ROLES OF COPPER AND FREE RADICALS IN THE INACTIVATION OF CATALASE BY ASCORBATE

by

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Role of Copper and Free Radicals in the Inactivation of Catalase by Ascorbate.

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ABSTRACT

The mechanisms involved in the inactivation of catalase by ascorbate and ascorbate plus $Cu^{2+}(His)_2$ were investigated in an attempt to gain a clearer understanding of the cytotoxic effects of ascorbate. Previous workers have postulated that inactivation of catalase by ascorbate is mediated by either hydrogen peroxide or superoxide and the hydroxyl radical. The accelerating effect of metal ions was presumed to be a direct result of an increase in ascorbate autoxidation and consequently, an increase in the generation of these activated species of oxygen. The results presented in this study contradict these postulates and the following alternative mechanisms are proposed :

(i) Ascorbate and/or semidehydroascorbate reversibly inhibit catalase by reducing compound I to inactive compound II.

(ii) Ascorbate plus $Cu^{2+}(His)_2$ inactivates catalase through site-specific production of semidehydroascorbate and the subsequent oxidation of ferricatalase to compound II. Compound II is then irreversibly inactivated by modification of the histidine residue distal to haematin iron at the active site of catalase.

DEDICATION

This thesis is dedicated to my best friend Lesley. Without her constant love, friendship, support and sacrifice my dream would never have come true.

Thanks Les

ACKNOWLEDGMENTS

I would like to extend my thanks to Dr Allan Davison, my senior supervisor, for his guidance, patience, and help in bringing this thesis to completion. Also, I would like to extend special thanks to my friends Barb, Judy, Karen, Rob and Tony and all the people in the Kinesiology department for making Lesley's and my stay here in Vancouver extremely enjoyable.

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CHAPTER 1

GENERAL INTRODUCTION

There are a wide variety of mechanisms that can initiate disease processes. One set of mechanisms, which are rapidly gaining prominence, are those that involve free radical intermediates (1,2). In order to develop effective protection against injury that involves free radical intermediates, a knowledge of the basic biochemical mechanisms involved is essential. This thesis attempts to address this problem and in doing so it is hoped that the mechanisms responsible for free radical mediated toxicity will be better understood.

Free radicals are molecules or molecular fragments that contain an unpaired electron (3). Characteristic properties of free radicals are paramagnetism and usually a high reactivity. A wide variety of biological processes, such as the catalytic action of many enzymes and the functioning of electron transport pathways, contain free radicals as reactive intermediates. However, an organism can be exposed to other sources of free

radicals which, because of their high reactivity and propensity to undergo chain reactions, promote cellular and tissue injury. Sources of these damaging free radicals include antibiotics, radiation and xenobiotics. Due to the ubiquitous nature of dioxygen in aerobic organisms, and its ability to readily scavenge electrons, many of these damaging radicals are oxygen-centered free radicals.

Defenses that exist to eliminate these toxic oxygen-centered free radicals primarily include the antioxidant α-tocopherol, and the enzymes superoxide dismutase, catalase and glutathione peroxidase. These enzymes scavenge superoxide (O_2^-), hydrogen peroxide (H_2O_2) and peroxides (ROOH), respectively (4). Disease states, xenobiotics and other enviromental stresses can overwhelm these defense mechanisms and cause the propagation of oxidative damage. Accumulating evidence now indicates that all the deleterious free radical reactions occurring continuously within the cells and tissues contribute significantly to the aging process (5,6).

Ascorbic acid, in the presence of metal ions, is an example of an agent that can promote free radical reactions in vivo (7,8). The biological damage it can induce includes protein destruction and lipid autoxidation. These two factors are presumed to promote cell lysis (9) and inactivation of enzymes. Lipid autoxidation can also cause the propagation of free radical reactions to membrane associated nucleic acids and proteins (10), as well as influencing enzyme control mechanisms

through alterations in membrane fluidity (11).

There is general agreement that ascorbic acid promotes oxidative insult by providing a source of H_2O_2 through its autoxidation. H_2O_2 can then react with metal ions, in a Fenton type reaction (reaction (1)), to produce the highly damaging hydroxyl radical.

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)+} + HO^- + HO\bullet \quad (1)$$

A potential aspect of the toxicity of ascorbate that has received little attention is its ability to inactivate catalase. The characterization of the mechanism in the inhibition of catalase by ascorbate is of extreme importance. Before ascorbate can induce cytotoxity, through the production of H_2O_2, it must first inhibit catalase and thereby allow sufficient levels of H_2O_2 to accumulate and give rise to damaging hydroxyl radicals. Therefore, the effect of ascorbate on the activity of purified catalase was investigated in an attempt to answer the following questions :

(i) What is the mechanism responsible for the inhibition of catalase by ascorbate?

(ii) Does ascorbate inhibit catalase by a different mechanism in the presence of metal ions, chiefly Cu^{2+}?

(iii) Are free radicals involved in either of these mechanisms?

This thesis consists of two main parts. The first part concerns the inhibition of catalase by ascorbate and is described in Chapter 2. Because it became apparent that the mechanism by which ascorbate plus $Cu^{2+}(His)_2$ inactivates catalase is different to that of ascorbate alone, this mechanism was investigated further and is outlined in Chapter 3.

CHAPTER 2

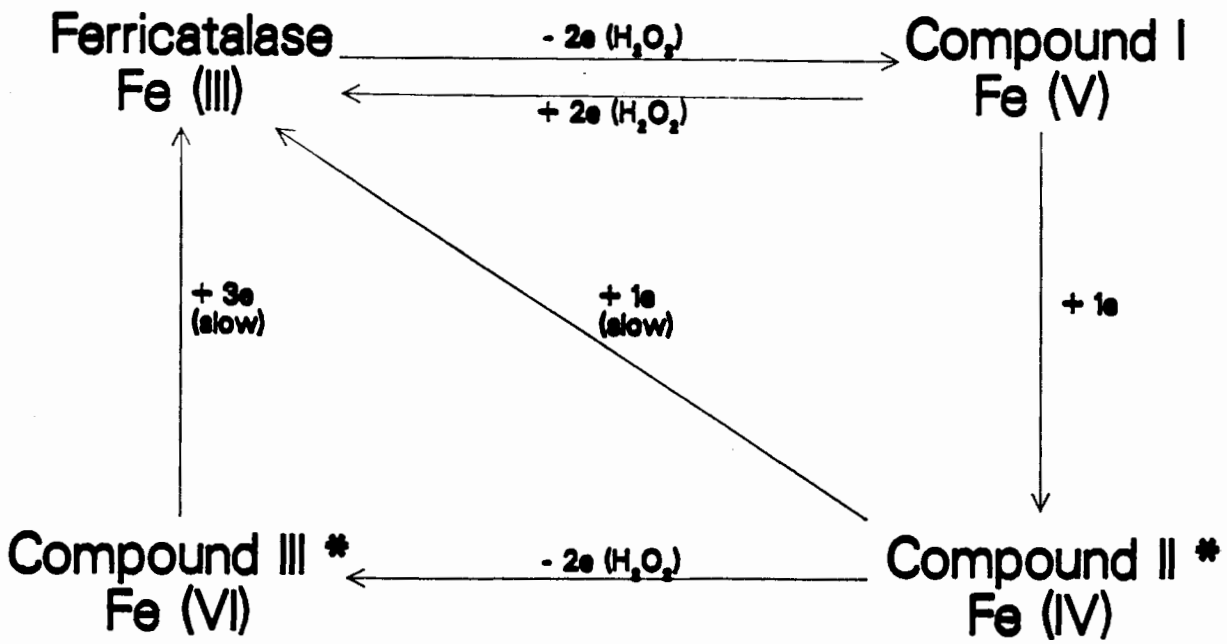
ROLES OF ASCORBATE AND FREE RADICAL INTERMEDIATES IN THE REVERSIBLE INHIBITION OF CATALASE BY ASCORBATE

INTRODUCTION

Catalase is an iron-porphyrin enzyme found in almost all animal cells. It functions, in part, to protect cells from the cytotoxic effects of hydrogen peroxide (H_2O_2). Ascorbate is among the compounds that are known to inhibit catalase. To date, however, the mechanism responsible for the inhibition of catalase by ascorbate has not been fully characterised.

An understanding of the inhibitory action of ascorbate is contingent upon a knowledge of the reaction mechanism of catalase. The resting Fe(III)-enzyme binds H_2O_2 at its active site and undergoes a two electron oxidation liberating H_2O.

Fig. 1. Possible redox transformations involved in reactions of catalase. Formal oxidation states of iron are shown in parentheses. * denotes an inactive form of catalase.


Ferricatalase
Fe (III)
- 2e (H_2O_2)
+ 2e (H_2O_2)
Compound I
Fe (V)
+ 3e
(slow)
+ 1e
(slow)
+ 1e
Compound III *
Fe (VI)
- 2e (H_2O_2)
Compound II *
Fe (IV)

The enzyme-peroxide complex so formed (compound I) binds and oxidizes a further molecule of H_2O_2 regenerating ferricatalase and liberating H_2O and O_2. In addition, at low concentrations of H_2O_2 compound I can act peroxidatically, catalysing two electron oxidations of compounds such as ethanol and formate. Alternatively, compound I can regenerate ferricatalase by two successive one electron reductions. The first of these univalent reductions forms inactive compound II which is incapable of decomposing H_2O_2. Compounds such as ethanol, methanol, NADH and NADPH, however, can transfer a single electron to it and thereby restore active catalase. Another inactive form of catalase is the enzyme-peroxide complex called compound III. It can be formed through the divalent oxidation of compound II by H_2O_2. A simplified summary of these reactions is given in Fig. 1. For more comprehensive reviews see (12) and (13).

From the limited number of investigations into the inhibition of catalase by ascorbate two quite different mechanisms have been proposed. Originally, Chance (14) demonstrated the spectrum of compound II in ascorbate treated catalase. He attributed the formation of compound II to the presence of H_2O_2 from ascorbate autoxidation, because the notatin system, which produces a continuous supply of H_2O_2, also caused the formation of compound II. However, the amount of compound II formed in the presence of ascorbate considerably exceeded that formed in the presence of the notatin system at pH 7.0. This lead Chance to speculate that the equilibrium between

compounds I and II may be affected by the ascorbate molecule itself. In a similar investigation, Keilin and Hartree (15) also showed that formation of compound II, when catalase was incubated with ascorbate, required oxygen and was greatly accelerated by copper salts.

Contrary to these results, Orr (16) could not show formation of compound II when catalase was incubated with ascorbate. Orr's results (16,17,18) indicate that ascorbate inactivates catalase through peroxy or hydroxl radical attack on the protein. This concept was substantiated by Orr's (16) finding that ascorbate caused degradative changes, actually cleaving the molecule to smaller peptide fragments. More recently, however, inactivation of catalase in haemosylate was reversed by ethanol or NADPH which suggests that the inactivated form of catalase was compound II (19). Thus, the two mechanisms proposed for the inactivation of catalase by ascorbate are (i) the formation of compound II by saturating levels of H_2O_2 and (ii) free radical attack on the enzyme.

Ascorbate has been excluded as the inhibiting species because inactivation requires oxygen (15) and is greatly accelerated by metal ions (15,17). Also, failure of pre-incubation of ascorbate with Cu^{2+} to lead to a stable inhibitory product (17) suggests that a transient intermediate in the oxidation of ascorbate is responsible for inactivating catalase. Intermediates in the oxidation of ascorbate to dehydroascorbate and hydrogen peroxide (20) include

semidehydroascorbate (21) and superoxide (22), while ascorbate can react further with hydrogen peroxide to produce free radical intermediates (23). At least in part, this involves the reduction of H_2O_2 (cyclically mediated by metals in a Fenton type reaction) to produce highly damaging hydroxyl radicals (24). Any one or a combination of these radical species may be responsible for inactivating catalase.

Although semidehydroascorbate is relatively unreactive and decays mainly by disproportionation (25) it reacts faster with cytochrome c than does ascorbate (26). Futhermore, the semiquinone radicals of 1,4-naptho-quinone-2-sulphonic acid and 6-hydroxydopamine have been implicated in the inactivation of catalase (27). Superoxide, the other transient intermediate of ascorbate autoxidation, inhibits catalase either by a rapid reaction that is prevented and reversed by superoxide dismutase (SOD) or by a slow reaction that is prevented by SOD or ethanol and reversed by ethanol only (28,29). The rapid inhibition was reportedly due to the formation of unstable compound III while the slow inhibition was attributed to production of compound II through the reduction of compound I by superoxide. It is unlikely that Fenton derived hydroxyl radicals are involved in inhibiting catalase because catalase is so efficient (30). In its presence H_2O_2 levels should be kept too low to give rise to substantial quantities of hydroxyl radicals via the Fenton reaction.

By applying the current knowledge of the actions of scavengers of activated oxygen and transition metals, this investigation into the inactivation of catalase by ascorbate was undertaken to clarify the discrepancies between previous investigations, to determine the species responsible for inactivation and to describe the reaction pathways through which they act.

MATERIALS and METHODS

Materials

L-Ascorbic acid, Analar grade, was from BDH Chemicals. Catalase (65,000 units/mg) was obtained from Boehringer because this commercial source has been shown to be free of superoxide dismutase (31). Crystalline, bovine blood superoxide dismutase (2,800 units/mg) and ascorbate oxidase (350 units/mg) were obtained from Sigma. Desferrioxamine was a gift from the Ciba Pharmaceutical Company. All other reagents were of analytical grade.

Procedures

Deionised, distilled water was used for preparation of stock buffer solutions. Ion contamination of the water was negligible (>10 Mohm cm^{-3}). Ascorbic acid stock was always freshly prepared in phosphate buffer (50mM, pH 7.0) and neutralized with 0.1M NaOH. A stock solution of $Cu^{2+}(His)_2$ was prepared by dissolving $CuSO_4$ and L(+)histidine monohydrochloride, in the molar ratio 1:2, in phosphate buffer (50mM, pH 7.0). In all cases, reagents, excepting catalase and $Cu^{2+}(His)_2$, were added to phosphate buffer (50mM, pH 7.0) to give the desired concentrations in a final volume of 2.025ml and pre-incubated at 37°C in sealed virtis vials for 10 minutes.

When used, $Cu^{2+}(His)_2$ was added immediately before the reaction was initiated by the addition of 0.1mg of catalase. The incubate was thoroughly mixed, aliquots were withdrawn and assayed for catalase activity at predetermined intervals for 1 hour. Atmospheres of nitrogen (Linde high purity grade < 5 ppm oxygen) or oxygen (Linde high purity grade) were induced in rubber capped vials, using a Virtis gas manifold, by five cycles of repeated evacuation to boiling and reintroduction of the desired gas to a slight positive pressure. Rubber caps were carefully degassed prior to use. All reagents added to incubates under nitrogen atmospheres were prepared under atmospheres of nitrogen.

Catalase assay

Catalase activity was measured using an LKB spectrophotometer coupled to a micro-computer. The assay procedure was as outlined by Aebi (32) with the following modifications. An aliquot was withdrawn from the reaction mixture and rapidly mixed into a cuvette containing enough H_2O_2 in phosphate buffer(50mM, pH 7.0) to give an initial absorbance between 0.550 and 0.520 at 240nm. The rate of change in absorbance between 0.450 and 0.400 was taken as a measure of catalase activity. Aliquot volumes between 8μl and 25μl were chosen to give a rate of change in absorbance of between 0.02 and 0.10 absorbance units per 15 seconds.

Ascorbate analysis

The method used for ascorbate analysis is a modification of that given by Chevion and Navok (33). A heating step was introduced because the reaction was not instantaneous, taking several hours to reach maximum absorbance. This finding was similar to that of Schilt and Di Tusa's (34) who used PPTS as a ferroin type chromagen to assay ascorbate. This analysis involves the reduction by ascorbate of ferriphenanthroline to ferrophenanthroline and then measuring the change in absorbance at 515nm due to the production of the ferrous complex. A stock solution of ferriphenanthroline (1.8mM) was adjusted to pH 5 with HCl. Before assaying ascorbate the stock was diluted 1:10 in imidazole buffer (0.1M, pH 8.0) and a 2ml aliquot was transfered to an air tight screw cap cuvette. Nitrogen was first scrubbed with 5% sodium sulfite solution to remove any traces of oxygen, then bubbled through the buffered ferroin solution for 10 minutes. A 25µl aliquot was injected into the air tight cuvette which was then heated to 90°C for exactly 10 minutes. Absorbance was recorded immediately. A reagent blank prepared in the same manner, except for the addition of ascorbate, gave negligibly small absorbance values. All assays were performed in duplicate. Ascorbate concentrations were calculated from the regression line of a graph of absorbance as a function of ascorbate concentration. The correlation coefficient of the regression line was 0.994 and the standard error of the estimated variance was 0.0275, giving a maximum error of ± 4.8%.

Optical Absorption Spectra

Optical absorption spectra were recorded with the above mentioned spectrophotometric equipment. Absorbance measurements were recorded at 0.9 nanometer intervals at a scanning speed of 1 nanometer per second. The molecular weight of catalase was assumed to be 240,000 (30). The spectra are presented in Appendix 1.

Numerical Analysis

The initial activty of catalase was taken to be the mean value for catalase incubated alone in phosphate buffer for 1 hour. Each treatment effect was compared with the control for that particular set of experiments. Positive results (treatments which showed significant deviation from the control) were replicated. Estimates of the experimental error were ± 6.9% based on 8 experiments performed on different days and ± 2.2% based on 3 experiments performed on the same day.

The data were fitted to the exponential decay equation

$$y = a.e^{-b.x} + c$$

where a is the initial value minus the final value c and b is the time decay constant. The unknown coefficients were calculated by an iterative non-linear regression

procedure (35,36). This provided the initial rates (-a.b) and final values (c) of enzyme activity with their associated standard errors. The initial rate is obtained by differentiating y with respect to x and setting x equal to zero. The values of the coefficients were tested with a one-tailed t test (35) to determine the significance ($p<0.05$) of differences between treatment effects.

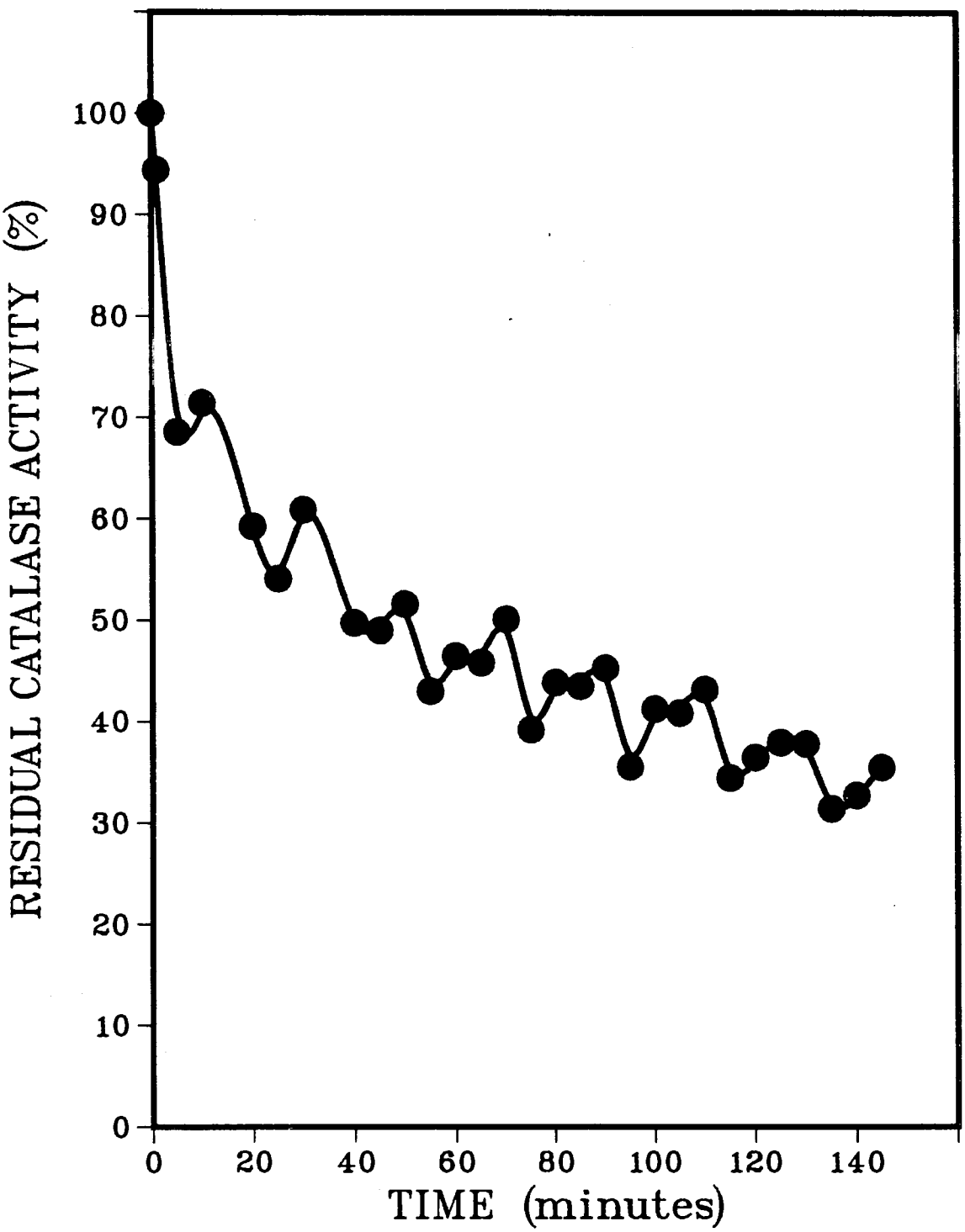
RESULTS

Inhibition of catalase by ascorbate

When catalase was incubated with ascorbate there was a progressive loss of catalase activity (Fig. 2). In the initial 25 minutes catalase lost 45% of its activity. Subsequent to this rapid inhibition there was a further slow net inhibition of 25% over the following 125 minutes. This slow phase of inhibition displayed oscillatory behaviour. In all further experiments catalase activity was measured over 1 hour and consistently displayed a single oscillation phase (Figs. 3-8). In contrast, the varation in catalase activity, when catalase was incubated alone, was random. Fluctuations in the temperature of the water bath were minimal ($<0.2°C$), therefore, they were considered unlikely to evoke the observed oscillations.

Trend analysis (37) was used to test the statistical significance of these oscillations. The method of orthogonal polynomials was applied to the oscillatory data of Fig. 3 and significant ($p<0.001$) contributions of the cubic and quartic components were revealed. This indicates that the trend in the decay of catalase activity is best described by a fourth order polynomial, i.e. a function that is oscillatory in nature.

Fig. 2 Inhibition of catalase by ascorbate. The final incubate contained 50mM phosphate buffer, pH 7.0, 0.20μM catalase and 2mM ascorbate at 37°C. Aliquots were withdrawn and residual catalase activity was measured in terms of the rate of decrease in absorbance between 0.450 and 0.400 at 240nm due to H_2O_2 decomposition. The estimated S.D. was ± 2.2% (see materials and methods).


RESIDUAL CATALASE ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
TIME (minutes)

Effects of $Cu^{2+}(His)_2$ and metal chelates

In order to ascertain the effects of metal ions, catalase and ascorbate were incubated with $Cu^{2+}(His)_2$ or chelating agents. Ascorbate alone decreased catalase activity to approximately 60% of its original activity within 1 hour (Fig. 3). The addition of $Cu^{2+}(His)_2$ enhanced this effect, decreasing activity to about 20% of the original value. Neither desferrioxamine nor diethylenetriaminepentaacetic acid (DTPA) modified the inactivation induced by ascorbate, although DTPA reduced the effect of asorbate plus $Cu^{2+}(His)_2$ decreasing the inhibition to that of ascorbate alone.

Effects of superoxide dismutase

SOD had no significant effect on the inactivation of catalase induced by ascorbate (Table 1) when added to a final concentration of 0.15μM. Such a high concentration was used because reportedly lower concentrations fail to exert an effect on ascorbate oxidation (38,39). In these studies, due to the difficulty of permanently denaturing SOD by boiling, DTPA was included as a control for the possible chelating effects of SOD. Bovine serum albumin (BSA) was used as a control to reveal any non-specific protein effects.

Fig. 3 Effects of $Cu^{2+}(His)_2$ and metal chelators on inhibition of catalase by ascorbate. Incubation and assay conditions were as described in Fig. 2. When present, $Cu^{2+}(His)_2$, DTPA and desferrioxamine were added to final concentrations of 40μM, 0.1mM and 0.1mM respectively. The lines plotted represent (○) catalase only; (●) catalase plus ascorbate; (▽) catalase plus ascorbate and DTPA; (■) catalase plus ascorbate and desferrioxamine; (△) catalase plus ascorbate and $Cu^{2+}(His)_2$; (□) catalase plus ascorbate, Cu^{2+}-(His)2 and DTPA. The estimated S.D. was ± 2.2% (see materials and methods).

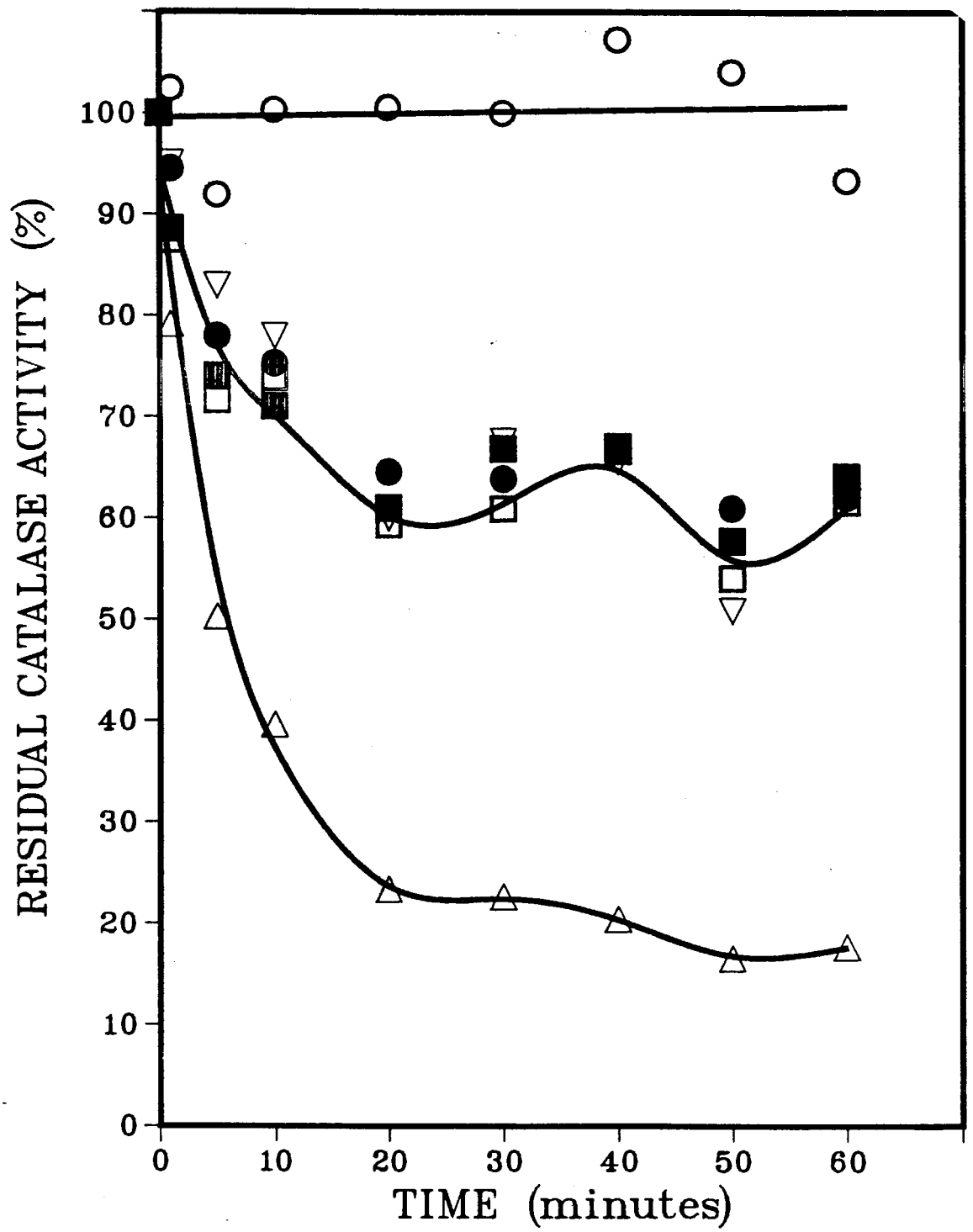

RESIDUAL CATALASE ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (minutes)

Table 1. Effects of oxy-radical scavengers on the initial rate of inhibition of catalase by ascorbate. Incubation and assay conditions were as described in Fig. 2. DTPA was included in the SOD and BSA trials at a final concentration of 0.1mM. The initial rates of reactions with their associated standard errors were calculated by non-linear regression (see materials and methods). None of these values was significantly ($p < 0.05$) different from the control.

Scavenger	Initial rate of inhibition -% enzyme activity / minute
None	5.27 ± 1.2
SOD (0.15μM)	3.75 ± 0.9
BSA (100μg/ml)	5.62 ± 1.8
Thiourea (1mM)	3.39 ± 1.8
Mannitol (1000mM)	4.76 ± 1.3

Effects of hydroxyl radical scavengers

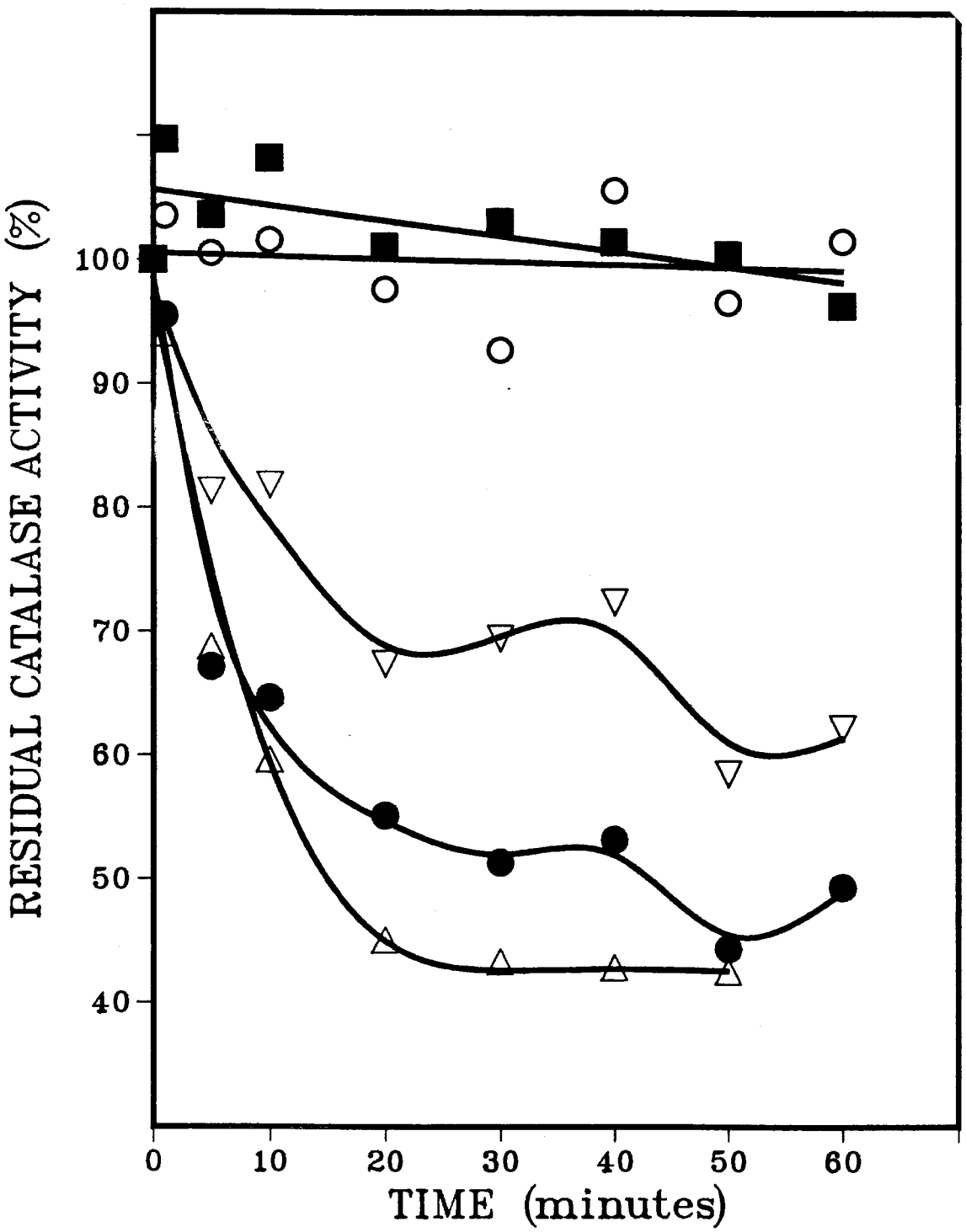
To test the hypothesis that hydroxyl radicals are responsible for the inactivation of catalase, thiourea or mannitol were used as hydroxyl radical scavengers to see if inactivation could be prevented. Urea was used as a control for thiourea because it is a structural analog of thiourea but it is a poor hydroxyl radical scavenger (40).

The lack of significant effects of hydroxyl radical scavengers (Table 1) contrasts with Orr's (17) result that dimethylsulphoxide decreased the extent of catalase inhibition induced by ascorbate. This discrepancy can be explained by traces of metal ion contaminants in Orr's preparations and will be more completely discussed in Chapter 3.

Effects of nitrogen and oxygen atmospheres

In agreement with the results of Keilin and Hartree (15) nitrogen prevented inactivation of catalase induced by ascorbate alone (Fig. 4). The protection offered by nitrogen, however was relieved by 2mM H_2O_2. In this reaction, to exclude the formation of O_2 from the decomposition of H_2O_2 by catalase and reaction of H_2O_2 with ascorbate, H_2O_2 was initially incubated with catalase for 5 minutes. The reaction vial was then evacuated and placed under nitrogen before the reaction was started by the addition of ascorbate.

Fig. 4. Effects of nitrogen and oxygen atmospheres on inhibition of catalase by ascorbate. Incubation and assay conditions were as described in Fig. 1 while atmospheres of the desired gas were induced into rubber capped vials as outlined in materials and methods. The lines plotted represent (O) catalase alone under air; (●) catalase plus ascorbate under air; (■) catalase plus ascorbate under nitrogen; (▽) catalase plus ascorbate under oxygen; (△) H_2O_2 treated catalase and ascorbate under nitrogen. The estimated S.D. was ± 2.2% (see materials and methods).

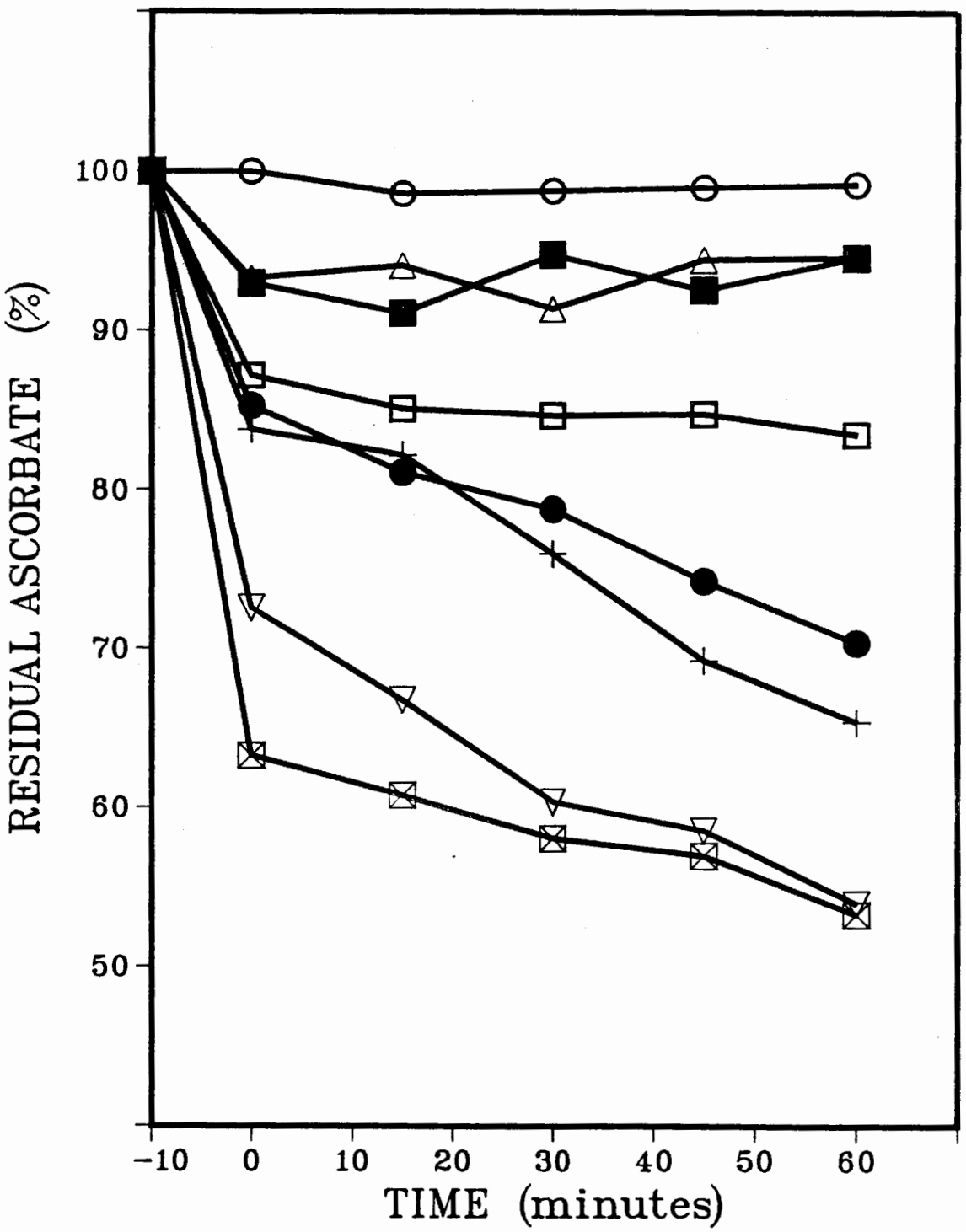

RESIDUAL CATALASE ACTIVITY (%)
100
90
80
70
60
50
40
0
10
20
30
40
50
60
TIME (minutes)

This procedure was employed to maintain anaerobic conditions and hinder the formation of semidehydroascorbate. When 2mM H_2O_2 was incubated with catalase alone for one hour there was no significant difference between the initial (100%) and final (105%) values of catalase activity. Surprisingly, an atmosphere of oxygen also offered some protection, decreasing the initial rate of inhibition by 45%.

Effects on ascorbate oxidation

In an attempt to correlate the oxidation of ascorbate with the inhibition of catalase, the rate of oxidation of ascorbate in the presence of selected scavengers was measured (Fig. 5). When catalase was added after 10 minutes of pre-incubation of ascorbate, the rate of ascorbate oxidation was dramatically decreased. DPTA in combination with catalase completely blocked ascorbate oxidation. Desferrioxamine was less effective in this regard. When SOD was added along with DTPA no additional protection could be observed in the initial phase of oxidation, compared to that with DTPA alone. An atmosphere of nitrogen completely blocked oxidation of ascorbate while ascorbate oxidase, $Cu^{2+}(His)_2$ (both added immediately before catalase) and an atmosphere of oxygen all accelerated oxidation compared to their respective controls. Interestingly, the rate of ascorbate oxidation in the presence of catalase was approximately the same for 20% and 100% oxygen atmospheres.

Fig. 5 Effects of oxygen, metal chelating agents, $Cu^{2+}(His)_2$, ascorbate oxidase and SOD on the oxidation of ascorbate in the presence of catalase. Incubation conditions were as described in Fig. 2. Aliquots of 25μl were withdrawn and residual ascorbate concentration was determined by measuring the increase in absorbance at 515nm due to the production of ferrophenanthroline. When present, $Cu^{2+}(His)_2$, ascorbate oxidase, SOD, DTPA and desferrioxamine were added to final concentrations of 40μM, 1.7 units/ml, 0.15μM, 0.1mM and 0.1mM respectively. The lines plotted represent (●) catalase plus ascorbate under air; (■) catalase plus ascorbate and DTPA; (□) catalase plus ascorbate and desferrioxamine; (▽) catalase plus ascorbate and $Cu^{2+}(His)_2$; (△) catalase plus ascorbate, Cu^{2+}-(His)2 and DTPA; (+) catalase plus ascorbate and ascorbate oxidase; (O) catalase plus ascorbate under N_2; and (⊠) catalase plus ascorbate under O_2.


RESIDUAL ASCORBATE (%)
100
90
80
70
60
50
−10
0
10
20
30
40
50
60
TIME (minutes)

Effects of ascorbate concentration on the initial rate of inhibition

The relationship between the concentration of ascorbate and the initial rate of inhibition was investigated primarily to determine whether or not the protection offered by oxygen (see above) was due solely to a decrease in the initial ascorbate concentration. Inhibition of catalase by ascorbate displayed saturation kinetics (Fig. 6), i.e. no further increase in the initial rate of inhibition of catalase occurred above an ascorbate concentration of 1mM. The initial rate of inhibition of catalase under an atmosphere of oxygen was 55% (Fig. 4) of that under air, which corresponds to an initial ascorbate concentration of approximately 0.4mM. However, under an atmosphere of oxygen ascorbate concentration never dropped below 1mM (Fig. 5).

Effects of ethanol

Because ethanol is a substrate of compounds I and II it was added to see if it could modulate the inhibitory action of ascorbate toward catalase. Ethanol (20mM) completely prevented catalase inactivation induced by ascorbate and moreover, when added during the reaction, to concentrations of 20mM or 40mM, it reversed inactivation (Fig. 7).

Fig. 6 Effects of ascorbate concentration on the initial rate of inhibition of catalase. Incubation and assay conditions were as described in Fig. 2. The maximum initial rate of inhibition was based on the rate of inhibition induced by 2mM ascorbate. Error bars represent ± the S.E. of the % of maximum initial rate of inhibition as calculated by non-linear regression.

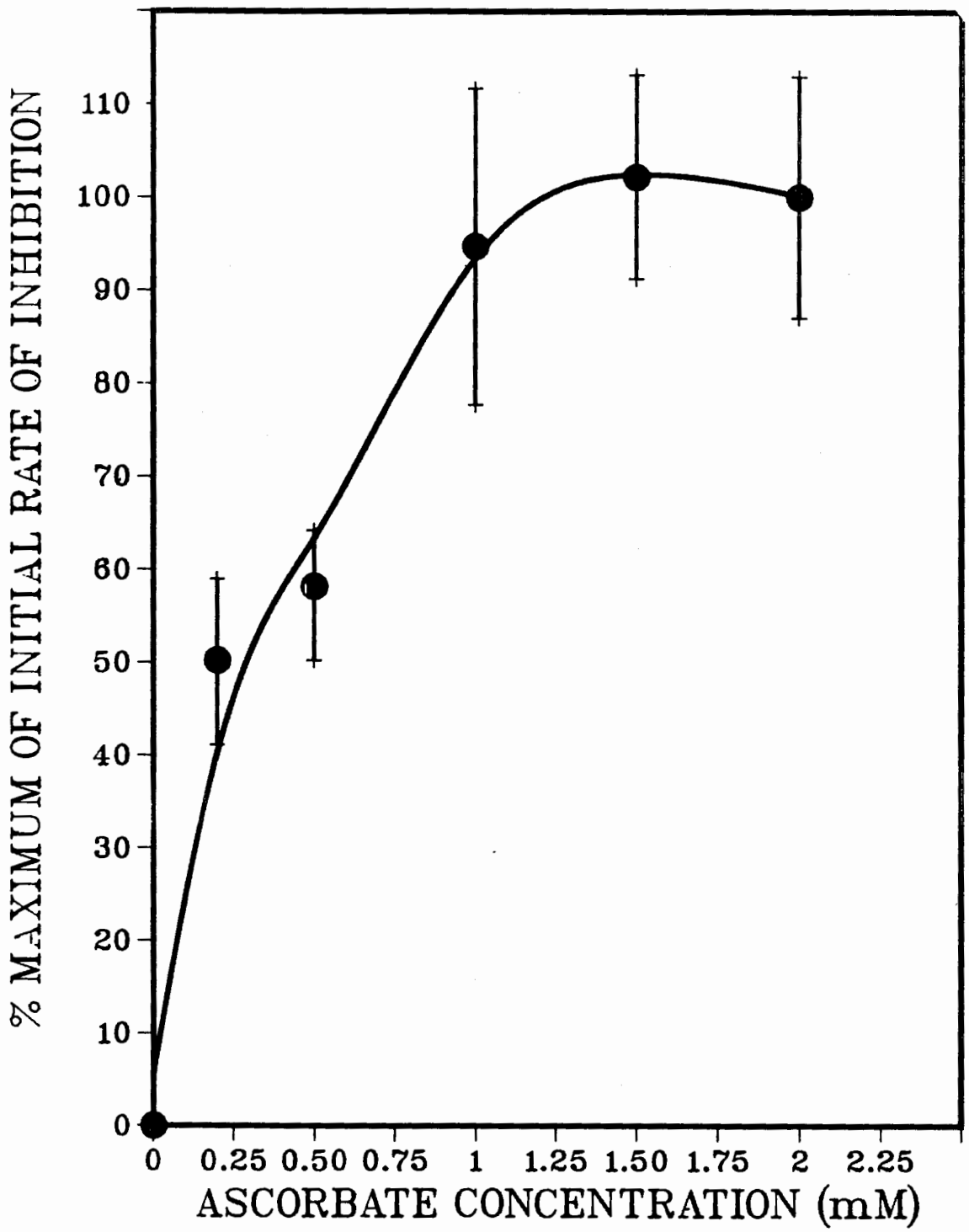
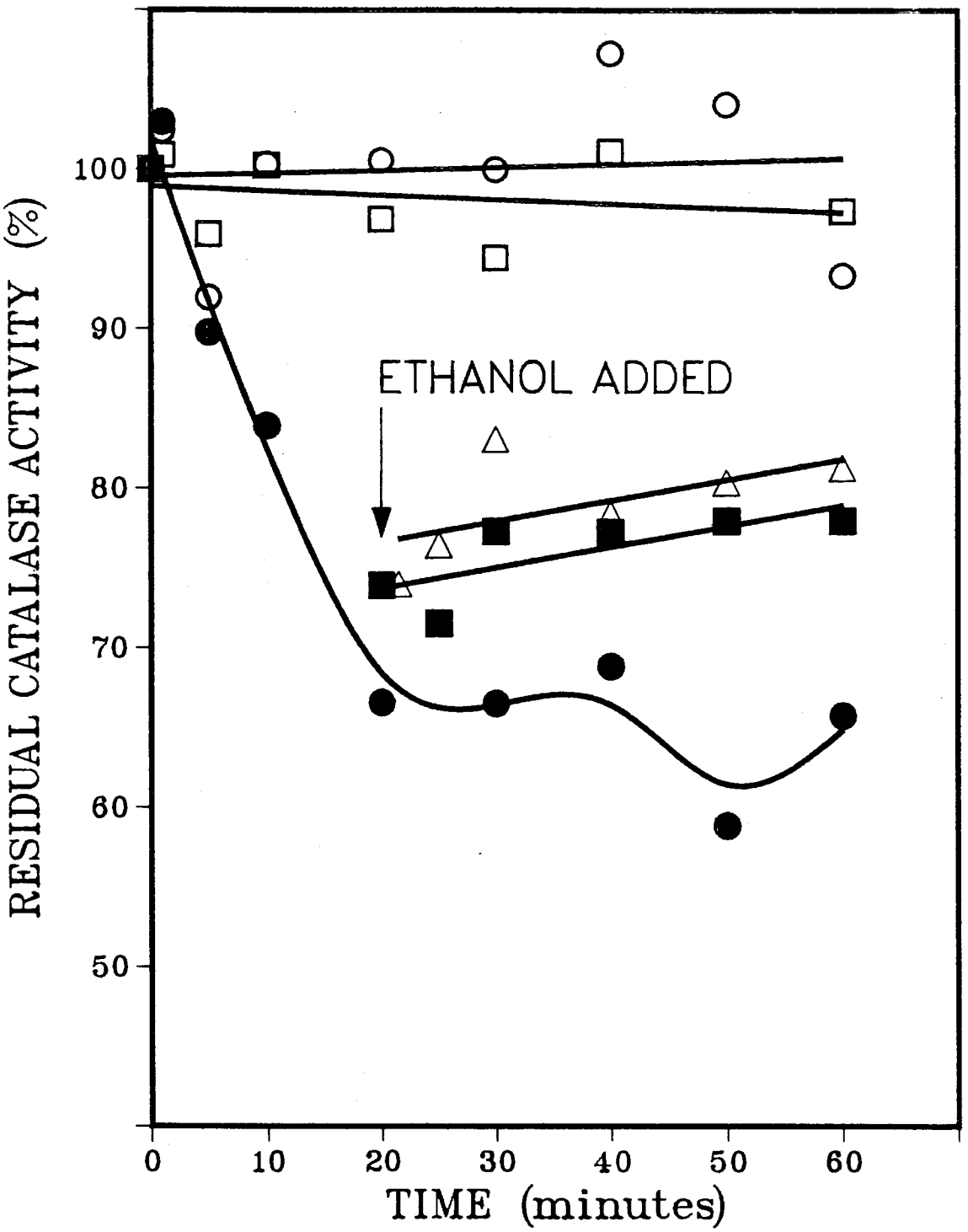

% MAXIMUM OF INITIAL RATE OF INHIBITION
110
100
90
80
70
60
50
40
30
20
10
0
0
0.25
0.50
0.75
1
1.25
1.50
1.75
2
2.25
ASCORBATE CONCENTRATION (mM)

Fig. 7. Effects of ethanol on the inhibition of catalase by ascorbate. Incubation and assay conditions were as described in Fig. 2. The lines plotted represent (○)catalase alone; (●) catalase plus ascorbate; (□) catalase plus ascorbate and 20mM ethanol; (■) catalase plus ascorbate and 20mM ethanol added at 20 minutes; (△) catalase plus ascorbate and 40mM ethanol added at 20 minutes. The estimated S.D. was ± 2.2% (see materials and methods).


RESIDUAL CATALASE ACTIVITY (%)
100
90
80
70
60
50
ETHANOL ADDED
0
10
20
30
40
50
60
TIME (minutes)

Effects of ascorbate oxidase

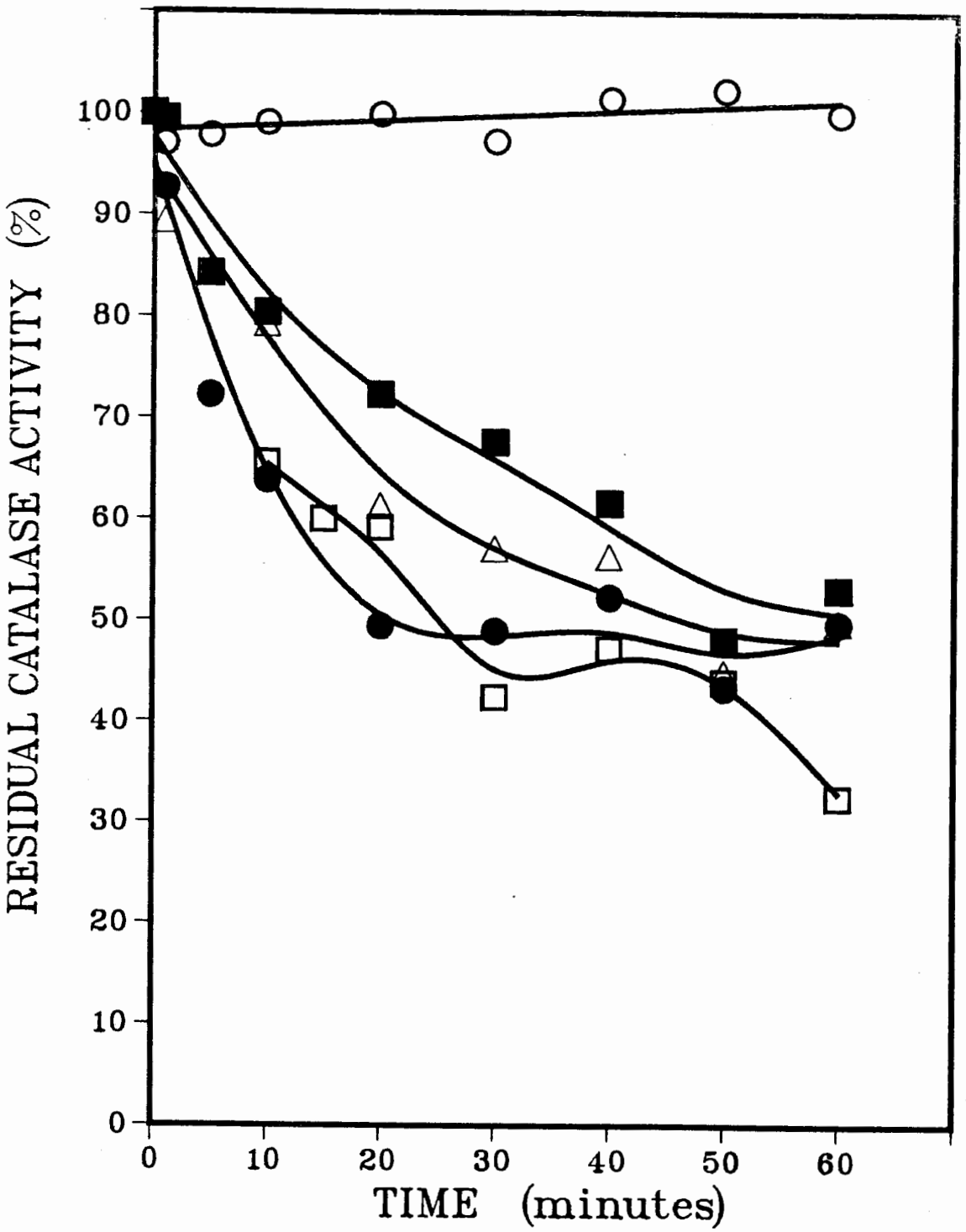
Ascorbate oxidase oxidizes ascorbate without generating H_2O_2 while liberating semidehydroascorbate as a reaction product (41). Because this enzyme can modify these three reactants in our system it was included to see how it might influence the effect of ascorbate. High concentrations of ascorbate oxidase (10 units/ml) decreased the inhibition of catalase by ascorbate but simultaneous addition of 2mM H_2O_2 relieved this protection slightly (Fig. 8). Similar addition of H_2O_2 to catalase incubated with ascorbate only had no effect on the extent of inhibition (data not shown). When low concentrations of this enzyme (1.7 units/ml) were added 10 minutes after the commencement of the reaction the final extent of catalase inhibition was increased by 18% compared to ascorbate alone.

Optical absorption spectra

The extinction coefficient at 404nm decreased when catalase (2μM) was incubated with ascorbate (2mM) in phosphate buffer (50mM, pH 7.0) at 20°C, indicating that compound I was formed (14). Also, there was a slight shift of 2nm toward higher wavelengths in the Soret region, which agrees with the earlier results (14,15) that compound II is formed in this reaction. Orr's (16) inability to detect these small changes is presumably

because he used much lower concentrations of catalase.

Fig. 8. **Effects of ascorbate oxidase on the inhibition of catalase by ascorbate.** Incubation and assay conditions were as described in Fig. 2. The lines plotted represent (O) catalase alone; (●) catalase plus ascorbate (■) catalase plus ascorbate and 10 units/ml ascorbate oxidase; (Δ) catalase plus ascorbate, 10 units/ml ascorbate oxidase and 2mM H_2O_2; (□) catalase plus ascorbate and 1.7 units/ml ascorbate oxidase added at 10 minutes. The estimated S.D. was ± 2.2% (see materials and methods).


RESIDUAL CATALASE ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (minutes)

DISCUSSION

Exclusion of activated species of oxygen

Clearly, the inhibition of catalase is independent of ascorbate oxidation because metal chelating agents virtually blocked ascorbate oxidation in the presence of catalase (Fig. 5) but they had no effect on the inhibition of catalase (Fig. 3). Therefore, transient intermediates and products of ascorbate oxidation are not the inhibiting species. Further support for this finding is the lack of significant effects on the initial rate of catalase inhibition by the respective scavengers of superoxide and hydroxyl radicals (Table 1). The potentiation by $Cu^{2+}(His)_2$ refutes this argument. In a Chapter 3 it will be shown that ascorbate plus $Cu^{2+}(His)_2$ inactivates catalase by a mechanism that is quite different from that involved here. Although inhibition of catalase is independent of ascorbate oxidation, semidehydroascorbate cannot be excluded because it can be produced by comproportionation between ascorbate and dehydroascorbate (42) formed in the pre-incubation phase of ascorbate oxidation.

H_2O_2 can also be excluded as the inhibiting species because it had no effect when added to incubates of catalase alone or to catalase plus ascorbate. Furthermore, an atmosphere of oxygen increased the initial rate of ascorbate oxidation, presumably

increasing H_2O_2 production, but actually protected rather than aggravated catalase inhibition. However, because H_2O_2 relieved the protection offered by nitrogen or by high concentrations of ascorbate oxidase, it must be implicated in the mechanism of inhibition of catalase. Nevertheless, from these results alone it is clear that neither of the two mechanisms proposed previously is completely valid.

Identification of reactive intermediates

The data support the concept that either ascorbate or semidehydroascorbate is the active species that inhibits catalase. Ascorbate alone can inhibit catalase because under anaerobic conditions that were adjusted to minimize semidehydroascorbate formation, catalase was still inhibited. The increases in extents of catalase inhibition and ascorbate oxidation in the presence of ascorbate oxidase, however, suggest that semidehydroascorbate can also inhibit catalase. Furthermore, the protection offered by O_2 is not fully accounted for by the oxidation of ascorbate. Thus oxygen must be reacting with an intermediate of ascorbate oxidation that can inhibit catalase, most plausibly semidehydrascorbate.

The reaction between O_2 and semidehydroascorbate is kinetically (25) and thermodynamically (43) unfavourable (the respective reduction potentials for the couples $O_2/O_2^{-}\bullet$ and $A/AH^{-}\bullet$ being -0.33 and -0.21) but the rate of its reaction is

nevertheless non-zero. From a simple calculation using the data of Bielski et al. (25) it is evident that the rate of reaction of semidehydroascorbate with O_2, at saturating levels of O_2, is approximately 50% of the rate of disproportionation of this radical. The simplest mechanism by which high oxygen concentrations protect against catalase inhibition is by oxidation of semidehydroascorbate, i.e.

$$A\bullet^- + O_2 \text{ ----> } A + O_2\bullet^- \quad (1)$$

Spectral evidence plus the result that ethanol can reverse inhibition indicates that compound II is the inactive form of catalase produced in this reaction. The requirement for H_2O_2 implies that compound I is also involved in the reaction mechanism.

Mechanisms of inhibition of catalase

On the basis of the above results the reaction pathway involved in inhibition of catalase by ascorbate can be written as :

$$AH_2 + O_2 \text{ ----> } A + H_2O_2 \quad (2)$$

Ferricatalase + H_2O_2 -----> Compound I (3)
Fe(III) Fe(V)

Compound I + AH^- -----> Compound II + $A\bullet^-$ (4)
Fe(V) Fe(IV)

Compound I + $A\bullet^-$ -----> Compound II + A (5)
Fe(V) Fe(IV)

This mechanism is analogous to that proposed by Yamazaki et al. (44) for the peroxidatic action of peroxidase, except that free radical intermediates appeared to be poor substrates for peroxidases. The relative extents to which ascorbate and semidehydroascorbate reduce compound I cannot be determined from this investigation. It can be concluded, however, that because semidehydroascorbate will be present in much lower concentrations than ascorbate, the rate constant for its reaction with compound I must be considerably greater than that of ascorbate.

Protection by Ethanol

Ethanol prevents inhibition by preferentially reacting with

compound I to regenerate ferricatalase, i.e.

$$\underset{Fe(V)}{\text{Compound I}} + CH_3CH_2OH \longrightarrow \underset{Fe(III)}{\text{Ferricatalase}} + CH_3CHO + H_2O \quad (6)$$

In addition, ethanol reverses inhibition by reducing compound II back to active catalase, i.e.

$$2\underset{Fe(IV)}{\text{Compound II}} + CH_3CH_2OH \longrightarrow 2\underset{Fe(III)}{\text{Ferricatalase}} + CH_3CHO \quad (7)$$

Reasons for Oscillations

In support of the observed oscillatory behaviour, Yamasaki and coworkers (45,46) have demonstrated that the analogous oxidation of NADH by horseradish peroxidase involves the coupled oscillations of molecular oxygen and compound III. Furthermore, the requirements for a reaction to display oscillatory behaviour (47), namely that oscillations only occur in extensively coupled multivariate systems, that they affect changes of state far from equilibrium and that oscillatory chemical systems contain unstable states (e.g. compound II), are all meet in the system under study.

If compound II is reduced back to active catalase by ascorbate or semidehydroascorbate (reaction (8)) then the series of oscillations (Fig. 2) can be explained by fluctuations in the relative concentrations of compounds I and II as the reaction progresses to equilibrium.

$$\underset{\text{Fe(IV)}}{\text{Compound II}} + AH^-/A\bullet^- \longrightarrow \underset{\text{Fe(III)}}{\text{Ferricatalase}} + A\bullet^-/A + H^+ \quad (8)$$

V, the reaction velocity of the catalase assay, is defined by the equation :

$$V = k.([\text{ferricatalase}] + [\text{compound I}]).[H_2O_2] \quad (i)$$

The initial concentration of H_2O_2 is necessarily always constant so that :

$$V = k'([\text{ferricatalase}] + [\text{compound I}]) \quad (ii)$$

However, the concentrations of ferricatalase and compound I can be expressed in terms of the total enzyme concentration, '[E]', and the concentration of compound II by the equation :

$$[\text{ferricatalase}] + [\text{compound I}] = [E] - [\text{compound II}] \quad (iii)$$

taken that the concentration of other possible enzyme-peroxide complexes is negligble. By substitution one obtains the differential equation :

$$dV/dt = -k'd[\text{compound II}]/dt \quad \text{(iv)}$$

From reactions (5) and (8) equation (iv) can be expressed as:

$$dV/dt = k'_{8}.[\text{Compound II}] - k'_{5}.[\text{compound I}] \quad \text{(v)}$$

The term [AH] has been incorporated into the constants because in this system the reductant was always present in saturating amounts, i.e > 1mM for ascorbate. Hence, whenever $k'_{5}.[\text{compound I}]$ is greater than $k'_{8}.[\text{compound II}]$, such as the beginning of the reaction, there will be a net drop in catalase activity. Catalase activity will rise though when the net reaction favours reduction of compound II. However, the series of oscillations will die out as $k'_{8}.[\text{compound II}]$ approaches $k'_{5}.[\text{compound I}]$.

It is recommended that these oscillations are investigated further so as to establish their exact cause.

Implications of reversible inhibition of catalase by ascorbate

In living cells it is likely that the ability of catalase to be reduced by one-electron donors has been utilized by evolution to serve useful ends. Our results indicate that

catalase may not only remove H_2O_2 catalytically but it may also remove H_2O_2 peroxidatically, by utilizing ascorbate as a substrate. Also, on the basis that semidehydroascorbate is a major species responsible for inactivating catalase, it is interesting to speculate that catalase can act peroxidatically toward this and related radicals, such as semiquinones, preventing free radical chain propagation and subsequent cellular damage. In both these instances electrons supplied by glycolysis and the hexose monophosphate shunt, in the form of NADH and NADPH, which can reduce compound II (48) would maintain catalase in its active state. These conclusions extend the hypothesis of Sullivan et al. (49) that O_2, SOD and catalase form a metabolic pathway that protects against oxidative damage, to also include protection against semidehydro radicals of enediols like ascorbate. Whether the rate of removal of radicals of this type by catalase is rapid enough to provide a useful contribution to their elimination, must await estimation of ambient concentrations of semidehydroascorbate and semiquinones in cells. Also, estimates of their rates of reaction with catalase under varying concentrations and reaction conditions must be obtained.

SUMMARY

Copper accelerated inhibition of catalase induced by ascorbate but the metal chelating agents desferrioxamine and DTPA were without effect. The inhibition of catalase by ascorbate was prevented and reversed (80%) by ethanol (20mM or 40mM). Superoxide dismutase, thiourea (1mM) and mannitol (1M) had no protective effect, so that the involvement of superoxide and hydroxyl radicals is excluded. A nitrogen atmosphere protected completely against ascorbate-induced inhibition. This protection, however, was relieved by the inclusion of H_2O_2 prior to the addition of ascorbate. H_2O_2 alone did not induce inactivation of catalase. Increasing oxygen concentration from ambient to 100% also slowed inhibition. The protection by oxygen could not be explained solely by an increased oxidation of ascorbate, implicating an intermediate of ascorbate oxidation. High concentrations of ascorbate oxidase retarded inhibition of catalase by ascorbate. This effect was due to a decline in H_2O_2 production. In contrast, low concentrations of ascorbate oxidase, added after the start of the reaction, increased the final extent of catalase inhibition by 18%. The optical absorption spectra of catalase incubated with ascorbate revealed the conversion of active catalase to inactive compound II. Spectral changes and the exclusion of the major alternative damaging radicals suggest that ascorbate and/or semidehydroascorbate reversibly inhibit catalase by reducing

compound I to inactive compound II.

CHAPTER 3

ROLES OF FREE RADICAL INTERMEDIATES IN THE IRREVERSIBLE INACTIVATION OF CATALASE BY ASCORBATE PLUS $Cu^{2+}(His)_2$

INTRODUCTION

Ascorbate, in the presence of copper, has been reported to promote biological damage by two quite different mechanisms. In the mechanism proposed by Chevion and coworkers (50,51), ascorbate plays a dual role by reducing Cu^{2+} to Cu^{1+} and by providing H_2O_2 through its autoxidation. In turn, Cu^{1+} bound to macromolecules reacts with H_2O_2 in a Fenton reaction to produce highly damaging hydroxyl radicals, which react preferentially with the macromolecule at the site of their generation. In the second of these mechanisms it is postulated that Cu^{2+} binds histidine residues and subsequently chelates O_2 and semidehydroascorbate (52). This complex is then thought to

promote specific degradation of histidine, damaging proteins and inactivating enzymes.

Catalase is known to be irreversibly inactivated by ascorbate plus Cu^{2+}. An understanding of the mechanism involved is of paramount importance because before ascorbate plus Cu^{2+} can induce damage to a wide spectrum of macromolecules, catalase must first be inactivated to allow sufficient levels of H_2O_2 to become available for site specific production of hydroxyl radicals.

Keilin and Hartree (15) were the first to investigate the inactivation of catalase by ascorbate in the presence of Cu^{2+}. Their absorption spectra revealed that inactivation was due to formation of compounds II and III. They proposed that these inactive forms of catalase were produced from the reaction between active catalase and saturating levels of H_2O_2. Orr (16) investigated this reaction further but was unable to detect formation of these inactive compounds. This was possibly a result of incubating low concentrations of catalase at pH 7.0 where formation of compounds I and II is slow in comparison to pH 5.0 (4). However, Orr demonstrated that in the simultaneous presence of ascorbate and Cu^{2+}, catalase was extensively degraded to smaller peptide fragments. Because of this result he speculated that catalase is inactivated by peroxy or hydroxyl radical attack on the protein.

In both of the above investigations (15,16) it was presumed that Cu^{2+} exerted its effect by accelerating the reaction in

which catalase is inactivated by ascorbate alone. However, ascorbate alone does not inactivate catalase by either of these mechanisms (see Chapter 2). Rather, it reversibly inhibits catalase through the univalent reduction of compound I to compound II by either ascorbate or semidehydroascorbate. Moreover, the acceleration by $Cu^{2+}(His)_2$ was in contradiction to the results that inhibition of catalase was independent of either transition metal ions or oxidation of ascorbate. Thus the mechanism of inactivation of catalase by ascorbate plus $Cu^{2+}(His)_2$ is different from that involving ascorbate alone. For this reason, the current study was undertaken to investigate the inactivation of catalase by ascorbate plus $Cu^{2+}(His)_2$ more fully to see in what respects the mechanism differs from that of ascorbate alone, and to determine the mechanism of inactivation.

MATERIALS and METHODS

Materials

L-Ascorbic acid, Analar grade, was from BDH Chemicals. Catalase (65,000 units/mg) was obtained from Boehringer because this commercial source has been shown to be free of superoxide dismutase (31). Crystalline, bovine blood superoxide dismutase (2,800 units/mg) was obtained from Sigma. All other reagents were of analytical grade.

Procedures

Deionised, distilled water was used for preparation of stock buffer solutions. Ion contamination of the water was negligible (>10 Mohm cm^{-3}). Ascorbic acid stock was always freshly prepared in phosphate buffer (50mM, pH 7.0) and neutralized with 0.1M NaOH. A stock solution of $Cu^{2+}(His)_2$ was prepared by dissolving $CuSO_4$ and L(+)histidine monohydrochloride, in the molar ratio 1:2, in phosphate buffer (50mM, pH 7.0). In all cases, reagents, excepting catalase and $Cu^{2+}(His)_2$, were added to phosphate buffer (50mM, pH 7.0) to give the desired concentrations in a final volume of 2.025ml and pre-incubated at 37°C in sealed Virtis vials for 10 minutes. $Cu^{2+}(His)_2$ was added immediately before the reaction was initiated by the addition of 0.1mg of catalase. The incubate was

thoroughly mixed, aliquots were withdrawn and assayed for catalase activity at predetermined intervals for 1 hour. Atmospheres of nitrogen (Linde high purity grade < 5 ppm oxygen) or oxygen (Linde high purity grade) were induced in rubber capped vials, using a Virtis gas manifold, by five cycles of repeated evacuation to boiling and reintroduction of the desired gas to a slight positive pressure. Rubber caps were carefully degassed prior to use. All reagents added to incubates under nitrogen atmospheres were prepared under atmospheres of nitrogen.

Catalase assay

Catalase activity was measured using an LKB spectrophotometer coupled to a micro-computer. The assay procedure was a modification of that outlined by Aebi (32). Briefly, the rate of change in absorbance between 0.450 and 0.400 at 240nm, due to the decline in H_2O_2, was taken as a measure of catalase activity.

Ascorbate analysis

The method used for ascorbate analysis was a modification of that given by Chevion and Navok (33). This analysis involves the reduction of ferriphenanthroline to ferrophenanthroline by ascorbate, and measuring the change in absorbance at 515nm due to the production of the ferrous complex. All assays were

performed in duplicate. Ascorbate concentrations were calculated from the regression line of a graph of absorbance as a function of ascorbate concentration. The correlation coefficient of the regression line was 0.994 and the standard error of the estimated variance was 0.0275, giving a maximum error of ± 4.8%. More detailed descriptions of the above two assays are given in Chapter 2.

Optical Absorption Spectra

Optical absorption spectra were recorded with the above mentioned spectrophotometric equipment. Absorbance measurements were recorded at 0.9 nanometer intervals at a scanning speed of 1 nanometer per second. The molecular weight of catalase was assumed to be 240,000 (30).

Electron Spin Resonance Spectra

ESR measurements were performed on a Varian E-4 spectrometer run at 20°C using a modulation amplitude of 0.025 Gauss and receiver gains of 20-50.

Numerical Analysis

The initial activty of catalase was taken to be the mean value for catalase incubated alone in phosphate buffer for 1 hour. Each treatment effect was compared with the control for that particular set of experiments. Positive results (treatments

which showed significant deviation from the control) were replicated. Estimates of the experimental error were ± 6.2% based on 4 experiments performed on different days and ± 2.2% based 3 experiments performed on the same day.

The data were fitted to the exponential decay equation

$$y = a.e^{-b.x} + c$$

and the unknowm coefficients were calculated by an iterative non-linear regression procedure (35,36) (see Chapter 2). This provided the initial rates (-a.b) and final values (c) of enzyme activity with their associated standard errors. The values of the coefficients were tested with a one-tailed t test (35) to determine the significance ($p<0.05$) of differences between treatment effects.

RESULTS

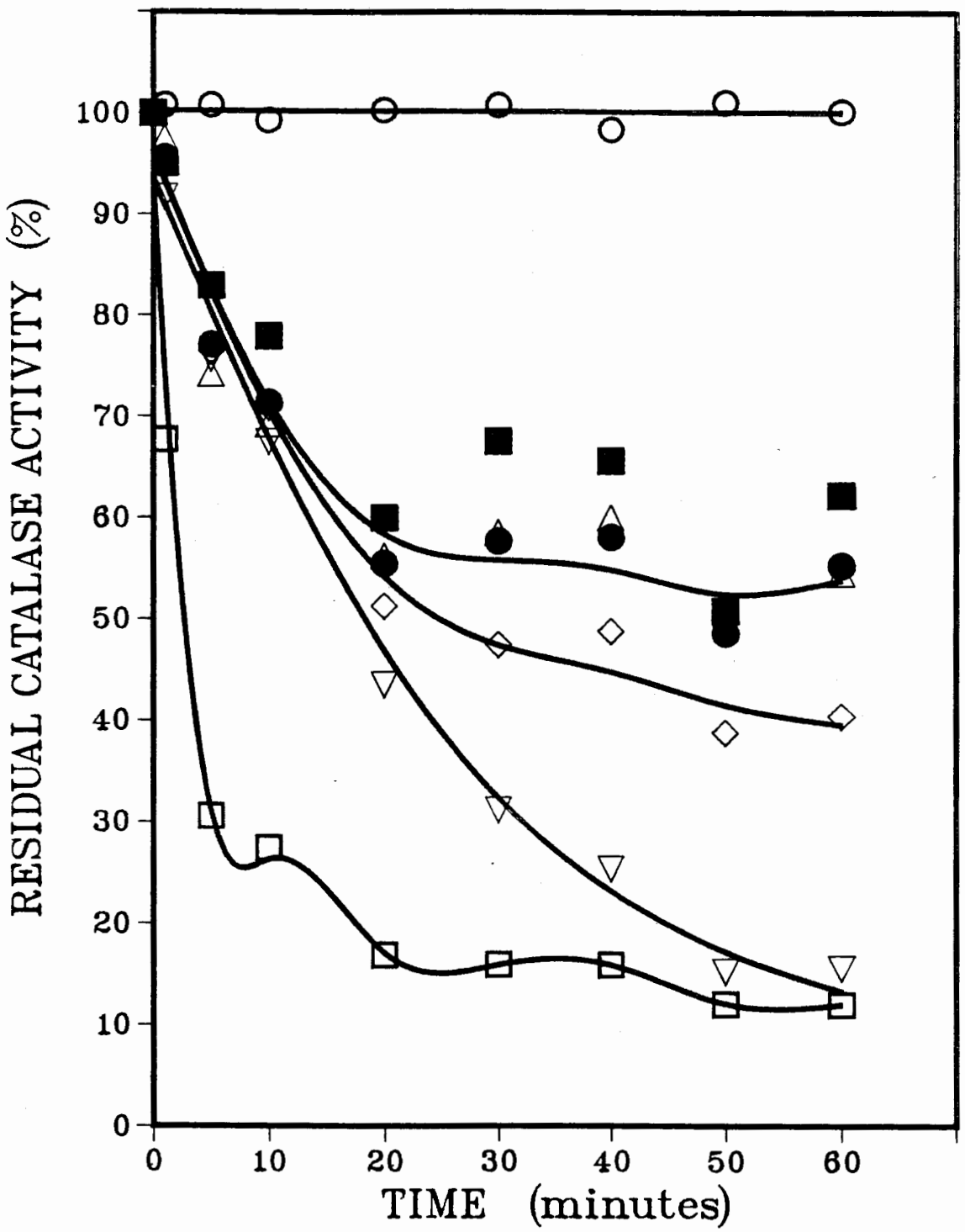
Effects of chelating agents

Incubation of catalase with ascorbate (2mM) and $Cu^{2+}(His)_2$ (40μM) resulted in rapid loss of enzymatic activity compared to that in the presence of ascorbate alone (Fig. 1). The resultant loss of activity was 80% after 20 minutes, while over the following 40 minutes there was a further slow loss of 5%. Under similar experimental conditions $Cu^{2+}(His)_2$ (40μM) alone caused a loss of catalase activity of 13% over 1 hour. Addition of the chelating agent diethylenetriaminepentaacetic acid (DTPA) decreased the rate and extent of inactivation to those values observed with ascorbate alone. Increasing the concentration of histidine from 40μM to 2mM resulted in a progressive decrease in the extent of catalase inactivation until at a histidine concentration of 2mM the rate and extent of inactivation were equivalent to those observed with ascorbate alone.

Effects of oxy radical scavengers

To determine whether or not superoxide and hydroxyl radicals are involved in the inactivation mechanism, scavengers of these radicals were incubated together with catalase and ascorbate plus $Cu^{2+}(His)_2$. Within the limits of experimental

Fig. 1 **Effects of ascorbate on the inactivation of catalase in the presence of $Cu^{2+}(His)_2$, DTPA and histidine.** Incubation mixtures contained 50mM phosphate buffer, pH 7.0, and 0.20μM catalase at 37°C. Ascorbate and $Cu^{2+}(His)_2$ were added to final concentrations of 2mM and 40μM respectively. Aliquots were withdrawn and residual catalase activity was measured in terms of the rate of decrease in absorbance between 0.450 and 0.400 at 240nm due to H_2O_2 decomposition. The lines plotted represent (○) catalase plus 2mM histidine; (●) catalase and ascorbate plus 2mM histidine; (□) catalase and ascorbate plus $Cu^{2+}(His)_2$; (■) catalase, ascorbate plus $Cu^{2+}(His)_2$ and 0.2mM DTPA; (▽) catalase ascorbate plus $Cu^{2+}(His)_2$ and 0.5mM histidine; (◇) catalase ascorbate plus $Cu^{2+}(His)_2$ and 1.0mM histidine; (△) catalase ascorbate plus $Cu^{2+}(His)_2$ and 2.0mM histidine. The estimated S.D. was ± 2.2% (see materials and methods).


RESIDUAL CATALASE ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (minutes)

error, superoxide dismutase (0.15μM), benzoate (1mM) and mannitol (1-1000mM) had no significant effect on the rate or extent of inactivation (Table 1). Nevertheless, the hydroxyl radical scavenger thiourea (1mM) gave significant protection, decreasing the initial rate of inactivation and increasing the final value of enzyme activity to approximately those values for ascorbate alone.

Effects of nitrogen and oxygen atmospheres

In order to determine the effects of oxygen concentration on the progress of inactivation, catalase was incubated with ascorbate plus $Cu^{2+}(His)_2$ under atmospheres of nitrogen, air or oxygen. An atmosphere of oxygen decreased the initial rate of inactivation by 82% ± 10% and increased the final activity of catalase by approximately 50% compared to that observed under an atmosphere of air (Fig. 2). A nitrogen atmosphere, however, failed to protect and actually slightly increased the final extent of damage. This effect was more evident at low concentrations of ascorbate (Fig. 7).

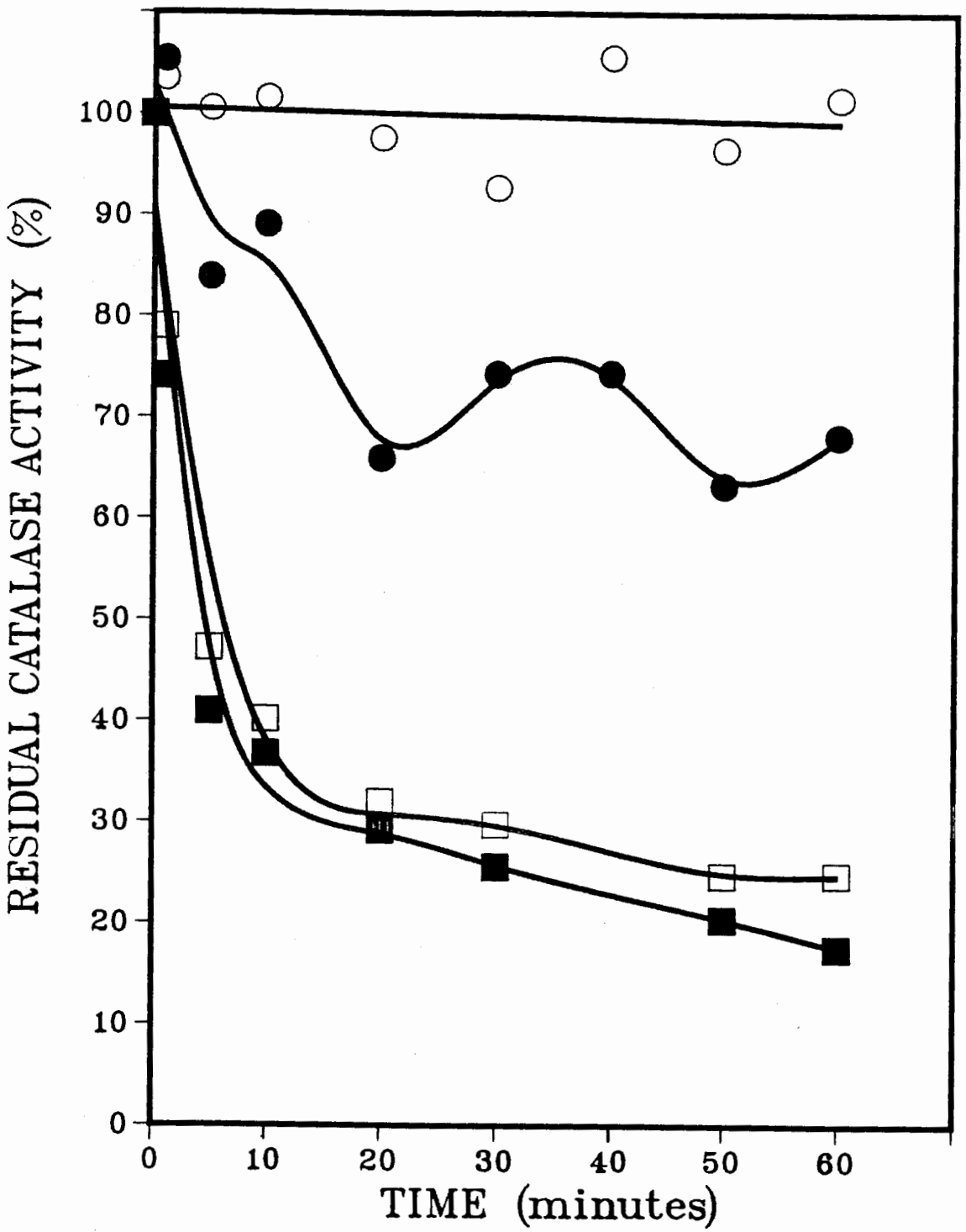
Effects on ascorbate oxidation

The decline in residual ascorbate concentration was measured (Fig. 3) in an attempt to correlate the progress of ascorbate oxidation with the inactivation of catalase. When catalase was added to the incubate,

Table 1. Effects of oxy-radical scavengers on the initial rates and final values of catalase inactivation by ascorbate plus $Cu^{2+}(His)_2$. Incubation and assay conditions were as described in Fig. 1. The initial rates of reactions (I.R.) and final values (F.V.) with their associated standard errors were calculated by non-linear regression (see materials and methods). Each of the three sets of results were performed on different days. A control (no scavenger) was performed for each set of experiments to which the treatment effect was compared. * indicates a significant difference from the control ($p<0.05$).

Scavenger	I.R. % activity/minute	F.V. % activity
None	13.0 ± 1.9	23.2 ± 1.9
SOD (0.15μM)	12.9 ± 1.9	19.7 ± 1.9
BSA (100μg/ml	13.4 ± 2.4	22.9 ± 2.7
Ascorbate only	4.0 ± 1.2*	56.9 ± 3.4*
None	23.5 ± 4.7	24.3 ± 2.3
Thiourea (1mM)	5.2 ± 0.8*	53.3 ± 1.7*
Urea (1mM)	16.5 ± 4.1	24.6 ± 2.6
None	19.9 ± 5.2	15.6 ± 2.7
Benzoate (1mM)	23.7 ± 5.9	17.7 ± 2.6
Mannitol (1mM)	20.6 ± 4.2	17.8 ± 2.2
Mannitol (125mM)	22.2 ± 3.6	19.6 ± 2.0
Mannitol (500mM)	23.7 ± 9.8	23.2 ± 3.6
Mannitol (1000mM)	14.5 ± 4.0	22.2 ± 3.0

Fig. 2. Effects of nitrogen and oxygen atmospheres on inactivation of catalase by ascorbate plus $Cu^{2+}(His)_2$. Incubation and assay conditions were as described in Fig 1. Atmospheres of the desired gas were induced into rubber capped vials as outlined in materials and methods. The lines plotted represent (○) catalase alone under air; (□) catalase plus ascorbate and $Cu^{2+}(His)_2$ under air; (■) catalase plus ascorbate and $Cu^{2+}(His)_2$ under nitrogen; (●) catalase plus ascorbate and $Cu^{2+}(His)_2$ under oxygen. The estimated S.D. was ± 2.2% (see materials and methods).


RESIDUAL CATALASE ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (minutes)

immediately after the addition of $Cu^{2+}(His)_2$, there was a substantial decrease in the rate of oxidation of ascorbate compared to that during the 10 minute pre-incubation period. DTPA (0.2mM) in combination with catalase virtually blocked ascorbate oxidation completely. However, histidine (0.5mM-2mM) was without effect, as was superoxide dismutase (0.15μM). An atmosphere of nitrogen completely blocked oxidation of ascorbate, while an atmosphere of oxygen decreased the residual concentration of ascorbate (at 1 hour) by 25% compared to that obtained under an atmosphere of air. In agreement the results in Chapter 2, in the presence of catalase the rate of ascorbate oxidation was similar for atmospheres of 20% and 100% oxygen.

Effects of ascorbate concentration on inactivation

The effect of ascorbate concentration on the initial rate of inactivation was investigated to determine if the protection offered by oxygen (Fig. 2) could be accounted for by the corresponding decrease in ascorbate concentration (Fig. 3). The initial rate of catalase inactivation displayed saturation kinetics with respect to ascorbate, tending toward a maximum initial rate at an ascorbate concentration of 2mM. The initial rate of inactivation under an atmosphere of oxygen was decreased to 18% ± 10% of that under an an atmosphere of air. For a fall in ascorbate concentration to account for this the initial ascorbate concentration would have to be approximately 80μM.

Fig. 3 Effects of oxygen, metal chelating agents, and SOD on the oxidation of ascorbate in the presence of catalase and $Cu^{2+}(His)_2$. Incubation conditions were as described in Fig. 1. Aliquots of 25μl were withdrawn and residual ascorbate concentration was determined by measuring the increase in absorbance at 515nm due to the production of ferrophenanthroline. The lines plotted represent (△) catalase plus ascorbate and $Cu^{2+}(His)_2$; (●) catalase plus ascorbate, $Cu^{2+}(His)_2$ and DTPA (0.2mM); (□) catalase plus ascorbate, $Cu^{2+}(His)_2$ and histidine (0.5mM); (■) catalase plus ascorbate, $Cu^{2+}(His)_2$ and histidine (2mM); (○) catalase plus ascorbate, $Cu^{2+}(His)_2$ and SOD (0.15μM); (○) catalase plus ascorbate and $Cu^{2+}(His)_2$ under nitrogen; (▽) catalase plus ascorbate and $Cu^{2+}(His)_2$ under oxygen.

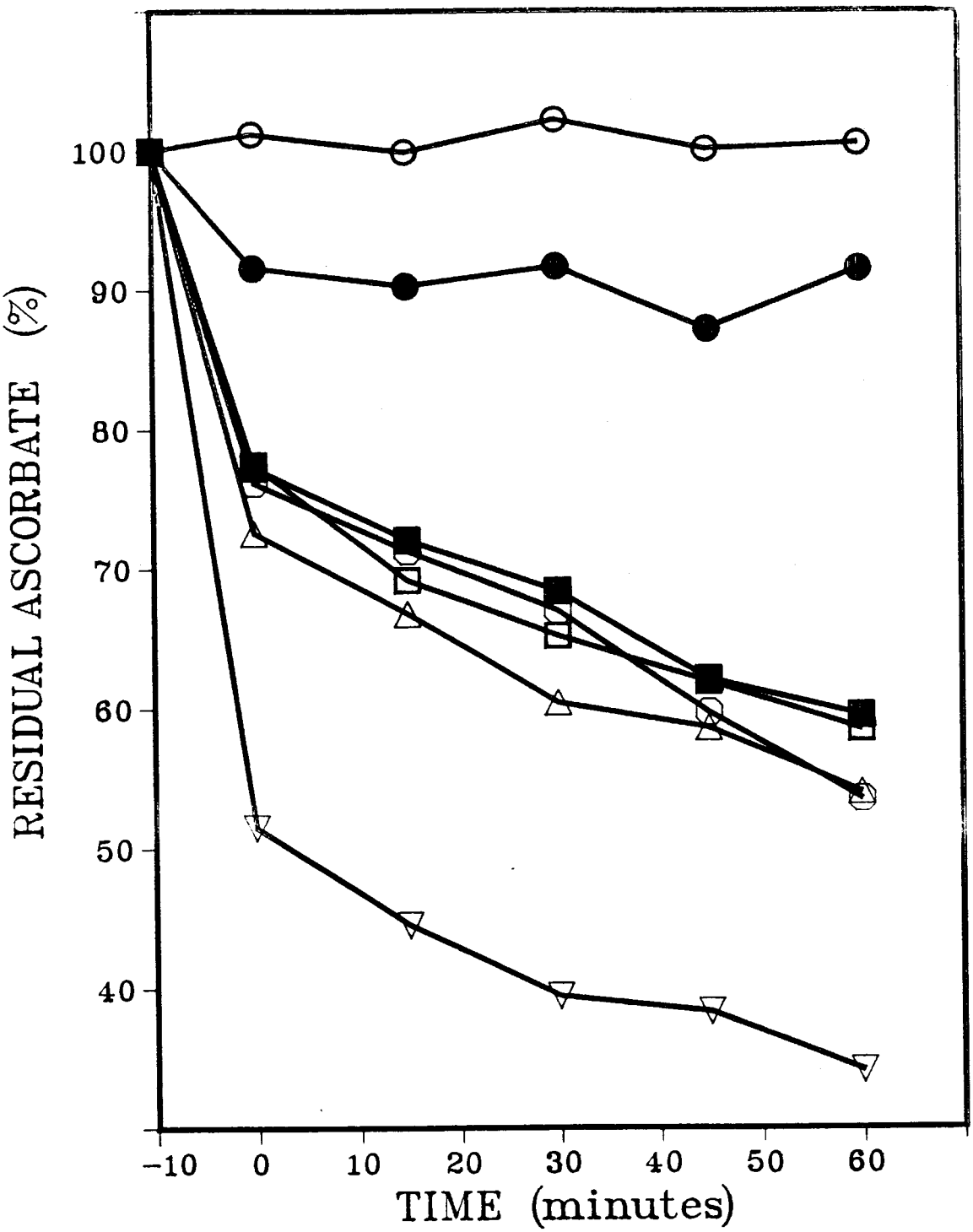
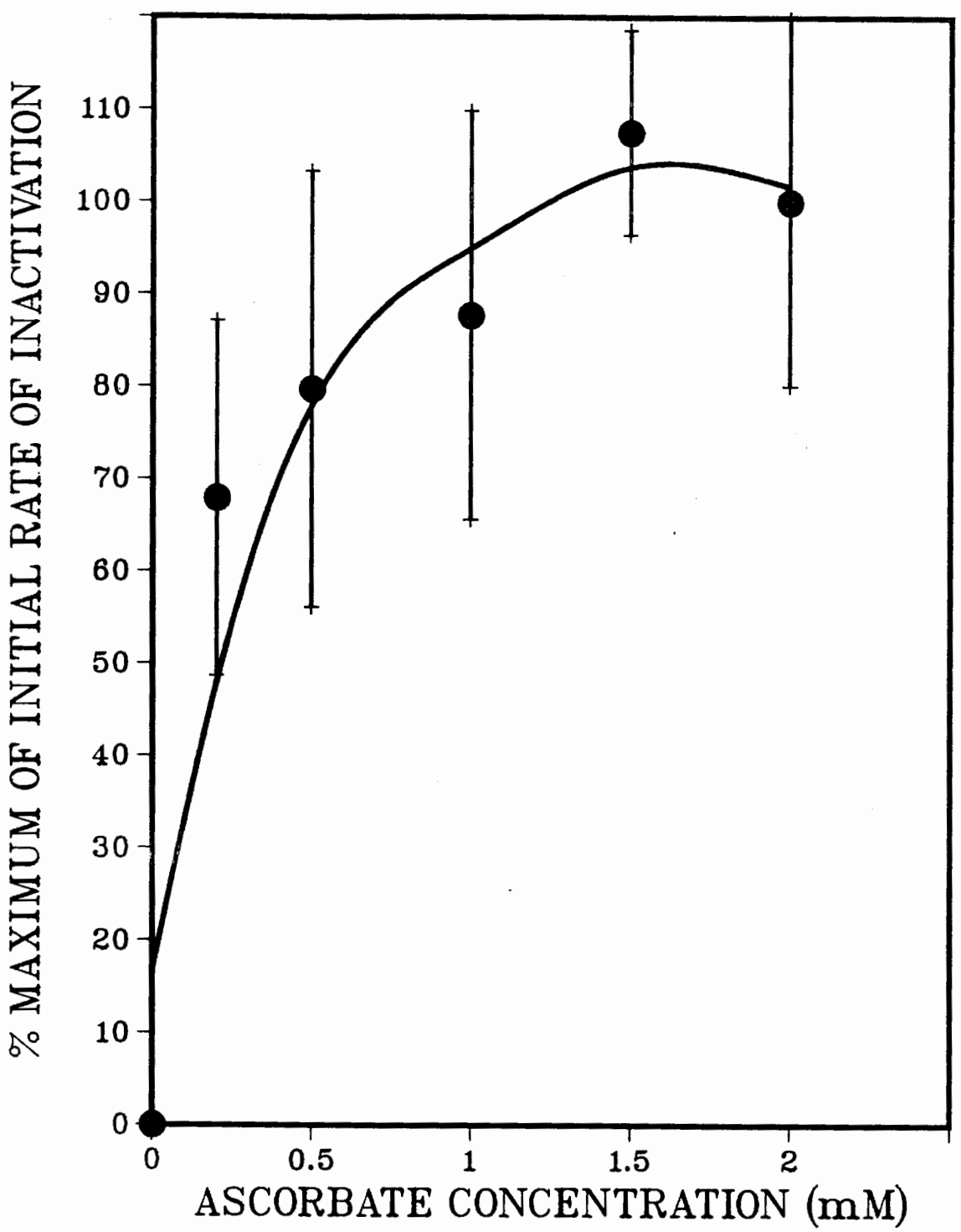

RESIDUAL ASCORBATE (%)
100
90
80
70
60
50
40
-10
0
10
20
30
40
50
60
TIME (minutes)

Fig. 4 Effects of ascorbate concentration on the initial rate of inhibition of catalase in the presence of $Cu^{2+}(His)_2$. Incubation and assay conditions were as described in Fig. 1. The maximum initial rate of inhibition was based on the rate of inhibition induced by 2mM ascorbate in the presence of 40μM $Cu^{2+}(His)_2$. Error bars represent ± the S.E. of the % of maximum initial rate of inhibition as calculated by non-linear regression.


% MAXIMUM OF INITIAL RATE OF INACTIVATION
110
100
90
80
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
ASCORBATE CONCENTRATION (mM)

Under the prevailing conditions, however, the initial concentration of ascorbate was approximately 1mM.

Effects of ethanol

As illustrated in Chapter 2, ethanol can prevent and reverse inhibition of catalase by ascorbate which indicates that compounds I and II are involved in the reaction mechanism responsible for the observed inhibition. To determine if similar enzyme-peroxide complexes are involved in inactivation induced by ascorbate plus $Cu^{2+}(His)_2$ ethanol was included in the incubate at the beginning and 20 minutes after the start of the reaction (Fig 5). In contrast to the results with ascorbate alone, ethanol neither prevented nor reversed inactivation of catalase, even under an atmosphere of nitrogen.

Effect of low concentrations of ascorbate

When concentrations of ascorbate were less than 1mM the steady state levels of active catalase oscillated in a manner similar to that observed with ascorbate alone (6). A typical example is given in Fig. 6 for 0.2mM ascorbate plus 40μM $Cu^{2+}(His)_2$. This reaction was accelerated under an atmosphere nitrogen. However, in contrast to 2mM ascorbate in the presence of 40μM $Cu^{2+}(His)_2$, ethanol slowed the initial rate of inactivation and also reversed inactivation by approximately 8% when added at 30 minutes.

Fig. 5 Effects of ethanol on inactivation of catalase by ascorbate plus $Cu^{2+}(His)_2$ under atmospheres of air or nitrogen. Incubation and assay conditions were as described in Figs. 1 & 2. The lines plotted represent (○) catalase alone; (△) catalase plus ascorbate and $Cu^{2+}(His)_2$ under air; (▽) catalase plus ascorbate, $Cu^{2+}(His)_2$ and 20mM ethanol under air (◇) catalase plus ascorbate, $Cu^{2+}(His)_2$ and 20mM ethanol added at 20 minutes under air; (●) catalase plus ascorbate and $Cu^{2+}(His)_2$ under nitrogen; (□) catalase plus ascorbate, $Cu^{2+}(His)_2$ and 20mM ethanol under nitrogen; (■) catalase plus ascorbate, $Cu^{2+}(His)_2$ under nitrogen and 20mM ethanol added at 20 minutes. The estimated S.D. was ± 2.2% (see materials and methods).

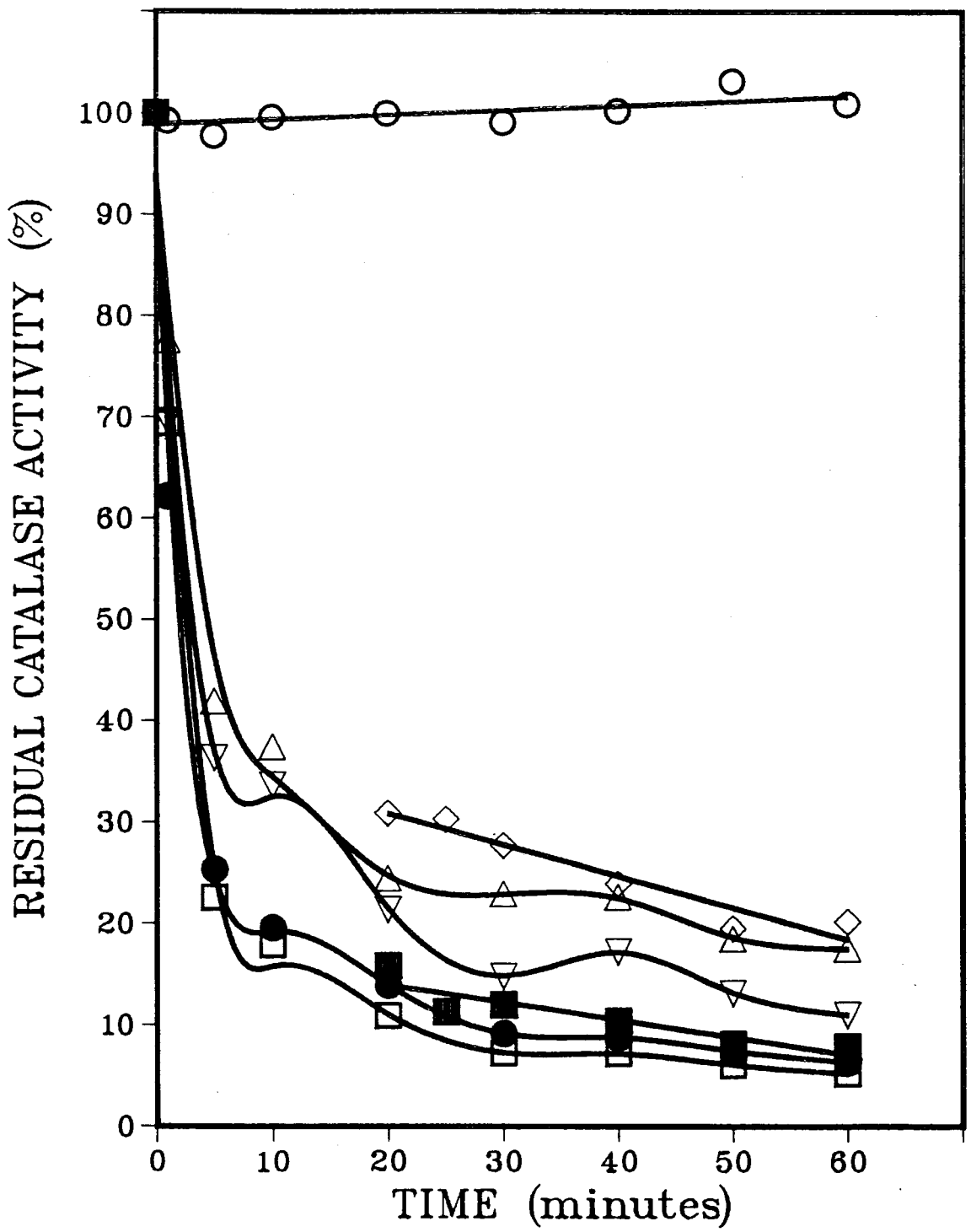
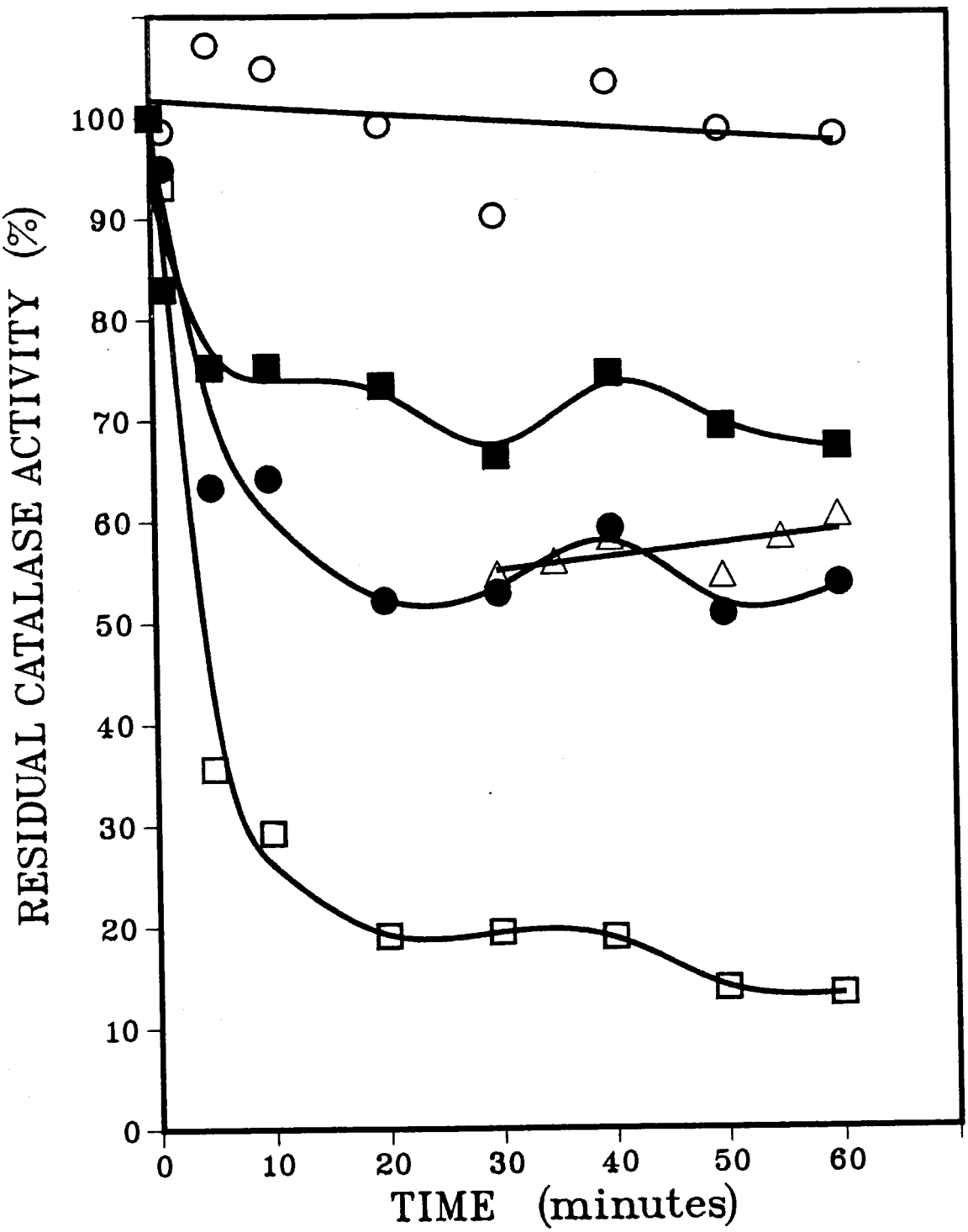

RESIDUAL CATALASE ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (minutes)

Fig. 6 Effects of low concentrations of ascorbate on the inactivation of catalase in the presence of $Cu^{2+}(His)_2$. Incubation and assay conditions were as described in Fig. 1 except ascorbate was present at 0.2mM. Lines plotted represent (○) catalase only; (●) catalase and ascorbate plus $Cu^{2+}(His)_2$; (□) catalase and ascorbate plus $Cu^{2+}(His)_2$ under nitrogen; (■) catalase, ascorbate plus $Cu^{2+}(His)_2$ and 20mM enthanol; (△) catalase ascorbate plus $Cu^{2+}(His)_2$ and 20mM ethanol added at 30 minutes. The estimated S.D. was ± 2.2% (see materials and methods).


RESIDUAL CATALASE ACTIVITY (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME (minutes)

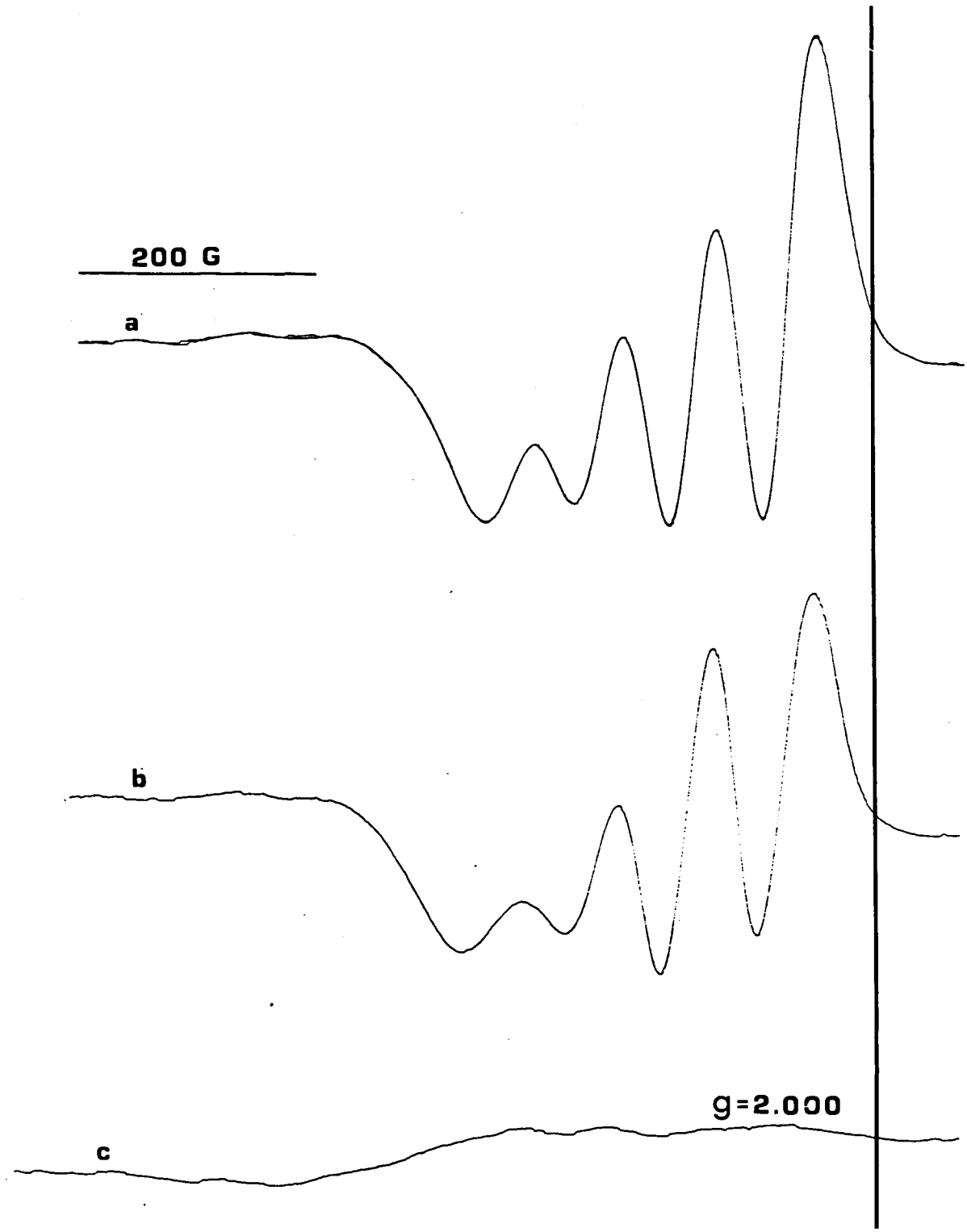
Optical absorption spectra

Optical absorption spectra revealed a large decrease in the extinction coefficient at 404nm as well as a shift of 3nm toward higher wavelengths in the Soret region. These results are similar to those reported earlier (14,15) for the spectrum of inactive compound II. In the current experiments addition of ascorbate (2mM) plus $Cu^{2+}(His)_2$ (40μM> to catalase (2.0μM) caused a progressive precipitation of catalase. A precipitate was not formed when $Cu^{2+}(His)_2$ was omitted from the incubate.

Electron spin resonance spectra

ESR spectroscopy was employed to study the binding of Cu^{2+} to catalase and the reaction of Cu^{2+} with ascorbate. The ESR absorption curve for 8mM $Cu^{2+}(His)_2$ (Fig. 8a) resembles that observed for various paramagnetic copper complexes at room temperature (53). In the presence of catalase (10mg/ml) the spectrum (Fig. 8b) clearly changed in a manner reflecting an association between copper and the added protein. Anaerobic addition of excess ascorbate resulted in almost complete disappearance of the ESR spectra associated with the Cu^{2+} ions (Fig. 8c).

Fig. 7 ESR spectra of $Cu^{2+}(His)_2$. All spectra were recorded using a Varian E-4 spectrometer operated at 20°C under the conditions outlined in materials and methods. All samples were prepared in phosphate buffer (50mM, pH 7.0). Spectra represent (a) $Cu^{2+}(His)_2$ (8mM); (b) (a) plus catalase (10mg/ml); (c) (b) plus ascorbate (20mM).


200 G
a
b
c
g=2.000

DISCUSSION

Effects of scavengers of various species of oxygen

As argued in the case of ascorbate alone, activated species of oxygen can be excluded as participants in the reaction mechanism leading to inactivation because an atmosphere of oxygen actually confers considerable protection, rather than enhancing damage. The lack of significant effects of superoxide dismutase, benzoate and mannitol support this arguement. The ability of the hydroxyl radical scavengers thiourea and dimethylsulphoxide (17) to decrease the rate of inactivation of catalase by ascorbate plus Cu^{2+} must then be attributed to their metal chelating properties (54), rather than their conventional roles as hydroxyl scavengers. Additional support for the exclusion of H_2O_2 is that an atmosphere of nitrogen failed to protect against inactivation although it completely blocked oxidation of ascorbate, and correspondingly H_2O_2 production. Clearly, the mechanism of inactivation of catalase by ascorbate plus $Cu^{2+}(His)_2$ does not involve superoxide, hydroxyl radicals or hydrogen peroxide, contradicting previous proposals (15,16).

Identification of reactive intermediates

A reaction between ascorbate and Cu^{2+} is an integral part of the inactivation mechanism because inactivation during the simultaneous presence of these two reactants was greater than the sum of their individual effects. Also, the metal chelating agent DTPA, which prevents the reaction between Cu^{2+} and ascorbate, decreased inactivation to that observed with ascorbate alone. The ESR spectra (Figs. 8a & 8c) suggest that this reaction involves the reduction of paramagnetic Cu^{2+} to non-paramagnetic Cu^{1+}, i.e.:

$$Cu^{2+} + AH^{-} \text{----->} Cu^{1+} + A\bullet^{-} + H^{+} \quad (1)$$

The most obvious explanation for the progressive protection of catalase by increasing concentrations of histidine is that this amino acid prevents Cu^{2+} from binding to catalase and exerting its effect. That is, high concentrations of histidine force the equilibrium of the ligand exchange reaction (reaction (2)) to the left, preventing formation of a catalase-Cu^{2+} complex.

$$Cu^{2+}(His)_2 + \text{Catalase} \text{----->} \text{Catalase-}Cu^{2+} + 2\ His \quad (2)$$

Direct evidence for the formation of a catalase-Cu^{2+} complex is provided by the ESR spectra (Figs. 8b) of $Cu^{2+}(His)_2$ in the presence of catalase. The known ability of Cu^{2+} to form high

affinity complexes with amino acids and proteins is additional evidence in support of this concept (55). These results are in complete agreement with those presented by Shinar et al (50) for the inactivation of acetylcholine esterase by ascorbate plus Cu^{2+}.

In view of the observed spectral changes, which are characteristic of inactive compound II, it must be concluded that this enzyme-peroxide complex is involved in the mechanism of inactivation. Compound I, however, can be excluded from the reaction mechanism because inactivation is independent of oxygen. That is H_2O_2 is not required to convert catalase to compound I before inactivation is observed. For this reason alone the reaction mechanism involved must be different from that proposed in Chapter 2 for the reversible inhibition of catalase by ascorbate alone.

The protective effect of oxygen provides indirect evidence for the involvement of semidehydroascorbate in the inactivation of catalase by ascorbate plus $Cu^{2+}(His)_2$. Involvement of semidehydroascorbate is based on the effect molecular oxygen has on metal-chelate catalysed autoxidations of ascorbate. As proposed by Martell (56), these reactions are expected to proceed through single inner sphere electron transfer steps within a metal-chelate-ascorbate complex. The first electron transfer is followed by dissociation of the reduced metal ion and recombination of semidehydroascorbate with another metal ion in the higher valence state. However, in the presence of excess

molecular oxygen, rapid re-oxidation of the metal in the ternary complex occurs before this complex can dissociate. This allows a second reduction of the metal, this time by semidehydroascorbate. Thus, elevated concentrations of molecular oxygen prevent semidehydroascorbate from becoming available for side reactions such as the inactivation of catalase.

Mechanisms of inactivation of catalase

On the basis of the above discussion, it is proposed that Cu^{2+} initially binds to catalase and is subsequently reduced by ascorbate. Any semidehydroascorbate so formed at the active site of catalase then oxidizes the haematin iron of ferricatalase to generate inactive compound II, i.e :

$$\text{Ferricatalase} + Cu^{2+}(His)_2 \text{ -----> } \text{Ferricatalase-}Cu^{2+} + 2\ His \quad (3)$$

$$\text{Ferricatalase-}Cu^{2+} + AH^- \text{ -----> } \text{Ferricatalase-}Cu^{1+} + A\bullet^- + H^+ \quad (4)$$

$$\text{Ferricatalase-}Cu^{1+} + A\bullet^- \text{ ----> } \text{Compound II-}Cu^{1+} + A^- \quad (5)$$

Reaction (5) is simply the reversal of the one electron reduction that regenerates active catalase from compound II (12). Normally reaction (5) is slow, but when copper is protein bound as in this case, the site specific production of semidehydroascorbate makes this radical's local concentration

extremely high, favouring formation of compound II and ascorbate. Production of compound II by reaction (5) has already been demonstrated in the analogous reaction of horseradish peroxidase with K_2IrCl_6 (57).

An explanation for the irreversible nature of inactivation

Clearly, the proposed mechanism does not explain the irreversible nature of the inactivation of catalase by ascorbate plus $Cu^{2+}(His)_2$. A possible explanation for the irreversible reaction comes from the effects 3 amino-1H-1,2,4 triazole, semicarbazide and cyanogen bromide have on catalase. These compounds all reportably inhibit catalase through modification a histidine residue (12) at the active site of catalase. This histidine residue has recently been demonstrated to be distal to the haematin iron at the active site of catalase (58). In addition, ascorbate plus Cu^{2+} can induce specific degradation of histidine residues (52). Thus, it is proposed that catalase is irreversibly inactivated by ascorbate plus $Cu^{2+}(His)_2$ through modification of the distal histidine at the active site of catalase.

The dependence of this irreversible reaction on ascorbate concentration implies that modification of the distal histidine occurs subsequent to formation of compound II. The activated form of ascorbate responsible for irreversible inactivation may be semidehydroascorbate because anion radicals can selectively react with methionine, cysteine and ring-containing amino acids

such as histidine (59). Activated forms of oxygen can be excluded for the reasons explained above.

Modification of the histidine distal to haematin iron would inactivate catalase as proposed by Dounce (60) because this residue may act by orientating or binding the second H_2O_2 molecule involved in the catalytic reaction. Any change in structure of the distal histidine would prevent decompostion of H_2O_2. In this situation catalase would become highly susceptible to oxidative damage through a Fenton reaction between bound Cu^{1+} and unreacted H_2O_2. In this model then, irreversible inactivation converts catalase into a suicide enzyme, in that its substrate (H_2O_2) brings about its destruction. This reasoning explains the degradative changes observed by Orr (6) and the precipitation of catalase in the presence of ascorbate and $Cu^{2+}(His)_2$.

Clearly, the above working hypothesis opens several approaches for future research into the specific mechanism involved in the irreversible inactivation of catalase by ascorbate plus $Cu^{2+}(His)_2$.

Implications

It follows from the above discussion that semidehydroascorbate should not be dismissed as biologically irrelevant. Despite its relatively low reactivity it is indeed capable of undergoing reactions that would be detrimental in vivo. As discussed by Swartz and Dodd (61), the low reactivity

of semidehydroascorbate allows it to migrate relatively long distances and eventually to react in a site specific manner with crucial cellular targets distant from its site of formation. These effects, however, are moderated by the scavenging effect of catalase and the spontaneous dismutation of semidehydroascorbate. Finally, it can be concluded that the extreme cytoxicity of ascorbate, in the presence of metal ions (62,63) is at least in part due to the irreversible inactivation of catalase and subsequent production of H_2O_2, hydroxyl radicals and semidehydroascorbate.

SUMMARY

$Cu^{2+}(His)_2$ enhanced the inhibition of catalase by 2mM ascorbate but the mechanism is different from that of ascorbate alone, although optical absorption spectra show that inactive compound II was produced in both cases. However, 20mM ethanol only prevented or reversed metal catalysed inhibition at concentrations of ascorbate less than 1.0mM. At higher concentrations of ascorbate (2mM) ethanol was without effect, even under an atmosphere of nitrogen. Surprisingly, oxygen protected whereas an atmosphere of nitrogen actually accelerated the reaction slightly. The protection offered by oxygen could not be accounted for by an increase in ascorbate oxidation. Superoxide dismutase, benzoate and mannitol had no effect on the initial rate of inactivation, excluding significant involvement of superoxide and hydroxyl radicals. For this reason, the ability of 1mM thiourea to decrease the initial rate of inactivation to that of ascorbate alone must be attributed to its metal chelating properties. Increasing the concentration of histidine from 80μM to 3mM had no effect on ascorbate autoxidation but progressively slowed inactivation of catalase to that observed with ascorbate alone. This result implicates the binding of Cu^{2+} to catalase as a prerequisite to inactivation of this enzyme by ascorbate plus $Cu^{2+}(His)_2$. ESR spectra support this result and show that in this system ascorbate reduces Cu^{2+} to Cu^{1+}. The most plausible explanation

for the inactivation of catalase by ascorbate plus $Cu^{2+}(His)_2$ is that initially catalase is directly oxidized by semidehydroascorbate, yielding inactive compound II. Further modification of this product by activated forms of ascorbate then leads to irreversible inactivation.

CHAPTER 4

GENERAL DISCUSSION

In Chapter 2 it was demonstrated that ascorbate and/or semidehydroascorbate can reversibly inhibit catalase by reducing compound I to compound II. In the presence of $Cu^{2+}(His)_2$, however, it became apparent that the mechanism of inactivation was different to that with ascorbate alone. This mechanism was investigated further, as described in Chapter 3, and it was found that site specific production of semidehydroascorbate results in the direct oxidation of ferricatalase to inactive compound II. Subsequent to formation of compound II, ascorbate plus $Cu^{2+}(His)_2$ irreversibly inactivates catalase. This irreversible inactivation was suggested to involve modification of the histidine residue distal to haematin iron at the active site of catalase.

It is unlikely that the inhibition of catalase by ascorbate alone is detrimental *in vivo* because active catalase can be regenerated by NADH or NADPH. The results in Chapter 2, however, may explain why glucose 6-phosphate dehydrogenase

(G6PDH)-deficient individuals cannot tolerate even moderate amounts of ascorbate in their diets (64). G6PDH is essential in maintaining the activity of glutathione peroxidase because it provides reducing equivalents for adequate levels of reduced glutathione (a required substrate of glutathione peroxidase). Without G6PDH, catalase is the only defence against peroxide mediated cytotoxicity. Conceivably, catalase can function effectively without reducing equivalents, in the form of NADPH, as long as it is not converted to its inactive forms. However, high levels of ascorbate would cause an increase in compound II formation and a consequent decrease in the ability to scavenge damaging peroxides and semiquinones. It is not surprising therefore, that diets high in ascorbate have proven fatal to G6PDH deficient individuals (65).

It is also unlikely that, under normal circumstances, ascorbate in the presence of metals can irreversibly inactivate catalase *in vivo*. Protein concentrations within cells are very high and if free metal ions were available they would rapidly become protein bound. Therefore, the likelihood of metals becoming bound to the active site of catalase is extremely low. Certain pathological conditions, however, warrant discussion in this regard.

Patients with iron overload, and healthy Bantu who drink acidic beer out of iron pots, have abnormally low contents of ascorbic acid in their blood and tissues. Supplementation of their diet with ascorbic acid, in the absence of chelating

agents, has often been detrimental and sometimes fatal. Halliwell and Gutteridge (66) have suggested that these detrimental results are due to increased lipid peroxidation and generation of hydroxyl radicals. In light of the results presented in Chapter 3, the additional hypothesis that catalase is irreversibly inactivated by ascorbate and iron, prior to lipid peroxidation and hydroxyl radical production, must be considered.

A more common source of ascorbate and iron salt mixtures are the frequently consumed multivitamin preparations. These multivitamin preparations are generally considered beneficial but in abnormally large amounts they cause severe damage to gastric mucosa of rats (67). In addition, Stich et al. (68) have tested the capacity of commercially available multivitamin tablets to induce chromosomal aberrations in cultured Chinese hamster ovary cells. Chromosomal breaks and exchanges were induced by all the tablets tested. The toxic and clastogenic effect of the vitamin preparations were correlated primarily with their content of ascorbic acid and iron. The results in Chapter 3 suggest that these toxic effects of multivitamin preparations are, at least in part, due to the irreversible inactivation of catalase and consequent production of damaging hydroxyl radicals.

In summary, this thesis has characterized the mechanisms responsible for the inactivation of catalase by ascorbate and ascorbate plus $Cu^{2+}(His)_2$. Through clarification of these

mechanisms a clearer understanding of the free radical mediated toxicity of ascorbate has become apparent.

APPENDIX 1

EFFECTS OF ASCORBATE AND ASCORBATE PLUS $Cu^{2+}(His)_2$ ON THE OPTICAL ABSORPTION SPECTRA OF CATALASE

Fig. 1. The optical absorption spectra of catalase. Catalase (2μM) was incubated in phosphate buffer (50mM, pH 7.0) at 20°C.

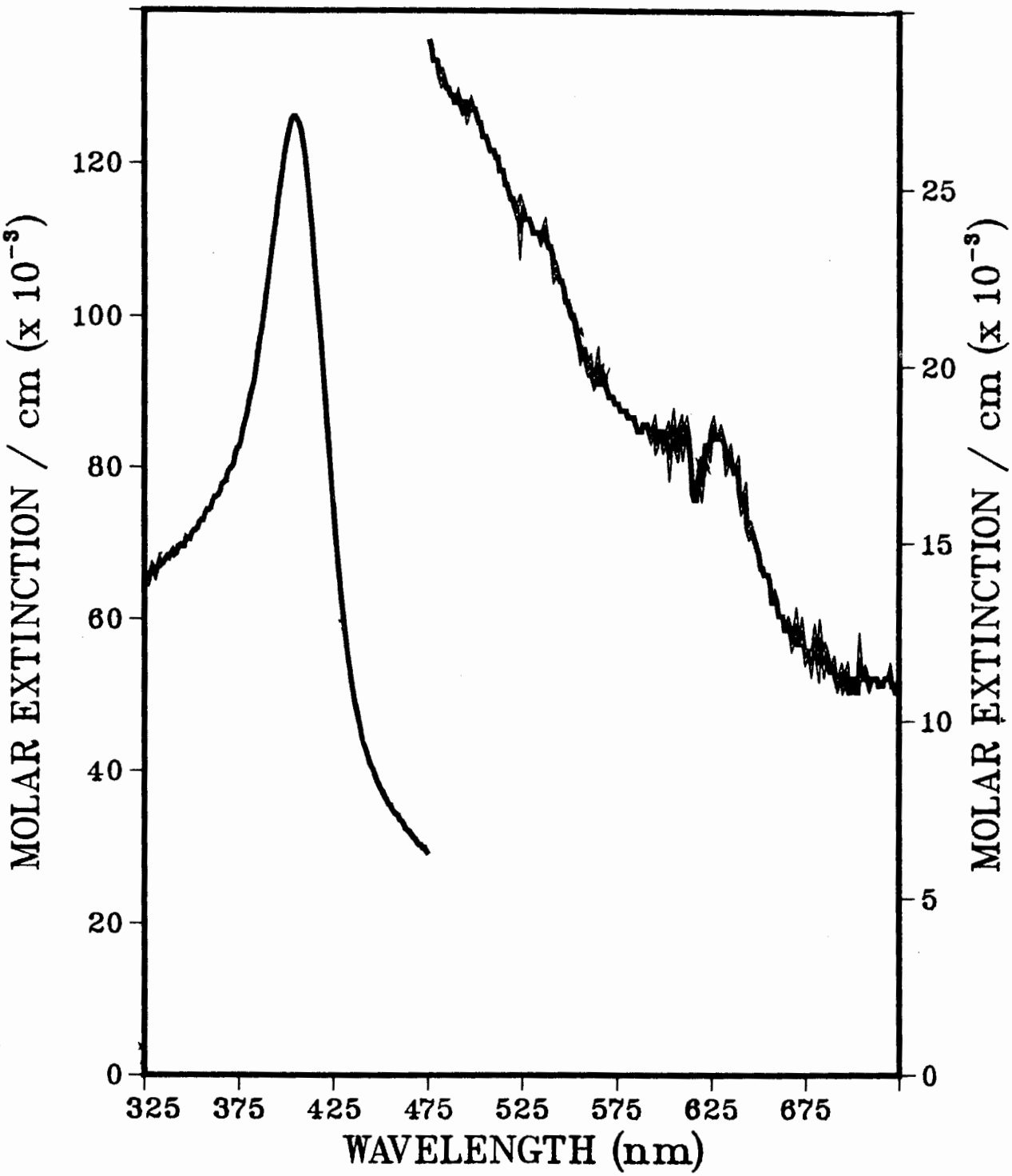

MOLAR EXTINCTION / cm (x 10⁻³)
120
100
80
60
40
20
0
MOLAR EXTINCTION / cm (x 10⁻³)
25
20
15
10
5
0
325
375
425
475
525
575
625
675
WAVELENGTH (nm)

Fig. 2. The effect of ascorbate on the optical absorption spectra of catalase. Catalase (2μM) was incubated with ascorbate (2mM) in phosphate buffer (50mM, pH 7.0) at 20°C.

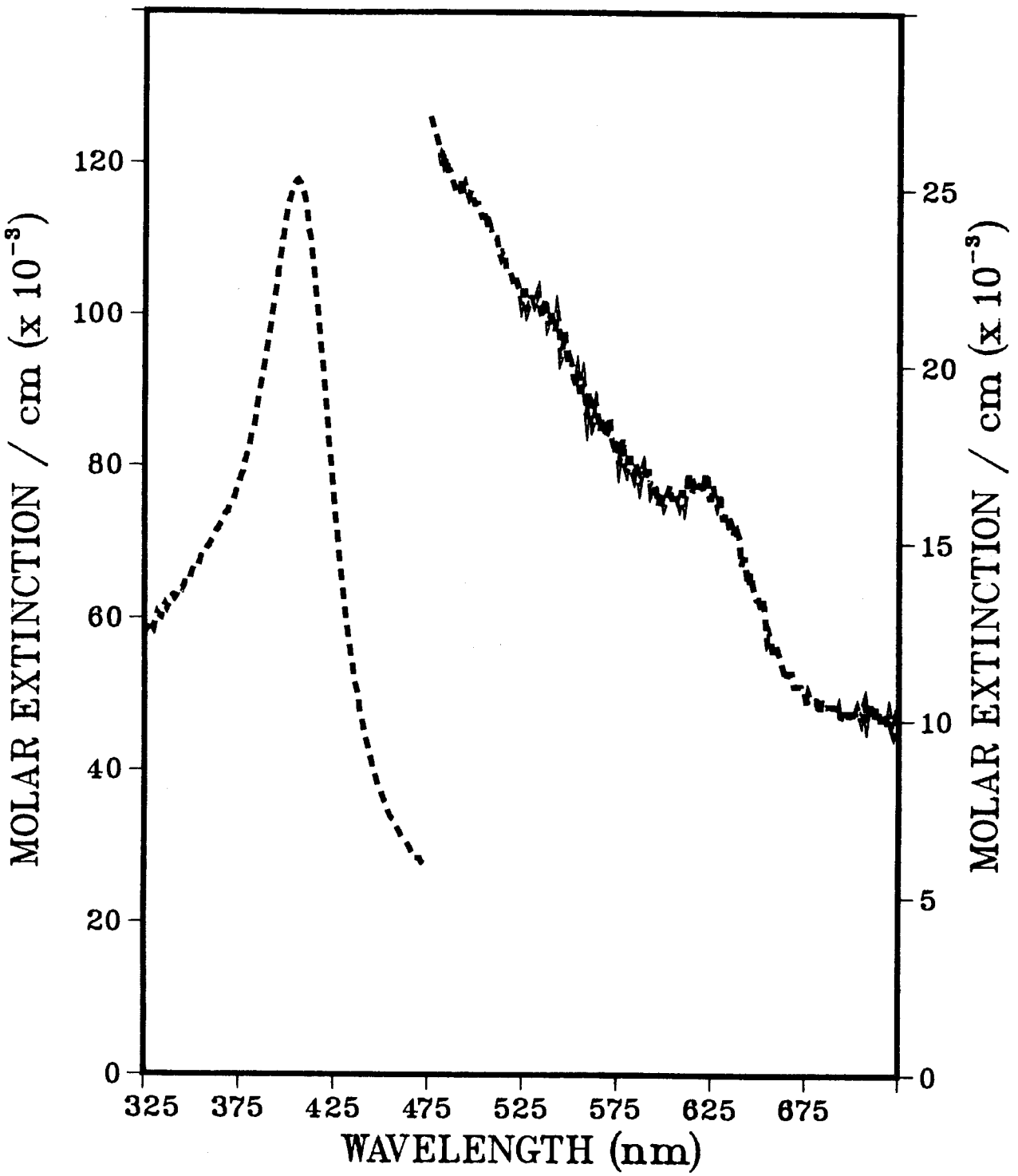
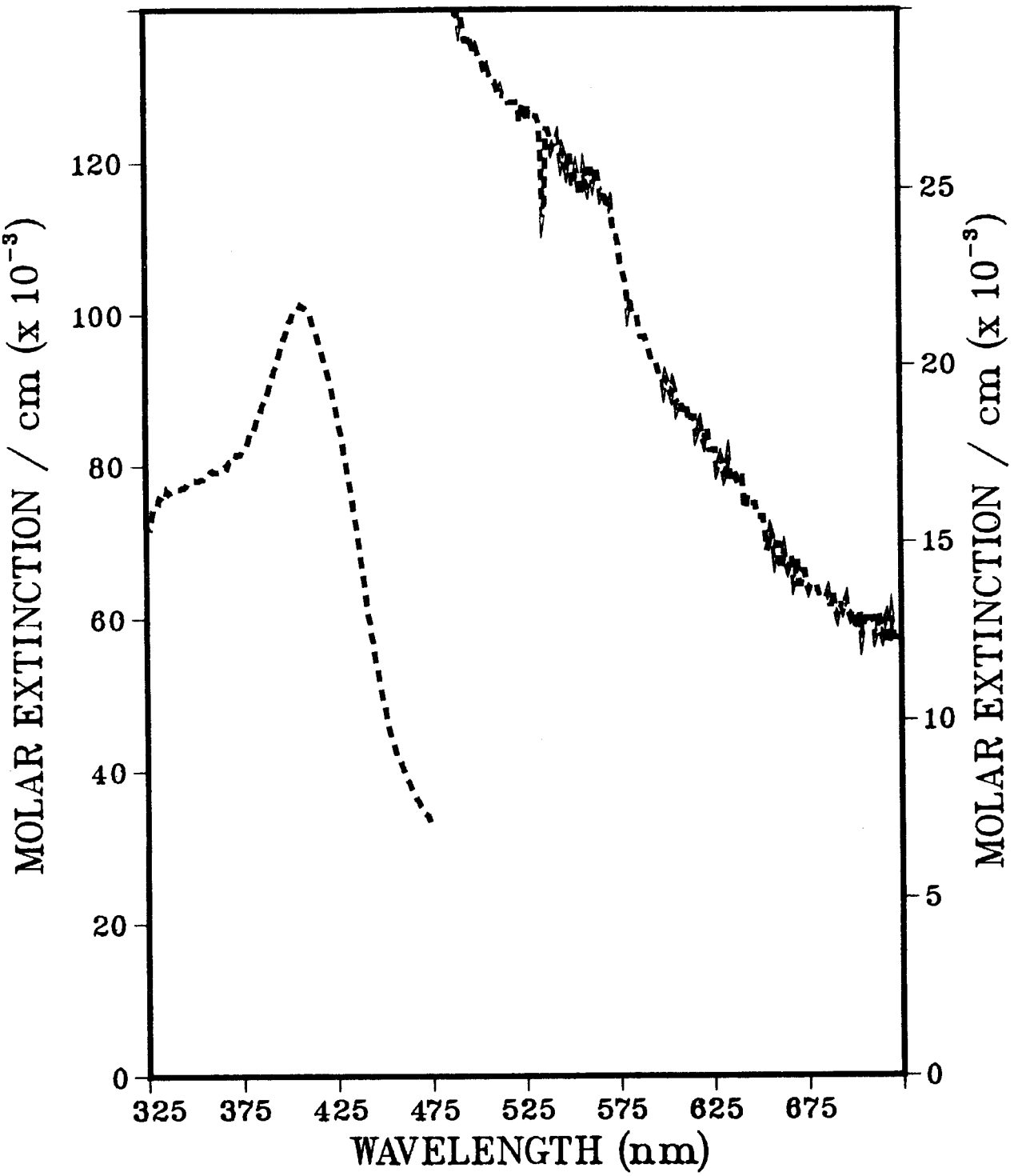

MOLAR EXTINCTION / cm (x 10⁻³)
120
100
80
60
40
20
0
MOLAR EXTINCTION / cm (x 10⁻³)
25
20
15
10
5
0
325
375
425
475
525
575
625
675
WAVELENGTH (nm)

Fig. 3. The effect of ascorbate plus $Cu^{2+}(His)_2$ on the optical absorption spectra of catalase. Catalase (2μM) was incubated with ascorbate (2mM) and $Cu^{2+}(His)_2$ (40μM) in phosphate buffer (50mM, pH 7.0) at 20°C.


MOLAR EXTINCTION / cm (x 10^{-3})
120
100
80
60
40
20
0
MOLAR EXTINCTION / cm (x 10^{-3})
25
20
15
10
5
0
325
375
425
475
525
575
625
675
WAVELENGTH (nm)

APPENDIX 2

RAW DATA

The following is the raw data plotted in the main body of the thesis. Units are given on the corresponding graphs.

CHAPTER 2
FIGURE 3.
'CATALASE'
0 100
1 102.43
5 91.953
10 100.33
20 100.57
30 100
40 107.29
50 104.07
60 93.352
'CATALASE • ASCORBATE'
0 100
1 94.522
5 77.963
10 75.26
20 64.55
30 63.849
40 66.927
50 60.916
60 62.062
'CATALASE • ASCORBATE • DTPA'
0 100
1 87.456
5 71.61
10 73.826
20 59.308
30 60.902
40 66.591
50 54.009
60 61.547
'CATALASE • ASCORBATE • DESFERRIOXAMINE'
0 100
1 88.626
5 74.026
10 71.035
20 60.984
30 66.819
40 67.041
50 57.708
60 64.094
'CATALASE • ASCORBATE • Cu'
0 100
1 79.107
5 50.248
10 39.594
20 23.349
30 22.609
40 20.373
50 16.527
60 17.646

'CATALASE • ASCORBATE • Cu • DTPA'
0 100
1 95.07
5 82.946
10 77.959
20 59.998
30 67.575
40 65.646
50 50.805
60 62.265
CHAPTER 2.
FIGURE 7.
'CATALASE'
0 100
1 102.43
5 91.953
10 100.33
20 100.57
30 100
40 107.29
50 104.07
60 93.352
'CATALASE • ASCORBATE'
0 100
1 102.98
5 89.766
10 83.923
20 66.589
30 66.512
40 68.855
50 58.853
60 65.759
'CATALASE • ASCORBATE • 20mM ETOH'
0 100
1 100.89
5 95.922
10 100.27
20 96.881
30 94.427
40 101.11
60 97.391
'CATALASE • ASCORBATE • 20mM ETOH'
20 73.932
25 71.54
30 77.313
40 77.244
50 77.978
60 78

'CATALASE • ASCORBATE • 40mM ETOH'
21.5 74
25 76.453
30 83.098
40 78.388
50 80.372
60 81.282
CHAPTER 2
FIGURE 4.
'CATALASE'
0 100
1 103.54
5 100.55
10 101.63
20 97.659
30 92.773
40 105.67
50 96.607
60 101.58
'CATALASE • ASCORBATE / AIR'
0 100
1 95.526
5 67.169
10 64.653
20 55.147
30 51.356
40 53.176
50 44.372
60 49.36
'CATALASE • ASCORBATE / N2'
0 100
1 106.21
5 103.26
10 101.55
20 92.44
30 94.568
40 100.09
50 97.228
60 88.799
'CATALASE • ASCORBATE / N2'
0 100
1 109.7
5 103.67
10 108.23
20 101.11
30 103.12
40 101.5
50 100.49
60 96.392

'CATALASE • H202/N2 • ASCORBATE / N2'
0 100
1 94.03
5 68.6
10 59.694
20 45.106
30 43.352
40 42.854
50 42.543
'CATALASE • ASCORBATE / 02'
0 100
5 81.352
10 81.843
20 67.43
30 69.496
40 72.398
50 58.531
60 62.231
CHAPTER 3
FIGURE 2.
'CATALASE '
0 100
1 103.54
5 100.55
10 101.63
20 97.659
30 92.773
40 105.67
50 96.607
60 101.56
'CATALASE • ASCORBATE • Cu / 02'
0 100
1 105.44
5 83.883
10 89.127
20 65.975
30 74.349
40 74.424
50 63.336
60 68.249
'CATALASE • ASCORBATE • Cu / AIR'
0 100
1 78.974
5 47.182
10 40.145
20 31.979
30 29.749
50 24.691
60 24.782

'CATALASE • ASCORBATE • Cu / N2'
0 100
1 74.099
5 40.894
10 36.741
20 29.176
30 25.58
50 20.35
60 17.528
CHAPTER 2
FIGURE 2.
'CATALASE • ASCORBATE'
0 100
1 94.39
5 66.553
10 71.445
20 59.27
25 54.096
30 60.906
40 49.725
45 48.984
50 51.577
55 43
60 46.454
65 45.818
70 50.096
75 39.196
80 43.871
85 43.5
90 45.271
95 35.527
100 41.246
105 40.848
110 43.159
115 34.419
120 36.456
125 37.928
130 37.795
135 31.397
140 32.705
145 35.457
CHAPTER 3
FIGURE 5.
'CATALASE'
0 100
1 99.254
5 97.766
10 99.6
20 100
30 99.1
40 100.26
50 103.2
60 100.93

'CATALASE • ASCORBATE • Cu /N2'
0 100
1 62.249
5 25.359
10 19.584
20 13.907
30 9.1206
40 8.7478
50 7.4085
60 6.3151
'CATALASE • ASCORBATE • Cu • ETOH / N2'
0 100
1 69.456
5 22.576
10 17.934
20 10.935
30 7.2399
40 7.1696
50 6.0261
60 5.1896
'CATALASE • ASCORBATE • Cu • ETOH / N2'
20 15.81
25 11.302
30 11.999
40 10.378
50 8.111
60 7.8574
'CATALASE • ASCORBATE • Cu / AIR'
0 100
1 77.641
5 41.962
10 37.487
20 24.48
30 22.927
40 22.613
50 18.486
60 17.534
'CATALASE • ASCORBATE • Cu • ETOH / AIR'
0 100
1 69.653
5 36.377
10 33.722
20 21.384
30 14.791
40 17.236
50 13.122
60 11

'CATALASE • ASCORBATE • Cu • ETOH / AIR'
20 30.899
25 30.31
30 27.7
40 23.891
50 19.518
60 20.2
CHAPTER 3.
FIGURE 1.
'CATALASE • 2mM His'
0 100
1 100.8
5 100.8
10 99.3
20 100.3
30 100.8
40 98.4
50 101.1
60 100.2
'CATALASE • ASCORBATE • 2mM His'
0 100
1 95.613
5 77.1
10 71.4
20 55.469
30 57.7
40 58.1
50 46.6
60 55.306
'CATALASE • ASCORBATE • Cu'
0 100
1 67.702
5 30.539
10 27.408
20 16.842
30 15.913
40 15.847
50 11.982
60 11.954
'CATALASE • ASCORBATE • Cu • DTPA'
0 100
1 95.07
5 82.946
10 77.959
20 59.998
30 67.575
40 65.646
50 50.805
60 62.265

'CATALASE • ASCORBATE • Cu • 2mM His'
0 100
1 97.5
5 74.23
10 69.239
20 56.208
30 58.545
40 60
50 51.196
60 54.514
'CATALASE • ASCORBATE • Cu • 0.5mM His'
0 100
1 91.7
5 75.7
10 67.521
20 43.476
30 31.137
40 25.325
50 15.207
60 15.539
'CATALASE • ASCORBATE • Cu • 1mM His'
0 100
1 95.3
5 76.448
10 70.897
20 51.25
30 47.443
40 48.76
50 38.77
60 40.414
CHAPTER 2
FIGURE 8.
'CATALASE • DES '
0 100
1 97.007
5 97.801
10 99.09
20 99.919
30 97.293
40 101.49
50 102.4
60 100
'CATALASE • ASCORBATE • DES'
0 100
1 92.659
5 72.2
10 63.848
20 49.441
30 48.952
40 52.307
50 43.367
60 49.731
'CATALASE • ASCORBATE • 1.7U A.O. • DES'
10 65.6
15 60.0
20 59.2
30 42.4
40 47.2
50 44.0
60 32.5
'CATALASE • ASCORBATE • 10U A.O. • DES'
0 100
1 99.464
5 84.209
10 80.39
20 72.289
30 67.629
40 61.603
50 48.109
60 53.123
'CATALASE • ASCORBATE • 10U A.O. • 2.0mM H202
0 100
1 89.379
5 84.5
10 79.248
20 61.333
30 57.124
40 56.359
50 44.927
60 49.851
CHAPTER 2
FIGURE 6.
'CATALASE • ASCORBATE '
0 0
0.2 50.2
0.5 58.2
1.0 94.7
1.5 102.2
2.0 100.0
' '
0.2 41.1
0.2 59.0
' '
0.5 50.2
0.5 64.2
' '
1.0 77.7
1.0 111.7
' '
1.5 91.2
1.5 113.2
' '
2.0 87
2.0 113

CHAPTER 3
FIGURE 4.
'CATALASE • ASCORBATE • Cu'
0 0
0.2 67.9
0.5 79.7
1.0 87.7
1.5 107.5
2.0 100.0
' '
0.2 48.7
0.2 87.10
' '
0.5 56.1
0.5 103.3
' '
1.0 65.6
1.0 109.8
' '
1.5 96.4
1.5 118.6
' '
2.0 80.1
2.0 119.9
CHAPTER 3
FIGURE 6.
'CATALASE'
0 100
1 98.626
5 107.18
10 104.89
20 99.193
30 90.135
40 103.3
50 98.62
60 98.048
'CATALASE • Cu • 0.2mM ASCORBATE '
0 100
1 95
5 63.465
10 64.32
20 52.175
30 52.836
40 59.237
50 50.773
60 53.701
'CATALASE • Cu • ASCORBATE /N2'
0 100
1 93.116
5 35.587
10 29.287
20 19.071
30 19.371
40 18.872
50 13.795
60 13.173
'CATALASE • Cu • ASCORBATE • 20mM ETOH'
0 100
1 83.018
5 75.277
10 75.337
20 73.402
30 66.377
40 74.5
50 69.244
60 67.166
'CATALASE • Cu • ASCORBATE • ETOH 20MIN'
30 54.675
35 55.917
40 58.3
50 54.524
55 58.279
60 60.443
CHAPTER 2
FIGURE 5.
'ASCORBATE • CATALASE'
-10 100
0 85.3
15 81.1
30 76.8
45 74.3
60 70.4
'ASCORBATE • CATALASE • DES/N2'
-10 100
0 100
15 98.6
30 98.8
45 99.0
60 99.2
'ASCORBATE • CATALASE • DES'
-10 100
0 87.2
15 85.1
30 84.7
45 84.8
60 83.5
'ASCORBATE • CATALASE • DTPA'
-10 100
0 93.0
15 91.1
30 94.8
45 92.6
60 94.6
'ASCORBATE • CATALASE • SOD • DTPA'
-10 100
0 93.3
15 94.1
30 91.4
45 94.5
60 94.6
'ASCORBATE • CATALASE • Cu'
-10 100
0 72.6
15 66.8
30 60.4
45 56.6
60 54.0
'ASCORBATE • CAT/02'
-10 100
0 63.3
15 60.8
30 56.1
45 57
60 53.3
'ASCORBATE • CATALASE • A.O. • DES'
-10 100
0 83.8
15 82.2
30 76.0
45 69.3
60 65.4
'ASCORBATE • CATALASE • Cu • DTPA'
-10 100
0 91.5
15 90.3
30 91.7
45 87.2
60 91.5
CHAPTER 3
FIGURE 3.
'ASCORBATE • CATALASE • Cu /N2'
-10 100
0 101.2
15 99.9
30 102.2
45 100.1
60 100.5
'ASCORBATE • CATALASE • Cu • 0.5mM HIS'
-10 100
0 77.5
15 69.2
30 65.2
45 61.9
60 58.5
'ASCORBATE • CATALASE • Cu • 2mM HIS'
-10 100
0 77.3
15 72.1
30 68.4
45 62.1
60 59.5
'ASCORBATE • CATALASE • Cu'
-10 100
0 72.6
15 66.8
30 60.4
45 58.6
60 54.0
'ASCORBATE • CATALASE • Cu / 02'
-10 100
0 51.5
15 44.5
30 39.4
45 36.3
60 34.1
'ASCORBATE • CATALASE • Cu • SOD'
-10 100
0 76.2
15 71.3
30 67.0
45 59.7
60 53.5

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