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

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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

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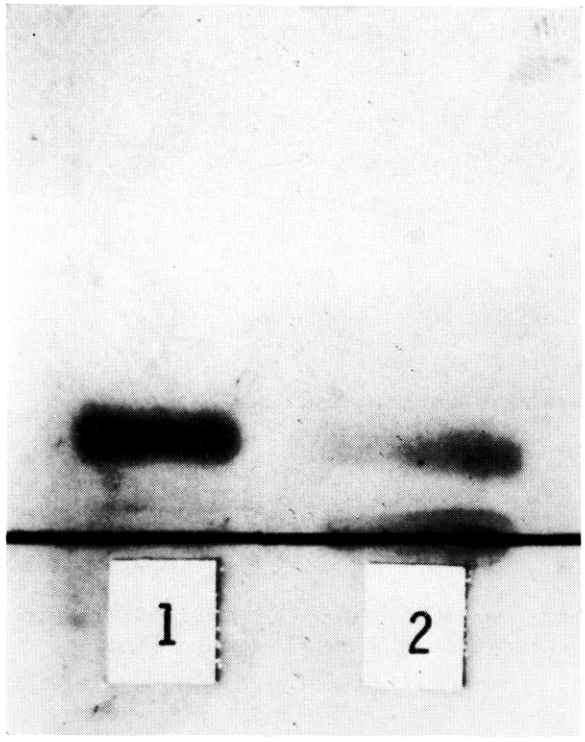

FIGURE 1: Electrophoretic profiles of catalase. (1) Catalase alone; and (2) catalase after incubation with Cu^{2+} and ascorbate; for details, see text.

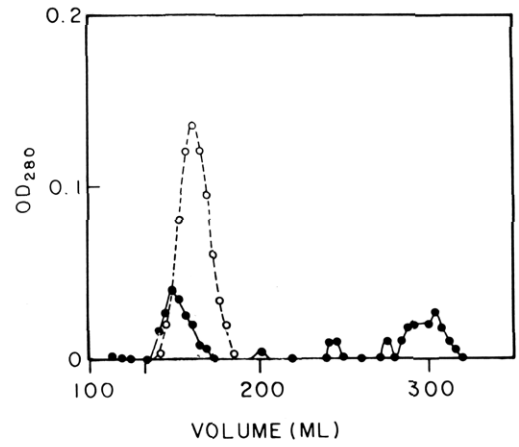

FIGURE 2: Chromatographic profiles of catalase on Whatman No. 1 Sephadex G-100. (--O--O--) Unincubated catalase control; (–●–●–) catalase incubated 35 min at 37° with 2×10^{-6} M Cu^{2+} and 2×10^{-4} M ascorbate.

Whatman No. 1 cellulose powder. The column was equilibrated and run in M/20 Tris (pH 7.4) at 4°. A flow rate of 7 ml/hr was maintained and fractions of 3 ml were collected.

L-[1-^{14}C]Ascorbic acid (sp act. 3.6 mc/mmole) was obtained from the Radiochemical Centre, Amersham Bucks., England. This material was found to be radiochemically pure; in ascending chromatography using butanol–acetic acid–water (4:1:5), it migrated as a single radioactive spot with the same R_F as the recrystallized ascorbate used in these studies. Radioactivity measurements were made in a Nuclear-Chicago scintillation counter.

Results

Electrophoresis and Chromatography. In preliminary experiments with the catalase preparations used in these experiments, it was found that 3–5 μg of catalase was readily detectable when stained in cellulose acetate paper with nigrosin (see Methods).

The effect of Cu^{2+} and ascorbate on the electrophoretic pattern of catalase has been examined. In this experiment, 310 Bu (Bergmeyer units, Bergmeyer (1955)) of catalase was incubated with 2×10^{-6} M Cu^{2+} and 2×10^{-4} M ascorbate in M/100 Tris (pH 7.0) in a total volume of 2 ml. A control tube containing 310 Bu of catalase in M/100 Tris (pH 7.0) was incubated concurrently. The tubes were incubated for 35 min at 37°. Aliquots were removed and assayed during the incubation. The inhibition (60%) was complete after 10 min. No inhibition of the control catalase occurred. After 35 min the tubes were chilled on ice and 20 μl from each tube was electrophoresed as described in Methods. The electropherogram is shown in Figure 1. The control catalase (Figure 1, 1) migrated as a distinct band toward the anode. The effect of Cu^{2+} and ascorbate is obvious (Figure 1, 2). The major band of native catalase is very much reduced in staining intensity, and a heavily stained area remains at, or near, the origin. It would appear that incubation of catalase with Cu^{2+} and ascorbate has caused major structural changes in the molecule.

Following the removal of the aliquot for electrophoresis, the catalase solution incubated with Cu^{2+} and ascorbate was chromatographed on the modified Sephadex G-200 column (see Methods) and the chromatographic profile is shown in Figure 2. Unfortunately, the contents of the control tube were lost; however, an aliquot of native, unincubated catalase containing the same Bu's as the experimental solution was chromatographed instead (Figure 2). The untreated catalase emerged from the column as a single uniform peak (on the basis of optical density at 280 mμ and enzymatic activity) between 140 and 160 ml. The profile of the treated catalase is very different. A small portion, corresponding roughly to the residual enzymatic activity, eluted in the same area as native catalase. This material was enzymatically active. The major fraction of the optical density applied to the column emerged as a series of small fragments scattered along the chromatographic profile with the major fraction of OD_{280} material emerging after about 300 ml.

The effect of ascorbate alone on the chromatographic profile was examined. In this experiment 235 Bu of catalase was incubated at 37° in M/100 Tris

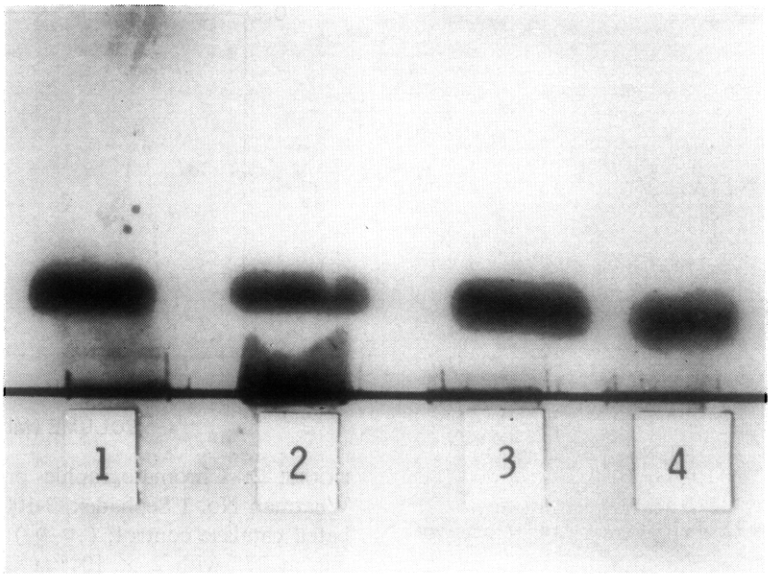

FIGURE 3: Electrophoretic profile of catalase. (1) Catalase alone; (2) catalase, 2×10^{-6} M Cu^{2+} and 2×10^{-4} M ascorbate; (3) catalase and 2×10^{-4} M ascorbate; and (4) catalase and 4×10^{-3} M ascorbate. For details, see text.

(pH 7.0) with 4×10^{-3} M ascorbate. After 1 hr the inhibition was 74.7% when compared to a control containing only 235 Bu of catalase in M/100 tris (pH 7.0). Chromatography of these solutions resulted in some very interesting effects. The catalase control, which had not decreased in its assayable activity, had a much less uniform profile than the unincubated control described above. A small fraction of the total material emerged after the main peak. Apparently, therefore, prolonged incubation at 37° dissociates a small fraction of the native molecules. The profile for the ascorbate-treated catalase indicated that, although some material emerged in the position of the native molecule, the major portion eluted between 200 and 300 ml. A small but significant amount of material, absorbing at 280 mμ, emerged prior to position of the native molecule. These data suggested that ascorbate itself cause not only extensive degradation of catalase, but that also some polymerization or aggregation had occurred.

Experiments with [^{14}C]*Ascorbate*. The initial experiments described above suggested that both Cu^{2+} and ascorbate, and ascorbate alone, affect the molecular architecture of catalase, but, apparently in different ways. The chromatographic profile of Cu^{2+}–ascorbate-treated catalase suggests that the molecule is extensively degraded to very small molecular weight fragments. On the other hand, ascorbate-treated catalase is degraded but not to such small fragments, and there is also evidence that some aggregation or polymerization had occurred.

In order to allow a more rigorous comparison of the effects of ascorbate, or ascorbate and Cu^{2+} on catalase, the incubations were run concurrently. Furthermore, it was of interest to determine if spectral changes in catalase occurred during the incubation and whether, as suggested by Lemberg and Foulkes (1948), there was evidence for complexing between ascorbate and catalase. To test these points the following incubation mixtures containing 1250 Bu of catalase in M/100 Tris (pH 7.0) were prepared (the final volume was 0.5 ml): (i) catalase alone; (ii) catalase, 2×10^{-6} M Cu^{2+}, and 2×10^{-4} M [^{14}C]ascorbate (sp act. 7.5×10^{6}); (iii) catalase, 2×10^{-4} M [^{14}C]ascorbate (sp act. 7.5×10^{6}); (iv) catalase, 4×10^{-3} M [^{14}C]ascorbate (sp act. 1.33×10^{5}); (v) catalase alone.

The incubation was run at 37°. The spectrum of tubes i–iv was recorded at approximately every 10 min between 10 and 75 min in 0.5-ml microcells (1-cm path length) between 200 and 700 mμ in a Unicam SP800 recording spectrophotometer. After 75-min incubation, the tubes were chilled on ice and an aliquot of 10 μl from tubes i–iv was electrophoresed and stained as described. The incubation mixtures were stored at 4° and 0.36 ml of each was chromatographed separately on the column described above. The order of chromatography of the samples was i–v. Immediately prior to chromatography, 1 ml of M/100 Tris (pH 7.0) was added to the aliquot to be applied to the column.

The electrophoretic patterns are shown in Figure 3. Some degradation of the untreated catalase molecule apparently occurs during the incubation since there is evidence of staining at the origin (Figure 3, 1). An intensely stained area, indicative of degraded molecules, extending from the origin can be seen in the catalase sample treated with Cu^{2+} and ascorbate (Figure 3, 2). As before, the staining representative of the native molecule is severely reduced. At low ascorbate concentrations (2×10^{-4} M) no significant differences from the electropherogram for the untreated catalase can be detected (Figure 3, 3). However, at elevated ascor-

bate concentrations (4×10^{-3} M), the staining of the principle band is much reduced (Figure 3, 4) and it is significant that there is no concomitant increase in staining at the origin.

The chromatographic profiles of test solutions i–iv are shown in Figure 4. In addition to recording at 280 mμ, readings have been made at 405 mμ, the Soret band of catalase. Also included in the figures are the data showing the distribution of radioactivity. In general, the results confirm the earlier observations noted above.

The profile of the control catalase solution (Figure 4A) contains an anomalous peak associated with the tailing end of the main catalase peak. The enhanced optical density in this tube is not correlated with increased activity; the activity is consistent with that of the tubes on either side. Four other tubes, collected later, contain OD_{280} material. Since three of these also contain OD_{405} material, the result can hardly be artifactual and it is concluded that they represent fragments of the parent molecule. There was no enzymatic activity in any of these tubes; indeed, in the description that follows, activity was only found in tubes collected where native catalase normally emerged.

The profile of tube ii (Figure 4B), which is the result of incubating catalase with Cu^{2+} and ascorbate, is similar to the data shown above (Figure 2, 2). The main native peak is much reduced and a series of smaller molecular weight fragments are evident. There is no evidence of polymerized material. The major concentration of the fragmented molecules is between 300 and 350 ml off the column and it is in this region that the radioactivity also emerges. This result demonstrates that the catalase molecule has been degraded to fragments the size of small dialyzable peptides. Aliquots were taken from those tubes that contained both radioactivity and ultraviolet-absorbing material and dialyzed overnight against 3 l. of water at 4°. It was found that 90% of the radioactivity was dialyzable in this time, suggesting that the association of counts and OD_{280} was fortuitous, *i.e.*, they coemerged from the column purely on the basis of their size. Activity was found only in the native catalase area and it represented 30% of the activity of the control catalase assayed after it emerged from the column.

The effect of low levels of ascorbate (2×10^{-4} M) on the chromatographic profile of catalase is shown in Figure 4C. The significant feature of this profile is the presence of a small peak that emerged prior to native catalase, *i.e.*, at 130 ml. This novel peak extended over three tubes (9 ml) and contained material absorbing at both 280 and 405 mμ. No enzymatic activity could be detected. However, it was interesting that two tubes contained radioactivity which was slightly above background (6 cpm/ml above background). Since the counts were so low, no attempt was made to determine whether they were dialyzable or not. All the catalactic activity was present in the main peak (155–190 ml) and it represented 90% of the activity in the control. The radioactivity emerged between 300 and 350 ml.

The effect of high levels of ascorbate (4×10^{-3} M) on catalase is quite dramatic (Figure 4D). The chromatographic profile resembles that of Figure 4B in that a considerable amount of degradation into smaller molecular weight species has occurred. However, the major portion of these molecules has not been completely degraded. Nearly all the tubes containing degraded fragments contain OD_{405} material. The profile is also distinct from Figure 4B in that polymerized material, emerging prior to the native peak, in approximately the same position as the material seen in the profile from Figure 4C, is present. Significant radioactivity was found in the tubes of the polymerized peak (22 cpm/ml above background). The material was dialyzed overnight at 4° against 3 l. of water and again assayed for radioactivity; 50% of the counts still remained. It is therefore considered that a significant amount of ascorbate must be bound to the polymerized catalase species. No other tubes containing counts were detected, except in the area where they are normally found, *i.e.*, between 300 and 350 ml. Assays for catalactic activity were made and, as before, only those tubes

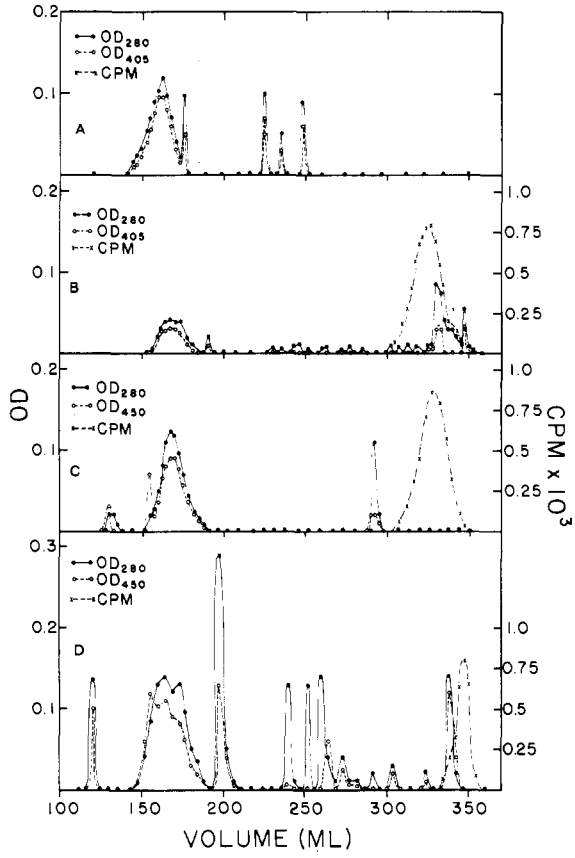

FIGURE 4: The chromatographic profiles of catalase incubated at 37° for 75 min. (A) Catalase alone; (B) catalase, 2×10^{-6} M Cu^{2+} and 2×10^{-4} M ascorbate; (C) catalase and 2×10^{-4} M ascorbate; and (D) catalase and 4×10^{-3} M ascorbate. (-●-●-) OD_{280}, (- -o- -o- -) OD_{405}, and (-···×···×··-) radioactivity. For other details, see text.

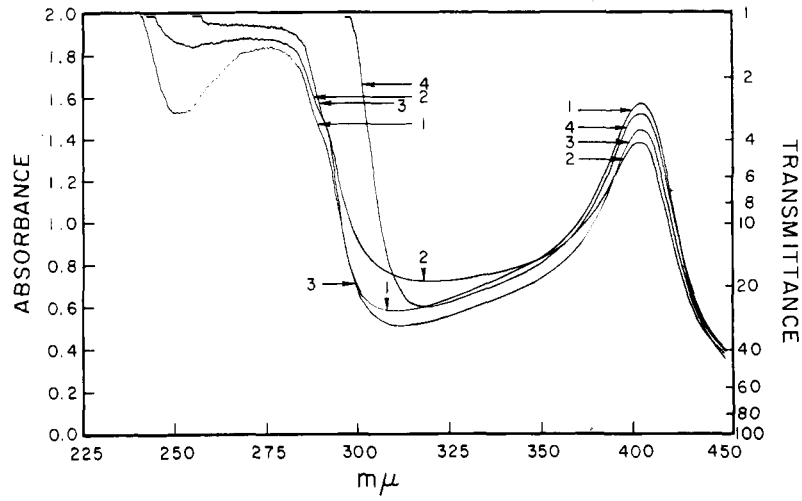

FIGURE 5: Spectrophotometric profiles of catalase incubated under varying conditions at 37° for 75 min. (1) Catalase alone; (2) catalase, 2×10^{-6} M Cu^{2+} and 2×10^{-4} M ascorbate; (3) catalase and 2×10^{-4} M ascorbate; and (4) catalase and 4×10^{-3} M ascorbate. For other details, see text.

collected where native catalase emerges had activity. The recovery of activity was 61% of the control.

The profile of catalase (incubated in M/100 Tris (pH 7.0) for 75 min at 37°) chromatographed immediately after mixing with 5 μl of [^{14}C]ascorbate (1.2×10^5 cpm) is not shown since the profile was almost identical with Figure 4A. However, it is important to note that no counts were found associated with the native molecule or any of the fragments. All the radioactivity added could be accounted for in the usual area (300–350 ml).

The spectrum of solutions i–iv was recorded as described above. Although there are small, possibly interpretable changes occurring above 450 mμ, they are not shown here because the differences are not sufficiently clear to draw firm conclusions. However, below 450 mμ, the changes in the spectra are obvious and are shown in Figure 5. It is clear, that under the experimental conditions described here, there is a decrease at OD_{405} (the λ_{max} for the Soret band of catalase) when catalase is incubated with ascorbate. The most marked decrease at 405 mμ is in the presence of both Cu^{2+} and ascorbate (tube ii). Low levels of ascorbate (2×10^{-4} M) appear to reduce the peak at 405 mμ to a greater extent than high levels (4×10^{-3} M).

It is apparent from the data in Figure 5 that there is no radical shift in the peak at 405 mμ as described by Chance (1950) and Keilin and Hartree (1951) for the formation of complex II of catalase by ascorbate or by ascorbate and Cu^{2+}. However, close examination of the spectra reveal that at high ascorbate concentrations there is evidence for a very slight shift to longer wavelengths creating as isobestic point at 425 mμ. Similarly, a small shift toward longer wavelengths with an isobestic point at 440 mμ is caused by the action of Cu^{2+} and ascorbate on catalase.

Below 350 mμ the spectrum of catalase in the presence of high ascorbate (4×10^{-3} M) is masked by the absorption from residual unoxidized ascorbate. In the presence of either low concentrations of ascorbate, or ascorbate and Cu^{2+} (where all the ascorbate is oxidized to nonultraviolet-absorbing material), the spectrum of catalase is very much altered. At 280 mμ and below, both curves show an increased absorption and flattening of the peak. Above 300 mμ, the curve for catalase and ascorbate alone follows the spectrum for the "native" molecule, but in the presence of Cu^{2+} and ascorbate, there is a large increase in the absorption between 300 and 350 mμ.

Discussion and Conclusions

Keilin and Hartree (1951) noted that, when complexes II and III of catalase were generated by Cu^{2+} and ascorbate, a precipitate was invariably formed; if either Cu^{2+} or ascorbate was omitted, no precipitate occurred. No precipitation of catalase has been witnessed during the course of the experiments recorded here or previously (Orr, 1966, 1967). However, the concentration of all the reactants was much lower than those used by Keilin and Hartree and therefore might have escaped detection. Nevertheless, the denaturation of catalase in the presence of Cu^{2+} and ascorbate was clearly evident from the electrophoretic studies reported here. The major portion of stained material remained as a diffuse region at or near the origin. A more definitive evaluation of the effect of Cu^{2+} and ascorbate on the structure of catalase was obtained from comparison of the chromatographic profiles of native catalase with that subjected to incubation with Cu^{2+} and ascorbate. The comparison made it obvious that during the incubation of catalase with Cu^{2+} and ascorbate the integrity of most of the catalase molecules was destroyed. A variety of smaller fragments together

with a small proportion of native material was found. Since the majority of these fragments is concentrated in the region where dialyzable small molecules elute from the column, it must be concluded that the denaturation of the molecule has extended to chain and peptide-bond scissions. No polymerized species was found.

The effect of ascorbate, in the absence of Cu^{2+}, on the physical properties of catalase is much less pronounced and is a function of the concentration of ascorbate. There was very little inhibition of catalase by the lower concentration of ascorbate (2×10^{-4} M) and no obvious change in the electrophoretic pattern could be detected. However, while the chromatographic profile of catalase incubated with 2×10^{-4} M ascorbate was essentially the same as the control, it contained one outstanding feature, *i.e.*, the presence of a significant amount of polymerized material which appeared to have ascorbate associated with it. At the higher ascorbate level (4×10^{-3} M) substantially more inhibition of catalase occurred and this was reflected in the reduced intensity of staining in the main catalase band and the obvious degradation of the catalase molecule as indicated by the chromatographic profile. Unlike the effect of Cu^{2+} and ascorbate on catalase, the molecule appears to be only partially broken down since the main concentration of ultraviolet-absorbing material occurs between the native molecules and dialyzable fragments. Furthermore, polymerized material, containing significant amounts of nondialyzable radioactivity, emerged prior to the native molecule. The presence of counts associated with catalase suggests that at least a small proportion of the ascorbate molecules bind to catalase. This suggestion is not novel (Lemberg and Foulkes, 1948); however, Chance (1950) and Keilin and Hartree (1951), do not share this view. They attribute the effects described by Lemberg and Foulkes (1948) to the formation of complex II of catalase. While the ascorbate–catalase association described here appears to be real, it represents an extremely small fraction of the total ascorbate present in the system. Possibly, the association of ascorbate with catalase only occurs during the polymerization process since no radioactivity was found bound to any of the degraded fragments at either high or low ascorbate concentrations.

The absorption spectrum of catalase between 260 and 300 mμ was significantly affected following incubation with low levels of ascorbate or ascorbate and Cu^{2+} (as noted above, the catalase spectrum at high ascorbate concentrations could not be evaluated due to the absorption of unoxidized ascorbate). The shapes of both curves between 260 and 285 mμ are almost identical. They differ from the control spectrum in that the absorption is increased and that the peak exhibited by the native molecule at 280 mμ has flattened. The enhanced absorption is not due to ascorbate which under these conditions would be oxidized to nonultraviolet-absorbing material. Above 300 mμ the spectrum of catalase incubated with low ascorbate follows, but is lower than, the spectrum of native catalase. On the other hand, there is a marked increase in absorption above 300 mμ in Cu^{2+}–ascorbate-treated catalase. It seems reasonable to suggest that some of the changes in the absorption spectrum of catalase discussed above could be due to oxidation of tryptophan and/or tyrosine residues which are known to cause increased absorption in this region of the spectrum (Barron *et al.*, 1955). However, it is not possible to rule out a contribution from free heme molecules which absorb maximally in this region (300–400 mμ) (Osbahr and Eichhorn, 1962). The obvious spectral changes in catalase treated with low ascorbate are surprising since there is almost no change in the activity or chromatographic profile of the molecule. It is tempting to speculate that a few extremely labile residues are oxidized and that these contribute to an absorption change out of all proportion to their concentration.

A reduction in absorption at the Soret band of catalase (405 mμ) was found under all conditions where ascorbate was present in the reaction. Chance (1950) has shown that incubation of catalase with ascorbate results in the formation of complex II of catalase which is characterized by a reduction at 405 mμ and a shift to longer wavelengths resulting in an isobestic point at 425 mμ. An isobestic point between catalase and high ascorbate-treated catalasec an be detected at 425 mμ, but there is no perceptible shift at 405 mμ. Possibly only traces of complex II were formed. There is also an isobestic point between native catalase and Cu^{2+}–ascorbate-treated catalase, but here it is at 425 mμ (indicating the presence of complex I (Chance, 1950)). It would appear that under the experimental conditions described here, very little, if any, complex II is formed. Although this conclusion is based on the data presented, *i.e.*, after incubation for 75 min at 37°, spectra were taken approximately every 10 min during the incubation and at no time was any shift in the Soret band observed. It is evident from these data that the inhibition of enzymatic activity cannot be due to complex II formation; rather the inhibition appears to be due to disintegration of the catalase molecule. This interpretation is at variance with the data and conclusions of Chance (1950), who showed that at pH 4.0 in acetate buffer complex II was formed. When catalase and ascorbate were incubated together, the conditions of pH may well be very important since at low pH the rate of ascorbate autoxidation is very slow (Weissburger *et al.*, 1943) and the H_2O_2 formed at pH 4.0 could preferentially react with catalase rather than with ascrobate or its oxidation products. The importance of the pH at which the reaction is run was emphasized by Keilin and Hartree (1951), who indicated that the formation of complex II occurred rapidly only within the pH range 4.0–5.5. In view of these considerations, it seems that two quite different mechanisms are possible, both of which result in the inhibition of catalase.

Irradiation of protein solutions under aerobic conditions results in a variety of effects, some of which find their parallel in the present report. The initial event is presumed to be the production of radicals due to the radiolysis of water which then react with the protein

to cause oxidative deamination of certain amino acids (Barron *et al.*, 1955). Disulfide bond breakage and the consequent aggregation (polymerization) and/or scission of the protein is also known to occur (Ambe *et al.*, 1961).

Of particular pertinence to the present study are the reports of Okada (1957) and Slobodian *et al.* (1962). Both authors were able to demonstrate, using two very different proteins (DNase and RNase, respectively), that the effects of irradiation could be completely mimicked by $\cdot OH$ generated by Fenton's system (Fe^{2+} plus H_2O_2), *i.e.*, both result in protein denaturation. It is considered, therefore, that the results presented in this paper confirm and extend the hypothesis presented previously (Orr, 1966, 1967), *i.e.*, that the inhibition of catalase activity by ascorbate either in the presence or absence of Cu^{2+} under the experimental conditions described, is due to free-radical ($\cdot OH$ and/or $\cdot O_2H$) attack and the resultant physical perturbation of the molecule.

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