CCXXXIII. THE AUTOXIDATION OF DIALURIC ACID.

BY GEORGE MAXWELL RICHARDSON.

*From the Department of Physiology and Biochemistry, University College,
and the Biochemical Department, Imperial College of Science, London.*

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OF recent years, biological studies of oxidation-reduction have been chiefly concerned with the rate and specificity of the oxidative reactions between metabolites. At the same time, many oxidation-reduction systems have been precisely defined electrometrically by measurements at an inert electrode. It will therefore be interesting to co-ordinate, where possible, the kinetics of oxidation with the oxidation-reduction potentials of the reacting systems.

With this end in view, a previous description of the electrode potentials of the dialuric acid-alloxan system:

included some preliminary data describing the atmospheric oxidation of dialuric acid [Richardson and Cannan, 1929]. The extent to which the reactants would associate to the dimeride alloxantin and interfere in the system was also formulated, but was found to be scarcely significant in dilute solution. The rapid, roughly linear velocity of oxidation obtained was therefore not an unlikely one. At the same time the manifestation in the data of some correlation in acid solution between electrode potential and rate of oxidation made an extended investigation using improved technique highly desirable.

While such experiments were being initiated with a Warburg gas absorption apparatus, the first of a series of three papers dealing with this autoxidation [Hill, 1930; 1931; 1932] appeared. His observations, however, would not submit to an orderly interpretation, and agreed neither with our published findings nor with such gasometric data as were then available. His calculations regarded alloxantin as the end-product of the oxidation, an assumption irreconcilable with the actual direct observations of Thunberg [1915]. Consequently, a much more thorough investigation of this autoxidation than was at first anticipated is now to be reported.

EXPERIMENTAL.

The rate of diffusion of oxygen into a reaction mixture effectively limits the technique available for measuring atmospheric oxidation velocities. In previous experiments we added solutions already saturated with oxygen to dialuric acid at high dilution to overcome this limitation, but the increased liability to error at such dilutions made a different technique desirable. It was also clear that oxidation in other than acid solution was too rapid to allow the accurate withdrawal of samples for titrimetric estimation of velocity. Yet current indicator methods were felt to be poorly suited to defining a continuous series of points during the oxidation. Consequently the gasometric measurement of oxidation in a set of six Warburg absorption manometers was adopted, small quantities of less dilute solutions being exposed to as large a surface of air as possible. Although the apparatus is not well suited to following oxidations with the rapidity that was attempted, the application of extensive controls should have minimised fortuitous errors. An abundance of fairly reproducible observations has been accepted as the practical criterion of reliability.

The preparations of dialuric acid, H_2O ; alloxan, H_2O ; and alloxantin, $2\text{H}_2\text{O}$ used were identical with those employed for the previous investigation. Stock solutions of dialuric acid and alloxantin in 0.05 *N* HCl were made up in sufficient quantity to last 2 or 3 days and were stored under hydrogen for immediate delivery from a burette into the absorption flasks. Solutions were approximately saturated at room temperature (0.025 *M* dialuric acid, and 0.0083 *M* alloxantin), and 2 or 3 times daily were titrated directly against 0.01 *M* iodine solution with starch indicator [*cf.* Büllmann and Bentzon, 1918]. Titratable reducing power never changed by more than 3.5 % on storing, and was seldom smaller than 95 % of the value calculated by weight. In the calculation of results, the iodine titre is accepted as the measure of dialuric acid concentration.

Stock buffer solutions adopted were 0.1 *M* and 0.05 *M* HCl, 0.1 *M* citrate, and Prideaux and Ward's universal buffer mixture 0.1 *N* with respect to each constituent group (this is of greater strength than is customary, care being necessary in cold weather lest the phenylacetic acid should become super-saturated in acid stocks). Buffer p_{H} values were measured with a Hildebrand hydrogen electrode, a saturated KCl calomel half-cell and a Cambridge portable potentiometer.

Experiments were conducted in the Warburg apparatus under the following conditions.

Temperature of thermostat, 18°; manometer liquid, Brodie's fluid (density 1.032₂); average capillary diameter 0.35 mm.; amplitude of swing, about 6 cm.; number of oscillations per min., about 120; capacity of flasks to manometer level, (1) 20.1 cc., (2) 19.5 cc., (5) 23.2 cc., (6) 20.3 cc. (by weight of mercury); volumes of experimental solutions, 2.4 and 1.2 cc.; average exposed

area of base of flasks 8.7 cm.²; average depth of liquid, 2.8 and 1.4 mm. All flasks in use were of the same general dimensions, and were constructed in the usual conical shape with side-arm and NaOH tube.

For each experiment, the stock solutions were delivered directly into the flasks from micro-glassware. The proportions were decided so that the reacting mixtures should contain: dialuric acid of the required concentration, the exact equivalent of 0.1 *M* NaOH to bring the dialuric acid solution to the desired p_H , as large an excess of buffer solution as possible, comparable concentrations of total salts, added catalytic solutions where desired, and, finally, distilled water to the required volume. Table I describes the solutions used between p_H 4 and 11. At more acid p_H values, the proportions were suitably modified

Table I. *Constitution of reaction mixtures.*

	Conc. of dialuric acid					Conc. of alloxantin	
	0.0166 <i>M</i>	0.0125 <i>M</i>	0.0066 <i>M</i>	0.0063 <i>M</i>	0.0021 <i>M</i>	0.0055 <i>M</i>	0.0021 <i>M</i>
Reductant (cc.)	1.6	1.2	0.4*	0.6	0.2	1.6	0.6
0.05 <i>M</i> HCl (cc.)	—	—	—	0.6	1.0	—	0.6
0.1 <i>N</i> buffer (cc.)	0.6	0.75	0.5	0.75	0.75	0.6	0.75
0.2 <i>M</i> NaOH (cc.)	—	0.45	0.14	0.375	0.325	—	0.35
0.8 <i>M</i> NaOH (cc.)	0.15	—	—	—	—	0.133	—
Water + catalyst (cc.)	0.05	0	0.16	0.075	0.125	0.067	0.1
Total volume (cc.)	2.4	2.4	1.2	2.4	2.4	2.4	2.4
Buffer strength	0.025 <i>N</i>	0.031 <i>N</i>	0.042 <i>N</i>	0.031 <i>N</i>	0.031 <i>N</i>	0.025 <i>N</i>	0.031 <i>N</i>
Ionic strength (p_H 8)	0.15	0.16	0.19	0.16	0.15	0.14	0.15

* 0.02 *M* dialuric acid stock.

for concentration of HCl un-neutralised and of dialuric acid un-ionised at the desired p_H (dialuric acid $p_K = 2.8$). Where catalyses were attempted, the quoted catalytic strengths were obtained by dilution from stock solutions of 0.02 *M* ferrous sulphate in 0.005 *M* HCl, 0.1 *M* KCN (from 95 % KCN solid), and 0.01 *M* barbituric acid (all by weight). The acidity or alkalinity of the added solutions was included in estimating the NaOH additions required above.

In initiating an experiment the main body of the flask received the dialuric acid, HCl and distilled water, the other constituents being added to the side compartment. Thus dialuric acid solutions were stored at optimum stability awaiting immediate admixture after temperature equilibration. Where it was necessary, the NaOH tube received 0.05 cc. 40 % NaOH on filter-paper (of vol. displacement 0.025 cc.) as CO₂ absorbent. After temperature equilibration, the manometers were reset to the standard levels, the gas system closed, and the solutions mixed by tilting at zero time. The method, allowing the rapid admixture of fully aerated solutions not confined in narrow tubes, permits estimation of zero time to within a few seconds. It also obviates the presence of metal parts of doubtful catalytic inertness. At the same time the degree of vagueness as to final p_H which it certainly permits is overcome by the large number of experiments conducted.

The course of the reaction was followed by manometer readings at intervals appropriate to the rapidity of absorption, barometric fluctuations being ascertained from a control manometer. As the half-life period of dialuric acid varied from more than 75 min. down to 1 min., the faster reactions were necessarily followed only singly or in pairs. Nevertheless, certain observations obviously refer to rates outside the limits of effective measurement of the apparatus and can be traced as such in the data.

The maximum oxygen requirement for the formation of alloxan and H_2O_2 from the solutions is theoretically 0.9 cc. This is 24 % of the total oxygen of the flasks. Actually, however, unregulated initial oxidation will have somewhat diminished this requirement. Nevertheless, since the acid solutions were exposed to air before buffer admixture only for some 7 to 10 mins., during which time oxidation with even vigorous agitation will be found not to exceed 20 to 30 %, preliminary oxidation with the apparatus stationary cannot have been large.

CALCULATION OF RESULTS.

Notation.

T	=absolute temperature in °C.
α_{18}	=solubility of the reactive gas in cc. per cc. solution.
V_s, V_a, V_g	=volumes in cc. of the solution, the CO_2 absorbent, and the gas space (empty), respectively.
C	=a constant for each flask and each value of $(V_s + V_a)$.
h	=alteration of pressure in mm. Brodie's fluid.
$X = hC$	=oxygen uptake in cc. (at 0° and 760 mm.).
n	=g.-mol. oxygen absorbed per g.-mol. dialuric acid.
m	=intended molarity of dialuric acid.
y	=correction factor determined by iodine titration.
x	=degree of oxidation = $\frac{X}{\text{theoretical O}_2 \text{ uptake}} = \frac{X}{22.4V_s n m y}$.
t	=time in minutes.
$k = \frac{x}{t}$	= $\frac{X}{22.4V_s n m y t}$ = linear velocity constant of oxidation.

Procedure.

In the Warburg (constant volume) method using Brodie's fluid in the manometer, the gas absorption is related to the pressure change by the following formula:

$$X = \frac{h}{10,000} \left[(V_g - V_s - V_a) \frac{273}{T} + V_s \alpha_{18} \right] = hC.$$

For flask No. 1, using 2.4 cc. solution and the adopted CO_2 absorbent, this reduces to:

$$X = \frac{h}{10,000} \left[(20.1 - 2.4 - 0.075) \frac{273}{291} + 2.4 (0.031^*) \right] = 1.660h \times 10^{-3}.$$

* Since the partial pressures of nitrogen and water vapour are constant during the reaction, this term should comprise just the solubility of oxygen in the aqueous buffer. It is a small magnitude (0.4 %) in the whole expression. Since Randall and Failey's solubility correction [1927] for the variable ionic strength of the experimental solutions ($\mu = 0.06$ to 0.23 according to p_{H}) is not greater than ± 3 % of α_{18} , it is not significant in these experiments.

When, during an experiment, a control manometer and flask containing 2.4 cc. water has been used to compensate readings of h for barometric fluctuations, the constant C becomes entirely independent of atmospheric pressure.

Observed pressure changes may therefore be converted directly into cc. of oxygen absorbed. In those cases where the manometers had been rapidly reset to cover pressure changes in excess of 250 mm., conterminous readings are simply totalled together, the point of resetting being appropriately indicated in the Figures and Tables. The oxygen uptake is then, after adjustment for y , plotted against time. Since the curve is approximately linear, the reaction may be defined directly by reading the appropriate absorption constant X/yt from the graph. Table II and Fig. 1 illustrate the procedure for a typical set of straightforward oxidations.

Table II. *Calculation of data. Variation of k with dialuric acid concentration.*

Citrate buffer; p_H 2.5.

m	0.0166 M		0.0125 M		0.0066 M		0.0063 M		0.0021 M	
V_s	2.4 cc.		2.4 cc.		1.2 cc.		2.4 cc.		2.4 cc.	
y	0.946		0.986		0.946		0.946		0.983	
Flask No.	(6)		(3)		(1)		(2)		(6)	
t	h	X/y	h	X/y	h	X/y	h	X/y	h	X/y
(mins.)	(mm.)	(cc.)	(mm.)	(cc.)	(mm.)	(cc.)	(mm.)	(cc.)	(mm.)	(cc.)
2	-22	0.039	-18	0.027	-6	0.011	-12	0.021	-3	0.005
4	51	0.091	44	0.066	17	0.032	29	0.049	9	0.016
6	86	0.153	78	0.117	29	0.054	52	0.088	15	0.026
8	120	0.214	111	0.167	43	0.081	76	0.129	21	0.036
10	151	0.269	148	0.223	55	0.103	99	0.168	27	0.046
13	200	0.356	200	0.302	67	0.126	132	0.224	36	0.062
16	240	0.427	242	0.364	70	0.131	151	0.256	42	0.072
20	298	0.530	302	0.455	"	"	159	0.270	41	0.071
25	346	0.616	333	0.501	"	"	158	0.268	"	"
30	357	0.635	332	0.500	"	"	"	"	"	"
35	359	0.639	"	"	"	"	"	"	"	"
40	360	0.641	"	"	"	"	"	"	"	"
X/yt (mean)	0.029		0.026		0.012		0.019		0.005	
k	0.032		0.039		0.067		0.056		0.044	
$\log k$	-1.50		-1.41		-1.17		-1.25		-1.36	

The constant X/yt , however, describes only a volume change, and will not reflect the molecular extent of the oxidation until it is converted into k , the degree of oxidation per minute ($\frac{X}{yt} \times \frac{1}{22.4 V_s n m}$). Since $22.4 V_s n m$ is a constant in any one reaction, the calculation of k follows directly. k is utilised in its logarithmic form for the ultimate tabulation against temperature, concentration, p_H , etc.

It might be well to indicate that comparative tabulation of $\log k$ is quite valid whatever value is postulated for n . The expression $\frac{1000X}{\bar{V}_s m y t}$ (cc. oxygen per g.-mol. dialuric acid per min.) involves no assumption about the reaction,

but differs from k only in the constant, $22,400n$, which is characteristic of the reaction in general. In the comparative, as distinct from the absolute, evaluation of constants, no presumption is implicit as to the molecular proportions of the reaction.

Fig. 1.

Fig. 3.

Fig. 1. Rate of absorption of oxygen. 2.4 cc. p_H 2.5. Citrate buffer. 18°.

Fig. 3. A. Effect of rate of oscillation. $k' = k \sqrt{m} \sqrt{H^+}$. 2 min. readings. • p_H 7.9, + p_H 3.9 (1.2 cc., 0.0066 M , CO_2 -free). ○ 0.005 and 0.01 M KCN (assorted). B. Effect of discontinuous oscillation. k' as above. 2.4cc. 0.0166 and 0.0125 M . CO_2 -free. 120 oscillations per min. • p_H 7.9 and 9.0, + p_H 6.0.

RESULTS.

Stoichiometric proportions.

If the autoxidation represents solely the production of alloxan, it should require two equivalents of oxidising agent, or 11.2 cc. oxygen per millimol. dialuric acid. In Fig. 2 the total oxygen absorbed experimentally is depicted, the included data being a selection, calculated as cc. oxygen per millimol. dialuric acid, from measurements which utilised not less than 0.03 millimol. dialuric acid in each experiment. The data differ in no significant fashion from those for the smaller amounts of dialuric acid. In those cases where the oxidation initiates CO_2 production, the CO_2 has been suitably removed. In Fig. 2 the maximum loss of reductant during temperature equilibration has also been inserted, after calculation from the rate of oxidation of dialuric acid at p_H 1.4 ($\log k = -1.6$, see later). It is clear that, with the possible exception of the region p_H 3 to 4, the molecular proportion oxygen/dialuric acid is undoubtedly double the expected ratio. In other words, the reactants are combining in equimolecular proportions, so that $n = 1$. This value is embodied in subsequent calculation of k .

Since it was probable that the technique adopted by Hill [1930; 1931] had measured only the later stages of the reaction, Fig. 2 gives a comparison with a recalculation of Hill's data upon the same basis. The probability is confirmed.

Fig. 2. Variation of total absorption with p_H . 2.4 cc. 0.0166 and 0.0125 M . • not CO_2 -free, + CO_2 -free, ○ data of Hill (10 cc., 0.018 and 0.0062 M).

Control of technique.

The rapidity of the reaction made it essential to determine the effect of varying technique upon the velocity measurements. In particular, such agitation of the reacting solutions was required that oxygen concentration would not be limited by its rate of diffusion. The rapidity of oscillation, therefore, was increased to the maximum speed of the apparatus by reducing the amplitude of swing to its limit (6 cm.). Unfortunately, this amplitude was still too great to permit the continuous reading of the manometers, and cessation of movement for a few seconds was ultimately necessary to obtain final readings after each approximate setting. Fig. 3 represents the definition of optimum conditions.

The data primarily represent alkaline oxidations, where it was expected that the speed of reaction would accentuate the possible inherent defects of technique. They represent a variety of values of p_H and m , and are therefore corrected to a common ordinate for convenient presentation by applying the conclusions of later sections. The separate portions of Fig. 3 represent (A) the conditioning of oxidation velocity by frequency of oscillation, and (B) the influence of discontinuous oscillation, interrupted at intervals of 2, 1 and 0.5 min., upon the observations. Since experiments, excepting those with added KCN, were conducted in the presence of a CO_2 absorbent, the efficiency of CO_2 absorption by 40% NaOH on a cylinder of filter-paper must be known as a control. This has, however, already been examined by Dixon and Elliott [1930].

It is clear in section A of Fig. 3 that the rapid reaction is very considerably conditioned by the frequency of oscillation. It is probable that k' varies as some power of the frequency, and that, although an approximate maximum was achieved at the adopted frequency of 120 complete oscillations per minute (limited by the inertia of the apparatus) faster absorptions would obtain at greatly accelerated frequencies. The increase within realisable frequencies, however, would not greatly exceed the somewhat generous experimental error. The inserted curve expresses the arbitrary relation $k' = C'(\text{frequency})^{1.33}$. It may be noted that the entirely distinctive reaction which occurs in the presence of 0.01 M and 0.005 M KCN, yet shows an effect of the degree of agitation which does not differ from the effect in the absence of KCN. In section B, it is apparent that interruption of oscillation becomes significant only for intervals of shorter duration than 0.5 min., and that 1 min. readings record the rapid reactions more closely than 2 min. readings. The variability is not appreciably greater than experimental error.

The velocity of autoxidation.

Fig. 4 depicts the gas volume change in a set of direct observations on dialuric acid solutions of varying p_H . The curves are clearly the resultant of two separable effects, namely, the effect of p_H on the rapidity of oxygen absorption (apparent more clearly from p_H 1.1 to 2.0), and the effect evident between p_H 4.5 and 11 of some opposing volume change which culminates

Fig. 4. Variation of absorption rate with p_H . 1.2 cc. 0.0066 M . Not CO_2 -free. Universal buffer.

about p_H 6. The opposing reaction is slower in developing than is the oxidation, and is only able to reverse the volume change when oxidation is far advanced and somewhat slow. The effect is not large in extent, and would appear to have no appreciable influence on oxidation velocity during the

initial stage of the reaction. It is maximum between p_H 6 and 7. Fortunately it is completely eliminated by an effective CO_2 (or other volatile acid) absorbent, and under such conditions the measured volume change is, as has been noted in Fig. 2, closely similar to that of acid reactions and is in simple stoichiometric ratio with the dialuric acid present. All data now to be quoted, unless otherwise indicated, have been obtained in the presence of a CO_2 absorbent if alkaline to p_H 4-7. The further description of the opposing reaction is reserved for a subsequent paper.

Fig. 5 is an assembly of data obtained at varying p_H values and concentrations of dialuric acid, where each point represents a separate experimental

Fig. 5. $\log k:p_H$ curves. 2.4 cc. 18°. (Alkaline data by readings at 0.5 or 1 min. intervals.)
 • not CO_2 -free, + CO_2 -free, o iodimetric data, □ $TiCl_3$ data.

curve of the type depicted in Figs. 1 and 4. The agreement is not as poor as might have been expected from the limited adaptability of the apparatus for rapid oxidations, and is quite sufficient to describe the situation with little uncertainty. It is seen that the effect of p_H is separable into three regions,

first, p_H 1 to 2, a region of rapidly increasing reaction velocity, next, p_H 2 to 4, one of uncertain interpretation which, significantly, corresponds with the incomplete oxidations of Fig. 2, and then finally, on the alkaline side of p_H 4, a region of steady increase of velocity as the 5th or 6th root of $[H^+]$, terminated by velocities too rapid for measurement. Log k in the inserted curves varies inversely as $[H^+]$ from p_H 1 to 2, and above p_H 4 inversely as $\sqrt[6]{[H^+]}$.

The fragmentary data included for 0.0007 M and 0.0044 M dialuric acid solutions are those already referred to [Richardson and Cannan, 1929] and are now included in full for reference. The 0.0007 M solutions were oxidised in a closed chamber by saturated solutions of oxygen (final conc. O_2 , ca. 0.0011 M) and the reaction followed titrimetrically in two ways: firstly, by depressing the autoxidation of appropriate 25 cc. samples with excess HCl and immediate titration under nitrogen with 0.005 M iodine, and secondly the less satisfactory addition of excess $FeCl_3$ solution and back-titration with titanous chloride. The 0.0044 M solutions were oxidised by agitation by a stream of air and estimated iodimetrically. The data are not incompatible with the gasometric data now presented. In fact, the seemingly erratic behaviour and inconsequent relation to p_H that was then confusing is, between p_H 2 and 5, now found to be characteristic. Under both conditions of measurement, the reaction is found to have a linear velocity constant.

It is interesting finally to note that a stream of nitrogen produced, during a period of 45 min. at p_H 2.5, no change in the dialuric acid titre.

Effect of temperature.

The effect of temperature, as indicated by several experiments at 25° and 30° respectively, may be defined by the relation:

$$\log k_{t_2} = \log k_{t_1} + 0.027 (t_2 - t_1).$$

Fig. 6 (A) portrays the variation of the velocity of oxidation of 0.0066 M dialuric acid with temperature. The full curve is the curve at this concen-

Fig. 6. A. Effect of temperature. $\circ$ 25°, $\bullet$ 30°. B. Effect of added catalysts, $\circ$ cyanide, $\bullet$ iron (data of Hill $\blacksquare$), $\square$ glass rods, $\times$ barbituric acid, $\triangle$ oxidised solution. log k : p_H curve (18°) from Fig. 5. 1.2 cc. 0.0066 M .

tration from Fig. 5, and the broken curves are the calculated curves for 25° and 30°, assuming the above relation to hold throughout the p_H range. It is noticeable that, whereas the p_H ranges 1 to 2 and 2 to 11 are separately differentiated with regard to catalysis or change of concentration, both p_H ranges are satisfactorily described by the one temperature coefficient.

Catalytic agents.

The roughly linear progress of oxidation is general evidence of a catalytic reaction embodying a constant concentration of catalyst. Further light upon the nature of this catalysis has been sought. In the first place, experiments were conducted at similar p_H values with different buffer solutions. Dilute HCl was used for the range p_H 0.7 to 1.7, citrate buffer was used for p_H 1.4 to 5, and universal buffer for p_H 2 to 11. No consistent differentiation was apparent, and no distinction is therefore made in Fig. 5 as to the buffers used in the respective experiments.

Although this in itself is probably sufficient evidence that catalysis was not due to the fortuitous presence of iron, definite observations on the catalytic effect of Fe and KCN on the oxidation are presented in Fig. 7 (A). It is clear

Fig. 7. A. Effect of iron and cyanide ions. $\bullet$ $\circ$ not CO_2 -free, $+$ CO_2 -free. B. Effect of glass surface. p_H 7.9. $\bullet$ plain, Δ glass rods. C. Effect of constituent impurities. p_H 2.0. 1 plain, 2, 3 and 4, 0.005, 0.004 and 0.0016 M added oxidised solution, respectively. 1.2 cc. 0.0066 M .

from this that there is, except in acid solution, no catalytic effect of iron (or inhibitory effect of KCN) upon the reaction. This finding is especially noteworthy since the formation of the well-known blue iron complex in slightly alkaline solution (0.0016 M Fe, p_H 7.9) is thus found to be entirely without catalytic influence. In the case of acid solution, addition of cyanide was found to have no effect, whereas addition of iron led to steadily increasing velocities, defined in Fig. 7 (A) by the arbitrary straight line, $k = \sqrt[5]{[Fe]}$.

The total oxygen absorptions in the presence of smaller concentrations than 0.0001 *M* Fe or 0.001 *M* KCN, respectively, were of the usual extent. In the presence of 0.00016 *M* iron, however, oxygen absorptions were less than this, and with 0.0016 *M* iron were quite definitely one-half the usual extent even assuming partial oxidation by added iron (hence $n = 0.5$). In alkaline Fe solution a certain confusion appears to arise from liberation of CO₂. Conversely, in the presence of 0.0083 *M* KCN, at p_H above 6, absorptions up to double the usual extent ($n = 2$) were obtained, although normal uptakes occurred at p_H values acid to 6.

Since the identity of velocity in different buffer preparations suggested the absence of catalytic impurity from the buffer, the dialuric acid preparation was next examined. The possibility of catalysis by a pyrimidine of similar structure was tested by the addition of barbituric acid,

possibly a contaminant by excessive reduction during preparation. No effect was observed, nor was barbituric acid itself oxidised. Next, a 0.02 *M* solution of dialuric acid in 0.001 *M* HCl was completely oxidised in a Warburg flask, and added, in final concentration 0.005 *M*, 0.004 *M*, and 0.0016 *M*, to fresh 0.0066 *M* dialuric acid solutions for oxidation experiments. Although the presumed catalyst must have been present in increased concentration at the initiation of these oxidations, the curves of Fig. 7 (C) show perfectly clearly that there has been no acceleration of the reaction.

The final possibility that catalysis was a surface phenomenon due to the glass container was tested by adding fine glass rods to increase the surface area of glass. Although the increase in wetted surface was from about 15 to about 30 cm.², no increased velocity was measured (Fig. 7 (B)).

In Fig. 6 (B), values of $\log k$ under different "catalytic" conditions in the oxidation of 0.0066 *M* dialuric acid are assembled, and compared with the mean curve for this concentration transcribed from Fig. 5.

The notable absence of catalysis induced extensively by any of the adopted procedures makes the linear order of reaction difficult to explain on any assumption that does not consider as a limiting factor the rate of permeation or of activation of oxygen. The fact that the one effective catalysis observed (Fe at p_H 1.3) has increased $\log k$ just so far as to align it with its more alkaline values while these values cannot themselves be increased, is itself rather significant in this respect. It appears that values greater than this cannot be obtained by catalytic acceleration of the oxidative reaction.

Effect of concentration.

A final indication as to the nature of the catalysis should be procurable from the relation of k to m . If z be g.-mol. of dialuric acid oxidised in time t , then when the reaction is limited by constant extraneous factors, such as

rate of activation of oxygen or catalysis by a constant impurity of the glass or buffer, the molar velocity constant should be $= z/t$. On the other hand, if the extraneous factors vary with m (e.g. catalysis by a constituent of the dialuric acid solution), the constant should be z/mt ; this situation, however, has been rendered very improbable by Fig. 7 (C). Now k has actually been calculated as $x/t (= z/mt)$. k is therefore likely to vary, in accordance with the first situation, as $1/m$.

In Fig. 8, the comparison of velocity with concentration of dialuric acid is made by transcribing the curves of Fig. 5 upon a common ordinate. It is clear that at no p_H does $\log k$ vary as $-\log m$ in accordance with the requirements for constant catalytic activation. Indeed, between p_H 1 and 2, $\log k$ is very approximately non-variant with m , whereas on the alkaline side of p_H 4, it corresponds notably with the unforeseen relationship, $k = C'' (1/\sqrt{m})$, indicated by the broken lines in Fig. 8.

Fig. 8. Variation of $\log k$ with concentration of dialuric acid. $\bullet$, $\circ$ Data of Hill (0.018 and 0.0062 M, respectively). $\times$ Data of Clark, Cohen and Gibbs for leucomethylene blue.

It is interesting to observe the excellent agreement of the gasometric data with the data from titration of fully oxygenated mixtures. At the same time, however, the data obtained by titration of aerated solutions are definitely low, being included merely to show a general analogy between the curves. Yet it is a pointer to an unexpected correlation with tentative observations of Clark, Cohen and Gibbs [1925] on the autoxidation of leucomethylene blue by aeration; but even though their data as presented in Fig. 8 are convincingly close to the square root relation, it would be premature to press the analogy.

The data of Hill, replotted and recalculated according to the conventions of this paper, are included in Fig. 8 for reference. As is to be expected, the velocities measured fell considerably short of the velocities determined in the present work.

Of further interest in Fig. 8 is the possibility that the decline of the alkaline points from the $\sqrt[6]{[H^+]}$ relationship may be conditioned more by the acid dissociation of dialuric acid [Richardson and Cannan, 1929] than by the technical difficulty of measurement. The dotted line represents the maximum attainable velocities on the assumption that velocity varies directly as the

concentration of the ion dial.- and is definable by the actual measurements between p_H 1 and 2. In regions not greatly alkaline, however, it is presumed that this maximum velocity is not attained owing to its limitation by the external situation already mentioned. Although the half-life of dialuric acid is, as Fig. 8 indicates, so brief that uncertainty of measurement precludes any positive conclusion, it is at least clear that this interpretation would align itself remarkably with the tendency towards constant oxidation velocities in the more alkaline regions.

The autoxidation of alloxantin.

The experiments in which oxidised dialuric acid was added to solutions awaiting measurement (Fig. 7 (C)) were analogous, in effect, to measuring the autoxidation of alloxantin. Nevertheless, distinctive reaction velocities in these cases were not obtained. In seeking to confirm the observation, reactions were now conducted with freshly prepared alloxantin solutions, using an identical technique with that for dialuric acid. Owing, however, to the limited solubility of alloxantin, reaction mixtures more concentrated than 0.0055 M could not (with the exception of measurements in HCl) be obtained. Needless to say, alloxantin solutions of this strength remain only slightly associated, and are actually equivalent, as calculated from the association constant, to 0.0050 M solutions of dialuric acid. Fig. 9 depicts the results in the usual

Fig. 9. Autoxidation of alloxantin. A. Total absorption. B. $\log k$. 18° . $\circ$ 0.0067 M , $\bullet$ 0.0055 M , $\times$ 0.0021 M alloxantin.

fashion upon a basis of curves already defined, where, however, the curve for 0.0055 M solutions is calculated from the observed relation $k = C(1/\sqrt{m})$ ($m = 0.005 M$ dialuric acid). The results clearly differ in no essential respect from those for dialuric acid. The oxygen uptakes were also of similar magnitude,

a selection of data for the more concentrated solutions being depicted in Fig. 9 upon a separate ordinate. Thunberg [1915], in two experiments on unbuffered saturated alloxantin solutions, measured uptakes in both cases in excess of two equivalents of oxygen, at rates such that the half-life of alloxantin was about 4 to 5 mins.

Alternative velocity constants.

The very striking variation of k as $1/\sqrt{m}$ over the p_H range 4 to 11 (Fig. 8) made it desirable to investigate the velocity equation

$$\frac{dz}{dt} = k_s' \sqrt{m - z},$$

from which by integration

$$k_s' = \frac{2\sqrt{m}}{t} \left(1 - \sqrt{1 - \frac{z}{m}} \right).$$

Fig. 10. Alternative velocity curves. 2.4 cc. p_H 1.4. HCl buffer. $\frac{dx}{dt} = k_s \sqrt{1 - x}$.

Using x instead of z , these relations reduce to

$$\frac{dx}{dt} = k_s \sqrt{1 - x} \quad \text{and} \quad k_s = \frac{2}{t} (1 - \sqrt{1 - x}),$$

in which k_s is non-variant with m . Fig. 10 shows that the expression fits the data for the slower and better defined oxidations with a fair degree of precision. Since it may be also calculated that

$$k = k_s \left(1 - \frac{k_s t}{4} \right),$$

or, provided the constants are calculated from early points such that kt is small compared with 4, that

$$k = k_s = \frac{k_s'}{\sqrt{m}},$$

variation of k_s with p_H is already satisfactorily defined in Fig. 5. These relations of k_s would derive chemically from the not impossible assumption that the

reaction is catalysed by both charges of the ion dial.--, presumably by way of a double peroxide from two molecules of added oxygen.

Yet the formulation provides no better explanation of the region p_H 4 to 11 than does the simple linear reaction. In both cases, limitation of the velocity below the maximum formulated rates appears to operate. Again, whereas both velocity constants theoretically remain non-variant with concentration, the variation actually is as $1/\sqrt{m}$ in either case. In general, indeed, the relation

$$\frac{dx}{dt} = k_n (1 - x)^n$$

shows that k_n , for any order of reaction calculated in terms of x (which is non-variant with m), should itself be non-variant with m . Consequently, for the region p_H 4 to 11, where explanation in terms of linear constants has already been found inadequate, no explanation in terms of the ordinary velocity constants can be provided.

DISCUSSION.

The fact that at p_H 7 oxidation was complete in considerably less than 10 mins. means that the experiments of Hill [1930; 1931] were conducted on solutions already considerably oxidised. This was initially suspected in a technique which exposed neutral solutions of dialuric acid to the atmosphere for an undetermined time before measurement, and which yielded thereafter plainly erratic total absorptions of oxygen. It was, however, an unfortunate circumstance that the few duplicates described in the second paper gave a general confirmation of the unduplicated findings of the first. In both cases, nevertheless, absorptions appear to have been very considerably below the maximum simply by reason of this initial oxidation (*cf.* Fig. 2).

It cannot be held therefore that alloxantin is the end-product of the reaction (an event unlikely by reason both of the large dissociation to its constituents in dilute solution, and of the existing evidence of Thunberg), since autoxidation has now been shown to proceed to completeness in definite stoichiometric proportions. The velocity equations of Hill are therefore in error. Again, for the reasons that the volume of solution was too large for efficient aeration (10 cc. in 33 cc. flasks), and that oxidation was in any case already far advanced, the actual velocities measured do not represent oxidation of dialuric acid under reproducible conditions, and can have no final significance (*cf.* Fig. 8). It is thus impossible to continue using this evidence to relate the oxidation-reduction in any way with the metabolic oxidation of cell material.

On the other hand, the oxidation of amino-acids in the presence of dialuric acid described in Hill's third paper [1932] is of considerable interest. But whether this is due merely to products of autoxidation, or to a definite reversible oxidation-reduction of dialuric acid has not yet been sufficiently defined. H_2O_2 is known to oxidise amino-acids in the given manner [Negelein,

1923; Breinl and Baudisch, 1907], while Hill discusses the similar reactions of alloxan. Unfortunately, however, the stoichiometry of either reaction near p_H 7 is not known. But it is conceivable that the end-products of oxidation of each equivalent of dialuric acid, may oxidise of themselves at least two equivalents of amino-acid. Therefore, since Hill measured greater values than this in only 2 of 11 experiments in reactions exceeding 5 hours, his evidence for catalysis by repeated oxidation-reduction of dialuric acid, remains inconclusive. In any event, it should be noted that alloxan as an individual constituent of cell material would have a half-life period not exceeding 2 mins. at any cell p_H alkaline to 6.5¹.

The possibility that both electrode potential and velocity of oxidation vary as a function of the dissociation constant of dialuric acid has been neither discounted nor more definitely manifested by the results of these experiments. But general correlation between potential and velocity has not been attained. The nature of the reaction appears to have been obscured by extraneous factors which the rapidity of oxidation allows to become operative.

Before considering these effects therefore the limitations of the technique should be briefly examined. Gasometric experiments have been conducted at 18°, the lowest temperature consistently available, and with air instead of oxygen as used previously, to minimise the rate of oxygen absorption. Nevertheless, the average absorption was at the rate of 1.5 to 3.0 cc. per hour, with a maximum of 12 to 15 cc. per hour. Dixon and Elliott [1930] state that under their conditions (32 cc. gas space, paraffin manometer fluid) measurements at rates limited to 1 cc. per hour are perfectly reliable, and they measure rates of 2.4 and 3.0 cc. per hour for cysteine and yeast without difficulty. Indeed, for rates of oxidation of pyrogallol of 18, 26 and 31 cc. per hour, only in the last case was there clearly discernible inconsistency. It is therefore probable that the rates measured herein are not greatly in error technically. Of this, the satisfactory concord between gasometric and titrimetric data is added evidence. This concordance of data is also evidence that aeration under the optimum conditions defined in Fig. 3 was actually equivalent to the complete oxygen saturation employed for the titrimetric data.

The extent of the error thought by Dixon and Elliott to arise from autoxidation of filter-paper under 40 % NaOH, does not appear to have been large under the present conditions, since no great discrepancy between oxygen absorption in the presence and absence of filter-paper-NaOH appears in Fig. 2.

As to stoichiometry, there is no doubt from Fig. 2 that, whereas only two electron equivalents are necessary for oxidation to alloxan, actually four equivalents of oxygen have reacted. Presumably this reaction is represented by: dialuric acid + O_2 = alloxan + H_2O_2 . In the presence of iron, however, only two equivalents were absorbed; but since such conditions imply that H_2O_2

¹ Fig. 2, Section A of the previous publication [Richardson and Cannan, 1929] portrays, as is clear from Table V, the variation of $\log 10k$ with p_H , not of $\log k$ as indicated.

is not likely to be a stable end-product owing to its enhanced oxidising activity the reaction may be expected to proceed with one-half the oxygen absorption and no production of H_2O_2 . It is interesting that the iodine titre of dialuric acid after oxygen absorption at p_{H} 5, 9 or 10 and acidification had entirely vanished. In such conditions, iodine does not react spontaneously with H_2O_2 [Liebhafsky, 1931].

The actual linear progress of the reaction characteristic between p_{H} 2 and 11 (cf. Figs. 2 and 4) is difficult to interpret. Not only did it occur where air-saturation was maintained by agitation, but even under conditions where the solution was pre-saturated with oxygen. It is therefore not a function of the rate of permeation of oxygen. Yet no change in the catalytic environment was able to point to its catalytic causation. It seems probable that the reaction is some obscure function of the rate of activation of oxygen, a conclusion supported by the positive temperature coefficient of the reaction, and by the unusual relations with $[\text{H}^+]$ and m . These relations may be defined by the equation $k' = k \sqrt{m}^6 \sqrt{[\text{H}^+]}$ or $k \sqrt{m}^5 \sqrt{[\text{H}^+]}$, where k' is a universal constant for the reaction between p_{H} 4 and 11. It is appropriate to recall that the tentative observations of Clark, Cohen and Gibbs [1925] described k for the autoxidation of leucomethylene blue also as a linear constant which varied as $\sqrt{[\text{H}^+]}$, and that the magnitude of k is found in Fig. 8 to be very little removed from the $\sqrt{m}$ relationship described for dialuric acid.

Between p_{H} 1 and 2, the progress of oxidation is not so definitely linear, and may be described by an alternative velocity equation. k , by either derivation, is found to be non-variant with m and to vary inversely as $[\text{H}^+]$, relations which would follow directly from the chemical assumption that dialuric acid is oxidisable only in the form of its ion. It is interesting that addition of iron, which had no effect alkaline to p_{H} 2, between p_{H} 1 and 2 causes the reaction to become definitely linear and to proceed at velocities similar to those in more alkaline solution.

It is therefore suggested that in the autoxidation of dialuric acid under the observed conditions, the rate of activation of the reactants is of finite magnitude and varies between p_{H} 4 and 11 as $\frac{1}{\sqrt{m} \sqrt{[\text{H}^+]}}$; further, that where, by reason of p_{H} or of iron catalysis, the rate of reaction is greater than the rate of activation, the observed oxygen uptakes are conditioned in speed by the rate of activation. The velocity of the true reaction is therefore only observed where oxygen uptake proceeds slowly, a condition which is not fulfilled except between p_{H} 1 and 2.

SUMMARY.

The rate of atmospheric oxidation of dialuric acid has been studied with reference to p_{H} , temperature, concentration of dialuric acid and influence of catalysts.

The reactants have been shown to combine in equimolecular proportions. In the presence of iron as catalyst, the proportion of oxygen was halved.

Alloxantin behaves as though completely dissociated to its components, dialuric acid and alloxan.

The suggestion that the oxidation-reduction has an important rôle in biological respiration has been rejected.

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APPENDIX.

A selection of the data from which k has been calculated for 0.0166 M solutions is given below:

2.4 cc.; 18°.

p_H	Buffer	y	mm. ³ /min.	Total uptake (mm. ³)	p_H	Buffer	y	mm. ³ /min.	Total uptake (mm. ³)
1.1	HCl	0.97	12	625	4.8	Citr.	0.98	31	560
1.4	"	0.95	27	695	6.0	U.B.	1.00	50	705
1.4	Citr.	0.95	29	660			0.96	54	735
2.0	U.B.	0.99	32	700	6.8	"	0.98	59	760
2.5	Citr.	0.95	28	640			0.97	62	745
		0.99	27	650	7.9	"	0.97	76	780
2.9	U.B.	0.97	21	590			0.97	90	775
3.3	Citr.	0.99	22	500			1.00	130	730
		0.99	26	550	9.0	"	0.97	150	780
3.9	U.B.	0.97	24	600			1.00	180	740
		0.96	23	505	9.7	"	0.99	220	765
4.7	"	0.98	32	625	11.0	"	0.99	275	755