anol (1.0 ml.) and then from ethanol (0.5 ml.) to yield 95 mg. (44%) of hygroscopic crystals, insoluble in carbonate but soluble in sodium hydroxide solution, which were dried at 100° *in vacuo* prior to analysis; m.p. 179–181°, $[\alpha]^{25}D$ +222° (*c* 0.50, ethanol).

Anal. Calcd. for $C_{16}H_{17}O_4N$: C, 66.9; H, 6.0; N, 4.9. Found: C, 66.8; H, 6.0; N, 5.0.

B.—Optimum conditions for the direct dehydration of the acid amide (100 mg., 0.33 millimole) to the imide were found to be fusion under nitrogen at 188–193° and atmospheric pressure for six hours, followed by sublimation at 0.1 mm. and 193°. The sublimate (95 mg., 101%) possessed $[\alpha]^{25}D$ +194° (*c* 0.50, ethanol), indicative of an imide content of 84%. Recrystallization two times from ethanol (0.5 ml., 1.0 ml.) gave 56 mg. (60%) of thebedioicimide, m.p. 177–178°, mixed m.p. with imide (m.p. 179–181°) prepared by method A, 177–180°.

Attempted Cyclization of Epi-thebedioic Acid Monoamide (XIVe) A.—The attempted preparation of the epi-imide by fusion of the ammonium salt of epi-thebedioic acid monoamide (200 mg., 0.66 millimole) in a manner identical with that used on the salt of thebedioic acid amide yielded a white crystalline sublimate. This was dissolved in chloroform and extracted successively with 1 *N* sodium carbonate and 1 *N* sodium hydroxide. Isolation of material from both extracts gave 85 mg. of carbonate soluble material, m.p. 210–214°, identified as recovered epi-thebedioic acid monoamide; and 7 mg. of an alkali soluble brown oil which could not be characterized. A large non-volatile residue was obtained.

B.—The direct fusion of epi-thebedioic acid monoamide was carried out exactly as for the natural series, except that, because of the high volatility of the starting material, hourly washings with chloroform were employed during the fusion to return volatile material from the cold finger to the melt. In addition, the compound was initially melted at 225° and the melt then kept at 193°. The sublimate of 27 mg., 49% of the starting material, was carbonate soluble and was identified as epi-thebedioic acid monoamide; $[\alpha]^{25}D$ −13.4° (*c* 0.54, ethanol). A non-volatile residue of 25 mg. also was obtained.

BERKELEY, CALIFORNIA

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE]

The Oxidation of L-Ascorbic Acid by *o*-Iodosobenzoic Acid[1,2]

BY WENDELL T. CARAWAY[3] AND LESLIE HELLERMAN[4]

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In non-buffered aqueous solutions, ascorbic acid is oxidized by *o*-iodosobenzoic acid according to a second-order reaction expressed by the differential equation: $-d[AA]/dt = k_2[AA][o\text{-RIO}]$. The value of k_2 is 1.3 liters mole^{-1} min.$^{-1}$ at 25° over the *p*H range 4 to 8 (half-life of 13 hours for 0.001 *M* solutions). The reaction exhibits general acid catalysis in agreement with the assumption that *o*-iodosobenzoic acid and the acid catalyst combine reversibly through hydrogen bonding to form a complex which then reacts with ascorbic acid in a rate-controlling step. The dissociation constant (K_c) of the complex was shown to vary inversely as the dissociation constant (K_a) of the ligating acid. *o*-Iodobenzoic acid catalyzes the reaction in acetate buffer at *p*H 4.6 but not in phosphate buffer at *p*H 7. Dehydroascorbic acid has no effect on the rate of the reaction in either case. In phosphate buffer at *p*H 7, the reaction proceeds partially at a rate independent of the concentration of *o*-iodosobenzoic acid but directly proportional to the concentration of the buffer: $-d[AA]/dt = k_2[AA][o\text{-RIO}] + k'[AA][\text{buffer}]$. The second term in the rate equation does not appear in other buffer systems at *p*H 7. Cupric ion markedly catalyzes the reaction while ferrous ion is only about one-tenth as effective. Ascorbic acid is oxidized practically instantaneously by *m*-iodosobenzoic acid or *p*-iodosobenzoic acid. *o*-Iodoxybenzoic acid is rapidly reduced to *o*-iodosobenzoic acid by ascorbic acid. Iodide ion is also oxidized by *o*-iodosobenzoic acid according to a second-order reaction which exhibits general acid catalysis in agreement with that observed for the oxidation of ascorbic acid. Oxidized ascorbic acid, in non-buffered aqueous solution, undergoes transformation to an equivalent molar quantity of a product which is readily oxidized by iodine but not by *o*-iodosobenzoic acid. In phosphate buffer more extensive degradation occurs with formation of and up to three equivalent molar quantities of products capable of reducing *o*-iodosobenzoic acid. The reaction should serve as a model system for further studies on the end products of oxidation of ascorbic acid.

The kinetics of oxidation of ascorbic acid has been investigated repeatedly,[5] with respect particularly to vitamin C preservation in foods, the determination of its role in oxidation–reduction processes, and the mechanism of its action in the living organism.

The introduction of *o*-iodosobenzoic acid[6] as a reagent for the estimation of certain sulfhydryl compounds prompted a study of the oxidizing action of this compound toward ascorbic acid. This had been observed to be oxidized *slowly*[7] to dehydroascorbic acid while *o*-iodosobenzoic acid was reduced to *o*-iodobenzoic acid. The slow rate of reaction here is unique in that either *m*- or *p*-iodosobenzoic acid now has been found to oxidize ascorbic acid practically instantaneously. Further study demonstrated that the rate of the reaction is affected by the nature and the concentration of the buffer system in a manner suggestive of a rather unusual type of general acid catalysis. A thorough study of the kinetics of oxidation of ascorbic acid by *o*-iodosobenzoic acid was undertaken to elucidate the mechanism of the reaction.

Experimental

Materials.—Solutions of L-ascorbic acid were prepared and standardized daily. Dehydro-L-ascorbic acid and diketogulonic acid were prepared by the methods of Kenyon and Munro.[8] *o*-Iodosobenzoic acid, purified according to Loe-

(1) (a) From the doctoral dissertation of Wendell T. Caraway, The Johns Hopkins University, 1950. (b) Presented before the Annual Meeting of the American Society of Biological Chemists, Atlantic City, N. J., April, 1950.

(2) One of several investigations supported in part by a research grant from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

(3) Predoctorate Research Fellow of the National Institutes of Health, 1949–1950.

(4) To whom inquiries should be directed.

(5) (a) E. S. G. Barron, R. H. DeMeio and F. Klemperer, *J. Biol. Chem.*, **112**, 625 (1936); (b) H. Schümmer, *Biochem. Z.*, **304**, 1 (1940); (c) A. O. Dekker and R. G. Dickinson, THIS JOURNAL, **62**, 2165 (1940); (d) R. W. Peterson and J. H. Walton, *ibid.*, **65**, 1212 (1943); (e) E. Silverblatt, A. L. Robinson and C. G. King, *ibid.*, **65**, 137 (1943); (f) A. Weissberger, J. E. LuValle and D. S. Thomas, Jr., *ibid.*, **65**, 1934 (1943); (g) A. Weissberger and J. E. LuValle, *ibid.*, **66**, 700 (1944).

(6) (a) L. Hellerman, F. P. Chinard and P. A. Ramsdell, *ibid.*, **63**, 2551 (1941); (b) L. Hellerman, F. P. Chinard and V. R. Deitz, *J. Biol. Chem.*, **147**, 443 (1943).

(7) L. Hellerman and A. Lindsay, unpublished work.

(8) J. Kenyon and N. Munro, *J. Chem. Soc.*, 158 (1948).

venhart and Grove,[9] was shown to be stable in solution in the presence of a slight excess of potassium hydroxide. Under similar conditions, *m*-iodosobenzoic acid[10,11] and *p*-iodosobenzoic acid[10] were found to undergo dismutation to iodo- and iodoxy derivatives. An increase in yield and purity of *p*-iodosobenzoic acid was effected by mild hydrolysis of the parent iodochloride in phosphate buffer solution. *o*-Iodoxybenzoic acid was prepared in crystalline form.[12] Porphyrindine was synthesized according to the method of Piloty and co-workers[13,14] with modifications.[15-17] Solutions prepared fresh daily with ice-cold water and kept below 5° during use, were standardized at one- to two-hour intervals. Spirocyclohexylporphyrexide, which had been synthesized by Porter and Hellerman,[18] was adapted for spectrophotometric analysis. Maximum absorption occurred at 425 mμ.

Doubly distilled water, produced by a Barnstead conductivity-water still, was used to recrystallize buffer salts and to prepare solutions. Results obtained by use of these solutions were shown repeatedly to check with results obtained by use of triply distilled water produced by an all-Pyrex Yoe distilling apparatus. Pure, dry nitrogen (Linde, water-pumped) was found suitable for use without further purification; in its presence no measurable oxidation of ascorbic acid occurred in the absence of *o*-iodosobenzoic acid during the reaction time used. Nitrogen, purified by passage over finely divided copper wire at 450°, was used in numberous double-check experiments with equal results. Great care was taken to exclude traces of metallic catalysts, especially copper.

Rate Measurements.—Measured volumes of *o*-iodosobenzoic acid and buffer solution were mixed in a reaction flask; the solution was brought to constant temperature (±0.02°) in a water-bath and deaerated by passage of washed nitrogen for an hour. At zero time a sample of ascorbic acid solution was added from a pipet; the reaction flask was agitated and samples were withdrawn at intervals for titration with porphyrindine. Passage of nitrogen was continued throughout the run. Alternatively, the samples were discharged into a solution containing a slight excess of spirocyclohexylporphyrexide and the residual dye was determined spectrophotometrically. The rate of oxidation of iodide ion by *o*-iodosobenzoic acid was measured spectrophotometrically by following the appearance of iodine. Results were interpreted graphically and expressed as initial rates (r_0, moles per minute), first-order constants (min.$^{-1}$) or second-order constants (l. mole^{-1} min.$^{-1}$).

The *p*H of all reaction mixtures was determined routinely with a Beckman glass electrode assembly.

Results and Discussion

Oxidation of Ascorbic Acid in Non-buffered Solution.—Ascorbic acid (AA) is very slowly oxidized by *o*-iodosobenzoic acid (RIO) in aqueous solution in the absence of buffer salts or metallic catalysts. Reaction proceeds at a rate directly proportional to the concentration of each of the reactants, following the rate equation: $-d[AA]/dt = k_2[AA][RIO]$, where $k_2 = 1.3 \pm 0.1$ liters mole^{-1} min.$^{-1}$ at 25° over the *p*H range from 4 to 8. Variation in the initial concentration of *o*-RIO was limited to fourfold owing to its low solubility below *p*H 7. In additional experiments with various buffer concentrations it was found by extrapolation to zero concentration of buffer that k_2 had an average value of 1.3 at 25°, confirming the results obtained in non-buffered solutions. Table I lists the results of an experiment conducted at 35°. The values for k_2 are in good agreement up to 260 minutes at which time 84% of the ascorbic acid had been oxidized.

TABLE I

OXIDATION OF ASCORBIC ACID IN NON-BUFFERED SOLUTION

$dx/dt = k_2(a - x)(b - x)$; $a = 9.45 \times 10^{-4}$ *M* (ascorbic acid); $b = 20.91 \times 10^{-4}$ *M* (*o*-iodosobenzoic acid); temp. 35.0°, *p*H (initially) 7.55; k_2 = liters mole^{-1} min.$^{-1}$

Min.	$(a - x) \times 10^4$	k_2	Min.	$(a - x) \times 10^4$	k_2
0	9.45	...	65	5.54	4.4
2	9.30	4.0	76	5.12	4.4
5	9.12	3.2	87	4.70	4.4
9	8.73	4.2	100	4.29	4.4
13	8.52	3.9	115	3.84	4.5
18	8.06	4.4	130	3.50	4.4
24	7.70	4.3	150	3.07	4.4
30	7.29	4.4	170	2.70	4.4
37	6.95	4.2	200	2.25	4.4
45	6.53	4.2	230	1.86	4.4
56	5.93	4.4	260	1.52	4.5

It is concluded that under these conditions *o*-iodosobenzoic acid does not react appreciably with any products that might form through decomposition of dehydroascorbic acid; otherwise, the reaction would deviate from second-order kinetics.

Oxidation of Ascorbic Acid in Acetate Buffer.—Experiments conducted in acetate buffer at *p*H 4.6 provided a method for evaluation of buffer effects under conditions where dehydroascorbic acid is relatively stable. Rate studies conducted at 25.0° in acetate buffer (*p*H 4.6, equimolar in acetic acid and sodium acetate) demonstrated that the initial rate of the reaction is directly proportional to the concentrations of ascorbic acid and *o*-iodosobenzoic acid, and conforms to the rate equation

$$r_0 = k''[AA][RIO] \quad (1)$$

By variation of the total concentration of acetate buffer employed, it was shown that the rate of the reaction was dependent also upon the concentration of buffer but not in a linear fashion. The value of the constant k'' is considered to be equal to the sum of the rate constants for the non-catalyzed (k_2) and the catalyzed reactions; hence

$$r_0 = k_2[AA][RIO] + k'[AA][RIO] \quad (2)$$

where k' is some function of the concentration of buffer. Empirical evaluation of the data led to the equation

$$r_0 = 1.1[AA][RIO] + 2.9[AA][RIO]\frac{[HOAC]}{0.044 + [HOAc]} \quad (3)$$

where [HOAc] is the molar concentration of acetic acid in the acetate buffer employed. The value of 1.1 for k_2 agrees with the value for the non-catalyzed reaction velocity constant reported in the preceding section. Then, from 1 and 3

$$k'' = 1.1 + \frac{2.9[HOAc]}{0.044 + [HOAc]} \quad (4)$$

Figure 1A illustrates the relation between k'' and

(9) A. S. Loevenhart and W. E. Grove, *J. Pharmacol. Exptl. Therap.*, **3**, 101 (1911).
(10) C. Willgerodt, *Ber.*, **27**, 2326 (1894).
(11) G. Ortoleva, *Gazz. chim. ital.*, **30** II, 1 (1900).
(12) C. Hartmann and V. Meyer, *Ber.*, **26**, 1727 (1893).
(13) O. Piloty and B. G. Schwerin, *ibid.*, **34**, 1863, 1870, 2354 (1901).
(14) O. Piloty and W. Vogel, *ibid.*, **36**, 1283 (1903).
(15) R. Kuhn and W. Franke, *ibid.*, **68B**, 1528 (1935).
(16) C. C. Porter and L. Hellerman, THIS JOURNAL, **61**, 754 (1939).
(17) H. A. Lillevik, R. L. Hossfeld, H. V. Lindstrom, R. T. Arnold and R. A. Gortner, *J. Org. Chem.*, **7**, 164 (1942).
(18) C. C. Porter and L. Hellerman, THIS JOURNAL, **66**, 1652 (1944).

[HOAc]. The smooth curve is the theoretical curve represented by equation 4; the experimental points are designated by circles.

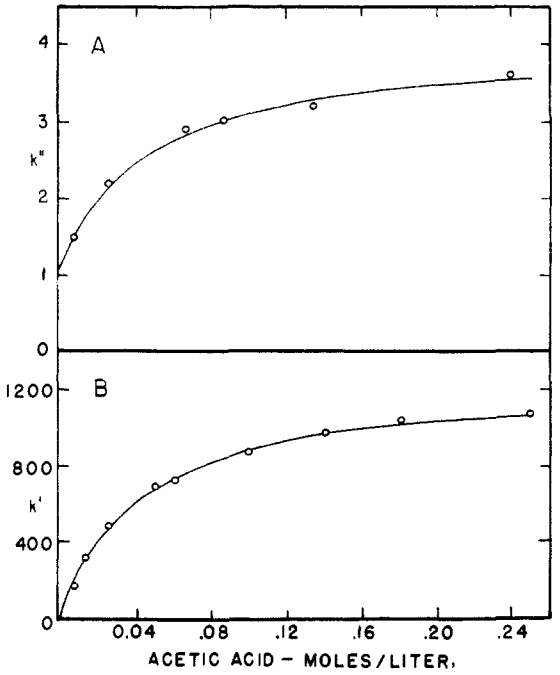

Fig. 1.—Effect of acetic acid on the rates of oxidation of ascorbic acid (A) and iodide ion (B).

Experiments to be described in the following sections indicate that the acidic component of all buffer systems exerts a catalytic effect on reactions where *o*-iodosobenzoic acid is the oxidizing agent. Most cases of general acid catalysis present a mechanism in which the acid functions as a proton donor in a rate-controlling step; consequently, the rate is linearly proportional to the concentrations of the catalyst and one of the reactants. In the present case the rate is non-linear with respect to the concentration of the catalyst and both reactants appear in the rate-controlling step. The only type of general acid catalysis consistent with the present observations is that suggested by Hammett,[19] *i.e.*, "a reversible hydrogen bonding between substrate and acid followed by a non-protolytic reaction of the complex thus formed."

Reversible complex formation may be formulated as

$$\mathrm{RIO} + \mathrm{HA} \overset{K_c}{\rightleftharpoons} \mathrm{C} \qquad (5)$$

where K_c is the dissociation constant of the complex C formed from the acid catalyst (HA) and *o*-iodosobenzoic acid (RIO); hence

$$[\mathrm{RIO}][\mathrm{HA}]/[\mathrm{C}] = K_c \qquad (6)$$

The total concentration of iodosobenzoic acid species is given by

$$[\mathrm{RIO}]_{\mathrm{total}} = [\mathrm{RIO}] + [\mathrm{C}] \qquad (7)$$

where $[\mathrm{HA}]_{\mathrm{total}} >> [\mathrm{RIO}]_{\mathrm{total}}$

$$[\mathrm{C}] = [\mathrm{RIO}]_{\mathrm{total}} \left(\frac{[\mathrm{HA}]}{K_c + [\mathrm{HA}]}\right) \qquad (8)$$

(19) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 241.

If the rate-controlling step is a reaction between the complex C and ascorbic acid, the rate equation becomes

$$r = k[\mathrm{AA}][\mathrm{C}] = k[\mathrm{AA}][\mathrm{RIO}]_t \left(\frac{[\mathrm{HA}]}{K_c + [\mathrm{HA}]}\right) \qquad (9)$$

Equation 9 is the same form as the second term in the empirical equation 3. Assumption of such complex formation leads to a logical and satisfactory explanation of the behavior of *o*-iodosobenzoic acid in various buffer systems.

A study of the entire course of the reaction between ascorbic acid and *o*-iodosobenzoic acid specifically in acetate buffer revealed that the reaction was autocatalytic. By independent variation of the initial concentrations of reactants and products, the nature of the catalyzed reaction was determined and its rate was shown to be proportional to the concentrations of *o*-iodosobenzoic acid and *o*-iodobenzoic acid. The over-all reaction is described, therefore, by the sum of the initiating second-order reaction and the subsequent catalyzed reaction

$$dx/dt = k''(a - x)(b - x) + k_z(b - x)(x) \qquad (10)$$

where a is initial concentration of ascorbic acid, b is initial concentration of *o*-iodosobenzoic acid, and x is concentration of *o*-iodobenzoic acid formed from the reaction between ascorbic acid and *o*-iodosobenzoic acid. The integrated form of equation 10 is

$$t = \frac{2.303}{(k_z - k'')b + k''a} \log \frac{k''b(a - x) + k_z bx}{k''a(b - x)} \qquad (11)$$

From experiments conducted at 25.0° in 0.200 *M* acetate buffer (*p*H 4.60, equimolar in acetic acid and sodium acetate) the values of the constants were determined: $k'' = 3.2$ l. mole^{-1} min.$^{-1}$ and $k_z = 25$ l. mole^{-1} min.$^{-1}$.

With the reactants present in equal concentrations, the rate of oxidation of ascorbic acid follows a curve as shown in Fig. 2. Addition of a portion of completely reacted solution catalyzed the reaction as required by equation 10. When solutions of *o*-iodosobenzoic acid and *o*-iodobenzoic acid were

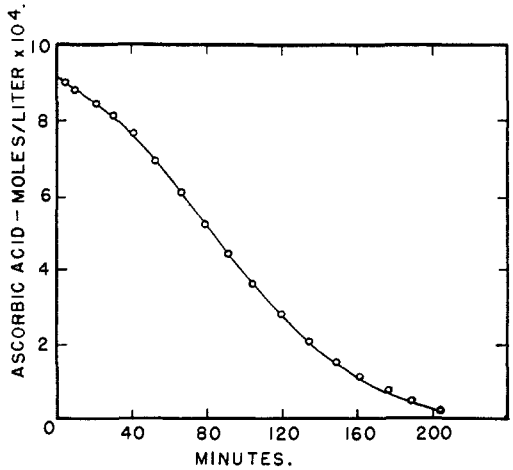

Fig. 2.—Oxidation of ascorbic acid by *o*-iodosobenzoic acid in acetate buffer. Initial concentrations: ascorbic acid, 9.21×10^{-4} *M*; *o*-iodosobenzoic acid, 9.46×10^{-4} *M*; acetate buffer, equimolar in acetic acid and sodium acetate, 0.200 *M*; temperature 25.0°, *p*H 4.60.

mixed and allowed to stand for 2 hours prior to addition of ascorbic acid, the initiating second-order reaction in equation 10 was abolished and the reaction proceeded entirely according to the kinetics of the catalyzed reaction. Addition of dehydroascorbic acid to the reaction mixture had no effect on the rate of the reaction.

A sample of the reaction mixture was taken at the end of each of the experiments and discharged into excess iodide. Determination of the iodine liberated provided a measure of the residual *o*-iodosobenzoic acid in solution. In every case it was found that one mole of *o*-iodosobenzoic acid had been reduced for each mole of ascorbic acid oxidized indicating that dehydroascorbic acid did not undergo decomposition with liberation of reducing compounds under the conditions used.

Oxidation of Iodide Ion by *o*-Iodosobenzoic Acid.—This reaction provided a convenient method by which the specific effects of buffer systems were more fully investigated. Preliminary experiments in which the concentration of iodide ion and *o*-iodosobenzoic acid were varied independently, demonstrated that the reaction is second-order, *i.e.*, first-order with respect to each component. Addition of cupric or ferrous ion had no effect on the rate of the reaction.

The results of experiments conducted in acetate buffer are shown in Table II. In experiments 1–6 the *p*H was held constant while the concentration of buffer was increased. A resultant increase in the rate of the reaction occurred as reflected in the values for k'. In experiments 7–11, where the total concentration of buffer was held constant while the relative proportions of its components were varied, it is observed that the rate is proportional to the acetic acid moiety only of the buffer.

TABLE II

OXIDATION OF IODIDE IN ACETATE BUFFER

o-Iodosobenzoic acid, 1.071×10^{-3} *M*; sodium iodide, 3.378×10^{-3} *M*; temp. 25°; $-d[RIO]/dt = k'[RIO][NaI] = k_2[RIO][NaI][HOAc]/(0.040 + [HOAc])$; k' and k_2 in liters mole^{-1} min.$^{-1}$

Expt.	[Buffer] mole per l.	[HOAc] mole per l.	*p*H	k'	k_2
1	0.0135	0.0068	4.7	178	1230
2	.025	.0125	4.7	323	1290
3	.050	.025	4.7	483	1260
4	.100	.050	4.7	699	1260
5	.200	.100	4.7	875	1230
6	.500	.250	4.7	1077	1250
7	.200	.1815	3.7	1042	1270
8	.200	.140	4.4	969	1250
9	.200	.100	4.7	875	1230
10	.200	.060	5.1	730	1220
11	.200	.0185	5.7	350	1110

Figure 1B indicates that the rate of the reaction varies with the concentration of acetic acid in a manner similar to that observed for the oxidation of ascorbic acid. Evaluation of the effect of acetic acid led to the equation

$$r_0 = k_2[RIO][NaI]\left(\frac{[HOAc]}{K_c + [HOAc]}\right) \quad (12)$$

where [HOAc] is the concentration of acetic acid and K_c is a constant with a value of 0.040 mole per liter. Equation 12 is formally equivalent to equation 9 derived to account for the effect of acetic acid on the rate of oxidation of ascorbic acid in which the value for K_c was found to be 0.044. This close agreement for the values of K_c in two distinct reactions reinforces the conclusion that acetic acid exerts its catalytic effect on *o*-iodosobenzoic acid. Application of equation 12 to the data of Table II led to values of k_2 which were constant over fairly wide variations in buffer concentrations and changes in *p*H.

The rate of oxidation of iodide was also studied in phenylacetate buffer at *p*H 4.28. The general pattern of the reaction was exactly the same as that found in acetate buffer except for different numerical values of the constants. At 25° in buffer equimolar in phenylacetic acid and phenylacetate ion, the values for k_2 and K_c in equation 12 become 1080 l. mole^{-1} min.$^{-1}$ and 0.0223 mole per l., respectively.

Below *p*H 5.5, *o*-iodosobenzoic acid (pK' 7.4) is present in solution almost entirely as the undissociated acid. In phosphate buffer at higher ranges of *p*H it was observed (1) that the catalyst in phosphate buffer is the monovalent ion ($H_2PO_4^-$) and (2) that only the undissociated neutral molecule of *o*-iodosobenzoic acid is capable of oxidizing iodide ion at an appreciable rate. With the limitation that [RIO] in equation 12 represents only the concentration of undissociated *o*-iodosobenzoic acid, the values for k_2 and K_c in phosphate buffer at 25° were determined as 2300 l. mole^{-1} min.$^{-1}$ and 0.327 mole per l., respectively.

It was observed that the values of K_c for the various acids increased as the pK_a of the acid increased. Since K_c is interpreted to be the dissociation constant of the complex formed by *o*-iodosobenzoic acid and the acid catalyst, it is concluded that the weaker acids form the less stable complexes. This is in agreement with the assumption that the complex is formed through reversible hydrogen bonding. Figure 3 shows the approximately linear relationship existing between log K_c and pK. Values of K_c for phenylacetic acid and monovalent phosphate ion were determined from experiments on the rate of oxidation of iodide; for pyridinium ion from experiments on the rate of oxidation of ascorbic acid; and for acetic acid from experiments on the rate of oxidation of both iodide and ascorbic acid.

Oxidation of Ascorbic Acid in Pyridine, Veronal and Borate Buffer Systems.—Pyridine buffer was prepared from freshly distilled pyridine by addition of sufficient hydrochloric acid to convert one-half of the total pyridine to pyridinium ion ($PyrH^+$). Initial-rate studies conducted at 25.0° in pyridine buffer of *p*H 5.34 produced data which conformed closely to the equation

$$r_0 = 1.4[AA][RIO] + 5.2[AA][RIO]\left(\frac{[PyrH^+]}{0.071 + [PyrH^+]}\right)$$

This equation has the same form as that found for acetate buffer.

The order of the reaction in veronal buffer (5,5-diethylbarbituric acid) was found to be the same as in non-buffered solutions, but the absolute rates were higher. An increase in *p*H from 7.0 to 8.0 resulted in decrease in the rate of the reaction which

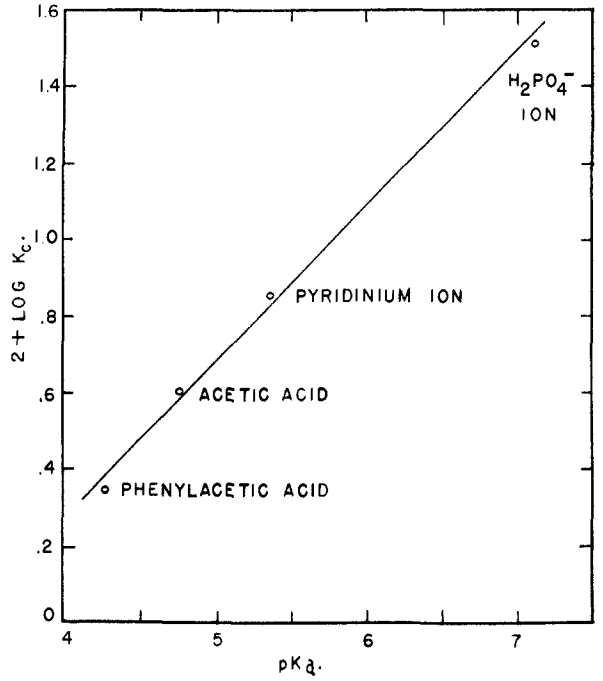

Fig. 3.—Comparison of complex dissociation constants K_c with acidity constants K_a.

correlated with the decrease in the concentration of non-ionized veronal in agreement with the mechanism of general acid catalysis presented for other buffer systems. Initial rate studies conducted at 25.1° in 0.05 *M* veronal buffer at *p*H 7.4 produced results in agreement with the rate equation: $r_0 = k''[AA][RIO]$ where $k'' = 2.4$ l. mole^{-1} min.$^{-1}$.

In 0.20 *M* borate buffer, *p*H 7.40, at 35°, the data conform to the kinetics of a second-order reaction. The value of the second-order constant for this reaction was 8.0 l. mole^{-1} min.$^{-1}$ which may be compared with the value of 4.4 obtained at the same temperature and *p*H but in the absence of buffer.

The observation that *o*-iodosobenzoic acid did not react further with any decomposition products of dehydroascorbic acid in borate buffer is in agreement with a similar behavior observed in non-buffered solutions. It is not necessary, therefore to assume that borate stabilizes dehydroascorbic acid although the possibility of such stabilization is not excluded by the present observations. Penny and Zilva[20] observed the rapid conversion of dehydroascorbic acid to diketogulonic acid in borate buffer at *p*H 7.4. The resulting diketogulonic acid was relatively stable and no reducing substances were formed. Militzer[21] has shown that borate does not inhibit the oxidation of ascorbic acid by oxygen.

Oxidation of Ascorbic Acid in Phosphate Buffer.—Attempts to study the entire course of the reaction between ascorbic acid and *o*-iodosobenzoic acid in phosphate buffer produced results which could not be interpreted by application of the usual kinetic techniques. The presence of phosphate appeared to exert a specific accelerating effect on the rate of decomposition of dehydroascorbic acid (or diketogulonic acid) with consequent formation of an indefinite amount of material capable of reducing *o*-iodosobenzoic acid. When ascorbic acid was mixed with a slight excess of *o*-iodosobenzoic acid in phosphate buffer at *p*H 7, the reaction proceeded smoothly for awhile and then stopped before all the ascorbic acid had been oxidized. Addition of iodide to the solution caused no liberation of iodine, thus indicating that *o*-iodosobenzoic acid had reacted with some decomposition product of dehydroascorbic acid. Preliminary studies demonstrated that the appearance of the reducing substance was considerably delayed and suggested that the early stages of the reaction could be investigated in the absence of this disturbing factor; consequently, only the initial rates of the reaction in phosphate buffer will be considered.

Limited investigation of the rate of oxidation of ascorbic acid in phosphate buffer at *p*H 6 indicated that the general picture was very similar to that already presented for the initial rate studies in acetate buffer. The reaction is first order with respect to both ascorbic acid and *o*-iodosobenzoic acid. The rate of the reaction is proportional to the concentration of phosphate buffer in agreement with the assumption that *o*-iodosobenzoic acid forms a reversible complex with dihydrogen phosphate ion analogous to that assumed for acetic acid.

The results of numerous experiments conducted in phosphate buffer at *p*H 7 demonstrated that the reaction was first order with respect to ascorbic acid but fractional order with respect to *o*-iodosobenzoic acid, *i.e.*, $r_0 = k[AA][RIO]^n$. The value of n lay between 0.5 and 0.6 for concentrations employed in the present investigations. In all other buffer systems, including phosphate at *p*H 6, the value of n was unity. It was concluded that the fractional order could be interpreted most adequately by addition of a second term containing ascorbic acid but not including *o*-iodosobenzoic acid, *i.e.*

$$r_0 = k_2[AA][RIO] + k_1[AA] \qquad (14)$$

From 19 separate experiments conducted at 25.0° in 0.20 *M* phosphate buffer, *p*H 7, the average values of k_2 and k_1 were determined as 4.25 l. mole^{-1} min.$^{-1}$ and 0.00377 min.$^{-1}$, respectively. The concentrations of both ascorbic acid and *o*-iodosobenzoic acid were varied independently several fold. The observation that the presence of 0.001 *M* potassium cyanide in a number of the experiments had no effect on the rate indicates that catalysis by traces of copper here is negligible.

An increase in the concentration of phosphate buffer resulted in an almost linear increase in the rate of the reaction over the range investigated. If the concentrations of ascorbic acid and buffer are held constant while the concentration of *o*-iodosobenzoic acid is varied, the rate equation 14 reduces to the equation of a straight line with slope $k_2[AA]$ and intercept $k_1[AA]$. By this method, the values for k_1 and k_2 at 25.0° determined for several different buffer concentrations are

Buffer, mole per liter	k_2, l. mole^{-1} min.$^{-1}$	k_1, min.$^{-1}$
None	1.4	0
0.05	2.2	0.0009
.10	3.0	.0021
.20	4.4	.0039
.50	9.2	.0099

(20) J. R. Penny and S. S. Zilva, *Biochem. J.*, **37**, 403 (1943).
(21) W. E. Militzer, *J. Biol. Chem.*, **158**, 247 (1945).

The dependence of k_2 on the concentration of buffer has been discussed in preceding sections. The nature of the reaction responsible for the second term of the rate equation is obscure. The possibility has been considered that this rate-determining step may be the formation of an intermediate of semiquinone-like structure from ascorbic acid and dehydroascorbic acid; however, direct addition of a solution of dehydroascorbic acid to the reaction mixture failed entirely to accelerate the rate. Similarly, the initial rate of the reaction was not significantly affected when *o*-iodobenzoic acid was added in a concentration of 0.001 *M* to a solution 0.001 *M* in both ascorbic acid and *o*-iodosobenzoic acid. Direct addition of a partially reacted solution also had no catalytic effect on the rate. There was no evidence of an induction period in any of the experiments to suggest autocatalysis in phosphate buffer at *p*H 7 similar to that observed in acetate buffer at *p*H 4.6. Variations in ionic strength were without effect on the rate. The rate was doubled when the concentration of phosphate buffer was increased from an ionic strength of 0.47 to 1.40 but remained unchanged when the ionic strength was brought to a similar level by addition of potassium chloride or potassium sulfate.

A catalytic effect of phosphate ion on oxidation reactions had been noted by a number of workers.[22-25] It should be emphasized that, in the present work, the fractional order with respect to *o*-iodosobenzoic acid was observed only in phosphate buffer at *p*H 7. At the same *p*H in the absence of buffer, or in veronal buffer, the reaction proceeded as first order with respect to *o*-iodosobenzoic acid. These observations indicate that phosphate ion exerts a specific effect on ascorbic acid which favors the oxidation of the latter by *o*-iodosobenzoic acid.

Catalysis by Metals.—The rate of oxidation of ascorbic acid by *o*-iodosobenzoic acid was accelerated markedly by traces of cupric ion under all conditions studied. In non-buffered solutions containing cupric ion in an amount such that only the catalyzed reaction assumed importance, the oxidation of ascorbic acid rapidly attained a steady state in which the rate followed a first-order reaction with respect to *o*-iodosobenzoic acid: $-d[AA]/dt = k'[RIO]$. That the concentration of ascorbic acid does not appear in the rate equation suggests that the rate-controlling step involves the reoxidation of cuprous ion by *o*-iodosobenzoic acid. In phosphate buffer at *p*H 7, addition of cupric ion to the reaction mixture had the effect of decreasing the order with respect to ascorbic acid and increasing the order with respect to *o*-iodosobenzoic acid, again indicating that the rate-controlling step involves the reoxidation of cuprous ion.

The order of the reaction with respect to the total concentration of cupric ion was investigated. It was assumed that the rate of the catalyzed reaction was superimposed on the rate of the reaction where no copper was added. The data, shown in Table III, indicated that the rate of the copper-catalyzed oxidation of ascorbic acid is proportional to the square root of the concentration of cupric ion. Similar results were obtained in 0.20 *M* phosphate buffer at *p*H 7. Our calculations based on data found in the literature[5a,g] indicate that the rate of the copper-catalyzed oxidation of ascorbic acid by oxygen follows a similar pattern.

Addition of ferrous ion (as $FeSO_4$) also increased the rate of the reaction but only approximately one-tenth as much as cupric ion under the same conditions. Manganese sulfate and magnesium sulfate at concentrations of 1×10^{-5} *M*, under the same conditions used for testing the effects of iron and copper, had no effect on the rate of the reaction.

TABLE III

EFFECT OF CUPRIC ION ON THE OXIDATION OF ASCORBIC ACID IN NON-BUFFERED SOLUTIONS

$r_c = r_0 - 1.1[AA][RIO] = k_2[RIO][Cu^{++}]^{0.5}$

temp. 25.0°; *p*H 4.54; no buffer

$[AA] \times 10^4$, moles per l.	$[RIO] \times 10^4$, moles per l.	$[Cu^{++}] \times 10^7$, moles per l.	$r_0 \times 10^6$, moles per min.	$r_c \times 10^6$, moles per min.	k_2
4.69	9.45	None	0.49	0.00	...
4.69	9.45	1.0	1.00	0.51	1.7
4.69	9.45	5.0	1.67	1.18	1.8
4.69	9.45	10.0	2.12	1.63	1.7
4.77	9.43	100.0	6.24	5.73	1.9

The effectiveness of potassium cyanide as an agent for suppression of copper catalysis was confirmed. With 0.001 *M* cyanide present (phosphate buffer, *p*H 7) the rate of oxidation of ascorbic acid was not changed when cupric sulfate was added to the extent of 5×10^{-7} *M*, while under similar conditions in the absence of cyanide, the rate was increased by 40%.

Effect of Temperature.—The rate constant of the reaction in non-buffered solution increased from 1.3 to 4.4 l. mole^{-1} min.$^{-1}$ when the temperature was increased from 25 to 35°.

Experiments were also conducted in 0.10 *M* phosphate buffer at *p*H 7 over the temperature range 13 to 55°. With the concentration of *o*-iodosobenzoic acid held constant, the rate equation 14 reduces to a first-order reaction with respect to ascorbic acid: $r_0 = k'[AA]$. Plot of log k' against the reciprocal of the absolute temperature produced a straight line in agreement with the Arrhenius equation: $k' = 6.0 \times 10^9 e^{-16,300/RT}$ where $R = 1.987$ cal. deg.$^{-1}$ mole^{-1} and k' is expressed in min.$^{-1}$.

Oxidation of Ascorbic Acid by *m*-Iodoso- and *p*-Iodosobenzoic Acids.—Under conditions similar to those used for the study of oxidation of ascorbic acid by *o*-iodosobenzoic acid, it was found that both *m*- and *p*-iodosobenzoic acids oxidized ascorbic acid practically instantaneously. This was demonstrated by mixing a solution of ascorbic acid with a slight excess of iodosobenzoic acid and, after about 30 seconds, adding a drop of porphyrindine. Since no reduction of the porphyrindine occurred, it was considered that all the ascorbic acid had been oxidized. It was observed also that a solution of ascorbic acid could be titrated in a quantitative manner with a solution of *p*-iodosobenzoic acid by use of porphyrindine as an internal indicator. The indicator remains in the leuco form until an excess

(22) E. J. Witzemann, *J. Biol. Chem.*, **45**, 1 (1920).

(23) M. Nicloux and H. Nebenzahl, *Comp. rend. soc. biol.*, **101**, 720 (1929).

(24) M. Barmore and J. M. Luck, *J. Gen. Physiol.*, **15**, 97 (1931).

(25) H. Lund and H. Lieck, *Skand. Arch. Physiol.*, **74**, 255 (1936).

of oxidizing agent has been added. The procedure is not recommended for analytical purposes since solutions of both the oxidizing agent and the indicator are very unstable.

Oxidation of Ascorbic Acid by *o*-Iodoxybenzoic Acid.—It might have been anticipated that one mole of ascorbic acid would require one-half mole of *o*-iodoxybenzoic acid (RIO_2) for complete oxidation to dehydroascorbic acid. When, however, ascorbic acid and *o*-iodoxybenzoic acid were mixed in molar ratios of 2:1, one-half of the total ascorbic acid was oxidized immediately but the remainder was oxidized at a very slow rate. When the reactants were mixed in equimolar proportions, complete oxidation of all the ascorbic acid occurred immediately. These observations suggested that *o*-iodoxybenzoic acid was reduced rapidly to *o*-iodosobenzoic acid by ascorbic acid.

To an approximately 0.015 molar solution of *o*-iodoxybenzoic acid in phosphate buffer at *p*H 6 was added an equimolar amount of ascorbic acid. A white flocculent precipitate formed almost immediately; this was collected, washed and dried, and was found by analysis to be *o*-iodosobenzoic acid of 99% purity.

The Effect of Phosphate on the Decomposition of Oxidized Ascorbic Acid.—Kinetic studies have shown that oxidized ascorbic acid undergoes decomposition in the presence of phosphate ion with formation of products capable of reducing *o*-iodosobenzoic acid. On the other hand, the formation of such reducing substances was not observed in non-buffered solutions at *p*H 7.7, in acetate buffer at *p*H 4.6 nor in borate buffer at *p*H 7.4. Additional preliminary experiments concerned with the nature and rate of formation of these reducing substances will now be described.

Solutions were prepared in which the concentration of *o*-iodosobenzoic acid was considerably in excess of the concentration of ascorbic acid. The reaction was allowed to proceed in the absence of oxygen; samples were withdrawn periodically and discharged into excess sodium iodide acidified with hydrochloric acid. The liberated iodine immediately oxidized all residual ascorbic acid and the excess iodine remaining was determined by titration with sodium thiosulfate. If no products were formed from oxidized ascorbic acid capable of reducing iodine (or *o*-iodosobenzoic acid), the concentration of excess iodine would have remained constant; however, the concentration of excess iodine invariably decreased with time. From the data obtained it was possible to calculate the number of moles of reducing agent formed per mole of ascorbic acid oxidized. The results of a series of experiments are shown in Table IV. Included is one run (2) in which dehydroascorbic acid was added initially. The strict agreement between the rate of formation of reducing substances from dehydroascorbic acid and ascorbic acid under the same conditions, (2) and (3), demonstrates that dehydroascorbic acid is the first oxidation product obtained when ascorbic acid is oxidized by *o*-iodosobenzoic acid. Under the conditions of experiment (3), the half-life of ascorbic acid is approximately 10 minutes.

TABLE IV

EFFECT OF PHOSPHATE ON THE DECOMPOSITION OF OXIDIZED ASCORBIC ACID

Temp. 41.8°; phosphate buffer; DAA = dehydroascorbic acid

	Initial concentrations, moles per liter				
	(1)	(2)	(3)	(4)	(5)
[Buffer]	None	0.177	0.177	0.177	0.177
[AA] × 10^4	4.42	....	4.42	4.42	4.42
[DAA] × 10^4	...	4.58	....	....	....
[RIO] × 10^4	26.20	26.20	26.20	26.20	26.20
*p*H	8	7.0	7.0	6.0	7.7

	Moles of reducing materials formed per mole of ascorbic acid oxidized				
Min.	(1)	(2)	(3)	(4)	(5)
1	0.00	0.00	0.00	0.00	0.00
60	.07	1.69	1.62	0.49	0.75
120	.22	2.13	2.30	1.20	1.35
210	.43	2.29	2.54	1.95	1.73
1140	.90	2.64	2.90	2.94	2.28

Experiment 1 shows that in non-buffered solutions approximately one mole of reducing material is formed per mole of ascorbic acid oxidized. This is in agreement with the assumption that dehydroascorbic acid has undergone hydrolysis to form diketogulonic acid, since Kenyon and Munro[8] have shown that one molecule of diketogulonic acid readily takes up two equivalents of iodine. The end-products of oxidation in this latter case are probably oxalic acid and threonic acid. It is unlikely that *o*-iodosobenzoic acid is capable of oxidizing diketogulonic acid at an appreciable rate in view of kinetic observations described for the reaction between ascorbic acid and *o*-iodosobenzoic acid in non-buffered solutions.

In the presence of phosphate ion, a limiting value is attained in which nearly three moles of reducing material have been formed per mole of ascorbic acid oxidized. This suggests that diketogulonic acid undergoes further decomposition, probably a decarboxylation, to form L-xylosone, since the presence of considerable carbon dioxide could be demonstrated in spent reaction mixtures. L-Xylosone might then undergo further degradation by a series of oxidations and decarboxylations.[26-28]

It was possible to show that dehydroascorbic acid was rapidly converted to diketogulonic acid by measurement of the rate of formation of reducing substances from the two compounds in phosphate buffer. We observed that, initially, diketogulonic acid takes up two equivalents of iodine while dehydroascorbic acid has no reducing action. Rapid conversion of dehydroascorbic acid occurs and the behavior of the two substances becomes parallel.

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