Studies on Ascorbic Acid. I. Factors Influencing the Ascorbate-Mediated Inhibition of Catalase*

Charles W. M. Orr†

ABSTRACT: The inhibition of catalase by ascorbate, and ascorbate plus Cu^{2+}, has been studied. In the presence of Cu^{2+}, the inhibition is very rapid. It is independent of substrate concentration and pH, but is strongly influenced by temperature. The temperature dependence reflects, at least in part, the rate of the reaction between Cu^{2+} and ascorbate. The inhibition can be prevented if solutions of sufficiently high ionic strength are used ($\pi/2 = 0.1$), but increases rapidly to a maximum when the ionic strength is reduced ($\pi/2 = 0.002$). Dimethyl sulfoxide reduces the inhibition by 50%. Inhibition studies with ascorbate alone have demonstrated clearly that ascorbate, by itself, inhibits catalase. The inhibition is potentiated by EDTA and by iron, and a synergistic action has been found when both iron and EDTA are used with ascorbate. Dimethyl sulfoxide reduces the inhibition by ascorbate. It is concluded that free-radical transients, known to be generated during the oxidation of ascorbate, are responsible for the inhibition of catalase.

The most likely candidates are $\cdot OH$ and $\cdot O_2H$; possibly both are involved

Foulkes and Lemberg (1948) concluded from a manometric study of the inhibition of catalase by ascorbate that Cu^{2+} was a necessary participant in the reaction. They did not, however, propose a mechanism for the reaction. The same authors have suggested that a ferric–ascorbate complex occurs between catalase and ascorbate (Lemberg and Foulkes, 1948). This view was challenged by Chance (1950) who demonstrated spectrophotometrically that an enzymatically inactive catalase species (complex II) is formed when ascorbate and catalase are incubated together. Chance concluded that ascorbate autoxidation occurred during the prolonged incubation of catalase with ascorbate resulting in a slow, continuous evolution of H_2O_2. Under these conditions, complex II is formed. More recently, it has been suggested (Orr, 1966) that the inhibition of catalase by ascorbate in the presence of Cu^{2+} could be due to the production of transient free-radical species formed in the Cu^{2+}-catalyzed autoxidation of ascorbate. In this report, data in support of this concept are considered and, in conjunction with data in a subsequent paper (Orr, 1967) where the relationship of these results to those of Chance (1950) are discussed, it is suggested that the inhibition of catalase not only by Cu^{2+} and ascorbate, but also by ascorbate alone (where any effect of Cu^{2+} is excluded), can be ascribed to free-radical attack of the protein.

Materials and Methods

The materials and methods have been described in a previous communication (Orr, 1966). Chemicals which were not referred to in the Experimental Section of the previous report were all analytical reagent grade quality.

Experimental Section

The Effect of Substrate Concentration, Temperature, and Inhibitor Concentration on the Kinetics of the Inhibition. Previously it was shown (Orr, 1966) that the inhibition of catalase by 2×10^{-4} M ascorbate is greatly potentiated by 2×10^{-6} M Cu^{2+}. At concentrations of Cu^{2+} higher than this the inhibition is almost too rapid to measure adequately under the conditions of the assay. The parameters of the inhibition have been examined in greater detail. The effect of varying the temperature, and the concentrations of H_2O_2 and ascorbate, at a constant Cu^{2+} concentration (2×10^{-6} M) was measured. The data from these experiments are shown in Table I. Quite clearly, the inhibition is unrelated to the concentration of substrate; over a 2.5-fold range of H_2O_2 concentrations, the inhibition for a given set of conditions remains unchanged.

The inhibition is markedly temperature dependent. When catalase solutions were incubated for 10 min at 0° (ice) in the presence of Cu^{2+} and ascorbate, and assayed immediately, essentially no inhibition occurred. However, it has been found by spectrophotometric assay of ascorbate at 265 mμ, that the reaction between Cu^{2+} and ascorbate at 0° is very slow. After 10-min incubation at 0°, almost no ascorbate has been oxidized, since the spectrum remains essentially unchanged.

* From the Industrial Hygiene Research Laboratories, I.C.I., Ltd., Alderley Edge, Cheshire, England. *Received June 12, 1967.*

† Present address: The McCollum-Pratt Institute, Department of Biology, The Johns Hopkins University, Baltimore, Md. 21218.

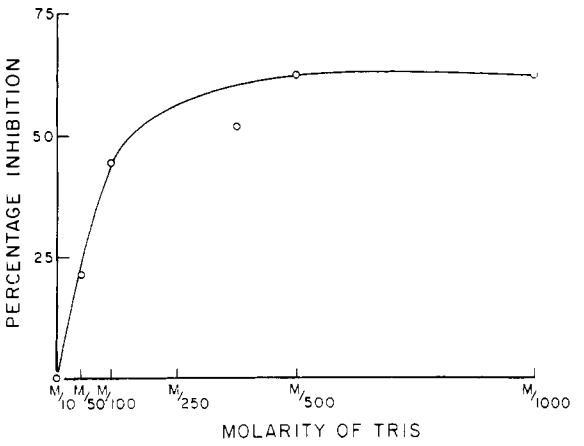

FIGURE 1: The effect of ionic strength on the inhibition of catalase by Cu^{2+} and ascorbate. The assay was run at 37° for 10 min at 2×10^{-6} M Cu^{2+} and 2×10^{-4} M ascorbate.

TABLE I: The Effect of Temperature, Ascorbate Concentration, and Substrate Concentration in the Presence of 2×10^{-6} M Cu^{2+} on the Inhibition of Catalase after 10-min Incubation.

Temp (°C)	Concn of Ascorbate (M)	% Inhibn: Concn of H_2O_2 (μmoles) 42	65	86	105
37	2×10^{-6}	15	22.3	25	30
25		29.5	25	25	23
0		0	0	7.5	0
37	2×10^{-5}	66.2	61.1	63.7	70
25		57.4	56	57.7	57.9
0		0	0	14	0
37	2×10^{-4}	69.7	69.8	70.7	76
25		59	53.5	59.5	53.5
0		7.5	0	14	0

The temperature dependence of the reaction can therefore be related to the rate of the catalysis of ascorbate by Cu^{2+}, which under these conditions is maximal at 37° and 2×10^{-5} M ascorbate.

The Effect of pH on the Inhibition. The effect of pH on the inhibition of catalase was tested using Sorenson phosphate buffers. These were prepared after shaking the respective phosphate solutions with 8-hydroxyquinoline and subsequent extraction of the 8-hydroxyquinoline with chloroform. No effort was made to test the effect of pH above 8 since it has been shown (Chance, 1954) that H_2O_2 decomposes spontaneously in this pH range.

Catalase was incubated in M/100 Sorenson's phosphate buffer with 2×10^{-4} M ascorbate and 2×10^{-6} M Cu^{2+} at pH 7.75, 7.3, 6.85, and 6.3. After 10-min incubation at 37°, an aliquot of each was assayed. No effect of pH could be demonstrated; the inhibition in all cases was approximately 40%.

The Effect of Ionic Strength on the Reaction. It has been claimed (Foulkes and Lemberg, 1948) that an increase in the ionic strength of phosphate buffer from M/2150 to M/105 completely prevents the inhibition of catalase by ascorbate. However, their data indicate that catalase in the absence of ascorbate is 40% less active in M/105 phosphate. No explanation for this effect or for the reduced inhibition is given; however, it is reasonable to suggest that residual Cu^{2+}, apparently present in Foulkes and Lemberg's preparation, is effectively removed from solution at the higher phosphate concentration.

It has been found here that Tris, which does not chelate Cu^{2+} (Good *et al.*, 1966), completely prevents the inhibition of catalase by Cu^{2+} and ascorbate when the incubation is run at M/10 Tris (pH 7.0). In fact, at this molarity, the activity of catalase was slightly higher in the presence of Cu^{2+} and ascorbate. As the molarity of Tris is decreased the inhibition increases rapidly and is maximal at M/500 (Figure 1).

The Effect of EDTA on the Inhibition of Catalase by Ascorbate. It has always been difficult to rule out the possibility that Cu^{2+} is present in trace amounts ($\leqslant 10^{-8}$ M), and that this level of contamination is responsible for the inhibition of catalase in supposedly uncontaminated solutions of ascorbate. In interpreting their results, Foulkes and Lemberg (1948) conclude that the presence of contaminating Cu^{2+} is essential for the inhibition of catalase by ascorbate. They draw this conclusion from the effect of the chelating agent diethyl dithiocarbamate[1] on the reaction. At a concentration of 2×10^{-4} M, DEDTC clearly reduces the inhibition by ascorbate, but by no means completely. If their data are calculated as per cent inhibition after incubation of catalase with 2×10^{-4} M ascorbate, the inhibition in the presence of DEDTC is still 40% after 20-min incubation at 37°.

In view of these results, it was felt that if contaminating Cu^{2+} was present and responsible for the ascorbate effect found here, a reduction in the per cent inhibition would be expected if DEDTC was included in the incubation mixture. It was found that at 2×10^{-4} M ascorbate, the degree of inhibition of catalase after 10 and 20 min of incubation was identical in the presence or absence of 2×10^{-4} M DEDTC (at this concentration, DEDTC has no effect on catalase activity). From these data, it can be concluded that ascorbate alone inhibits catalase.

However, as another check to demonstrate that the inhibition in the absence of Cu^{2+} was due only to ascorbate, EDTA was included in the reaction. Incubation mixtures were prepared containing catalase, 2×10^{-4} M ascorbate, and 2×10^{-4} M EDTA (sodium salt) in M/100 Tris buffer at pH 7.0. The incubation

[1] Abbreviations used: DEDTC, diethyl dithiocarbamate; DMSO, dimethyl sulfoxide.

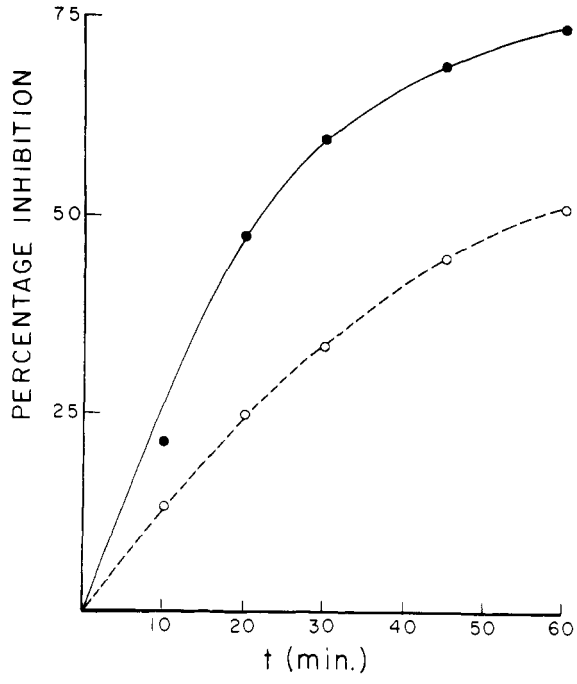

FIGURE 2: The effect of EDTA on the inhibition of catalase by ascorbate. (–O–O–O–) Catalase and 2×10^{-4} M ascorbate; (–●–●–●–) catalase, 2×10^{-4} M ascorbate, and 2×10^{-4} M EDTA.

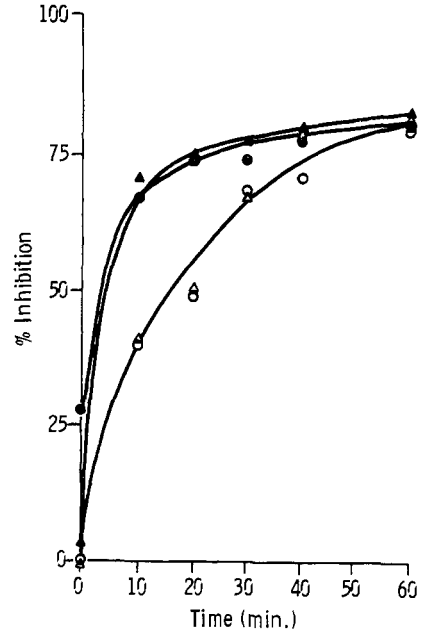

FIGURE 3: The inhibition of catalase by ascorbate with Fe^{2+} or Fe^{3+} in the presence or absence of EDTA. (–△–△–△–) Catalase, 2×10^{-4} M ascorbate, and 2×10^{-6} M Fe^{2+}; (–O–O–O–) catalase, 2×10^{-4} M ascorbate, and 2×10^{-6} M Fe^{3+}; (–▲–▲–▲–) catalase, 2×10^{-4} M ascorbate, 2×10^{-6} M Fe^{2+}, and 2×10^{-4} M EDTA; (–●–●–●–) catalase, 2×10^{-4} M ascorbate, 2×10^{-6} M Fe^{3+}, and 2×10^{-4} M EDTA.

was run at 37° and aliquots were removed and assayed. Control tubes without EDTA were run concurrently. The per cent inhibition was determined by comparison to catalase alone or to catalase with EDTA (the presence of EDTA did not affect the activity of catalase). The results are shown in Figure 2. Obviously, EDTA increases the ascorbate-mediated inhibition of catalase. In both cases (Figure 2) the inhibition is linear with time for 20 min and then slowly levels off. The increased inhibition due to EDTA is approximately 25%. In considering this result, the possibility that iron was present as a contaminant in the EDTA preparation could not be ruled out. It is known (Chalk and Smith, 1954) that the Cu^{2+}–EDTA and iron–EDTA chelates are different. In the latter case, it has been shown by Udenfriend *et al.* (1954) that such a chelate is active in promoting the hydroxylation of aromatic compounds by ascorbate. It seems reasonable to suggest such a mechanism here. The idea was tested by examining the effect of iron on the inhibition of catalase by ascorbate.

The Effect of Fe^{2+} and Fe^{3+} on the Inhibition of Catalase. In preliminary experiments it was found that either Fe^{2+} or Fe^{3+} (as their respective sulfates) inhibited catalase strongly (86%) at a concentration of 2×10^{-5} M. However, at 2×10^{-6} M the inhibition by either ion after incubation at 37° for 10 and 60 min was 11 and 18%, respectively. It was felt that the level of inhibition was tolerable in testing the effect of iron or EDTA-chelated iron on the inhibition of catalase by ascorbate.

The effect of Fe^{2+} or Fe^{3+} at a concentration of 2×10^{-6} M on the ascorbate-mediated inhibition of catalase was tested by incubating each ion separately with 2×10^{-4} M ascorbate and catalase. The reaction was run at 37° and aliquots were removed and assayed with time. The results are shown in Figure 3. It can be seen that both ions are equally effective in increasing the inhibition of catalase by ascorbate. If the data of Figures 2 and 3 are compared, it is apparent that after 30 min at 37° the presence of 2×10^{-6} M iron in the reaction mixture has resulted in a twofold increase in the degree of inhibition. When EDTA (2×10^{-4} M) is included in the reaction mixture with iron both the rate and amount of inhibition is dramatically increased (upper curves, Figure 3); after 10 min catalase is inhibited by 80% and the reaction is essentially complete. Consequently, the data support the hypothesis suggested above, *i.e.*, that the increased inhibition of catalase by ascorbate when EDTA is present could be due to traces of chelated iron in the reaction mixture.

The Effect of Dimethyl Sulfoxide. The results presented in this paper and in a preceding publication (Orr, 1966) strongly suggest that the ascorbate-mediated inhibition of catalase is due to free-radical attack of the protein. If this is true, it should be possible to reduce the inhibition by the introduction of a free-radical sink. Many radical quenchers are known, but a most

appropriate candidate would appear to be DMSO since it has recently been shown by Lohmann *et al.* (1965) that DMSO reduces the inhibition of catalase when the combination is exposed to X-ray irradiation.

Preliminary experiments demonstrated that DMSO (2.8×10^{-4} M) did not affect catalactic activity. To test the effect of DMSO on the inhibition of catalase by ascorbate, incubations were made at 25° and included 2×10^{-4} M ascorbate, catalase, and when present, 2.8×10^{-4} M DMSO. The results are given in Table II.

TABLE II: The Effect of DMSO on the Inhibition of Catalase by Ascorbate.

Time (min) of Incubn at 25°	% Inhibition		
	DMSO	Ascorbate	DMSO + Ascorbate
0	0	0	0
10	0	28.5	6.5
30	0	66.5	31.0
60	0	79.5	55

It is apparent that DMSO protects catalase quite dramatically from inhibition by ascorbate. Higher concentrations (2.19×10^{-3} M) of DMSO were tried in an attempt to increase the protective effect. No further reduction in inhibition occurred; in fact, at this concentration DMSO by itself is slightly inhibitory to catalase.

DMSO also protects catalase from inhibition by Cu^{2+} and ascorbate. When catalase was incubated with 2×10^{-6} M Cu^{2+} and 2×10^{-4} M ascorbate in the presence of 2.8×10^{-4} DMSO for 30 min at 25°, the inhibition was reduced by 50%.

Discussion

Foulkes and Lemberg (1948) were the first to demonstrate the inhibition of catalase by ascorbate and Cu^{2+}, but no mechanism for the reaction was proposed. The formation of an enzymatically inactive H_2O_2–catalase complex, known as complex II, was elegantly demonstrated by Chance (1950) when ascorbate was incubated with catalase, and Keilin and Hartree (1951) showed that the same complex occurred if Cu^{2+} and ascorbate were incubated with catalase. It will be shown in the succeeding paper (Orr, 1967) that complex II could not be demonstrated and in view of that result, and those presented in the present report, a second mechanism involving free-radical attack of the protein has been proposed. In support of this contention the following considerations are relevant. (1) The inhibitory species was a transient product of the Cu^{2+}-catalyzed autoxidation of ascorbate (Orr, 1966). (2) The production of free radicals in the Cu^{2+}-catalyzed autoxidation of ascorbate has been postulated by Dwyer (1964) on the basis of the work of Weissburger *et al.* (1943) and Weissberger and LuValle (1944). Dwyer (1964) considers that the perhydroxy radical, $\cdot O_2H$, would be formed on oxidation of the ascorbate semiquinone. (3) When Cu^{2+} and ascorbate are incubated with aniline, *o*-aminophenol is formed. This indicates that $\cdot OH$ or $\cdot O_2H$ are formed (C. W. M. Orr, in preparation). (4) Further evidence for the presence of free radicals formed during the interaction of Cu^{2+} and ascorbate is suggested from the work of Matsumura and Pigman (1965) and Barker *et al.* (1965) on the degradation of hyaluronic acid during incubation with Cu^{2+} and ascorbate. (5) It will be shown in a subsequent paper (Orr, 1967) that the inhibition is apparently due to physical degradation of the catalase molecule resulting in a number of lower molecular weight fragments.

In this report, the interaction between Cu^{2+}, ascorbate, and catalase has been investigated further. It has been shown that the inhibition was independent of pH and substrate concentration, but that it was weakly temperature dependent between 25 and 37° and that maximal inhibition occurred at 2×10^{-5} M ascorbate. The almost complete lack of inhibition at 0° has been found to be due to the lack of interaction between Cu^{2+} and ascorbate.

The inhibition is extremely sensitive to the ionic strength of the incubation mixture. At high ionic strengths (M/10 Tris), no inhibition occurs. Incubation at progressively lower ionic strengths resulted in increased inhibition reaching a maximum at M/500 Tris. Since the rate of ascorbate autoxidation is independent of ionic strength (as determined by the disappearance of OD_{265}, the λ_{max} for ascorbate), it must be concluded that the catalase molecule is altered. Possibly, it becomes unfolded (and presumably, therefore, more susceptible to free-radical damage) as the ionic strength is lowered. It should be noted that no change in activity as a function of ionic strength was recorded in control incubations except at M/10 Tris where a slight reduction in activity occurs (at M/10 Tris the activity of catalase in the presence of Cu^{2+} and ascorbate is actually greater than in their absence).

Finally, it was demonstrated that the inhibition by Cu^{2+} and ascorbate is reduced by 50% in the presence of DMSO. The latter compound is known to be a free-radical quencher. In this regard, it is pertinent that Lohmann *et al.* (1965) have shown that marked protection of catalase to X-ray irradiation was afforded by DMSO.

It appears that catalase is not unique in its sensitivity to inhibition by Cu^{2+} and ascorbate; Levvy and Marsh (1957) have shown that β-glucuronidase is strongly inhibited by Cu^{2+} and ascorbate and they concluded that the inhibition was due to an alteration in the structure of the protein. Thus, it is possible that the inhibitory activity of Cu^{2+} and ascorbate is a general phenomenon and the simplest interpretation of the effect is to evoke a free-radical mechanism.

Under conditions where no Cu^{2+} is added, ascorbate inhibits catalase but in a much more limited way.

Foulkes and Lemberg (1948), on the basis of the pronounced reduction in the ascorbate-mediated inhibition of catalase by DEDTC, felt that ascorbate alone had no effect on catalase and that the observed inhibition was solely due to trace contamination by Cu^{2+}. It should be noted, however, that the inhibition was far from being completely reversed; indeed, a simple calculation from their data (taken after 20-min incubation) reveals that 40% of the catalactic activity was still inhibited in the presence of DEDTC. It has been shown here that DEDTC has no effect on the inhibition of catalase by ascorbate, thus supporting the contention that ascorbate alone inhibits catalase.

In an attempt to substantiate the results of the DEDTC experiment, EDTA was included in the incubation mixture with ascorbate and catalase. It was found that EDTA potentiated the ascorbate-mediated inhibition of catalase. In considering this rather surprising result, it was noted that Tanner *et al.* (1959) had shown that certain chelating agents, among them EDTA, enhanced the rate of autoxidation of ascorbate. Since the inhibition is related to the rate of ascorbate autoxidation, the EDTA result becomes less puzzling. Nonetheless, it is still difficult to understand how EDTA, by itself, could promote the oxidation of ascorbate unless it is postulated that chelated iron is present. If such were the case, the well-known hydroxylating system (EDTA, ascorbate, and iron) of Udenfriend *et al.* (1954) would be present. This hydroxylating system is known to act through the production of $\cdot OH$ and/or $\cdot O_2H$ (Norman and Smith, 1964; Staudinger *et al.*, 1964) and would neatly fall in line with the free-radical mechanism formulated here. A study of the inhibitory effect of iron (either Fe^{2+} or Fe^{3+}) and ascorbate on catalase with and without EDTA was undertaken. It was shown that iron increases the inhibition of catalase by ascorbate. This result would be anticipated since the H_2O_2 produced during the autoxidation of ascorbate in the presence of ferrous iron gives rise to the well-known Fenton reagent (Fe^{2+} and H_2O_2) which is known to give rise to $\cdot OH$ and/or $\cdot O_2H$ (Kolthoff and Medalia, 1949). When EDTA was present together with iron and ascorbate the inhibition is extremely rapid; the rate of inhibition approaches that of Cu^{2+} and ascorbate.

Ascorbate at pH 7.0 undergoes autoxidation to dehydroascorbate with the production of H_2O_2. It is known that H_2O_2 can react with ascorbate to produce free-radical intermediates. White and Krupka (1965) suggest that this mechanism is responsible for the inhibition of fungal ascorbic acid oxidase. Furthermore, support for free-radical transients comes from experiments demonstrating the degradation of hyaluronic acid by ascorbate alone (Matsumura and Pigman, 1965) and particularly by ascorbate in the presence of added H_2O_2 (C. W. M. Orr, in preparation). Thus, the inhibition of catalase by ascorbate alone is considered to be due to the production of free radicals formed during its autoxidation. The reduction in the extent and rate of inhibition when compared to ascorbate plus Cu^{2+} can be attributed to the much slower rate of autoxidation and consequently, intensity of radical production. Perhaps the most convincing argument in favor of the radical hypothesis for the ascorbate effect stems from the results found when DMSO is included with ascorbate and catalase in the incubation mixture. Under these conditions, the inhibition by ascorbate is severely reduced.

Although there is substantial inferential evidence that the radicals involved in the inhibition of catalase are either $\cdot OH$ or $\cdot O_2H$ or both, it has not proved possible to demonstrate their existence specifically. Attempts to do this using electron spin resonance techniques have failed due to the intense noise from the long-lived semidehydroascorbate radical.

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Studies on Ascorbic Acid. II. Physical Changes in Catalase Following Incubation with Ascorbate or Ascorbate and Copper(II)*

Charles W. M. Orr†

ABSTRACT: Incubation of catalase with either ascorbate or ascorbate and Cu^{2+} results in degradative changes in the catalase molecule. The effect of ascorbate alone appears to be qualitatively distinct from that of ascorbate in the presence of Cu^{2+}. Electrophoretic and chromatographic analysis of catalase treated with Cu^{2+} and ascorbate revealed that the molecule is extensively degraded with the majority of the resultant fragments being dialyzable. A similar analysis of the effect of ascorbate alone indicated that the degraded fragments were substantially larger and that a small fraction of aggregated or polymerized material occurred. By using [^{14}C]ascorbate, significant nondialyzable radioactivity was found associated with the polymerized material suggesting that at least some ascorbate must be bound to catalase. After treatment with ascorbate, or ascorbate and Cu^{2+}, the spectrum of catalase is changed. While there is obvious reduction in the Soret band at 408 mμ the shift of this peak to longer wavelengths is almost undetectable. It is concluded that very little, if any, catalase complex II is formed under these conditions. Significant spectral changes occur at shorter wavelengths. These have been tentatively interpreted to represent oxidative changes in labile aromatic amino acids. The results strongly support previous data which indicated that the inhibition of catalase by ascorbate, or ascorbate and Cu^{2+}, was the result of $\cdot OH$ or $\cdot O_2H$ attack of the enzyme.

The inhibition of catalase by ascorbate has been demonstrated by Foulkes and Lemberg (1948), however, these authors concluded that the presence of trace amounts of Cu^{2+} were necessary for the inhibition. It has been shown quite clearly (Orr, 1966, 1967), that ascorbate alone inhibits catalase. It is also equally clear that both Cu^{2+} and Fe^{2+} or Fe^{3+} poteniate this effect.

Chance (1950) has shown that at relatively high ascorbate concentrations, a new, spectrophotometrically identifiable, catalase species is formed. This species, known as complex II, results from the saturation of the heme irons with H_2O_2. It is enzymatically inert. Complex II was also demonstrated to occur in the presence of high concentrations of both Cu^{2+} and ascorbate (Keilin and Hartree, 1951).

In a preliminary report (Orr, 1966) and in the preceding paper (Orr, 1967), it was proposed that the inhibition by either ascorbate alone, or in the presence of Cu^{2+}, was due to free-radical attack of the catalase molecule. Further evidence consistent with this proposal is presented here where it is shown that physical degradation of the catalase molecule occurs. Very little, if any, complex II was formed under the experimental conditions.

These observations and conclusions are obviously not in accord with those of Chance (1950) and Keilin and Hartree (1951). The reasons for this dichotomy are discussed and it is concluded that two different mechanisms are operative both of which result in enzymatically inactive catalase molecules.

Materials and Methods

The preparation of solutions and the assay procedure used have been described previously (Orr, 1966).

Electrophoretic analyses were made on Oxoid cellulose acetate paper (Oxoid, Ltd., England) using Oxoid buffer, pH 8.6. A conventional Shandon electrophoresis unit (Shandon Ltd., England) was used. After electrophoresis at 20 v/cm for 90 min, the catalase bands were stained overnight in 0.002% nigrosin in 2% acetic acid. The excess stain was removed by washing in tap water.

All chromatographic analyses were made using a column (100 × 2 cm) of Sephadex G 200 (Pharmacia Ltd., Great Britain) containing 10% by volume of

* From the Industrial Hygiene Research Laboratories, I.C.I., Ltd., Alderley Edge, Cheshire, England. *Received June 12, 1967.*

† Present address: The McCollum-Pratt Institute, Department of Biology, The Johns Hopkins University, Baltimore, Md. 21218.