ON THE REACTION INACTIVATION OF ASCORBIC ACID OXIDASE

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SUMMARY

1. The reaction inactivation of the copper protein, ascorbic acid oxidase, has been reinvestigated and new evidence concerning the cause of the inactivation has been obtained.

2. It has been found that the main products of the enzymic oxidation, *i.e.*, dehydroascorbic acid and water, are not responsible for the inactivation phenomenon.

3. The enzyme inactivation is dependent on the time required for the accumulation of a by-product of the reaction. This by-product appears to be hydrogen peroxide.

4. Only a very small amount of the by-product develops during the typical reaction, apparently as the result of a secondary and low-rate catalytic reaction.

5. Hydrogen peroxide is a very effective inactivating agent against ascorbic acid oxidase and only very small amounts are required to account for the experimentally observable enzyme inactivation.

INTRODUCTION

Several years after the discovery of ascorbic acid oxidase[1], the purified preparation of the enzyme which was obtained in these laboratories was found to become inactivated as it catalyzed the aerobic oxidation of ascorbic acid [2–4]. The rapid inactivation of the functioning enzyme, particularly apparent in the absence of protective agents such as gelatin or native catalase, was called "reaction inactivation", and the incomplete oxidation of the ascorbic acid resulting from the inactivation was indicated by an "inactivation total", *i.e.* an oxygen uptake lower than the theoretical value for the complete oxidation. It was also shown that environmental factors such as the pH of the system and the enzyme concentration were minor factors in the inactivation. Because earlier experiments[5] had indicated that hydrogen peroxide was not a terminal product of the ascorbic acid–AAO reaction, and the main product, dehydroascorbic acid, was not harmful to AAO, it was suggested[3] that the reaction inactivation might be due to some factor inherent in the ascorbic acid–AAO–oxygen system, possibly a highly reactive "redox form" of oxygen other than hydrogen peroxide or H_2O.

Abbreviations: AAO, ascorbic acid oxidase; BSA, bovine serum albumin.

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This type of reaction inactivation has also been observed with other enzymes; notably tyrosinase[6] and *p*-hydroxyphenyl pyruvic acid oxidase[7]. Recently ZANNONI *et al.*[8] have studied the reaction inactivation of *p*-hydroxyphenyl pyruvic acid oxidase in the presence of high substrate concentrations, but the mechanism of the phenomenon is not clear.

It is the purpose of this article to report that the reaction inactivation of AAO can be differentiated from substrate inhibition, and that the inactivation is caused by trace amounts of a product resulting from a secondary reaction. It is believed that the inactivating by-product is hydrogen peroxide.

EXPERIMENTAL

Materials

Ascorbic acid oxidase was prepared from the yellow crookneck squash (*c. pepo condensa*) essentially by the method previously described[9]. The specific activity of the most highly purified AAO preparation was 2000 units/mg protein.

The substrate, ascorbic acid, was obtained from Merck Company and used without further purification. The catalase (Kat. f. value $1 \cdot 10^6$ by manometric measurement) was obtained in crystalline form from the Worthington Company. The bovine serum albumin was the crystalline product supplied by Armour Company. The other chemicals, notably gelatin, metaphosphate, cupric sulfate, hydrogen peroxide and potassium cyanide were used as received from various manufacturers.

Assays

The rate of the ascorbic acid–AAO reaction was measured manometrically in the following way. The standard reaction mixture consisted of an appropriate volume of the enzyme solution, 0.5 ml of 0.4 *M* citrate–phosphate buffer (pH 5.5), various amounts of ascorbic acid and a sufficient amount of distilled water to make the final reaction volume 2.5 ml. When the specific activity of the enzyme was measured, the AAO in the main compartment of the reaction vessel with the buffer was "protected" with 0.25 ml of gelatin solution (5 mg/ml). The ascorbic acid solution (0.25 ml; 5 mg/ml), containing 0.25 mg of metaphosphoric acid, was placed in the side arm. The final reaction volume was always 2.5 ml and the final pH of the reaction mixture was always found to be 5.8.

The specific activity of the enzyme was expressed in terms of units described elsewhere[2]. The amount of protein was determined using the Folin–Ciocalteu phenol reagent[10].

RESULTS

The effect of substrate inhibition on reaction inactivation

Several different ways of accounting for the characteristic reaction inactivation of AAO can be proposed. Some examples are: (a) The known products of the reaction (or compounds secondarily derived from such products) might be responsible. (b) A by-product derived from the substrate or the enzyme by a secondary reaction might be responsible. (c) The enzyme–substrate complex might produce an intermediary substance that could inactivate the enzyme. (d) Any of the reaction parti-

cipants in activated form, *i.e.*, activated substrate, activated oxygen, activated copper ions, etc., might be responsible for the reaction inactivation.

Although earlier studies[5] have established that the products of the enzymic oxidation are not responsible for the enzyme inactivation, such studies were complicated by factors such as substrate inhibition and surface denaturation. As shown

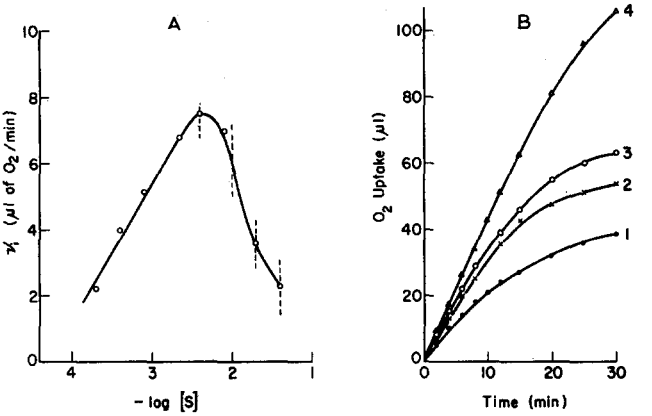

Fig. 1. A, variation of the apparent initial reaction velocity with the substrate concentration (expressed as $-\log S$). B, variation of the apparent initial reaction velocity and the reaction course with increasing substrate concentration. Curve 1, $4 \cdot 10^{-2}$ *M* ascorbic acid; curve 2, $2 \cdot 10^{-2}$ *M* ascorbic acid; curve 3, $1 \cdot 10^{-2}$ *M* ascorbic acid; curve 4, $4 \cdot 10^{-3}$ *M* ascorbic acid. Oxygen uptake was measured manometrically at 25°, pH 5.8. Each vessel contained AAO (1.0 µg), $4 \cdot 10^{-2}$ *M* citrate–phosphate buffer, ascorbic acid, and distilled water to make the final reaction volume 2.5 ml. The substrate concentrations involved in Fig. 1B are shown as dotted lines in Fig. 1A.

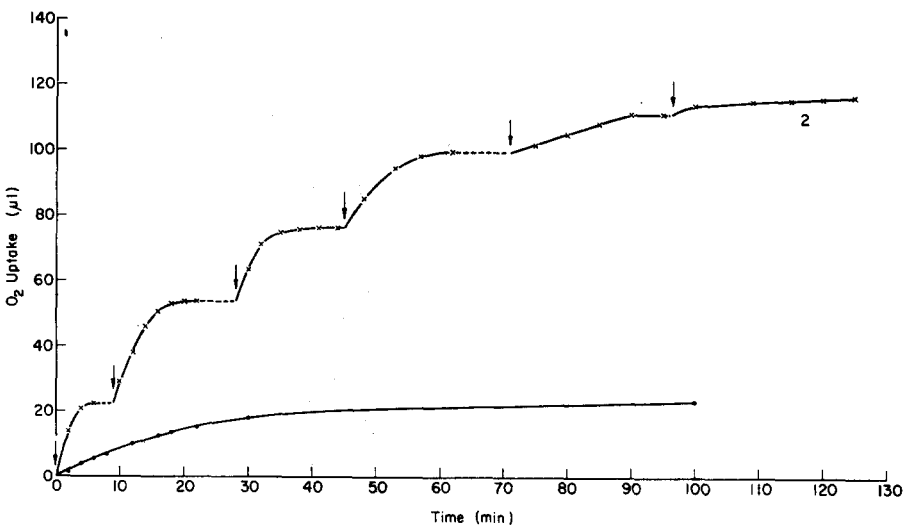

Fig. 2. Increase of apparent activity of the enzyme as the result of stepwise addition of concentrated substrate. Oxygen uptake was measured manometrically at 25°, pH 5.8. Each vessel contained AAO (0.4 µg), $4 \cdot 10^{-2}$ *M* citrate–phosphate buffer, $6 \cdot 10^{-3}$ *M* ascorbic acid and distilled water to make the final reaction volume 2.5 ml. In the experiment corresponding to curve 1, the total substrate was added at zero time, while in Expt. 2, the substrate was added in increments (total/6) as indicated.

in Fig. 1A, the activity of AAO is sensitive to the substrate concentration, *i.e.*, the enzyme exhibits a pronounced substrate concentration optimum. As shown in Fig. 1B, the higher the substrate concentration, the more pronounced and the more rapid is the inactivation. By comparing the data of Fig. 1B with those of Fig. 1A, it can be seen that the three lower curves in Fig. 1B involved inhibitory substrate concentrations.

The two curves in Fig. 2 show very strikingly the effect of substrate inhibition on the reaction inactivation. In the experiment represented by the upper curve, a given quantity of the enzyme catalyzed the ascorbic oxidation of several successive increments of ascorbic acid. Each increment of ascorbic acid was such that the substrate concentration in the reaction vessel never exceeded the optimum concentration (see Fig. 1A). The lower curve in Fig. 2 shows the much lower extent of oxidation that occurred when the same amount of enzyme was involved with a much larger amount of ascorbic acid; an amount equal to the sum of the increments used in the previous experiment. Although it is clear from these experiments that substrate inhibition increases the reaction inactivation, it is to be noted (upper curve) that the reaction inactivation is pronounced in the absence of substrate inhibition. Therefore, reaction inactivation is the result of other factors.

Reaction inactivation and the reaction rate

It has been suggested that the characteristic reaction inactivation of AAO might possibly be accounted for in terms of inactivating substances produced during the reaction. All such causative factors have one feature in common, *i.e.*, the effective concentration of the inactivating substance would be dependent on the rate of the ascorbic acid oxidation reaction. To study the relationship between the rate of the enzymic oxidation reaction and the extent of the reaction inactivation, the experiments indicated in Fig. 3 were performed.

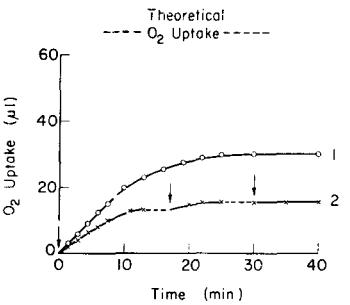

Fig. 3. Decrease of the apparent activity of the enzyme resulting from the stepwise addition of diluted substrate. Oxygen uptake was measured manometrically at 25°, pH 5.8. Each vessel contained AAO (0.2 µg), $4 \cdot 10^{-2}$ *M* citrate–phosphate buffer, $2.4 \cdot 10^{-3}$ *M* ascorbic acid, and distilled water to make the final volume 2.5 ml. In the experiment corresponding to curve 1, the total substrate was added at zero time, while in the Expt. 2, the substrate was added in increments (total/3) as indicated.

In the experiment corresponding to curve 1 in Fig. 3, a given amount of the enzyme and the substrate were involved in the aerobic oxidation reaction. It is to be noted that the amount of oxygen consumed before the enzyme became completely inactivated was approx. 50% of the oxygen required for complete oxidation of the substrate. The amounts of substrate involved in this experiment was such that the substrate concentration was somewhat lower than the optimum. In the experiment represented by curve 2, Fig. 3, the same amount of enzyme was reacted with three successive equal amounts of substrate so that the total amount of substrate used was finally equal to that used in the previous experiment (curve 1). It is to be noted that

the initial rate of oxidation in curve 2 was significantly lower than that in curve 1. Furthermore, the degree of the enzyme inactivation was significantly greater than for curve 1. This experimental finding, was also observed in several other experiments involving different substrate concentrations.

On the basis of these results it is clear that the reaction inactivation cannot be ascribed to the products of the enzymic reaction or to any intermediates, secondary substances, or any entities, whose concentration at any given time would be dependent on the initial enzymic reaction rate.

The by-product question

There remains the possibility that a by-product, a product of a secondary (possibly non-enzymic) reaction proceeding at a much lower rate than the main reaction, may cause the enzyme inactivation. This possibility is particularly attractive if only a very low concentration of the secondary product is required to inactivate the enzyme.

The results shown in Fig. 4 involved three different experiments. In the experiments corresponding to curves 1 and 2, the same amounts of ascorbic acid were used. The total amounts of enzyme used in these two experiments were also the same, but in Expt. 1 the total enzyme was added initially, whereas in Expt. 2 the enzyme was added at the periods indicated in increments of one fifth the amount used in Expt. 1. It is clear that only in the experiment involving the increment additions (curve 2) was enzyme inactivation apparent. Furthermore, the rate and extent of the apparent enzyme inactivation was about the same for the third, fourth and fifth enzyme increments. In the case of the first enzyme increment the rate of the oxidation was

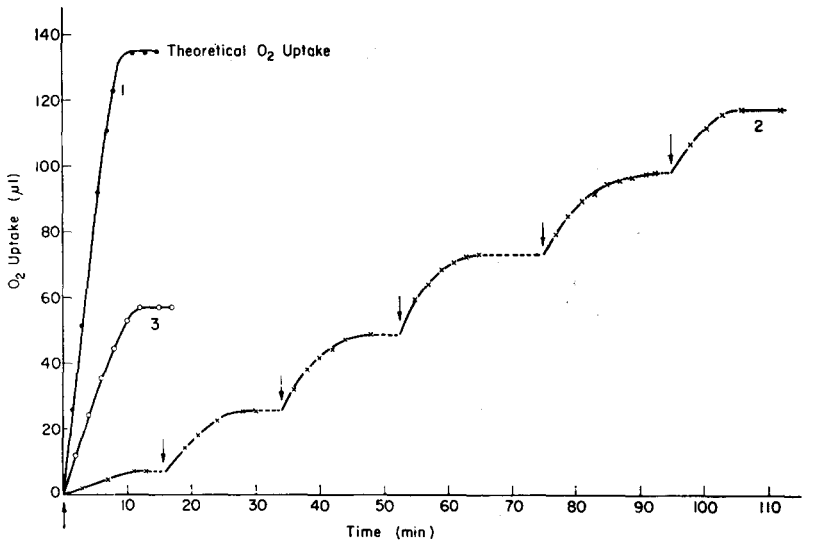

Fig 4. Dependence of the enzyme inactivation on the ratio of enzyme to substrate and the time of the reaction. Oxygen uptake was measured manometrically at 25°, pH 5.8. In Expts. 1 and 2, each vessel contained AAO (2 µg), $4 \cdot 10^{-2}$ *M* citrate–phosphate buffer, $4.8 \cdot 10^{-3}$ *M* ascorbic acid, and distilled water to make the final reaction volume 2.5 ml. In Expt. 1, the total enzyme was added at zero time while in Expt. 2, each increment of AAO (total/5) was added as indicated. In Expt. 3, the reaction vessel contained AAO (0.4 µg), $4 \cdot 10^{-2}$ *M* citrate–phosphate buffer, $2.4 \cdot 10^{-3}$ *M* ascorbic acid, and distilled water to make the final volume 2.5 ml. The enzyme (total/5) was added at zero time.

significantly lower than with the later increments. This was undoubtedly the result of substrate inhibition as discussed earlier.

In the experiment corresponding to curve 3, the amount of the substrate was one half that involved in Expts. 1 and 2. The same quantity of enzyme, however, was used as in each increment addition in Expt. 2. It is to be noted in curve 3 that the time required for enzyme inactivation was very similar to that required for each enzyme increment in Expt. 2. However, it can be seen that in Expt. 3 the enzyme accomplished much more oxidation before it became inactivated. This point is particularly apparent if curve 3 is compared with the oxygen-uptake curve resulting after the fifth enzyme increment addition in Expt. 2; where at the time of addition (75 min) the amount of residual unoxidized substrate was approximately one half the initial substrate concentration (comparable to that used in Expt. 3). The lower rate and extent of oxidation resulting after the fifth enzyme increment in Expt. 2 (as compared to Expt. 3) would suggest the accumulation of an effective inactivating agent (such as hydrogen peroxide) during the previous 75 min of the stepwise oxidation procedure used in Expt. 2.

In the case of Expt. 2, a sixth increment of the enzyme was added at the 95-min period and it was found that the extent of oxidation accomplished by this enzyme and the time required for its inactivation was less than the previous increments. Such results would be anticipated for a system gradually accumulating an enzyme-inactivating agent.

The values of the initial reaction velocity, the inactivation totals and the times required for the inactivation as observed in Expt. 2 are listed in Table I. The effects of substrate inhibition are apparent in the values listed for increments 1 and 2 but the higher increments show a constancy and then decrease as would be expected for a system gradually accumulating an inactivating by-product.

TABLE I

VARIATION OF THE APPARENT INITIAL REACTION VELOCITY (V_i), THE INACTIVATION OXYGEN TOTAL (T_i), AND THE TIME REQUIRED FOR THE INACTIVATION (t_i) WITH SUCCESSIVE ENZYME INCREMENTS DURING THE OXIDATION OF ASCORBIC ACID

All conditions were the same as described in the legend of Fig. 5.

Enzyme increment	V_i *(Nl/min)*	T_i *(Nl)*	t_i *(min)*
1	0.6	7.5	11
2	2.2	18	12
3	3.2	23	12
4	3.9	24	12
5	3.5	25	12
6	3.2	14	8

Evidence that by-products may develop during the enzymic oxidation of ascorbic acid may be obtained by investigating the stability of the residual substrate during the enzymic reaction. The results of such an investigation are presented in Fig. 5. Curve 1 of this figure shows that when ascorbic acid was rapidly oxidized with an excess of enzyme, the theoretical oxygen uptake was obtained. Curve 2 shows that when a much smaller amount of enzyme was initially employed with the same amount

of substrate, the oxygen uptake was determined by the enzyme inactivation. When more enzyme was added, finally in concentrated form, the total oxygen uptake was significantly lower than theoretical. In the experiment represented by curve 2, about a 12% loss in ascorbic acid occurred during a period of about 1 h. Curve 3, in which the enzyme (same amount as in curve 2) was reacted with a much smaller amount of ascorbic acid (one sixth that used in Expt. 2) shows that after the second addition of the similar sized increment of ascorbic acid, the enzyme became inactivated before the substrate was completely oxidized. This fact was established by the lack of oxygen.

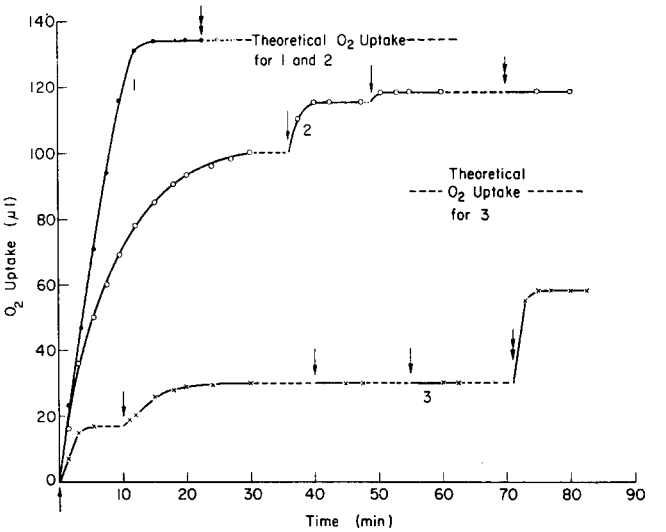

Fig. 5. Dependence of the amount of residual substrate remaining after enzyme inactivation on the extent of the reaction inactivation. Oxygen uptake was measured manometrically at 25°, pH 5.8. Each vessel contained AAO, $4 \cdot 10^{-2}$ *M* citrate–phosphate buffer, ascorbic acid and copper-free water to make the final volume 2.5 ml. In Expt. 1, the ascorbic acid ($4.8 \cdot 10^{-3}$ *M*) was mixed with the enzyme (2 µg) at zero time. In Expt. 2, each increment of enzyme (0.8 µg) was added to the ascorbic acid system ($4.8 \cdot 10^{-3}$ *M*) as indicated by single arrows. In Expt. 3, the enzyme (0.8 µg) was mixed with the substrate in increments (total/6) as indicated by the single arrows, the final substrate concentration being $3.2 \cdot 10^{-3}$ *M*. At the times indicated by the double arrows concentrated enzyme was added to determine the amount of residual substrate.

As can be seen, when an excess of concentrated AAO was added at about the 70-min period, the oxygen uptake was about 35% lower than expected.

These experiments (curves 2 and 3) reveal that when unoxidized (or residual) ascorbic acid is permitted to remain for a period of time in a system containing inactivated enzyme and the normal oxidation products of the enzymic oxidation, some of the residual ascorbic acid is lost, presumably by a secondary reaction.

On the autoxidation of the substrate

When the concentration of AAO is high, *i.e.*, the enzymic oxidation of ascorbic acid is finished in a short period, no reaction inactivation is observed, and the total amount of oxygen uptake is the theoretical value. However, as the enzymic oxidation of ascorbic acid proceeds over a longer period of time, enzyme inactivation becomes apparent. The fact that enzyme inactivation becomes the more apparent the slower the enzymic oxidation is conducted, suggests that a certain amount of time is required

for the accumulation of a by-product that may be responsible for the enzyme inactivation. For example, the accumulation of hydrogen peroxide by autoxidation of ascorbic acid would not be very significant in a fast enzymic oxidation but would become increasingly significant as the rate of the enzymic oxidation is decreased.

To check the possibility that simple autoxidation of the substrate might be responsible for the enzyme inactivation, the effect of exposing the substrate to the reaction conditions in the absence of enzyme for varying periods of time before initiating the enzymic oxidation (by adding the enzyme) was investigated. It was found through such preincubation experiments that the initial reaction velocities, inactivation totals and inactivation times (the time required for a complete enzyme inactivation) were not significantly different. In other words, it may be concluded that simple autoxidation of the substrate in the absence of the enzyme cannot account for the reaction inactivation phenomenon.

The effects of catalase and gelatin

As previously pointed out, earlier experiments carried out in these laboratories established that catalase was a much more effective agent than gelatin in protecting the enzyme against inactivation and neither agent appeared to affect the initial rate of ascorbic acid oxidation as catalyzed by the enzyme. In view of the accumulating evidence that hydrogen peroxide might after all be the factor responsible for the marked reaction inactivation of the enzyme, it seemed advisable to carefully reinvestigate the effects of adding catalase and gelatin (separately) before, during, and after the enzymic reaction.

It was found that the observable effect of catalase or gelatin on the initial reaction rate is dependent on the rate, *i.e.*, on the amount of AAO employed. This fact escaped the earlier investigators who did not study the effects of these protective agents at sufficiently low enzyme concentration. The new experimental results are shown in

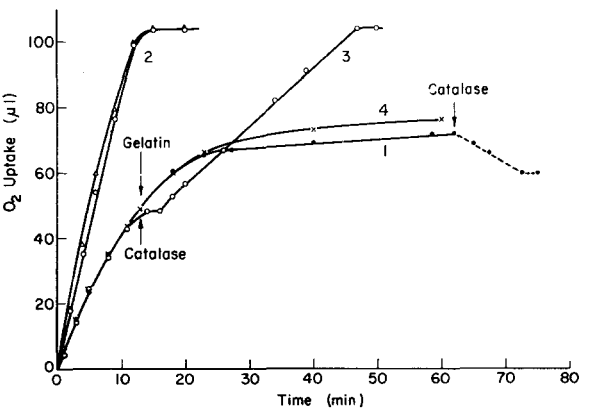

Fig. 6. Effects of adding catalase and gelatin at various times on the reaction course. Oxygen values were measured manometrically at 25°, pH 5.8. In addition to the components shown below, each vessel contained AAO (0.4 µg), $4 \cdot 10^{-2}$ *M* citrate–phosphate buffer, $4 \cdot 10^{-3}$ *M* ascorbic acid and copper-free water to make the final reaction volume 2.5 ml. Expt. 1, no catalase or gelatin initially. 18 µg catalase was added later as indicated. Expt. 2, 18 µg catalase or 1 mg gelatin was added to the buffered enzyme system before initiating the reaction by addition of substrate. Expt. 3, 18 µg catalase was added during the reaction as indicated. Expt. 4, 1 mg gelatin was added during the reaction as indicated.

Fig. 6. The control experiment (curve 1) shows that in the absence of protective agents, the enzyme was inactivated when only about 70% of the ascorbic acid had been oxidized. Curve 2 shows that the initial addition of a very small amount of catalase, or a considerably larger amount of gelatin, gave complete protection to the enzyme with the result that the theoretical oxygen uptake was rapidly obtained. When the control experiment was repeated and the same amount of catalase was added at about the 12-min period, a slight lag in oxygen uptake occurred (see curve 3) and then the rate became essentially linear (no further inactivation) until the ascorbic acid was completely oxidized. When a similar experiment with gelatin was conducted, the results were very different (see curve 4). There was little or not protective effect of gelatin when it was added during the course of the reaction. It should be noted that in the control experiment (curve 1) catalase was added at the 62-min period after the enzyme had been completely inactivated and a small evolution of gas resulted (presumably due to hydrogen peroxide decomposition). The addition of gelatin at this point was without effect. Furthermore, the addition of catalase after the rapid and complete oxidation of ascorbic acid by an excessive amount of ascorbic acid oxidase, never resulted in any evidence of hydrogen peroxide accumulation.

It is apparent from these experiments that the effectiveness of catalase as a protective agent against the reaction inactivation of AAO is very much greater than that of gelatin. It seems logical to conclude that the protective action of catalase as demonstrated in the above experiment is evidence for the production of a small amount of hydrogen peroxide which is produced by a slower and secondary reaction. The mechanism of the gelatin protective effect is considerably more obscure but it seems possible that the gelatin is simply neutralizing hydrogen peroxide by chemical reaction in a rather slow and ineffective manner. When gelatin is added initially, it can cope with the slow production of hydrogen peroxide and thereby protect the enzyme if a relatively large amount (in comparison to the enzyme protein) is present. However, when gelatin is added to the system after a certain amount of excess hydrogen peroxide has accumulated, the gelatin does not neutralize this hydrogen peroxide rapidly enough to prevent the continuing inactivation of the enzyme. As a consequence, little or no protection is observed (see curve 4).

The inhibitory effect of H_2O_2 on AAO

If it is assumed that the loss of the residual ascorbic acid is the result of a secondary oxidation reaction producing H_2O_2, then the amount of H_2O_2 involved can be estimated in the following way. The difference in O_2 uptake between the theoretical value (see Fig. 5, curve 1) and the observed value after the residual ascorbic acid had been completely oxidized in the presence of excess enzyme (see Fig. 5, curve 2) corresponds to a loss of n μmoles of ascorbic acid in about one hour. This loss in ascorbic acid is equivalent to the production of n μmoles of H_2O_2 during the same period. This type of calculation applied to many different systems similar to the case indicated in Fig. 5 has revealed that in a 2.5-ml reaction system containing initially 0.4 μg of AAO with the optimum ascorbic acid concentration, the production of H_2O_2 is about 1 μmole/h, *i.e.*, the concentration of H_2O_2 in this system can develop to about $4 \cdot 10^{-4}$ M in 1 h. In order to determine whether or not such small amounts of H_2O_2 would cause a recognizable inactivating effect on AAO during a 10-min period, the experiments reported in Table II and Fig. 7 were performed.

TABLE II

EFFECTIVENESS OF HYDROGEN PEROXIDE AS AN INACTIVATING AGENT

Oxygen uptake was measured manometrically at 25°, pH 5.8. Hydrogen peroxide in each concentration described in the table, was preincubated with shaking at 25°, for 10 min with AAO before initiating the reaction by the addition of substrate. Each vessel contained AAO as indicated, $4 \cdot 10^{-2}$ *M* citrate–phosphate buffer, $4 \cdot 10^{-3}$ *M* ascorbic acid, hydrogen peroxide, and copper-free water to make the final volume 2.5 ml.

Concentration of H_2O_2 ($M \times 10^4$)	*Expt. 1 (AAO: 0.4 μg)*			*Expt. 2 (AAO: 2 μg)*		
	V_i *(μl/min)*	T_i *(μl)*	t_i *(min)*	V_i *(μl/min)*	T_i *(μl)*	t_i *(min)*
4	0	0	—	1.5	27	3
2	0	0	—	1.5	36	5
0.4	0	0	—	1.5	75	10
0 (control)	8	60	20	15	104**	7*

* The time required to complete the oxidation of the ascorbic acid.
** Theoretical value.

It can be seen (Table II) that the inactivating effect of a given concentration of H_2O_2 as revealed by the values of V_i, T_i and t_i when compared to those of a control system, was strikingly apparent when the amount of enzyme involved was 0.4 μg in a 2.5-ml reaction volume (Expt. 1). When the amount of AAO used was enough to cause the complete and rapid oxidation of the ascorbic acid (see control, Expt. 2) the direct relationship between the inactivating effect of the H_2O_2 and its concentration became apparent in the values of T_i and t_i. It is to be noted that the enzyme concentration used in Expt. 1 was similar to that employed in the previously described inactivation experiments. It may be concluded, therefore, that AAO is very sensitive to hydrogen peroxide, and very small amounts of the latter which are not manometrically detectable by catalase* are sufficient to cause rapid and extensive inactivation of AAO.

When AAO is incubated for a 10-min period with a very small amount of H_2O_2 ($4 \cdot 10^{-5}$ *M*)* and then allowed to react with ascorbic acid, the results reveal

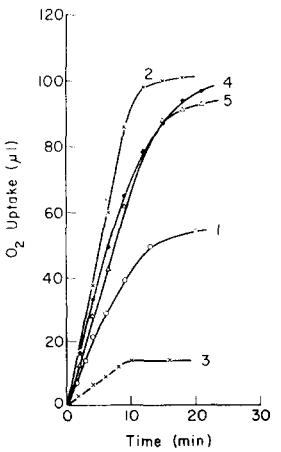

Fig. 7. Protective effects of gelatin and catalase against AAO inactivation by hydrogen peroxide. Oxygen uptake was measured manometrically at 25°, pH 5.8. In addition to the components described below, each vessel contained AAO (2 μg), $4 \cdot 10^{-2}$ *M* citrate–phosphate buffer, $4 \cdot 10^{-3}$ *M* ascorbic acid, and copper-free water to make the final reaction volume 2.5 ml. Expt. 1, no catalase or gelatin. Expt. 2, 1 mg gelatin or 18 μg catalase was preincubated with the AAO for 10 min. Expt. 3, $4 \cdot 10^{-5}$ *M* hydrogen peroxide was preincubated with the AAO for 10 min. Expt. 4, in the presence of 1 mg gelatin, $4 \cdot 10^{-5}$ *M* hydrogen peroxide was preincubated with the AAO for 10 min. Expt. 5, in the presence of 18 μg catalase, $4 \cdot 10^{-5}$ *M* hydrogen peroxide was preincubated with the AAO for 10 min.

* 10^{-4} *M* H_2O_2 in 2.5 ml reaction volume corresponds to 8.5 μg of H_2O_2. This amount of H_2O_2, when completely decomposed by catalase, would cause an evolution of 2.5 μl of oxygen, a volume close to the experimental error of the manometer.

extensive inactivation of the enzyme (compare curves 1 and 3, Fig. 7). When catalase or gelatin are present during the preincubation of the enzyme with H_2O_2 only a little destruction of the AAO is observed (compare curves 4 and 5 with 3 and 2, Fig. 7). The experiments of Fig. 7, therefore, support those of Fig. 6 in which the results of adding catalase and gelatin to the functioning AAO system were interpreted in terms of a very slow production of H_2O_2 by a secondary reaction.

Concerning copper and the H_2O_2 production

It has been demonstrated that the small amount of H_2O_2 produced by a slow and secondary reaction during the enzymic oxidation of ascorbic acid is sufficient to account for the enzyme inactivation observed during the reaction. Attention may now be turned to the role of the enzyme copper in this H_2O_2 production. To obtain information on this point, a number of experiments were performed to demonstrate that the production of H_2O_2 during a period of incubation, involving the substrate and a variety of modified enzymes or artificial enzyme models, was dependent on the presence of copper in the incubation system. Thus in the experiments corresponding to curves 1 and 2 of Fig. 8A the effect of preincubating the substrate with boiled AAO was evaluated. It can be seen that the boiled (denatured) enzyme, containing bound copper, produced sufficient H_2O_2 during a 30-min preincubation period with the substrate to cause rapid and extensive inactivation of the native AAO as soon as

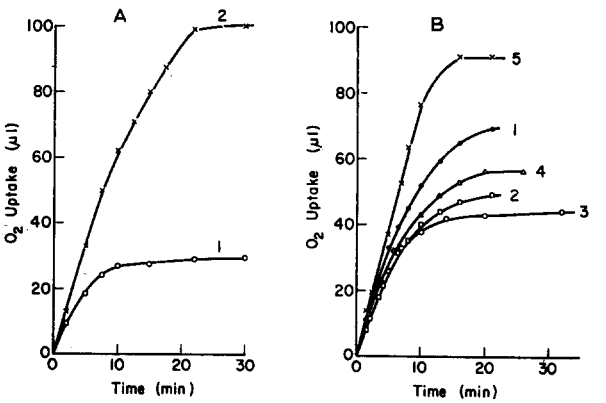

Fig. 8. A, Production of an inactivating or inhibitory substance by boiled AAO during preincubation. Oxygen uptake was measured manometrically at 25°, pH 5.8. Each vessel contained AAO (0.4 µg), boiled AAO (10 min, 4 µg), $4 \cdot 10^{-2}$ *M* citrate–phosphate buffer, $4 \cdot 10^{-3}$ *M* ascorbic acid and distilled water to make the final reaction volume 2.5 ml. In Expt. 1, the substrate was preincubated at 25° with the boiled AAO for 30 min with shaking and then the active AAO, which had also been thermostated at 25° for 30 min was added to the mixture to initiate the reaction. In Expt. 2, the active AAO, boiled AAO, and substrate were separately preincubated for 30 min and then mixed at zero time. B, Influence of the preincubation of ascorbic acid, under certain conditions that promote hydrogen peroxide production, on the subsequent enzyme inactivation. Oxygen uptake was measured manometrically at 25°, pH 5.8. Besides the following components described below, each vessel contained AAO (0.8 µg), $4 \cdot 10^{-2}$ *M* citrate–phosphate buffer, $4 \cdot 10^{-3}$ *M* ascorbic acid, and copper-free water to make the final volume 2.5 ml. In each case the substrate was preincubated with each of the components listed below for 30 min at 25° with shaking before adding the active enzyme at zero time. Expt. 1, none (substrate preincubated alone was added to the intact AAO at zero time). Expt. 2, 2 µg copper-containing boiled AAO. Expt. 3, $6 \cdot 10^{-8}$ *M* $CuSO_4$. Expt. 4, $6 \cdot 10^{-8}$ *M* $CuSO_4$ plus 4 µg bovine serum albumin. Expt. 5, 15 µg copper-free AAO obtained by KCN-treatment followed by dialysis against copper-free distilled water, or 4 µg bovine serum albumin.

TABLE III

PRODUCTION OF H_2O_2 BY VARIOUS SYSTEMS AS MEASURED BY CATALASE DECOMPOSITION

Oxygen output was measured manometrically at 25° within 5 min after the addition of 18 μg catalase which was added to $4 \cdot 10^{-3}$ M substrate preincubated with the indicated components in the presence of $4 \cdot 10^{-2}$ M citrate–phosphate buffer (pH 5.8). Preincubation was carried out at 25° for 30 min with shaking. The concentrations of Cu^{2+} (sulfate salt) used were $6 \cdot 10^{-9}$ M and $6 \cdot 10^{-8}$ M in series A and B, respectively. The amount of protein used in all cases was 2 μg and the total reaction volume was made 2.5 ml by adding copper-free water to each vessel.

	Components preincubated with substrate	*Amount of gas evolved (μl)*
Boiled AAO (for 10 min)		3
Copper-free AAO		0
Cu^{2+}	A	1.5
Cu^{2+}	B	2.7
Cu^{2+} + BSA	A	1.5
Cu^{2+} + BSA	B	2.6

it was added to the system. In Fig. 8B similar experiments are depicted involving preincubation of the substrate with cupric ion (curve 3), cupric ion plus BSA (curve 4) and copper free AAO or BSA alone (curve 5). A comparison of these curves with curve 1 (the control) and curve 2 (boiled AAO) makes it clear that the protein part of the AAO is not the cause of the H_2O_2 production but rather it is the copper.

The data in Table III showing how varying degrees of H_2O_2 production can be estimated by catalase decomposition, have been included to confirm that the preincubation of the substrate with boiled (denatured) AAO and with copper free AAO, cupric ion alone, and cupric ion with BSA, resulted in H_2O_2 production whenever copper was involved in the preincubation system.

DISCUSSION

As a basis for discussing the results obtained in this investigation, the following three equations may be postulated to account for the oxidation of ascorbic acid catalyzed by the copper protein (Cu-P), ascorbic acid oxidase.

Eqn. 1: Main, fast	$AH_2 + \frac{1}{2} O_2$	$\xrightarrow{\text{Cu-P (AAO)}}$	$A + H_2O$
Eqn. 2: Secondary, slow	$AH_2 + O_2$	$\xrightarrow{\text{Cu-P}}$	$A + H_2O_2$
Eqn. 3: Inactivation, fast	Cu-P (AAO) + H_2O_2	$\longrightarrow$	Inactive enzyme

Based on the amount of H_2O_2 calculated to be found during a 10-min period in the typical activity-measurement system, it can be estimated that the main reaction (Eqn. 1) is at least one hundred times as fast as the secondary reaction (Eqn. 2). Because of the marked sensitivity of the enzyme to a very small amount of H_2O_2, it is probably that a high percentage of the collisions between AAO and H_2O_2 in Eqn. 3 are effective in producing inactivation of the enzyme, but the very low concentrations of AAO and H_2O_2 involved in this reaction make the overall rate relatively low. Under these circumstances, therefore, the inactivation phenomenon shows no simple relationship to the rate of the enzymic oxidation but rather

varies directly with the amount of time that has elapsed in the oxidation process: time during which enough H_2O_2 may accumulate in the system to be detectable for catalase decomposition.

When catalase is added initially to the AH_2–AAO system, there is frequently little or no change in the initial reaction velocity. This fact has been used earlier[4] to argue against H_2O_2 involvement in the protection of AAO activity by catalase. However, in the view postulated above it becomes apparent that the amounts of H_2O_2 decomposed by catalase during the initial reaction would be too small to be experimentally significant in terms of oxygen uptake measurement and that under certain circumstances the destruction of such small amount of H_2O_2 by catalase could result in an apparent increase in the initial reaction velocity.

It has been known for some time that the copper content of AAO corresponds to six copper atoms per molecule of enzyme. However, it is still not known whether or not all of the copper atoms are simultaneously involved in the enzyme activity represented by Eqn. 1. Exchange experiments with radioactive ^{64}Cu (refs. 11, 12) have revealed that most of the enzyme copper (85% or more) undergoes exchange while the enzyme is functioning. However, it may be that a small fraction of the copper (15% or less) is not directly involved in the enzyme activity and might therefore be available for the secondary reaction producing H_2O_2 as represented by Eqn. 2.

At the present time, there is no information concerning the way in which the six copper atoms of the enzyme molecule are structurally located on the protein moiety. They may be located singularly at six different activity sites or a given activity center may involve a cluster of two or possibly more copper atoms. In either case it would appear to be possible that a given copper atom might function at one moment in the manner represented by Eqn. 1 and somewhat later be involved in the much slower reaction producing H_2O_2 as represented by Eqn. 2. It would seem that the manner in which the particular copper atom entered into the transitory complex with the approaching substrate molecule might be the determining factor concerning whether or not Eqn. 1 or 2 would take precedent.

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