the same temperature strongly emphasizes the difficulties involved in viscometry.

Summary

The densities, refractive indexes and viscosities of mixtures of methyl alcohol and dioxane were measured over a complete range of composition and at several temperatures. The densities, refractive indexes and viscosities of the pure substances were measured at several temperatures.

The composition coefficient equations for the densities, refractive indexes, and viscosities are given and the constants of these equations tabulated along with the numbers for plotting deviation graphs so that computed values agreeing with observed values may be obtained.

Temperature coefficient equations for densities, refractive indexes, and viscosities of the pure substances and their mixtures are derived and the constants of these equations are tabulated.

Comparison of our experimentally observed values of densities, refractive indexes and viscosities of pure methyl alcohol and pure dioxane is made with the values of these properties of the pure substances recorded in the literature.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

On the Mechanism of the Ascorbic Acid–Ascorbic Acid Oxidase Reaction. The Hydrogen Peroxide Question

BY HARRY G. STEINMAN AND CHARLES R. DAWSON

In 1937, St. Huszák,[1] while investigating the function of peroxidase in plants, came to the conclusion that since ascorbic acid and ascorbic acid oxidase are generally found associated with peroxidase they must play an integral part in the peroxidase system. In support of this hypothesis he cited experimental evidence which he interpreted as indicating that hydrogen peroxide was produced during the enzymatic oxidation of ascorbic acid by ascorbic acid oxidase. This hydrogen peroxide was assumed to be utilized by the peroxidase to oxidize an accompanying flavone derivative, the quinoid form of which then further oxidized a molecule of ascorbic acid. The proposed mechanism can be summarized as follows:

$$AH_2 + O_2 \longrightarrow A + H_2O_2$$
Catalyzed by ascorbic acid oxidase (1)

$$H_2O_2 + QH_2 \longrightarrow Q + 2H_2O$$
Catalyzed by peroxidase (2)

$$Q + AH_2 \longrightarrow A + QH_2$$
Spontaneous (3)

where AH_2 represents ascorbic acid, A dehydroascorbic acid, QH_2 the reduced form of the flavone and Q the oxidized form. This theory of ascorbic acid oxidation appears to have been widely accepted by other workers in the field.[2-5]

Since many oxidizing enzyme systems are known to produce hydrogen peroxide, *e. g.*, xanthine oxidase, *d*-amino acid oxidase and uricase,[6] and since the formation of hydrogen peroxide has been demonstrated in the oxidation of ascorbic acid catalyzed by cupric ion,[7,8] there was, until recently, little reason to question St. Huszák's theory. As soon as it was demonstrated, however, that ascorbic acid oxidase is a copper-protein,[9,10] it seemed desirable to reinvestigate the alleged formation of hydrogen peroxide during the enzymatic oxidation of ascorbic acid. Other metallo-protein oxidases have been shown to produce no hydrogen peroxide, *e. g.*, cytochrome oxidase,[6,11] and tyrosinase.[12] In fact, formation of hydrogen peroxide has been suggested as a criterion for the identification of respiratory systems lacking terminal metallo-protein oxidases.[13]

The study reported in this paper deals fundamentally with the question of hydrogen peroxide formation during the aerobic oxidation of ascorbic

(1) St. Huszák, *Z. physiol. Chem.*, **247**, 239 (1937).

(2) A. Szent-Györgyi, "On Oxidation, Fermentation, Vitamins, Health and Disease," The Williams and Wilkins Co., Baltimore, Md., 1939.

(3) E. A. H. Roberts, *Biochem. J.*, **33**, 836 (1939).

(4) M. F. Jayle, *Bull. soc. chim. biol.*, **21**, 14 (1939).

(5) T. Ebihara, *J. Biochem.* (Japan), **29**, 199 (1939).

(6) D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (London), **B119**, 114 (1936).

(7) C. M. Lyman, M. O. Schultze and C. G. King, *J. Biol. Chem.*, **118**, 757 (1937).

(8) A. O. Dekker and R. G. Dickinson, THIS JOURNAL, **62**, 2165 (1940).

(9) P. L. Lovett-Janison and J. M. Nelson, *ibid.*, **62**, 1409 (1940).

(10) E. Stotz, *J. Biol. Chem.*, **133**, c (1940).

(11) D. E. Green, "Mechanisms of Biological Oxidations," Cambridge University Press, Cambridge, 1940, p. 27.

(12) C. R. Dawson and B. J. Ludwig, THIS JOURNAL, **60**, 1617 (1938).

(13) C. A. Elvehjem and P. W. Wilson, "Respiratory Enzymes," Burgess Publishing Co., Minneapolis, Minn., 1939, p. 38.

acid when catalyzed by a highly purified ascorbic acid oxidase. We have attempted to ascertain whether hydrogen peroxide is formed as a terminal reaction product in a detectable amount when ascorbic acid is enzymatically oxidized by ascorbic acid oxidase in the absence of such factors as catalase, peroxidase and flavones.

Compatibility of Hydrogen Peroxide and Ascorbic Acid.—In order to learn whether hydrogen peroxide is formed as a reaction product of the enzymatic oxidation of ascorbic acid, the chemical properties of the various possible reactants were first studied, to determine whether hydrogen peroxide would be stable and thus detectable under the experimental conditions. All workers with ascorbic acid have noted the spontaneous decomposition of its aqueous solutions and have attributed this to the metallic ion content of the water acting as a catalyst for the autoxidation, copper being chiefly responsible.[14] There is a wide disagreement, however, as to the influence of secondary factors, such as *p*H, on the cupric ion catalyzed oxidation.[15,16]

It has been found in the study here presented that when highly purified buffer systems are employed, the nature of the buffer has a profound effect both on the rate of the copper catalyzed oxidation and its variation with *p*H. Because of this fact, pyrophosphate buffer has been used in the enzyme studies, for in this buffer very little autoxidation of ascorbic acid occurs even in the presence of appreciable amounts of cupric ion, as can be seen from the data compiled in Table I. The pyrophosphate causes no inhibition of the enzymatic reaction. This has also been noted by Krishnamurthy and Giri.[17]

It is clear from the data shown in Table I (also see Table IV) that the buffer systems used can apparently be divided into two classes. In one class, including pyrophosphate and citrate buffers, the oxidation proceeds at a relatively low rate. In the other group, including orthophosphate and acetate buffers, the oxidation proceeds at a relatively high rate. The results seem best explained on the basis that the ionic copper is inhibited in the pyrophosphate and citrate systems probably due to the formation of cupric ion complexes.[17,18]

TABLE I[a]

SHOWING THE EFFECT OF BUFFER ON THE INITIAL RATE OF OXYGEN UPTAKE OF ASCORBIC ACID WHEN CATALYZED BY CUPRIC ION AT *p*H 6.0. THE VALUES ARE GIVEN IN CU. MM. OF OXYGEN PER TEN MINUTES

Copper added, γ	Pyrophosphate buffer $Na_4P_2O_7$ + HCl	Citrate buffer NaOH + $H_3C_6H_5O_7$	Orthophosphate buffer Na_2HPO_4 + KH_2PO_4	Acetate buffer $NaC_2H_3O_2$ + $HC_2H_3O_2$
0	0	2	26	26
1	3	8	133	135
10	20	45	250	250
100	86	85	320	350

[a] Experiments conducted in 50-cc. Warburg respirometer flasks at 25°. Total reaction volume 8.0 cc., consisting of 2 cc. of 0.2 *M* buffer solution (0.1 *M* in the case of citrate), 1 cc. of the proper dilution of the copper solution, made by dissolving pure copper wire in nitric acid, 1 cc. of a freshly prepared aqueous ascorbic acid solution (2.5 mg.) and sufficient water to bring up to volume. Ascorbic acid placed in side arm of vessel prior to mixing at zero time. Salt components of the various buffers, and the citric acid purified by several recrystallizations from freshly redistilled water. Hydrochloric acid and acetic acid purified by distillation. 0.1 *M* sodium hydroxide solution prepared from reagent grade chemical. Ordinary distilled water redistilled in an all-glass (Pyrex) system used throughout for dilution of buffers, preparation of reagents and making up to volume of reaction mixtures. Special precautions taken in cleansing manometer vessels. Removed lubricant ("Lubriseal") and thoroughly rinsed with tap water. Filled with new sulfuric acid–dichromate cleaning solution for a short period, rinsed well and allowed to stand full of glass redistilled water for an hour. Steamed out for five minutes.

TABLE II[a]

SHOWING THE EFFECT OF HYDROGEN PEROXIDE ON THE RATE OF DISAPPEARANCE OF ASCORBIC ACID IN AQUEOUS SOLUTION AT VARIOUS *p*H VALUES. THE VALUES ARE GIVEN IN CC. OF 2,6-DICHLOROBENZENONE INDOPHENOL (0.00129 *M*)

Time, hours	*p*H 6.25 AH_2	*p*H 6.25 AH_2 + H_2O_2	*p*H 6.95 AH_2	*p*H 6.95 AH_2 + H_2O_2	*p*H 7.80 AH_2	*p*H 7.80 AH_2 + H_2O_2
0	2.75	2.75	2.75	2.75	2.75	2.75
1	2.65	2.65	2.70	2.70	2.70	2.69
2	2.55	2.54	2.65	2.60	2.65	2.62
4	2.46	2.44	2.60	2.40	2.55	2.33

[a] Experiments conducted in 125-cc. Erlenmeyer vessels at 25°. Concentrations of ascorbic acid and of buffer (pyrophosphate) kept the same as in manometric experiments (see legend, Table I), using 40 cc. total reaction volume. Hydrogen peroxide equivalent to 10 times the ascorbic acid present added at zero time. Two-cc. aliquots taken periodically and titrated with 2,6-dichlorobenzenone indophenol reagent in 1.0 *M* acetate buffer at *p*H 3.5. Tauber[20] has shown that ascorbic acid can be titrated quantitatively with this reagent in the presence of hydrogen peroxide. Values in the table are reproducible to about ±0.03 cc.

(14) C. G. King, *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 137 (1939).

(15) E. S. G. Barron, R. H. DeMeio and F. Klemperer, *J. Biol. Chem.*, **112**, 625 (1936).

(16) H. Schümmer, *Biochem. Z.*, **304**, 1 (1940).

(17) P. V. Krishnamurthy and K. V. Giri, *Ind. J. Med. Research*, **29**, 71 (1941).

(18) J. C. Ghosh and P. C. Rakshit, *Biochem. Z.*, **289**, 15 (1936).

Although hydrogen peroxide has been shown to accelerate the rate of autoxidation of ascorbic acid,[8] the data tabulated in Table II show that under the experimental conditions the rate of disappearance of ascorbic acid is very little increased by the presence of ten times the equivalent amount of hydrogen peroxide. The non-reactivity of hydrogen peroxide and ascorbic acid is further emphasized by the fact that there is no significant reaction between these two substances even in the presence of peroxidase.[1,19,20]

Compatibility of Hydrogen Peroxide and Dehydroascorbic Acid.—Aqueous solutions of dehydroascorbic acid are more unstable than those of ascorbic acid.[21,22] However, the initial stage of the decomposition which is pseudomonomolecular in character is not due to a metallic ion catalyzed oxidation. The rate of decomposition increases rapidly with increase in *p*H. The rate is also increased by the addition of hydrogen peroxide apparently because of a bimolecular reaction between the dehydroascorbic acid and hydrogen peroxide. This effect of hydrogen peroxide is more pronounced at *p*H 7 than at *p*H 5.

It can be seen from columns 3 and 5 of Table III that in thirty minutes, under the experimental conditions described, about 48% of the dehydroascorbic acid disappears whether prepared chemically or enzymatically when an equivalent amount of hydrogen peroxide is added to the buffered dehydroascorbic acid solution at zero time. The same is the case when the dehydroascorbic acid is rapidly formed (enzymatically) in the presence of hydrogen peroxide (see legend, Table III). The hydrogen peroxide disappears from the solution at a lower rate than does the dehydroascorbic acid since the latter disappears *via* two simultaneous reactions, only one of which involves the peroxide. The lower rate of disappearance of hydrogen peroxide (about 42% in thirty minutes) can be seen by a comparison of columns 6 and 7 with columns 3 and 5 of Table III. It is apparent that the oxidation product of ascorbic acid (dehydroascorbic acid) prepared enzymatically and that prepared by the use of *p*-quinone as an oxidizing agent show the same stability characteristics in aqueous solution and in dilute solutions of hydrogen peroxide (compare the data in columns 2 and 4 and columns 3 and 5 of Table III). Furthermore, the rate of disappearance of added hydrogen peroxide is independent of the manner of formation of the

TABLE III[a]

SHOWING THE EFFECT OF HYDROGEN PEROXIDE ON THE RATE OF DISAPPEARANCE OF DEHYDROASCORBIC ACID AT *p*H 6.30

The values are given as percentages of the original concentrations.

Time, min.	Dehydroascorbic acid: By quinone, Alone	By quinone, With H_2O_2	By enzyme, Alone	By enzyme, With H_2O_2	Hydrogen peroxide With dehydroascorbic acid: By quinone	By enzyme
0	100.0	100.0	100.0	100.0	100.0	100.0
15	92.2	71.4	90.0	72.0	75.9	74.0
30	84.3	51.7	82.0	52.0	57.9	58.0
45	79.8	38.2	78.0	40.2	50.0	52.0
60	73.6	32.0	74.2	32.0	43.8	44.2

[a] Dehydroascorbic acid prepared by oxidation of ascorbic acid with *p*-benzoquinone,[23] and also by enzymatic oxidation with ascorbic acid oxidase. Experiments conducted in 24 cc. reaction volume at 25°. Original concentration ascorbic acid same as previously indicated (legend, Table II). Oxidation to dehydroascorbic acid by *p*-benzoquinone carried out in slightly acid solution (0.2% acetic acid) and rapidly adjusted to *p*H 6.30 (pyrophosphate buffer) to initiate experiment. Slightly acid solutions of dehydroascorbic acid are stable for short periods of time. Hydrogen peroxide, in amount equivalent to the dehydroascorbic acid, added at zero time where indicated. Hydrogen peroxide and dehydroascorbic acid determined in 2.0-cc. aliquots from reaction mixture and controls at indicated time intervals. Dehydroascorbic acid determined by hydrogen sulfide reduction to ascorbic acid and then titration with indophenol reagent (0.00142 *M*) in 1.0 *M* acetate buffer at *p*H 3.5.[24] Hydrogen peroxide determined by titrating with 0.00282 *M* thiosulfate the iodine liberated from a sulfuric acid–potassium iodide solution containing pyrogallol.[25] To prepare the dehydroascorbic acid enzymatically, 12 units of an ascorbic acid oxidase preparation containing no catalase or peroxidase was used at *p*H 6.30. To account for the loss of dehydroascorbic acid during the five minute preliminary enzymatic oxidation (about 2–3%) a slight excess of ascorbic acid was used. The data obtained by adding an equivalent amount of hydrogen peroxide at the five minute period to the enzymatically produced dehydroascorbic acid, could also be reproduced by forming the dehydroascorbic acid in the presence of an equivalent amount of hydrogen peroxide, provided enough more enzyme was used to produce the dehydroascorbic acid within about one minute. The units of ascorbic acid oxidase are those proposed by Lovett-Janison and Nelson.[9] The enzyme preparation used was Preparation 10 described by them. This preparation contained 0.15% of copper and had an activity of about 430 units per γ of copper.

(19) A. Szent-Györgyi, *Biochem. J.*, **22**, 1387 (1928).

(20) H. Tauber, *Enzymologia*, **1**, 209 (1936).

(21) H. Borsook, H. W. Davenport, C. E. P. Jeffreys and R. C. Warner, *J. Biol. Chem.*, **117**, 237 (1937).

(22) E. G. Ball, *ibid.*, **118**, 219 (1937).

(23) M. O. Schultze, C. J. Harrer and C. G. King, *ibid.*, **131**, 5 (1939).

(24) O. A. Bessey, *ibid.*, **126**, 771 (1939).

(25) A. K. Balls and W. S. Hale, *J. Assoc. Off. Agric. Chem.*, **16**, 395 (1933).

oxidation product of ascorbic acid (compare columns 6 and 7). Crook[26] has previously pointed out that the same product of oxidation is formed by either enzymatic or chemical oxidation of ascorbic acid.

There is little reason to expect hydrogen peroxide as one of the reaction products of the oxidation of ascorbic acid by a chemical oxidizing agent such a *p*-quinone. Since it has been shown that hydrogen peroxide does accelerate the rate of disappearance of dehydroascorbic acid from aqueous solutions, the fact that dehydroascorbic acid prepared by the use of *p*-quinone is lost at the same rate as that prepared enzymatically is evidence against the view that hydrogen peroxide is a product of the enzymatic oxidation of ascorbic acid. Furthermore, since added hydrogen peroxide disappears from the solution at a lower rate than dehydroascorbic acid, it is evident that failure to detect hydrogen peroxide as a reaction product of the enzymatic oxidation of ascorbic acid cannot be ascribed to a reaction between the hydrogen peroxide and dehydroascorbic acid in any case where dehydroascorbic acid is found in appreciable amounts.

Not only is hydrogen peroxide compatible with ascorbic acid and reasonably so with dehydroascorbic acid, but it also must be relatively compatible with any intermediary "redox" forms that are produced during the enzymatic oxidation. This statement is supported by the fact that when a mixture of ascorbic acid and hydrogen peroxide is oxidized by a very powerful ascorbic acid oxidase solution, there is no detectable loss in hydrogen peroxide during the rapid production of dehydroascorbic acid. This, of course, also indicates that ascorbic acid oxidase has no peroxidatic function, *i. e.*, cannot utilize hydrogen peroxide for the oxidation of ascorbic acid.

The Effect of Catalase on the Reaction Course of the Enzymatic Oxidation of Ascorbic Acid.—When ascorbic acid is oxidized rapidly by an excess of catalase-free ascorbic acid oxidase, the oxygen consumption is one atom per mole of substrate, as shown in Curve I of Fig. 1. The rate of oxygen uptake is independent of the buffer system but is dependent on the *p*H, the optimum *p*H range being from about *p*H 5.6 to 7.0 (in the presence of gelatin) for the preparation in hand. In order to work as nearly as possible at the *p*H optimum of the enzyme it was decided to perform

(26) E. M. Crook, *Biochem. J.*, **35**, 226 (1941).

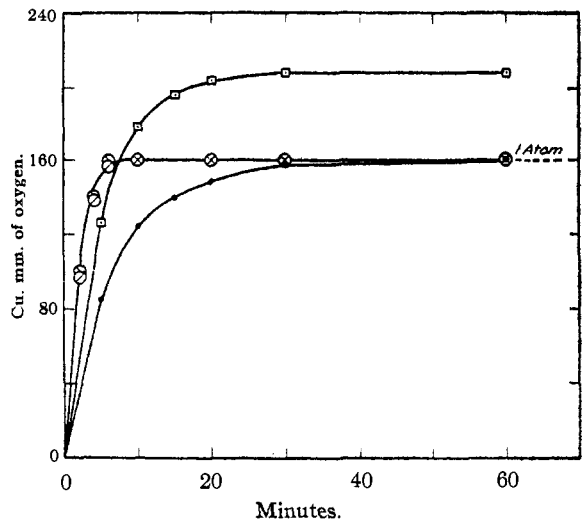

Fig. 1.—Curves showing the effect of catalase on the production and stability of the products resulting from the enzymatic and the cupric ion oxidation of ascorbic acid at *p*H 6.3. Experiments conducted as indicated in legend of Table I, using a pyrophosphate buffer. For 2.5 mg. of ascorbic acid 159 cu. mm. of oxygen uptake corresponds to 1 atom per mole of ascorbic acid. The systems corresponding to Curves I (⊘) and II (⊘) contained ten units of a catalase-free and peroxidase-free ascorbic acid oxidase (see legend, Table III), which was placed in the side arm prior to mixing at zero time, so as to keep the inactivating effect of the buffer at a minimum. Curves III (⊡) and IV (●) resulted from the use of 1 mg. of cupric ion instead of enzyme. The systems corresponding to Curves II and IV contained, in addition, 1.0 cc. of a dlluted (1:100) catalase preparation made from a solution of once crystallized catalase prepared according to the method of Sumner and Dounce.[27] The undiluted preparation had a Kat f. of about 14,000 (dry wt.: 4.94 mg./cc.) and could at 1:1000 decompose within one minute, under the experimental conditions, an amount of hydrogen peroxide equivalent to the ascorbic acid used. Both ascorbic acid and cupric ion cause some inhibition of catalase activity, as shown by Tria.[28] To demonstrate that the catalase remained active throughout the course of the oxidations, an amount of hydrogen peroxide equivalent to that theoretically producible from the ascorbic acid, as indicated in Equation (1), was added to the reaction systems at the end of the experiments. Before adding the hydrogen peroxide (via the side arm), the reaction mixtures were transferred to clean vessels to preclude any premature decomposition of hydrogen peroxide by residual catalase in the side arm. The added hydrogen peroxide was completely decomposed, as evidenced by a very rapid evolution of oxygen, in both the enzymatic and the cupric ion oxidation systems. The presence of alkali in the central well of the reaction flask, to absorb any carbon dioxide evolved during the reaction, had no effect on the results.

(27) J. B. Sumner and A. L. Dounce, *J. Biol. Chem.*, **127**, 439 (1939).

(28) E. Tria, *Ricera sci.*, **11**, 345 (1940).

all enzymatic studies at *p*H 6.2 to 6.3. It has already been shown that at *p*H 6.3 conditions are favorable for the detection of hydrogen peroxide in the presence of any of the "redox" forms of ascorbic acid likely to occur.

By the use of a sufficient amount of catalase, the decomposition of hydrogen peroxide can be effected at a rate so rapid as to be practically immeasurable. For this reason, if hydrogen peroxide is produced or plays any intermediary role during the enzymatic oxidation of ascorbic acid at *p*H 6.3, the presence of a relatively large amount of catalase in the reaction system should materially alter the course of the reaction, as measured manometrically. This statement is true provided the catalase remains active throughout the course of the reaction, and provided that any intermediary function of the hydrogen peroxide is not vastly more rapid than its rate of destruction by catalase. The latter possibility is very unlikely, as has been pointed out above.

Curve II of Fig. 1 shows the reaction course in the presence of an amount of catalase which is still active at the expiration of the experiment. Within the limits of the experimental error, the reaction course in the presence of catalase is indistinguishable from that in the absence of catalase, *e. g.*, Curves I and II of Fig. 1 are superimposable. Since the catalase remains active throughout the enzymatic oxidation (see legend of Fig. 1) and causes no change in the course of the reaction, as followed by this method, it seems very unlikely that the oxygen uptake values are in any part due to hydrogen peroxide formation. A further indication that no hydrogen peroxide ever existed during the course of the reaction is the fact that the amount of dehydroascorbic acid present at the end of the reaction is the same whether the ascorbic acid is oxidized in the absence or in the presence of catalase. If hydrogen peroxide had even a transitory existence, the dehydroascorbic acid values should be less in those experiments containing no catalase, due to reaction with the hydrogen peroxide simultaneously formed. This loss of dehydroascorbic acid was always found in the cupric ion-catalyzed oxidation of ascorbic acid in which hydrogen peroxide is produced.

The Effect of Catalase on the Reaction Course of the Cupric Ion Oxidation of Ascorbic Acid.—The fact that catalase has no effect on the rapid enzymatic oxidation of ascorbic acid is strong evidence that hydrogen peroxide is not formed during the reaction. Fortunately, the validity of this evidence can be tested by studying the effect of catalase on a system in which hydrogen peroxide is known to be formed, *i. e.*, the autoxidation of ascorbic acid catalyzed by cupric ion.[7,8] Care was taken to carry out such experiments under conditions as closely analogous to the enzymatic oxidation as possible. Curves III and IV of Fig. 1 show in graphical form a typical set of data, the latter curve being that obtained when catalase was present in the reaction mixture. It is at once apparent that in the copper-catalyzed oxidation of ascorbic acid catalase does have a pronounced effect upon the course of the reaction. This effect undoubtedly is due to the removal of hydrogen peroxide from the system by the catalase with the simultaneous return of oxygen, according to the reaction $2H_2O_2 \rightarrow 2H_2O + O_2$. Furthermore, ascorbic acid titration experiments are in agreement with the assumption that the excess oxygen uptake above 1.0 atom in the system containing no catalase (Curve III) is due to hydrogen peroxide formation, since the ascorbic acid disappears at about the same rate whether catalase is present or not. That the rate of oxidation of ascorbic acid in both cases is the same is also indicated by the fact that the oxygen absorptions are simultaneously completed at about thirty minutes in Curves III and IV, despite the greater oxygen uptake in the experiment performed in the absence of catalase. The hydrogen peroxide can be detected not only indirectly by the effect of catalase on the course of the reaction, but also directly by the addition of catalase at the end of the reaction (rapid evolution of oxygen) or by titration of the hydrogen peroxide with sodium thiosulfate (via the iodine produced on reaction with hydriodic acid).

If hydrogen peroxide were formed according to reaction (1), the extra oxygen uptake would be expected to correspond to another atom of oxygen per mole of substrate. The amount of extra oxygen consumption depends to a great extent on both the type of buffer system and the rate of oxidation, as shown in Table IV. It can be seen from the table that the total oxygen uptake never reaches two atoms of oxygen per mole of ascorbic acid during a reasonably rapid oxidation, *i. e.*, from 4.5 to 25 cu. mm. oxygen per minute. It appears that as the rate of oxidation increases, *i. e.*, as the amount of ionic copper increases, the

TABLE IV[a]

SHOWING THE EFFECT OF BUFFER ON THE TOTAL OXYGEN ABSORBED DURING THE CUPRIC ION CATALYZED OXIDATION OF ASCORBIC ACID AT *p*H 6.0 AS DEMONSTRATED BY THE AMOUNT OF CUPRIC ION NECESSARY TO PRODUCE THE SAME INITIAL RATE

Initial rate, cu. mm. O_2/min.	Pyrophosphate buffer Cu^{++}, γ	Pyrophosphate buffer Total, atoms	Citrate buffer Cu^{++}, γ	Citrate buffer Total, atoms	Acetate buffer Cu^{++}, γ	Acetate buffer Total, atoms	Orthophosphate buffer Cu^{++}, γ	Orthophosphate buffer Total, atoms
4.5	25	1.8	10	1.7	0.1	1.5	0.1	1.3
8.5	100	1.7	100	1.7	.4	1.5	.4	1.3
25	1000	1.4	1000	1.4	10	1.4	10	1.2

[a] Reaction mixtures and procedures same as indicated in legend Table I. Values reported in this table are reproducible to about ±0.05 atom. A part of the deviation arises from the fact that such systems tend to evolve carbon dioxide on long standing, the total absorption thus becoming a function of time. This was also observed by Lyman, Schultze and King.[7]

total oxygen uptake tends to decrease. This is true in both types of buffer. Furthermore, for a given rate of oxygen absorption, the total oxygen absorbed is less in those buffer systems where the copper is apparently free from inhibition by the buffer components, *e. g.*, the acetate and orthophosphate buffer systems.[29] It may be noted that these findings are in agreement with the often reported observation that traces of heavy metals result in the decomposition of hydrogen peroxide. It should be emphasized, however, that this observation cannot be used to explain the failure to detect hydrogen peroxide in the enzymatic reaction where the amount of copper involved is many times less (10 units = 0.023 γ Cu, see legends Fig. 1 and Table III) unless one ascribes a tremendous catalase activity to the copper protein oxidase. As previously pointed out, the oxidase preparation used was purified until free of catalase activity.

With the limitations previously pointed out, the presence of catalase in the reaction mixture during the aerobic oxidation of ascorbic acid catalyzed by ionic copper should remove hydrogen peroxide as fast as it is formed, restoring the excess oxygen to the system. In all instances where the catalase remained active throughout the duration of the experiment, as shown by its ability to decompose added hydrogen peroxide at the end of the experiment, the total oxygen uptake was just one atom per molecule of substrate, regardless of the rate of oxidation, the amount of copper present, the type of buffer, the presence or absence of gelatin and the presence or absence of a carbon dioxide absorption cup. Conversely, in every experiment in which catalase was used, and in which the oxygen uptake exceeded one atom, it was also found that the catalase had become inactivated before the end of the experiment.

These experimental results offer convincing evidence of hydrogen peroxide formation in the cupric ion oxidation of ascorbic acid. In view of the fact that this formation of hydrogen peroxide, when ascorbic acid is oxidized catalytically by ionic copper, is clearly demonstrated by the use of catalase, but there is no such indication of hydrogen peroxide formation when ascorbic acid is oxidized enzymatically by a specific copper-protein under exactly analogous conditions, it can be concluded that the oxygen uptake values during the enzymatic oxidation are in no part due to hydrogen peroxide formation.

On the Inactivation of Ascorbic Acid Oxidase.—An outstanding characteristic of the enzymatic oxidation of ascorbic acid by ascorbic acid oxidase is the pronounced inactivation of the enzyme that occurs during the course of the reaction, and which becomes especially evident when small amounts of enzyme are used (see Curve I, Fig. 2). During the course of these studies, it has been found that methemoglobin, peroxidase and catalase markedly protect ascorbic acid oxidase against this inactivation. Thus, as can be seen from the data graphically represented in Curves II, III and IV of Fig. 2, little or no inactivation of the ascorbic acid oxidase is evident when the reaction mixture contains a sufficient amount of methemoglobin, peroxidase or catalase. The amount of ascorbic acid oxidase used in these experiments was the same as that used to obtain the data for Curve I of Fig. 2. In the case of catalase (Curve II) the protective action is so great that an amount of ascorbic acid oxi-

(29) The hydrogen peroxide actually found at the termination of the experiment by manometric experiments with catalase or by direct titration is not equivalent to the extra oxygen consumption of the system. This is to be expected, since some of the hydrogen peroxide reacts with the dehydroascorbic acid simultaneously formed, in accordance with the finding shown in Table III. There is, however, reasonably satisfactory qualitative agreement between the hydrogen peroxide expected from the oxygen uptake values and the sum of the hydrogen peroxide actually found plus that calculated to have reacted with dehydroascorbic acid.

dase which, in the absence of catalase, can oxidize only about a fifth of the ascorbic acid present before becoming completely inactivated can, in the presence of catalase, completely oxidize the ascorbic acid. Furthermore, the oxidase protected in this manner can oxidize a second portion of the substrate at almost the original velocity.

It has been recognized for some time that hydrogen peroxide may be responsible for enzyme inactivation in certain cases.[30] That the protective action of the proteins noted above is not due to the removal of hydrogen peroxide as a reaction product, is evident from the fact that neither the peroxidase nor the methemoglobin preparations, in the experimental amounts used, possessed the ability to destroy hydrogen peroxide at any significant rate. Furthermore, that the protective action is not enzymatic in character, is evident from the fact that solutions of methemoglobin and of peroxidase which had been boiled for a long period of time also exhibited a marked protective action (see legend of Fig. 2).

It has been previously found that various hemochromogens can catalyze the autoxidation of ascorbic acid by undergoing a simultaneous coupled oxidation.[31] That no such phenomenon is responsible for the results observed above is shown by the fact that, under the experimental conditions and in the concentrations used, neither methemoglobin, peroxidase nor catalase was found to be a very effective catalyst for the autoxidation of ascorbic acid. The degree of protective action exhibited by these iron-porphyrin proteins is a function of the amount used, with catalase appearing to be the most efficient. The specific and quantitative aspects of this problem are under investigation in this Laboratory at the present time.

Effect of Quercetin.—According to the mechanism proposed by St. Huszák[1] the presence of quercetin in a system consisting of ascorbic acid, ascorbic acid oxidase and peroxidase should cause the ascorbic acid to disappear twice as fast as it does in a similar system lacking quercetin (see Reactions 1, 2 and 3). A comparison of Curves III and VI of Fig. 2 shows that quercetin has no observable effect when added to such a system. If hydrogen peroxide were responsible for any part of the oxygen uptake (as indicated in Reaction 1) any secondary removal of ascorbic acid by way of quercetin and peroxidase should be made readily apparent by a lowered total oxygen uptake.

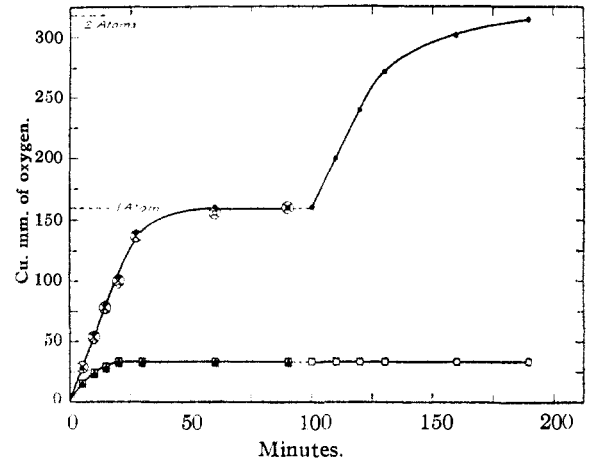

Fig. 2.—Curves showing the effect of methemoglobin, peroxidase, catalase, and peroxidase plus quercetin on the aerobic oxidation of ascorbic acid in the presence of a small amount of ascorbic acid oxidase, reaction mixtures and procedure same as indicated in legends of Table I and Fig. 1, one-half unit oxidase used: Curve 1 (□), oxidase in the absence of protective agents; Curve II (●) reaction mixture contained 1 cc. of a diluted (1:100) catalase preparation (see legend Fig. 1). At the one hundred minute period, an amount of ascorbic acid equivalent to that originally present was added to both systems, in order to verify that the enzyme was no longer active in reaction mixture I but was still active in II. The system corresponding to Curve III (⊘) differed from that of Curve II in that III contained, initially, 1.0 cc. of a diluted (1:10) peroxidase preparation in place of the catalase. The purified (undiluted) peroxidase preparation had a P. Z. of 590 (dry wt.: 2.74 mg./cc.) and could, at (1:100), in the presence of hydrogen peroxide oxidize within five minutes, via 1 cc. $M/1000$ quercetin, an amount of ascorbic acid equivalent to that used in the experiments. That the peroxidase was still active at the end of the experiment was shown qualitatively by its ability to oxidize catechol in the presence of hydrogen peroxide. Curve IV (⊘) was obtained with 1.0 cc. of diluted (1:10) methemoglobin preparation made from crystalline horse blood hemoglobin; dry wt. of undiluted preparation 18.6 mg. per cc. Solutions denatured by prolonged boiling as well as native solutions of methemoglobin and peroxidase gave identical results when used in the concentrations noted above. Curves V (▲) and VI (○) show the effect of 1 cc. of $M/1000$ quercetin on the normal oxidation and on the peroxidase protected reaction.

Discussion

The work that has been described above is contradictory to the view that hydrogen peroxide is produced as a reaction product during the enzymatic oxidation of ascorbic acid by ascorbic

(30) M. Dixon, *Biochem. J.*, **19**, 507 (1925).

(31) R. Lemberg, B. Cortis-Jones and M. Norrie, *ibid.*, **32**, 149 (1938).

acid oxidase. The possibility that a precursor of hydrogen peroxide, *i. e.*, a "redox" form of oxygen, has a transitory existence or some intermediary function in the enzymatic oxidation is suggested by some of the data. Certainly hydrogen peroxide *per se* can have no more than an evanescent existence, for, if it were present at all, it would have to perform some function at a rate greater than the rate of its decomposition by relatively large amounts of catalase; and the ascorbic acid oxidase must possess an enormous peroxidatic activity of which the experiments failed to disclose any trace.

The enzymatic oxidation of ascorbic acid differs from the cupric ion catalyzed reaction in that the former does not produce hydrogen peroxide as a terminal reaction product, whereas the latter does. It is possible that the same intermediary "redox" form of oxygen is produced initially in both cases and that hydrogen peroxide results when the intermediary is not efficiently utilized. Considering the evidence that has been presented above, there appears to be little reason at present for supporting the view that hydrogen peroxide is a reaction product of the enzymatic oxidation of ascorbic acid, and hence capable of entering into secondary reactions.

Summary

1. The enzymatic oxidation of ascorbic acid has been studied using an ascorbic acid oxidase preparation with negligible catalase and peroxidase activity.

2. In mixtures of ascorbic acid and ascorbic oxidase buffered within the *p*H range of 5.6 to 7.0, the initial oxidation product is formed by the absorption of one atom of oxygen per molecule of ascorbic acid.

3. The reaction between this oxidation product (dehydroascorbic acid) and hydrogen peroxide is not sufficiently rapid at *p*H 6.3 to account for the failure to detect hydrogen peroxide during the enzymatic oxidation of ascorbic acid by ascorbic acid oxidase.

4. At *p*H 6.3, the course of the aerobic oxidation of ascorbic acid by relatively large amounts of enzyme is unaffected by the addition of large amounts of catalase, peroxidase, methemoglobin or peroxidase plus quercetin.

5. At *p*H 6.3, the inactivation of the enzyme observed when relatively small amounts are added to ascorbic acid is largely prevented by the addition of catalase and native or denatured peroxidase or methemoglobin. However, these substances apparently do not affect the *initial* rate of oxygen uptake, and therefore do not act by removal of hydrogen peroxide *per se*.

6. Evidence has been presented to support the view that hydrogen peroxide is a terminal reaction product of the aerobic oxidation of ascorbic acid when catalyzed by cupric ion. In this case, catalase has a marked effect on the initial rate of the reaction, and the oxygen absorption totals are lowered to one atom per molecule of ascorbic acid.

7. It is concluded that hydrogen peroxide is not a reaction product of the aerobic oxidation of ascorbic acid when catalyzed by the copper-protein, ascorbic acid oxidase.

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