THE INHIBITION OF CATALASE BY ASCORBIC ACID

by C. W. M. Orr

Industrial Hygiene Research Laboratories
Imperial Chemical Industries Limited
Alderley Park, Nr. Macclesfield,
Cheshire, England.

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The activity of a crystalline preparation of catalase is inhibited by ascorbic acid. The inhibition occurs at levels as low as 2×10^{-6}M ascorbate. The rate and degree of inhibition of catalase by ascorbate is greatly increased by catalytic amounts of Cu^{++}. The inhibition of catalase by Cu^{++} alone, under the conditions of the experiments described here, is insignificant. Preincubation of Cu^{++} and ascorbate together, in the absence of catalase, does not lead to the accumulation of an inhibitory product. It is suggested that a transient free radical intermediate, formed during the interaction of Cu^{++} and ascorbate, and during the autoxidation of ascorbate, is responsible for the inhibition.

MATERIALS AND METHODS

A crystalline catalase preparation (lot * 26388) was obtained from Koch-Light Laboratories, Buckinghamshire, England. Before use, it was diluted 1 : 1 in M/20 tris pH 7.4 and chromatographed on a column (50 x 2 cm) of Sephadex G100. All fractions with an OD $\frac{405}{280} \geq 0.75$ were pooled and diluted appropriately in M/100 tris pH 7.0. Ascorbic acid (B.D.H., Poole, England) was recrystallised from ethanol. Tris was also recrystallised from ethanol. The middle fraction from redistilled reagent grade HCl was used to neutralise the tris buffers. $CuSO_4$ and hydrogen peroxide (100 volumes) were reagent grade. Before

use they were diluted appropriately in M/100 tris pH 7.0. The water used was first distilled, then deionized, and finally glass distilled.

Catalase assay. Catalase was assayed by a modification of the method of Chance (1954). Catalactic activity was measured in a Unicam SP800 recording spectrophotometer. A chart speed of 1 inch/10 seconds was used. The activity was calculated from the decrease in OD_{240} of a hydrogen peroxide solution. The amount of catalase used in the assays was adjusted so that a reduction of 0.06 OD units occurred in 5 seconds. This rate corresponds to 1.88 Bergmeyer units of catalase (Bergmeyer, 1956). The specific activity was 1300 Bergmeyer units/mg protein.

The assay solution comprised of 84 μ moles hydrogen peroxide, and 30 μ moles tris pH 7.0 in 3 ml water. An aliquot (0.02 ml) of the reaction mixture was pipetted onto the interior side-wall of the cuvette (which contained the assay solution) so that it remained as a suspended drop as long as the cuvette was held at an angle. The tilted cuvette was covered with parafilm, mixed, and inserted into the spectrophotometer. The time taken from mixing until the start of recording at OD_{240} was invariably 6-7 seconds. The OD decrease was recorded for 10 seconds. Under these conditions the slope was linear. The rate of reaction was proportional to the concentration of catalase.

The reaction mixture. When catalase was incubated with ascorbate, or ascorbate and Cu^{++}, the following procedure was followed. M/100 tris pH 7.0, catalase, and Cu^{++} (where used) were added in that order to a tube kept in ice. Immediately prior to including ascorbate in the reaction mixture, it was diluted appropriately from an M/10 solution in water (prepared fresh, before each assay), into M/100 tris pH 7.0. The desired concentration was then added to the reaction mixture, an aliquot of 0.02 ml removed, and the tube placed in a water bath at 37°C. The aliquot was assayed immediately.

The reaction mixture contained 0.5 μ moles tris pH 7.0, 47 Bergmeyer

units of catalase and either ascorbate, Cu^{++}, or Cu^{++} and ascorbate, in a final volume of 0.5 ml. The control tube contained only 0.5 μ moles tris pH 7.0 and 47 Bergmeyer units of catalase in 0.5 ml. Aliquots of 0.02 ml were removed when required and assayed.

Expression of results. The results have been expressed as the percentage inhibition of catalase brought about by any of the treatments mentioned, relative to the control rate. The percentage inhibition is calculated from the increase in time taken for an OD_{240} decrease of 0.06 units, relative to the control rate.

RESULTS AND CONCLUSIONS

The effect of ascorbic acid on the activity of catalase. In Figure 1, the inhibition of catalase activity by 2 x 10^{-6}M and 2 x 10^{-4}M ascorbate

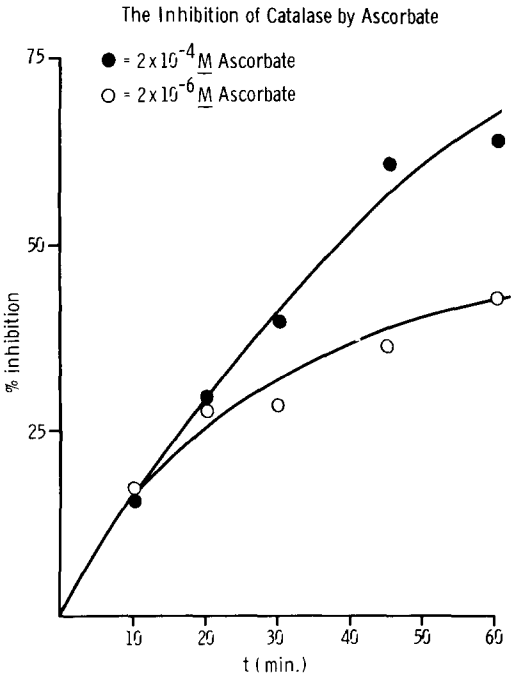

Fig. 1. The % inhibition of catalase by 2 x 10^{-6}M and 2 x 10^{-4}M ascorbate.

(final concentration) is plotted as a function of time. The data show clearly that the inhibition is concentration dependent. The time course of the inhibition is similar in the initial period (0 - 20 minutes) at both ascorbate concentrations. After 20 minutes the inhibition at 2×10^{-4}M ascorbate continues to increase, whereas at the lower concentration, the rate falls off. After 60 minutes, the difference in percentage inhibition at the two ascorbate concentrations is approximately 25%.

The inhibition of catalase by ascorbate in the presence of Cu^{++}. Catalase was incubated, as described in METHODS, with 2×10^{-4}M ascorbate at three

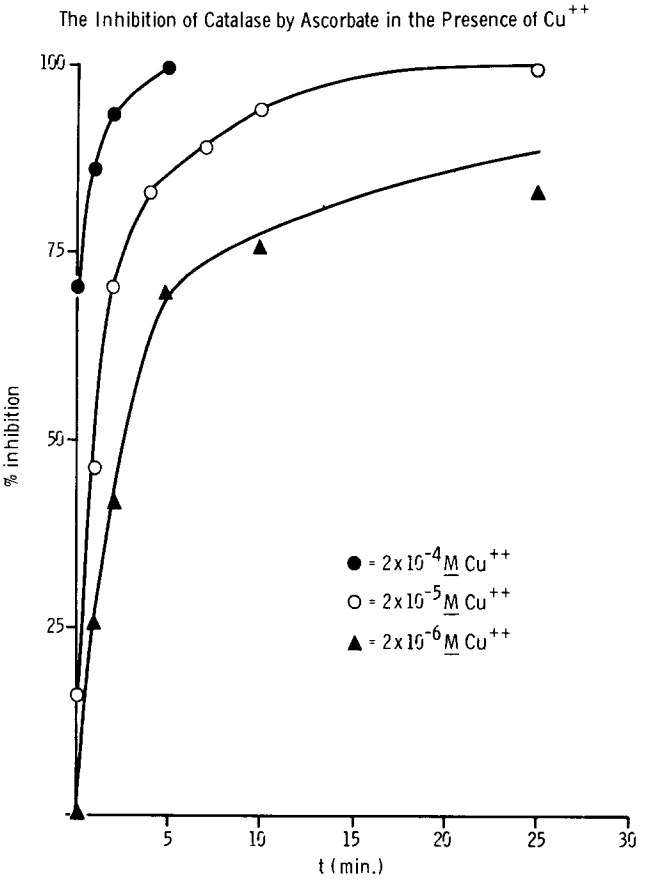

Fig. 2. The % inhibition of catalase by 2×10^{-4}M ascorbate in the presence of 2×10^{-6}M, 2×10^{-5}M, and 2×10^{-4}M Cu^{++}.

different Cu^{++} concentrations, i.e. 2×10^{-4}M, 2×10^{-5}M and 2×10^{-6}M. Aliquots of 0.02 ml were taken from the reaction mixtures at the times indicated in Figure 2. At all Cu^{++} concentrations, the inhibition is characterised by an extremely rapid initial phase followed by a slower lag phase.

At 2×10^{-4}M Cu^{++}, in the presence of 2×10^{-4}M ascorbate, the inhibition is so rapid that the enzyme is 70% inhibited in the time required to remove and assay an aliquot (approximately 20 seconds). The enzyme was completely inhibited, under these conditions, after 5 minutes incubation at 37°C.

At 2×10^{-5}M Cu^{++}, 100% inhibition is reached after 20 minutes, and at the lowest Cu^{++} concentration (2×10^{-6}M) the curve levels out at 90% inhibition (Figure 2).

The nature of the inhibitor. Cu^{++} alone inhibits catalase at high concentrations. Under the experimental conditions described here, 2×10^{-4}M Cu^{++} inhibits catalase by 35% after 30 minutes of incubation at 37°C. At 2×10^{-5}M Cu^{++} and 2×10^{-6}M Cu^{++}, the inhibition after 30 minutes incubation at 37°C is 9% and 0% respectively. Clearly, Cu^{++} alone is not the major factor in the inhibition.

The reaction between Cu^{++} and ascorbate is well-known (e.g. Weissburger and LuValle, 1944). Cu^{++} catalyses the autoxidation of ascorbate to dehydroascorbate. To test whether dehydroascorbate inhibits catalase, 2×10^{-4}M dehydroascorbate was incubated at 37°C with catalase under the conditions described above. No inhibition occurred. A variety of other degradation products of ascorbate occur. In order to determine if one of these species was inhibitory, Cu^{++} and ascorbate (2×10^{-5}M and 2×10^{-4}M, respectively) were preincubated together at 37°C, for 1, 2 and 5 minutes. The reaction mixture was that described in METHODS except that catalase was omitted. The volume of the preincubation mixture was 0.35 ml. Follow-

ing the preincubation period, the tubes were placed in ice and 0.15 ml (47 Bergmeyer units) of catalase was added to make the final volume 0.5 ml. An aliquot of 0.02 ml was immediately assayed. The reaction mixture was incubated on ice for a further 5 minutes and assayed again. The reaction mixture was transferred from ice to a water bath at 37°C and assayed after 2, 5 and 10 minutes. No inhibition of the controls occurred when treated in this way. The results are presented in Table 1.

Table 1. The percentage inhibition of catalase when added to a Cu^{++}-ascorbate system (for details, see text) preincubated for different periods of time.

Preincubation Period (min.)	% Inhibition of Catalase After Addition to Preincubation Mixture				
	0°C		37°C		
	0 min.	5 min.	2 min.	5 min.	10 min.
1	0	28.5	53.5	64.3	74.7
2	0	9	9	19.3	20
5	0	9	9	19.3	25

The data in Table 1 show that a stable inhibitory product is not formed during the interaction of 2×10^{-4}M ascorbate and 2×10^{-5}M Cu^{++}. The inhibitory activity of the preincubation mixture is related to the period of preincubation. After 2 and 5 minutes of preincubation, the inhibition of added catalase is slight. However, after 1 minute of preincubation substantial inhibitory activity remains (Table 1).

The data are interpreted as follows: Cu^{++} and ascorbate react together very rapidly. Once the interaction is complete (when no ascorbate remains) the inhibitory potency of the mixture is dissipated. In support

of this interpretation, it has been found that under the conditions of the preincubation experiment described above, approximately 50% of the ascorbate is oxidised after 1 minute and none remains after 2 minutes of incubation with Cu^{++} (as judged by the decrease in λ max (265 mμ) of ascorbate). It is speculated that, during the interaction of Cu^{++} and ascorbate, free radical formation occurs and that the inhibition of catalase is due to free radical attack of the protein. This contention is supported by two observations. Firstly, Cu^{++} and ascorbate form an active hydroxylating system (Orr, 1966) and, furthermore, Cu^{++} potentiates the ORD of hyaluronic acid by ascorbate (Matsumura and Pigman, 1965). Both of these phenomena have been interpreted as free radical effects. Secondly, chromatography of catalase on G200 Sephadex after incubation with Cu^{++} and ascorbate results in a number of small fragments with lower molecular weights than native catalase which has a single peak.

A more detailed report is in preparation.

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