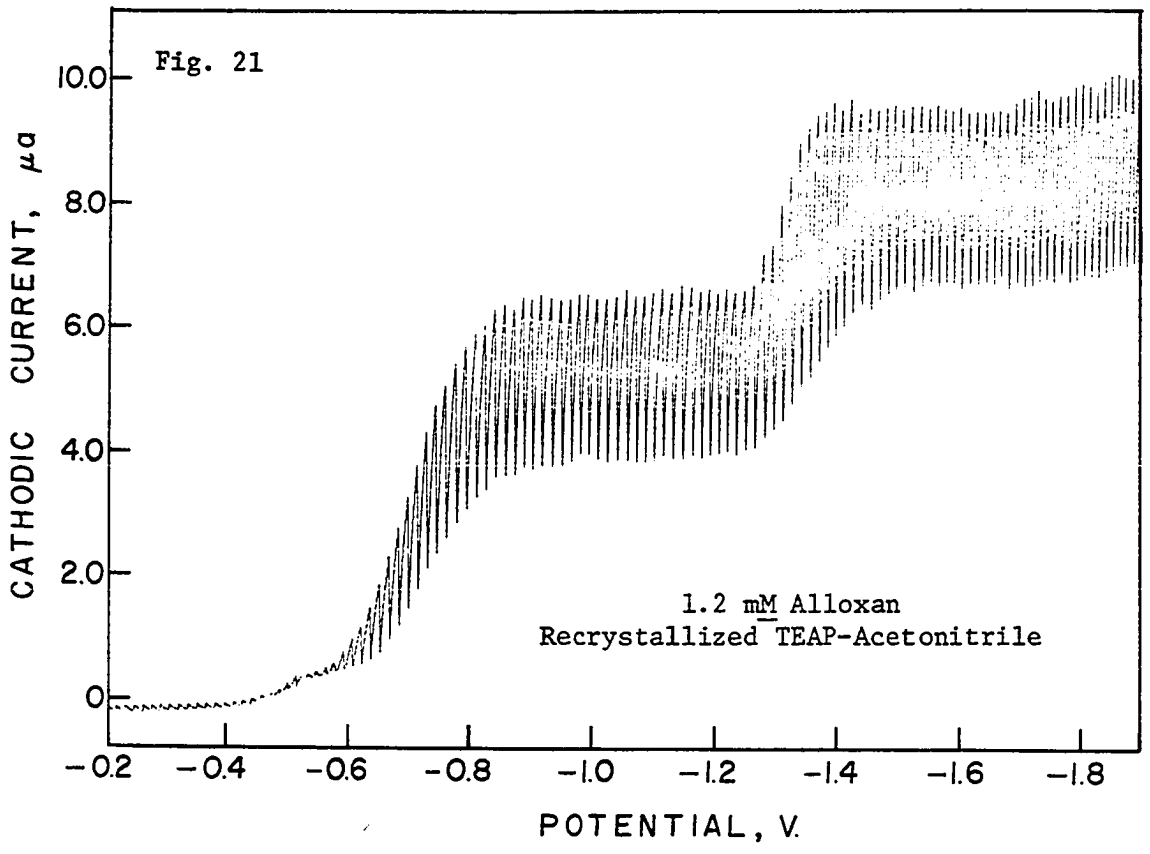


Fig. 21
CATHODIC CURRENT, μa
10.0
8.0
6.0
4.0
2.0
0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
-1.8
POTENTIAL, V.
1.2 mM Alloxan
Recrystallized TEAP-Acetonitrile

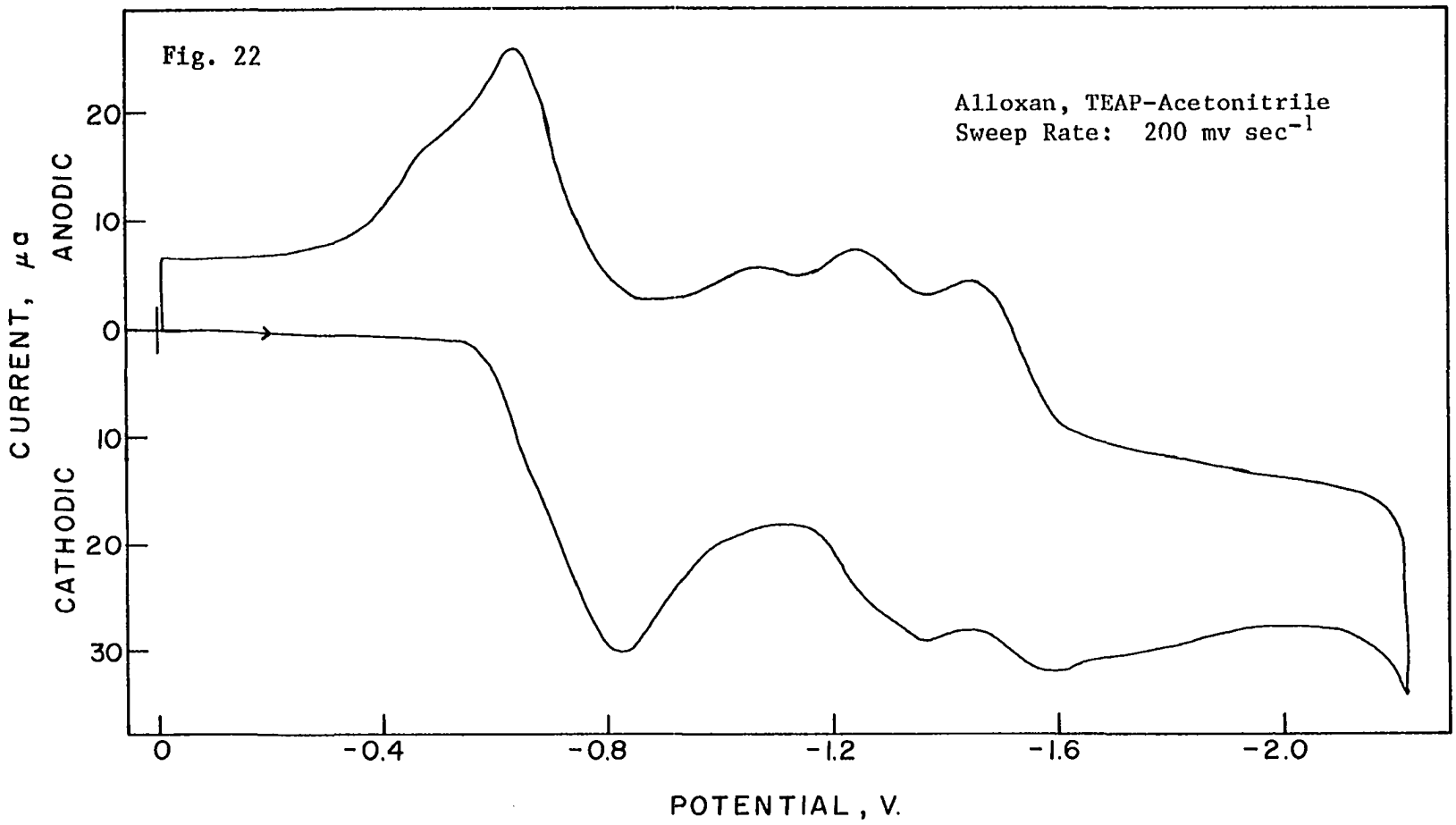


Fig. 22
Alloxan, TEAP-Acetonitrile
Sweep Rate: 200 mv sec^{-1}
CURRENT, μa
ANODIC
CATHODIC
20
10
0
10
20
30
0
-0.4
-0.8
-1.2
-1.6
-2.0
POTENTIAL, V.

reversible character of the product formed at the potential of the first reduction. $(E_p)_c$ = -0.84 v compares favorably with the reduction potential of -1.0 v used to obtain the faradaic n value of the Wave I + II reduction at the d.m.e.

It was observed that if traces of oxygen were present in the solution, the anodic peak heights diminished. In such cases, at slow scan rates, e.g., 2 - 10 mv sec^{-1}, no anodic peaks were distinguishable. This substantiates the belief that alloxantin and dialuric acid, and their intermediates, are very susceptible to air-oxidation.

Polarography of DMA in Acetonitrile Solutions

A very brief investigation of the behavior of DMA in 0.1 M TEAP in acetonitrile at the d.m.e. revealed that two cathodic waves are exhibited. The first, $E_{1/2}$ = -0.77 v is very distinct, the second, $E_{1/2}$ = ca. -1.0 v is only slightly higher. A 1.0 mM solution produced a total cathodic current of 3.3 μa with a diffusion current constant of 2.5. This compares favorably to the combined I + II process of alloxan reduction.

Upon electrolysis at -1.0 v, a polarographic analysis of the reduction product revealed a single anodic wave, $E_{1/2}$ = -0.74 v. The nature of the anodic wave, by comparison of $E_{1/2}$ and current size is closer to that of the alloxan reduction product which is identified by the -0.5 to -0.7 v plateau.

Two statements can be made on the basis of this data, (1) the first one-electron reduction of dimethyl alloxan occurs at a potential comparable to that of alloxan but the second electron is much more

difficult to transfer and, in fact, the reduction does not occur within the observable potential range (2) whatever the nature of the TEAP-product interaction which produces the anodic plateau in the 0 to -0.3 v region, it cannot form, or is not electroactive, in the case of DMA.

Proposed Mechanism for Reduction of Alloxan in Acetonitrile

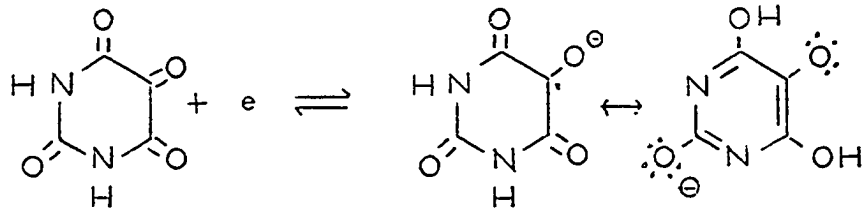
A mechanism for the reduction of alloxan in acetonitrile, very similar to that of quinones in non-aqueous solvents (Equations 30 - 32) is proposed in the light of the accumulated data. Coulometric electron number determinations substantiate that two one-electron transfer processes are involved. The 1600 cm^{-1} absorption in the IR spectra of the crude -1.0 v solid electrolysis product indicates that alloxantin is present in the product mixture. The UV absorbing species (λ_{max} = 274 mμ) in aqueous solution is proof that dialuric acid is formed in the reduction product solutions. In the -1.0 v reduction product, dialuric acid is no doubt formed by the disproportionation of alloxantin in the aqueous solution required for the spectrophotometric study. Dialuric acid is formed upon protonation of the primary product of the -1.8 v reduction. It should be noted that acetonitrile is not a perfectly aprotic solvent and also that since the alloxan monohydrate is the solid form dissolved in the solutions, there are some protons available.

It has already been shown that Wave I is the reduction of alloxan to the adsorbed one-electron reduction product, that Wave II is the reduction of alloxan to the dissolved form and that Waves III and IV are the further reduction of the adsorbed and dissolved product to the final product.

The proposed mechanism can be outlined as follows in Equations 34 - 40.

Primary Electrode Reaction of Waves I and II

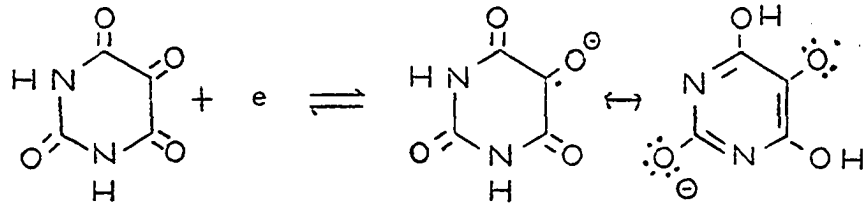
Wave I



(34)

Alloxan — Adsorbed Alloxan Free Radical Anion

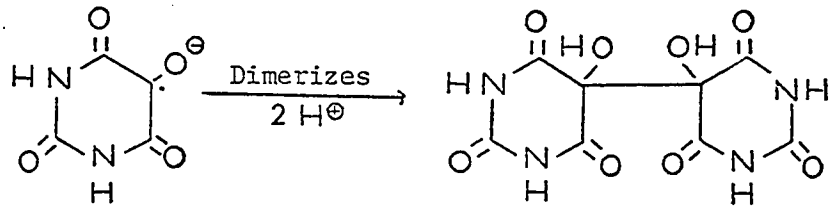
Wave II



(35)

Alloxan — Dissolved Alloxan Free Radical Anion

Subsequent Reactions of Primary Reduction Product

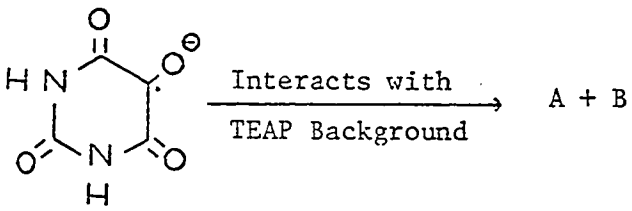


(36)

Alloxan Free Radical Anion

Alloxantin

or

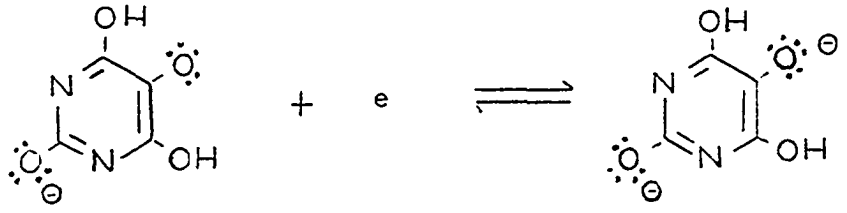


(37)

where: A = species oxidized at ca. -0.8 v

B = species oxidized at ca. -0.4 v

Primary Electrode Reaction of Waves III and IV

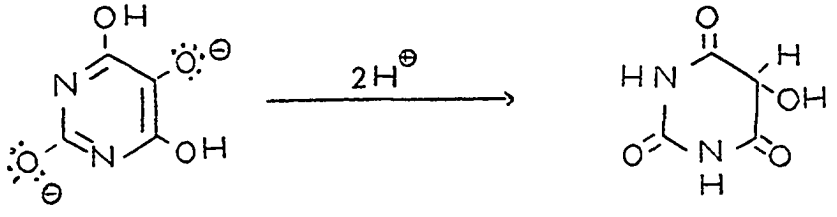


(38)

Alloxan Free Radical Anion

Dialuric Acid Dianion

Subsequent Reactions of Primary Reduction Product

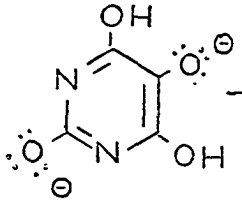


(39)

Dialuric Acid Dianion

Dialuric Acid

or



$$\xrightarrow[\text{TEAP Background}]{\text{Interacts with}} \quad A' + B' \qquad (40)$$

where: A' = species oxidized at ca. -0.8 v

B' = species oxidized at ca. -0.4 v

Since addition of protons did not affect the $E_{1/2}$ of the reduction steps, the protonation step must be slow by comparison with the primary electron transfer reaction. The protonation is therefore considered as a subsequent reaction.

Both the free radical anion and the dialuric acid dianion are aromatic. The observation that a similar aromatic structure cannot be written for DMA and the fact that the reduced DMA product solution did not contain the species identified as B in Equation 37 may link the TEAP-reduction product interaction to the aromaticity of the structure.

The proof of two one-electron reductions of alloxan in acetonitrile further substantiates the proposed mechanism for the reduction of alloxan and its N-methylated derivatives in aqueous solutions. It is indeed likely therefore that at least in part the primary reduction

product at the potential of the kinetic-controlled wave in aqueous solutions is the appropriate alloxantin, which disproportionates to the respective alloxan and dialuric acid.

EXPERIMENTAL

Synthesis of Dimethyl Alloxan

Dimethyl alloxan was prepared according to the method of Fischer.[49] Caffeine (25 g) and concentrated HCl (35 g) were mixed with 75 ml H_2O and heated over a water bath at ca. 50 °C for about two hours while gradually adding 9.7 g powdered $KClO_3$. The solution was cooled with cold water and then ice. The free chlorine gas was driven off by bubbling air through the solution. A precipitate formed after about two hours and was removed by filtration. The almost colorless filtrate was cooled again with ice and a cold solution of $SnCl_2$ added dropwise (prepared by dissolving 13.5 g crystalline $SnCl_2$ in 10 ml concentrated HCl and adding 10 ml H_2O). Mixing was performed by bubbling a stream of air through the solution. After about three hours, a precipitate was removed by filtration. The solid was placed in an evaporating dish, mixed with a small amount of water and warmed on a waterbath. A small amount of concentrated HNO_3 (enough to dissolve solid) was added and the solution placed in a flat crystallization dish in a vacuum desiccator. Crystallization took as long as two weeks. Recrystallization was carried out from water.

The freshly recrystallized product was clear though the surface rapidly became white. The behavior of the crystals on melting was not sharp. The crystals melted at around 80 °C and resolidified between 120 - 130 °C. The solid then melted at 270 °C. This follows the report

that the dehydrate melts "bei etwa 97 °C" and the reported melting point for the anhydrous dimethyl alloxan is 270 - 271 °C.[50]

Synthesis of Methyl Alloxan

Theobromine (20 g), concentrated HCl (32 g) and 50 ml water were mixed and heated as in DMA synthesis. The temperature was maintained under 50 °C. Gradually, 9.1 g $KClO_3$ were added. Upon cooling and driving off of chlorine, a colorless precipitate formed and was removed by filtration. The filtrate was cooled to 0 °C and a solution of 10 g crystalline $SnCl_2$ in 10 ml concentrated HCl added dropwise, maintaining the temperature at 0 °C. The solution was placed in an ice box over night as suggested by Fischer but it was found that frequently a much longer period was required before a precipitate formed. The solid product was then treated in the same manner as the DMA above, except that to the solution after addition of the concentration HNO_3, a quantity of water 1.5 times that of the solution volume was added plus a drop of concentrated HCl. Several attempts yielded only a small amount of clear diamond-like crystals. The melting point was in the 150 - 154 °C range. Mulliken[51] reported mp 156 °C with decomposition.

Other Chemicals

Alloxan was obtained from Nutrional Biochemicals, alloxantin and tetraethylammonium perchlorate from Eastman, dialuric acid from Calbiochem, murexide from Matheson, Coleman and Bell and lituim perchlorate from G. Frederick Smith Co. One batch, 500 gm, of TEAP was recrystallized from acetonitrile. The rest was used as received. Acetonitrile was either 'Baker analyzed' or Fisher certified reagent

grade. Unopened it contained from 0.04 to 0.23 per cent water as indicated on the analysis label.

Buffer solutions, prepared from analytical grade chemicals, had an ionic strength of 0.5 M as used. The buffered systems used were HCl - KCl, HOAc - NaOAc and citric acid - NaH_2PO_4 - KCl (McIllvaine buffer). 2 M H_2SO_4 and 1 M HOAc were also used.

Argon used for deoxygenation was equilibrated with water or acetonitrile in a bubbling chamber. Nitrogen, used for deoxygenation of the reference and counter cells in the macroscale and coulometric work, was used directly from the tank.

Apparatus

Most of the apparatus has been described in Part I. A Sargent Model A IR compensator was used in conjunction with the Sargent XV polarograph for the non-aqueous, high resistance solutions. For the non-aqueous work, the three-compartment cells described in Part I were used, but the salt bridges, inserted on the counter and reference side of the frits, were prepared by refluxing 50 ml of 0.1 M TEAP or $LiClO_4$ in dimethylformamide and gradually adding 2.7 g 15 centipoise methyl cellulose (Fischer). The reference electrode suspended in the background electrolyte acetonitrile solution consisted of a 9 mm diameter glass tube fitted with a fine porosity frit and salt bridge (described above) in which was contained a 0.1 M $AgNO_3$ solution in acetonitrile and a small coil (about 5 coils) #16 silver wire held in place by a small rubber stopper. This constitutes a Ag - 0.1 M $AgNO_3$ electrode which is 0.337 v versus S.C.E.[42] The counter electrode was a platinum

gauze also suspended in the background electrolyte acetonitrile solution.

Procedure for Height and Temperature Studies

Height and temperature studies were carried out in the normal way. The height of the mercury column was usually varied from 90 to 30 cm and triplicate polarograms recorded. The value of m at each height was obtained by collecting the mercury which dropped from the capillary tip immersed in water during a timed interval and with an open circuit. The mercury was dried and the mass recorded.

Temperatures of 25 ± 0.2 °C and 40 ± 0.2 °C were maintained and triplicate polarograms recorded.

Preparation of the Aqueous Solutions

Aqueous solutions of the alloxans were not stable so each test solution was made up in the desired buffer immediately prior to use. Solutions used for the pH study were in the 1 mM range. Buffered solutions were of 0.5 M ionic strength.

Procedure for Aqueous Polarographic Studies

The pH of the prepared solution was recorded and the solution placed in the polarographic cell. Deoxygenation was attained by bubbling a heavy stream of argon through the solution for five min.

A stream of gas was directed over the solution during analysis. Polarograms were obtained in triplicate for each solution. The same technique was employed to obtain polarograms of the background solution. Current values were calculated by subtraction of the average background current from the average total limiting current height.

In solutions of low pH, the presence of chloride ion in a solution masked the half-wave potential of Wave Ia. Several washings of the cell were therefore necessary whenever chloride solutions had been used if a polarogram in 1 M HOAc or 2 M H_2SO_4 was to be obtained.

Procedures for Faradaic n Value Determinations

Wave I

The reductions were carried out in the three compartment cell described in Part I for macroscale electrolysis. All the cells were totally deoxygenated by means of gas bubblers inserted in the rubber stoppers atop each cell. A magnetically stirred pool of mercury (30 ml) served as the cathode in the working cell. Electrical contact was made through a platinum wire sealed in glass tubing containing mercury. A temperature of 25 °C was maintained by circulating water from the constant temperature bath under the working electrode compartment atop the magnetic stirrer. The coulometer described in Part I was in the circuit.

A solution of 1 M HOAc (150 ml) was placed in the working electrode compartment and a potential of -0.4 v applied. The reduction was continued until the quantity of standard HCl titrated per unit time attained a constant value. This value was the background level, i.e., it was related to the number of electrons consumed per unit time by reduction of the background. This value was recorded and the potentiostat was turned off.

A weighed quantity of the appropriate alloxan was added to give a solution in the 1 mM concentration range. Upon dissolution the potential was again applied and the time required for reduction care-

fully noted. Titrations of standard HCl into the coulometer were performed about every 15 min, attaining a value of pH 7 for the coulometer solution, the volume of acid required was also noted. The course of the electrolysis could not be followed spectrophotometrically, hence the electrolysis was considered complete when the quantity of acid titrated per unit time achieved that of the background level noted above.

The entire cell was then placed under the d.m.e. for polarographic analysis of reduction product solutions. The products were highly susceptible to air oxidation and conditions of total deoxygenation were continued as long as analysis of the product solution was in process. The n values were calculated as described in Part I.

Wave II

Coulometric studies of the reduction occurring at higher potentials were complicated by the instability of the alloxans in the pH regions required to observe Wave II. It was therefore necessary to prepare a concentration calibration curve for solutions and to conduct only short term reductions.

Procedure for preparing the concentration calibration curve. Two consecutive concentration-decomposition studies were performed on each alloxan. Solutions of the alloxan under consideration was prepared (1.0 mM) in pH 4.9 or pH 5.1 McIlvaine buffer and 1,2,3,4 and 5 ml aliquots immediately transferred into volumetric flasks containing the background solution to give a total 10 ml volume. The solutions were allowed to stand for 30 min. Then, as rapidly as possible, polarographic scans from -1.2 to -1.6 v were obtained. The solutions were

scanned in the order 0.1, 0.5, 0.2, 0.4 and 0.3 mM. The background current at the potential of the limiting current of Wave II was obtained as the average from several background scans. This current subtracted from the average limiting current at each concentration gave the current value for the Wave II reduction. The current plotted versus concentration yielded a straight line through the origin.

Procedure for coulometry of Wave II reduction. The background solution (pH 4.9 or pH 5.1 McIllvaine buffer), 150 ml, was electrolyzed at -1.5 v to a constant level of standard HCl titration per unit time as described above and the electrolysis then continued for another 30 min, the volume of acid being carefully noted. The potentiostat was then turned off and a weighed quantity of the respective alloxan sufficient to produce a concentration of about 1 mM was added. The potential was then applied and a 30 min period of reduction allowed. The potentiostat was turned off, the coulometer solution pH adjusted to pH 7 and the entire cell placed under the d.m.e. Triplicate polarograms scanned from -1.2 - -1.6 v were obtained. The concentration of the alloxan present in the solution was determined by comparison of the average limiting current of the reduction product solution with the prepared calibration curve. Four such reductions were carried out on DMA, two on alloxan and due to the scarcity of material, only one on MA. The n values were calculated in the manner already described.

Spectrophotometric Procedure for Aqueous Solutions

Samples studied spectrophotometrically had to be maintained in an oxygen-free environment. This was best achieved by bubbling a stream of nitrogen through the background solution contained in a volumetric

flask. The air in the spectrophotometric cell was replaced with nitrogen and capped. Pipettes flushed with nitrogen were used for measurement and transference purposes. Nitrogen was used to force the solution from the pipette. Under these conditions transference of reduction product solutions from the electrolysis cell, or of dissolution of solid alloxantin or dialuric acid in a buffered solution was sometimes successfully performed without the introduction of air into the system.

Preparation of Reduction Product Solutions for Spectrophometric Studies

Due to the problem of excluding air from solutions of the electrolysis product, reductions were carried out on solutions of DMA sufficiently dilute to make dilution of the product solution unnecessary when observed spectrophotometrically. Therefore solutions 0.2 m$\underline{M}$ in DMA were reduced at -0.4 v and at -1.4 v in pH 4.7 acetate buffer and the electrolysis product solution, treated as described above, observed under UV light of 360 - 220 mμ wavelength. For comparative purposes, solutions 0.1 m$\underline{M}$ in alloxantin and 0.2 m$\underline{M}$ in alloxan were also reduced in pH 4.7 acetate buffer at -0.4 v and the reduction solutions observed spectrophotometrically.

Lyophlization

The apparatus used for lyophilization is detailed in Part I. Solutions of the DMA reduction products were transferred under air-free conditions into round bottom flasks of appropriate size, which had previously been flushed with nitrogen. The solution was shell frozen to the walls of the flask while a stream of nitrogen flowed into the

flask.

Procedure for Alloxantin Dissociation Study

A solution 0.2 mM in alloxantin was prepared in pH 4.7 acetate buffer according to the following method. An appropriate weighed quantity of solid alloxantin was placed in a volumetric flask which had been flushed with nitrogen. A deoxygenated solution of buffer was added, the flask stoppered and placed in a hot water bath to aid in dissolution. When dissolved and cooled an air-free transfer was made to the polarographic cell and two scans from -1.2 to -1.60 and then +0.4 to -0.2 v obtained. The entire process was performed twice and the results were comparable.

Preparation of Alloxan-Acetonitrile Solutions

Alloxan was difficultly soluble in acetonitrile-TEAP background solution and required considerable shaking to accomplish dissolution. Solutions 2.0 to 0.1 mM were made up in 0.1 M TEAP and 0.8 M $LiClO_4$ respectively in acetonitrile. Warming of the solutions over water was prohibited since this increased the water content of the solvent considerably as observed in polarographic analysis.

Preparation of Solutions for Polarographic Studies

Each solution placed in the working electrode compartment was carefully deoxygenated and a very vigorous stream of argon passed over the solution throughout the analysis. Similarly, the counter and reference compartments were deoxygenated at intervals and maintained stoppered. Oxygen is very soluble in acetonitrile; trace amounts are readily discernible under polarographic analysis.

Drop behavior of mercury was frequently erratic due to the tendency of the drop to cling to the glass around the capillary tip. Dipping the tip into water solution until single drops formed and dropped was found to remedy the problem. Careful drying of the electrode with tissue was necessary before replacing it in the acetonitrile solutions.

Procedure for Addition of Proton Sources

Addition of Benzoic Acid

A 2.0 mM solution of alloxan in TEAP acetonitrile was the stock solution. To 20 ml portions of stock solution, solid benzoic acid (reagent grade) was added giving concentrations of 0.1, 0.2, 0.8, 1.0, 2.0, 4.0 and 22.0 mM in benzoic acid. Each solution was prepared immediately prior to use with as identical as possible time intervals involved for mixing and deoxygenation upon transferring the solution to the polarographic cell. Triplicate scans from 0 to -2.0 v and -1.56 to -2.4 v at scan rates of 2 v/ 10 min and 3 v/10 min respectively.

Addition of Water

Each solution was made up in a 25 ml volumetric blask. A total volume of 15 ml was used for each test solution and the water content varied from 0.05 to 1.0 ml as measured by pipettes. Weighed quantities of solid alloxan (2 to 3 mg) was added to each 15 ml solution, shaken until dissolved and transferred to the polarographic cell. Each solution was prepared immediately prior to use. Total deoxygenation time was 5 min; the time interval of the mixing and transference was maintained as constant as possible. The polarographic analysis was per-

formed as described for benzoic acid.

Procedure for n Value Determinations in Acetonitrile

The working electrode was 30 ml triply distilled mercury which was magnetically stirred. All compartments were totally deoxygenated. The working electrode compartment was insulated from the heat of the magnetic stirrer by several layers of paper gauze toweling. Temperatures under 30 °C were thereby maintained.

The background solution, 150 ml of totally deoxygenated 0.1 M TEAP in acetonitrile was reduced at -1.0 and -1.8 v respectively with the coulometer in the circuit and the background level determined as previously described. The potentiostat was then turned off.

Alloxan in a weighed quantity to produce a concentration of about 1 mM was then added, dissolved and the potential applied. Completion of the electrolysis was indicated when the quantity of acid titrated per unit time equalled that observed for the background reduction. The calculation of n was performed as described previously.

Procedures for Acetonitrile Spectrophotometric Studies

The UV absorption of the TEAP solution prohibited spectrophotometric studies on the reduction product solutions. However, small quantities of the acetonitrile reaction solution were added to deoxygenated water contained in a spectrophometric cell and qualitative spectral behavior observed.

Separation and Analysis of Solid Reduction Product

The solid material observed to form in macroscale reductions of

alloxan in acetonitrile was separated from the reduction product solution in the following manner. A dry bag continuously flushed with nitrogen was used to assure an air-free working atmosphere. The entire electrolysis cell was transferred to the bag upon termination of the electrolysis with the gas dispersion tubes still connected to the gas sources. Once inside the bag, the bubblers were disconnected. The solid was transferred by means of pipettes to a sintered glass filter and flask system under vacuum. The solid was washed with deoxygenated acetonitrile and placed in a vacuum desiccator.

When dry, the solid was removed from both the desiccator and the bag, and a KBr pellet prepared for IR analysis.

Other Methods Employed

The solutions of DMA studied were treated in every respect as described above for alloxan. The methods of short term electrolysis in the polarographic cell for elimination of mercury, silver and TEAP are described in the Results section. Voltammetric studies were carried out in the polarographic cell. The graphite electrode was treated as described in Part I except that upon resurfacing the electrode was worked with acetonitrile instead of water.

SUMMARY

Polarography, voltammetry and controlled-potential electrolysis of alloxan, methyl alloxan and dimethyl alloxan in aqueous solutions of varying pH, and of alloxan in acetonitrile was extensively studied. The results from the aprotic solvent support those of the aqueous solution and clarify the electrode mechanism.

At the potential of the first cathodic wave (Wave Ia) observed in aqueous solutions, the respective alloxans are reduced in a two-electron process, which is kinetically-controlled by the rate of dehydration of the reducible carbonyl site. The variation of $E_{\frac{1}{2}}$ with pH is $E_{\frac{1}{2}} = 0.060 - 0.03$ pH for alloxan, $E_{\frac{1}{2}} = 0.08 - 0.027$ for methyl alloxan and $E_{\frac{1}{2}} = 0.10 - 0.036$ pH for dimethyl alloxan. This cathodic wave was observed by previous workers[4,5] and is a two-electron, one-proton reduction to the dialuric acid anion, which may in part occur by way of the dimerization to alloxantin followed by rapid disproportionation.

A second pH independent reduction (Wave Ib) is observed for 1.0 mM solutions of DMA and MA and 10.0 mM solutions of alloxan in the pH range 0 to 3. It is proposed that the respective protonated alloxan molecule is reduced in a manner identical to that of the first cathodic wave.

Wave II, which can be detected only in the limited pH range of ca. 4.5 - 5.9, was also observed by the earlier workers. It is

a two-electron, one-proton reduction of the hydrated alloxan molecule to the appropriate dialuric acid anion.

Polarography of alloxan solutions in 0.1 M TEAP in acetonitrile revealed four cathodic waves. Wave I is shown to be an adsorption wave, representing the reduction of alloxan to the adsorbed reduction product. Wave II represents the reduction to the dissolved reduction product. The nature of Waves III and IV are not clear. The combined waves (I + II) and (III + IV) represent two one-electron reductions of alloxan to the alloxan free radical anion and dialuric acid dianion respectively. Subsequent reactions produce alloxantin and dialuric acid respectively, i.e., upon protonation of the primary reduction products and dimerization in the case of alloxantin, and an unknown interaction product thought to be due to the tetraethylammioum perchlorate. The primary reduction is very similar to that observed for quinones in acetonitrile[45] and affirms the benzoid-quinoid character of alloxan.

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