The Function of Catalase-bound NADPH*

(Received for publication, July 29, 1986)

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Catalase (H$_2$O$_2$:H$_2$O oxidoreductase, EC 1.11.1.6) is of historical interest for having been the subject of some of the earliest investigations of enzymes. A feature of catalase that has been poorly understood for several decades, however, is the mechanism by which catalase remains active in the presence of its own substrate, hydrogen peroxide. We reported recently that catalase contains tightly bound NADPH. The present study with bovine and human catalase revealed that NADPH both prevents and reverses the accumulation of compound II, an inactive form of catalase that is generated slowly when catalase is exposed to hydrogen peroxide. Since the effect of NADPH occurs even at NADPH concentrations below 0.1 μM, the protective mechanism is likely to operate in vivo. This discovery of the role of catalase-bound NADPH brings a unity to the concept of two different mechanisms for disposing of hydrogen peroxide (catalase and the glutathione reductase/peroxidase pathway) by revealing that both mechanisms are dependent on NADPH.

During studies of human erythrocytes, earlier investigators noticed that activity of catalase (H$_2$O$_2$:H$_2$O oxidoreductase, EC 1.11.1.6) declined under conditions in which erythrocytes or hemolysates had not only exposure to peroxides but also limitations in ability to generate NADPH (1, 2). The cause for the decline in activity was unknown. We recently studied the role of NADP-binding proteins in the regulation of intracellular glucose-6-phosphate dehydrogenase (d-glucose 6-phosphate:NADP$^+$ 1-oxidoreductase, EC 1.1.1.49) (3, 4). Those studies led to the unexpected finding that catalase contains tightly bound NADPH (5). Each tetrameric molecule of the enzyme from human erythrocytes and bovine liver was found to bind four molecules of NADPH with a dissociation constant for NADPH that was less than 10 nM. The order of NADPH concentrations below 0.1 μM, the protective mechanism is likely to operate in vivo. This discovery of the role of catalase-bound NADPH brings a unity to the concept of two different mechanisms for disposing of hydrogen peroxide (catalase and the glutathione reductase/peroxidase pathway) by revealing that both mechanisms are dependent on NADPH.

NADPH was that NADPH protects the enzyme against inactivation by its own substrate, H$_2$O$_2$ (5). Simultaneously with the submission of the present report, Jouve (6) reported that dithioerythritol induced changes in the absorption spectrum of bovine catalase comparable to the formation of compound II, an inactive form of catalase (6). Some reversal of the change was achieved with NADPH and NADH at micromolar concentrations. They suggested that NADPH may be involved in reversing the inactivation of catalase by its substrate, H$_2$O$_2$. The effect of NADPH, however, was only partial; and in porcine catalase, for example, thiol compounds are known to induce changes in the activity and absorption spectrum of catalase that do not represent conversion of the active enzyme to compound II (7).

This report confirms the hypothesis that NADPH protects catalase against inactivation by H$_2$O$_2$ and offers certain details on how the protection may be accomplished. Except for inclusion of observations on the presence and function of NADP, some of the experiments of this report are repetitions of experiments 36 years ago by Britton Chance (8). He provided one of the earliest identifications of an enzyme-substrate complex, and his efforts, along with those of D. Keilin, E. F. Hartree, and P. Nicholls, led to much of the present understanding of the mechanism of action of catalase (8–10). In considerations of the action of catalase to follow, only the substrates of the present study (H$_2$O, and ethanol) are included. Compound III, which did not seem to be formed in the present study, is omitted from consideration.

As the first step in the catalase cycle, interaction between H$_2$O$_2$ and ferricatalase (catalase before exposure to H$_2$O$_2$) causes the enzyme to become compound I, a spectroscopically distinct and enzymatically active form of catalase. Reaction of compound I with a second molecule of H$_2$O$_2$ results in ferri-catalase, O$_2$, and water. An alternative substrate for this second part of the cycle is ethanol, which reacts with compound I to form ferricatalase, acetaldelyde, and water. Although the rate constant is much greater with H$_2$O$_2$ than with ethanol, the latter can be added to a concentration in vitro that is several orders of magnitude higher than that of H$_2$O$_2$ in vivo and in the experiments to follow. Under this condition, compound I is almost imperceptible, whereas detectable amounts of compound I are present when catalase has H$_2$O$_2$ as the only substrate. Whenever compound I is present, the enzyme undergoes inactivation by the gradual conversion of compound I to compound II through a one-electron reduction of compound I by unidentified reductants (10). Compound II is an inactive form of catalase that has a different absorption spectrum from that of ferricatalase and compound I. A decrease in the concentration of compound I, from either (a) the cessation in generation of H$_2$O$_2$ or (b) the addition of ethanol, results in spontaneous reactivation of catalase by an apparent one-electron reduction of compound II to ferricatalase. As with the formation of compound II, the nature of the
reducing substances is poorly understood. The reaction rate varies with concentrations of catalase in a manner suggesting that the reducing substances are within the molecule of catalase itself (10). These various reactions involving catalase are illustrated and further described under "Discussion."

EXPERIMENTAL PROCEDURES

A homogeneous preparation of human catalase was obtained from erythrocytes by the method of Morikofer-Zwez et al. (11), as previously described (5). The final preparation had a specific activity of 3.5 x 10^4 U/mg and had 4 heme groups and 3.5 molecules of NADPH per molecule of catalase. Crystalline catalase from bovine liver was prepared from Boehringer Mannheim. The following sequence was added: 0.05-0.15 ml of a Krebs-Ringer solution/Tes' buffer, pH 7.4 (3), were added 3.2 mg of the bovine catalase. The suspension was mixed and then allowed to stand at room temperature for 10 min, during which time the crystals dissolved. The catalase was concentrated to a volume of 0.05-0.15 ml by centrifugation at 2 C for 10 min at 1,000 x g in a CF-25 ultrafiltration cone (Amicon). The concentrate was resuspended in 6 ml of 0.01 m (sodium) phosphate buffer, pH 6.5. Again ultracentrifuged, then adjusted to a volume of 0.8 ml. Particles were removed by centrifugation at 15,000 x g for 10 min. NADPH-saturated bovine catalase was prepared in the same manner except that NADPH was present in the 6 ml of Krebs-Ringer solution/Tes' buffer and the 6 ml of phosphate buffer at a final concentration of 1 m.

The addition of phosphate buffer, without NADPH, to the concentrate after the second centrifugation brought the volume to 0.8 ml, and the unbound NADPH content to 0.027 pmol of NADPH per molecule of catalase. The dissolved bovine catalase (unexposed to NADPH) had a specific activity of 2.1 x 10^4 U/mg and had 3.1 molecules of NADPH per molecule of catalase. Extinction coefficients of the catalase indicated that each molecule contained only 3 heme groups, as has been observed with mammalian liver catalase by others (9, 10). Yeast glucose-6-phosphate-dehydrogenase was obtained from Boehringer Mannheim.

Glucose oxidase (6-D-glucose:oxygen l-oxidoreductase, EC 1.1.3.4) from Aspergillus niger was a product of Sigma.

The addition of glucose oxidase and glucose to solutions of catalase served to expose the catalase to H_2O_2 at steady-state concentrations of H_2O_2 as described by Chance (8). Preliminary assays of the glucose oxidase permitted a choice to be made of the concentration of glucose oxidase that would allow continuous generation of H_2O_2 at the desired rate. The assays for glucose oxidase were carried out under conditions identical to those for the incubations with catalase: 0.01 m sodium phosphate buffer, pH 6.5, 2 mM glucose at 37 C in a reaction volume of 500 ml in a cuvette having a light path of 1 cm and a width of 4 mm. In addition, the assay mixture contained horseradish peroxidase, phenol, and 4-aminoantipyrine as specified for the spectrophotometric determination of H_2O_2 by the method of Green and Hill (12). Under these conditions, the rate of generation of H_2O_2 was constant for 1 h at concentrations of glucose oxidase up to 6.7 nm, which resulted in a generation rate of 2 nmol ml^-1 min^-1. Assays for catalase activity were by observation of decreasing absorbance of the H_2O_2 at 240 nm (13).

NADP and NADPH were assayed by the enzymic cycling methods of Burch et al. (4) and of Lowry and Passonneau (15), as described earlier (16). Readings of blanks in the cycling assay indicated that the exchangeable NADP content of the glucose-6-phosphate dehydrogenase was less than 0.8 pmol/ml. Determination of glucose-6-phosphate was by a fluorometric and enzymic method (15).

RESULTS

Effects of H_2O_2 and NADPH on the Absorption Spectra of Catalase—Curve A of Fig. 1 is the absorption spectrum of ferricatalase. Curve B is the absorption spectrum after 90 min of exposure of ferricatalase to H_2O_2, which was generated at a rate of 1 nmol ml^-1 min^-1. After the exposure, catalase exhibited the increased absorbance at 430-440 nm that is characteristic of compound II and the reduced absorbance at 405 nm that is a property of both compound I and compound

\(^1\) The abbreviation used is: Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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**Fig. 1. Absorption spectra of bovine liver catalase.** All incubations and measurements of spectra were of 2 uM catalase, 0.01 m phosphate buffer, pH 6.5, at 37 °C. A, curve A, before exposure to H_2O_2. B, curve B, after exposure for 30 min to H_2O_2 generated at a rate of 1 nmol ml^-1 min^-1 by the presence of glucose oxidase (3.4 nm) and glucose (2 mM). C, curve C, as with B but the presence of an NADPH-regenerating system formed by the addition to the 0.5-ml reaction mixture of glucose-6-phosphate dehydrogenase (5 uM), glucose 6-phosphate (0.2 mmol), and NAD^+ (2 mmol). D, curve D (superimposed on curve A), as with B but with the presence of ethanol at a final concentration of 2 mM. *Inset,* tracings of the recordings of absorbance at 405 nm, versus time, of reactions identical to those of B and C except that the concentration of glucose oxidase was 26.7 nm. The ordinates are offset for graphic clarity. The lefthand scale is for reaction B; the righthand, for C'.

II (8, 10). From the known molar extinction coefficients of ferricatalase, compound I, and compound II (10), 62% of the catalase of curve B was estimated to be compound II. Repetition of the experiment revealed that the activity of the catalase of curve B had dropped to 54% of the activity of untreated enzyme and that the NADPH/NADP ratio of bound NADP had dropped from 0.98 to 0.48. The presence of an NADPH-regenerating system throughout the incubation (curve C) allowed the absorbance at 405 nm to decrease less than when the regenerating system was omitted (curve B). In the presence of the NADPH-regenerating system (curve C), activity of the catalase did not diminish. Absorbance values at 405 and 435 nm indicated that the catalase of curve C was 86% ferricatalase and 14% compound I. When ethanol was present throughout the incubation, the catalase (curve D) retained the absorption spectrum of untreated catalase. Similar spectra, and changes in spectra, were obtained with human catalase. The NADPH bound to human catalase, however, underwent complete conversion to NADP^+ during exposure to the H_2O_2-generating system under the conditions of reaction B.

Determinations of the absorption spectra of 10 uM catalase from 500 to 680 nm confirmed the presence of ferricatalase, compound I, and compound II in the proportions found at the lower wavelengths and under the conditions of Fig. 1. None of the spectra at the higher wavelength had the twin absorption peaks at 545 and 585 nm that are characteristic of compound III (10, 17). Compound III is an inactive form of catalase that is produced under more drastic conditions of H_2O_2 exposure than were used in this study (10, 17).

A reaction mixture consisting of catalase, glucose oxidase, and glucose was analyzed with a recording spectrophotometer that rapidly and repeatedly plotted the absorption spectrum of the reaction mixture. Within 1-2 min, the spectrum became
almost identical to curve C of Fig. 1, then gradually became that of curve B. This effect can be appreciated from observations of the change in absorbance at a fixed wavelength of 405 nm with reactions containing catalase, glucose oxidase, and glucose in either the presence or absence of an NADPH-regenerating system (Fig. 1, inset). The absorbance of both reaction mixtures at 405 nm dropped quickly, and then either remained almost stable (when a source of NADPH was present) or declined gradually. The intercept of the the latter slopes with the ordinate provided a measure of the drop in absorbance resulting from the generation of compound I. The drop was corrected for the small decrease in absorbance resulting from the 2% increase in volume when the solution of glucose was added. These observations indicated that NADPH did not greatly affect the steady-state concentration of compound I formed early in the reaction but that NADPH did largely prevent the accumulation of compound II.

The corresponding changes in absorbance at 435 nm during H2O2 generation are shown in Fig. 2. Since the spectra of ferricatalase and compound I are essentially isosbestic at 435 nm (8), the increase in absorbance at 435 nm corresponds to inactivation of catalase (formation of compound II). In the absence of an NADPH-regenerating system, compound II was formed (reaction A of Fig. 2). The addition of a continuous source of NADPH, however, caused the accumulation of compound II to be not only prevented (reaction C) but also reversed (reaction B).

Prevention of the Formation of Compound II—The following experiments provided a maximum estimate of the rate at which NADPH was oxidized in the process of preventing the formation of compound II. The incubations resulting in curves A and C of Fig. 2 were repeated with the following modifications: (a) glucose oxidase was present at three different concentrations, and (b) the rate of oxidation of NADPH was determined from the amount of 6-phosphogluconate generated during 1 h of H2O2 production in reaction mixtures containing glucose-6-phosphate dehydrogenase, glucose 6-phosphate, and NADP+ (as with reaction C). The rate of generation of compound II was estimated from the initial slope following the addition of glucose to the mixture without an NADPH-regenerating system (Fig. 3). The conversion of ferricatalase or compound I to compound II was considered to be accompanied by an increase in millimolar extinction coefficient (per heme group) of 33 at 435 nm. Plots of absorbance at 435 nm indicated no accumulation of compound II in the presence of the NADPH-regenerating system. Repetition of the experiment allowed estimation, by the method depicted in Fig. 1 (inset), of the amount of compound I formed. Over the range of H2O2 production used in the experiments of Fig. 3, the initial concentration of compound I and the rate of compound II formation varied with the rate of H2O2 production. Table I contains the rates of production of compound II and the rates of oxidation of NADPH (as reflected by rates of formation of 6-phosphogluconate). A rate of 6-phosphogluconate production of 3.5-3.8 nmol ml⁻¹ h⁻¹ was observed when glucose oxidase or glucose was omitted from the reaction mixture (Table I). Rates in this range were assumed to result from the action of glucose-6-phosphate dehydrogenase in reducing the 4 μM of added NADP+. With the correction of 3.5, the rate of formation of compound II, at each of the three rates of H2O2 production, corresponded to 30-40% of the rate of NADPH oxidation, but the rate of NADPH oxidation was only 7-25% of the rate of H2O2 production (Table I). The presence of ethanol in the reaction mixture reduced the rate of 6-phosphogluconate production to 3.6 nmol ml⁻¹ h⁻¹. Thus, ethanol abolished the net oxidation of NADPH in the reaction mixtures. This abolition of NADPH oxidation by ethanol provided assurance that NADPH was not directly oxidized appreciably by glucose oxidase or by H2O2 under the conditions of this study.

![Fig. 2. Kinetics of the formation and disappearance of compound II, as measured by absorbance at 435 nm. Each cuvette contained 0.49 ml of 0.01 M phosphate buffer, pH 6.5, 2.2 μM bovine liver catalase, 6.7 nM glucose oxidase. Reaction C also contained the NADPH-regenerating system described for reaction C of Fig. 1. The reaction temperature was 37 °C. Absolute absorbances at 435 nm were measured. The recordings were then offset by approximately 0.05 absorbance for graphic clarity. At the first arrow, the generation of H2O2 at a rate of 2 nmol ml⁻¹ min⁻¹ was started by the addition of 10 μl of 0.1 M glucose. At the second arrow, components of the NADPH-regenerating system were added to reaction B. An equivalent volume (20 μl) as phosphate buffer was added to reactions A and C.](image1)

![Fig. 3. Formation of compound II at different rates. The reaction mixtures consisted of 0.49 ml of 2 μM bovine catalase, 0.01 M phosphate buffer, pH 6.5, in which the final concentrations of glucose oxidase were (A) 26.7 nM, (B) 6.7 nM, and (C) 1.7 nM. The temperature was 37°C. Arrows, addition of 10 μl of 0.1 M glucose.](image2)
Function of Catalase-bound NADPH

TABLE I

Rates of formation of compound II and of oxidation of NADPH at various rates of generation of H₂O₂

<table>
<thead>
<tr>
<th>Glucose oxidase (nM)</th>
<th>Rates of formation of compound II (nmol ml⁻¹ h⁻¹)</th>
<th>Rates of oxidation of NADPH, total (nmol ml⁻¹ h⁻¹)</th>
<th>Rates of oxidation of NADPH, net (nmol ml⁻¹ h⁻¹)</th>
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<tr>
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<tr>
<td>1.7</td>
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<tr>
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<tr>
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<td>0</td>
<td>3.8</td>
<td>0.3</td>
</tr>
<tr>
<td>6.7</td>
<td>120</td>
<td>3.6</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*Glucose absent.

In the presence of an NADPH-regenerating system, the absorbance at 435 nm failed to increase over several minutes even when the concentration of glucose oxidase was increased to 2 μM, which corresponded to a rate of H₂O₂ production of 10 nmol ml⁻¹ s⁻¹. The formation of compound II was prevented in the reaction of curve C of Fig. 2 by the addition of NADP in the proportion of 4 nmol/ml to the 2 μM solution of catalase. Because this lot of bovine catalase was only 78% saturated with NADPH, however, the addition of NADP in the proportion of 4 nmol/ml raised the concentration of unbound NADP in the reaction mixture only to approximately 2 μM. The studies of Fig. 2 were repeated with NADPH-saturated bovine catalase (Fig. 4). The catalase of Fig. 4 had been suspended in a solution of 1 μM NADPH and then concentrated by ultrafiltration. The addition of glucose-6-phosphate dehydrogenase and glucose 6-phosphate protected the catalase against H₂O₂-induced formation of compound II not only when the NADP concentration was raised by 1 μM (curve C of Fig. 4) but also when no NADP was added (curve B). Addition of the concentrated catalase to the reaction mixture resulted in an unbound NADPH concentration of less than 60 nM.

**Conditions Affecting the Disappearance of Compound II**—The addition of a continuous source of NADPH caused the accumulation of compound II to be reversed (curve B of Fig. 2). Reversal failed to occur when only one component of the NADPH-regenerating system was added (glucose-6-phosphate dehydrogenase, glucose 6-phosphate, or NADP⁺). This observation permitted exclusion of the possibility that the reversal was the result of contamination of the components with ethanol. The experimental leading to curve B of Fig. 2 was repeated, except that the addition at the second arrow was either ethanol, the NADPH-regenerating system, or both (Fig. 5). Readings of absorbance, above each asymptote, were transferred to semi-log graphs. This replotting revealed that the decay of compound II followed first-order kinetics. Since NADPH is a substrate at constant concentration, the constants must be considered pseudo-first order for reactions having the NADPH-regenerating system. Least-squares estimates (18) of the first-order rate constants were made from the equation for first-order kinetics and from eight absorbance readings at intervals of 8–12 min. The means and standard deviations from five consecutive incubations are given in Table II. The results in Table II indicate that the decay with the NADPH-regenerating system (k₅) is significantly greater than the decay with ethanol (kₜ) (t = 10.7; 8 degrees of freedom; p < 0.001). Table II also contains the decay constants for compound II when the production of compound II was stopped by removal of glucose (and consequently the means for generation of H₂O₂) by rapid ultrafiltration/dilution of the reaction mixture. This latter approach caused the compound II to decay spontaneously at a rate comparable to the rate with ethanol (kᵣ = kₗ = kₜ) (Table II). The rate with an
NADPH-regenerating system \((k_d)\), however, was decidedly faster than the spontaneous rate \((k_s)\) \((t = 8.7; 8\) degrees of freedom; \(p < 0.001)\) Table II). Since \(k_d > k_s\), NADPH must enhance the rate of disappearance of compound II. The similarity between \(k_s\) and \(k_d\) \((\text{Table II})\); however, indicates that NADPH also has some ability to retard the conversion of compound I to compound II.

The catalase-bound NADPH of bovine catalase was stripped of 96% of enzymically detectable NADP through 48 h of exposure to low concentrations of \(H_2O_2\) by the gaseous diffusion method previously described \((5)\). The sample was then carried through the sequence indicated for curve A of Fig. 5. The absorbance at 435 nm rose during the generation of \(H_2O_2\). This result was interpreted to mean that NADPH was not essential for the inactivation of catalase by reduction of compound I to compound II. Moreover, the addition of ethanol to a final concentration of 2 mM resulted in a drop in absorbance at 435 nm as with reaction A of Fig. 5. The NADPH bound to human catalase became entirely NADP⁺ during exposure to \(H_2O_2\) under conditions of reaction A of Fig. 5. As with the bovine enzyme, however, the addition of ethanol resulted in the disappearance of compound II of this NADPH-depleted catalase. This subsequent incubation with ethanol did not change the amount or NADPH/NADP ratio of the catalase-bound NADP. Thus, the presence of catalase-bound NADPH was not necessary for the frequently cited "spontaneous" disappearance of compound II that accompanies a decrease in concentration of compound I.

Similar rates of disappearance of compound II were observed following the addition of the full NADPH-regenerating system to reaction mixtures at pH 7.5 or at KCl concentrations of 0.1 M. Reversal of the accumulation of compound II was also achieved by the direct addition of NADPH or NADP (Fig. 6). In the experiment of Fig. 6, the concentration of compound II fell, only to rise again, presumably after the NADPH or NADH had been consumed.

**DISCUSSION**

Earlier investigators found that: (a) catalase is slowly inactivated in the presence of its substrate, \(H_2O_2\); (b) the loss of activity results from the conversion of compound I, a catalase-oxygen intermediate in the catalase cycle, to compound II, an inactive form of catalase; and (c) stopping the exposure to \(H_2O_2\), or otherwise reducing the steady-state concentration of compound I, leads to reactivation of catalase through the slow and spontaneous disappearance of compound II \((8, 10)\). The present study revealed that NADPH is not required for \(b\) or \(c\) but is effective in preventing and reversing \(a\). An exception to \(a\) occurs when certain alcohols are present at many times the concentration of \(H_2O_2\). This disproportion reduces the steady-state concentration of compound I and therefore reduces the rate of formation of compound II \((8, 10)\). A few compounds, such as methanol and ethanol, are capable of such action \((10)\). Uncertainty exists, however, as to whether compounds of this type exist intracellularly at concentrations that are significant for this effect on catalase \((19, 20)\). We now suggest that the hypothesis for their existence, for preventing the inactivation of catalase in vivo, is unnecessary, since the accumulation of compound II can be prevented by NADPH at concentrations that are known to exist within cells.

That NADPH allows catalase to remain active in vivo is made likely by the very low concentrations of NADPH needed for preventing the accumulation of compound II and by these experimental conditions, which were roughly comparable to conditions within cells. The concentration of unbound NADP in human erythrocytes is approximately 2 \(\mu\)M \((4)\). The concentration of catalase within human erythrocytes \((5)\) is similar to that used in the present study but is slightly less than the concentration in rat liver \((19)\). Chance \((8)\) estimated that the concentration of \(H_2O_2\) with the catalase/glucose oxidase system is of the order of 1 nM. The concentration in vivo may be 1–100 nM \((18)\). An upper limit to the normal rate of generation of \(H_2O_2\) in human erythrocytes can be estimated from the rate of NADPH generation in the pentose phosphate pathway of erythrocytes from people with a genetic deficiency of catalase. That rate, 4 nmol ml⁻¹ min⁻¹ \((21)\), is less than the 380 nmol g⁻¹ min⁻¹ rate of \(H_2O_2\) production estimated in rat liver \((20)\). Limiting the rate of \(H_2O_2\) production to 2 nmol ml⁻¹ min⁻¹ in most of the present experiments prevented the depletion of dissolved oxygen in the cuvettes and allowed the rate of production to be essentially constant for 1 h (see "Experimental Procedures"). Greater rates of \(H_2O_2\) generation were used for some experiments in which conclusions could be reached from observations of 3–15 min.

NADPH is oxidized in the process of preventing the accumulation of compound II. Because bovine and human cata-

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**Table II**

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>NADPH (μM)</th>
<th>Ethanol (mM)</th>
<th>Rate constants (10^4 \times K, s^{-1})</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.D.</td>
<td>Designation</td>
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<td>(k_a)</td>
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**Fig. 6. Effect of NADPH and NADH on compound II.** The reaction mixtures consisted of 0.49 ml of 2.1 μM bovine catalase, 0.01 M phosphate buffer, pH 6.5, 0.7 mM glucose oxidase. The temperature was 37 °C. First arrow, addition of 10 μl of 0.1 M glucose; second arrow, addition, to a final concentration of 20 μM, of ethanol to reaction A, NADPH to reaction B, and NADH to reaction C.
NADPH, even at concentrations of unbound NADPH of less than 0.1 mM, strongly binds NADPH (dissociation constant less than 10 nM) and have a greater affinity for NADPH than for NADP⁺ (5), the bound NADP⁺ is readily displaced by NADPH, even at concentrations of unbound NADPH of less than 0.1 μM. NADH also reverses the accumulation of compound II (Fig. 6). But catalase binds NADP⁺ to the exclusion of NADH when presented with the two reduced dinucleotides at equimolar concentrations (5). Moreover, cytoplasmic NADP is largely in the reduced form, whereas NAD is largely in the oxidized form. NADPH, therefore, is the more likely of the two reduced dinucleotides to be involved in the protection of cytoplasmic catalase.

Although ethanol prevents the accumulation of compound II, the mechanism involving ethanol is different from that involving NADPH. Under the conditions of the present study, ethanol greatly decreases the steady-state concentration of compound I (Fig. 1) (8, 10), thereby decreasing the rate of conversion of compound I to compound II. Moreover, ethanol is known to be consumed at a rate approaching the rate at which the source of H₂O₂ (glucose oxidase and glucose) is producing H₂O₂ (10). In contrast, NADPH does not greatly decrease the concentration of compound I (Fig. 1, inset), and the NADPH is oxidized at a rate that is only a fraction of the rate of generation of H₂O₂ (Table I). The rate of oxidation of NADPH when catalase is exposed to H₂O₂ is greater than the rate of formation of compound II in the absence of NADPH (Table I). Consequently, a possibility exists that NADPH is utilized to some extent in a manner similar to ethanol (reaction 2b of Fig. 7). The results of the present study neither confirm nor refute that possibility.

Fig. 7 is the traditional scheme for catalase (10, 22) with H₂O₂ and ethanal as substrates. AH denotes one-electron donors (reducing substances) either extrinsic to catalase (22) or within the catalase molecule (10). The scheme of Fig. 7 has served well to explain the catalytic cycle of the enzyme (the portion represented by curved arrows). To the extent that AH might denote compounds added in vitro, the scheme also accounts for one-electron reduction by substances such as ferrocyanide or nitrite (10). The scheme suggests however, to account for the ability of catalase to remain active in the face of continual generation of H₂O₂ within cells. As illustrated by the experiment of Fig. 3, the production of H₂O₂ and formation of compound I, even at a slow rate, results in the conversion of most of the catalase to compound II in the absence of NADPH. Earlier authors have suggested that AH could represent reducing groups within the catalase molecule and that the enzyme is endowed with a "good supply" of such reducing equivalents (10). For the situation of constant intracellular exposure to H₂O₂, however, this explanation amounts to an assumption that catalase has an unlimited number of internal reducing equivalents. In addition, NADPH is difficult to fit into the existing scheme (Fig. 7), since the scheme requires one-electron reductions and NADPH is a two-electron reducing substance. Retention of the traditional scheme, in modified form, would be possible if reaction 4 could be represented by:

\[
\text{Compound II + A'} + \text{NADPH} \rightarrow \text{ferricatalase + AH + NADP}^+ \\
\text{MODIFIED REACTION 4}
\]

As with Fig. 7, A' and AH represent groups within the catalase molecule itself, and compound II is at an oxidation level of IV, whereas ferricatalase is at a level of III (Fig. 7, legend). Modified reaction 4 therefore is balanced with respect to oxidation and reduction, although it leaves unexplained how the NADPH could result in two one-electron reductions. A feature of possible relevance is the ability of NADP to participate in one-electron reactions when NADP exists as a free radical.

The ability of catalase to recover activity in the absence of H₂O₂ and NADPH could be explained if reaction 3 were reversible.

\[
\text{Compound I + AH} \rightleftharpoons \text{compound II + A'} \\
\text{MODIFIED REACTION 3}
\]

As a result of the similarity between k₁, k₃, and k₄ in Table II, an ability of NADPH to inhibit reaction 3 must be assumed if reaction 3, or a modified form of reaction 3, is assumed to exist.

Whether the traditional scheme can be retained in such a revised form, or will need to be replaced by a different scheme, must await future studies. Fita and Rossmann (23) found that the NADPH is close to the surface of catalase, whereas the heme group is buried roughly 20 Å below the molecular surface. The two are connected by a tunnel, but the smallest aperture of the tunnel is less than 3 Å (23). The current difficulty in fully explaining the mechanism of action of NADPH, however, should not detract from the observation that NADPH, in biologically realistic concentrations, effectively prevents and reverses the accumulation of compound II.

Catalase is ubiquitous among aerobic forms of life, and formation of compound II is a common property of catalases from a single heme group (10). Of interest, therefore, is whether the utilization of NADPH (or NADH) by catalase is an early or recent development in evolution. NADPH is present on bovine and human catalase (6). We find that it is present also on canine catalase.² The catalase of A. niger neither contains nor binds NADPH (5). A re-evaluation of crystallographic data confirmed the presence of NADPH on bovine catalase but provided no evidence for the presence of NADPH on the catalase of P. vitale (24). To date, therefore, the binding of NADPH by catalase has been shown to be present in three species of mammals and to be absent in two species of molds. The novel function of catalase-bound NADPH, relative to the function of NADPH in dehydrogenases, suggests that the binding site for the NADPH may have evolved independently from the sites that bind NAD or NADP in other proteins. This independent evolution, in turn, could explain why the configuration of the NADPH-binding site in bovine catalase differs from that of other NADP-binding and NAD-binding enzymes (24).

²H. N. Kirkman, S. Galano, and G. F. Gaetani, unpublished observations.
Earlier in this century, the action of catalase was thought to be the principal intracellular means for degrading H$_2$O$_2$. Largely through the studies of Cohen and Hochstein (25), however, the disposal of H$_2$O$_2$ in certain mammalian cells began to be attributed to the enzymic utilization of NADPH for the reduction of oxidized glutathione, followed by the action of glutathione peroxidase on H$_2$O$_2$. Supporting the importance of the NADPH/glutathione mechanism were observations that genetically determined deficiencies of glucose-6-phosphate dehydrogenase lead to either chronic hemolytic disease or susceptibility to acute hemolytic anemia. This susceptibility, which affects over 100 million people (26), is attributable to inability of affected erythrocytes to withstand certain peroxidative stresses. Discovery of the presence and role of catalase-bound NADPH, however, brings a unity to the concept of two different mechanisms for disposing of hydrogen peroxide (catalase and the glutathione reductase/peroxidase pathway) by revealing that both mechanisms are dependent on NADPH.

REFERENCES