The Inhibition of Cow-Liver Catalase by 3-Nitropropionate

By KO SAWAI, Toshiyasu MAEDA and Tokuji SHIMOMURA

Department of Agricultural Chemistry, Faculty of Agriculture,
University of Hokkaido, Sapporo

Received March 30, 1966

The cow-liver catalase was inhibited by 3-nitropropionate (3-NPA) at every pH tested. The 50% inhibition degree, ϕ , of 3-NPA under experimental condition was 8.7×10^{-4} M, which was almost that of phenol (1.0×10^{-3} M). Among 3-NPA, 1-nitropropane, and propionate, 3-NPA had the most effective inhibitory effect. The maximum absorption spectrum of free catalase in the Soret region was not shifted clearly by an addition of 3-NPA, but the intensity of it was decreased. The inhibition type of 3-NPA was not definitely classified as the cyanide type. Results indicate that this inhibition essentially be due to reaction between the prosthetic group and anion form of nitro group in 3-NPA, and that the inhibitory action of nitro group may be stimulated by carboxylic group. Finally, this inhibition could be thought as a partial toxic cause in vivo.

INTRODUCTION

The toxic component of *Indigofera endecaphylla* Jacq. has been identified as 3-NPA by Morris et al.¹⁾ Dosage of 3-NPA has resulted in methemoglobinemia in rats. Matsumoto et al.²⁾ have pointed out that this phenomenon is not produced by 3-NPA directly, but by nitrite presumably formed from 3-NPA in vivo. In other words the amounts of methemoglobin produced by lethal dosages of 3-NPA are too low to account for the fatal effects. Therefore, it can be thought that 3-NPA makes animals sick by its own toxicity. To explain the toxic mechanism, some investigators suggested that enzymic studies are necessary, because mortality of animals could be resulted when 3-NPA inhibits some enzyme systems of vital importance. The succinate dehydrogenase has been inhibited weakly by 3-NPA in vitro,³⁾ but no information about other enzymes has been obtained. In the present investigation, 3-NPA was found to be

the catalase inhibitor; and inhibitory behavior of which was examined and discussed.

MATERIALS AND METHODS

Chemicals. 3-NPA was synthesized from 3-propiolactone by the method of Hass et al.⁴⁾ Its m.p. was 68°C after three recrystallizations from chloroform. Other chemicals used were of extra pure grade except that hydrogen peroxide was of guaranteed grade. Solutions of 3-nitropropionic acid, hydroxylamine hydrochloride, 1-nitropropane, and propionic acid were adjusted to pH 7.0 with sodium hydroxide and a solution of potassium cyanide was adjusted to pH 7.0 with hydrochloric acid.

Enzyme Preparation. Catalase was prepared from cow liver by the method of Shirakawa.⁵⁾ The enzyme could not be obtained in crystalline form, and the value of "Katalasefähigkeit" was about 600.

Enzyme Assay. Catalase units were expressed by the same definition described by Lück.⁶⁾

Enzyme Reaction. The spectrophotometric procedure of Beers et al.⁷⁾ was applied. A mixture of

1) M. P. Morris, C. Pagán and H. E. Warmke, *Science*, **119**, 322 (1954).

2) H. Matsumoto, J. W. Hylin and A. Miyahara, *Toxicol. Appl. Pharmacol.*, **3**, 493 (1961).

3) J. W. Hylin and H. Matsumoto, *ibid.*, **6**, 168 (1964).

4) H. B. Hass, H. Feuer and S. M. Pier, *J. Am. Chem. Soc.*, **73**, 1858 (1951).

5) M. Shirakawa, *J. Agr. Chem. Soc. Japan*, **23**, 361 (1950).

6) H. Lück, in H. U. Bergmeyer (editor), "Methods of Enzymatic Analysis", Academic Press Inc., New York, 1963, p. 885.

7) R. F. Beers and I. W. Sizer, *J. Biol. Chem.*, **195**, 133 (1952).

FIG. 1. Rate of Decrease of Hydrogen Peroxide at Different pH Values.

----, with 3-NPA; —, without 3-NPA (control). Experimental conditions: 0.1 M phosphate buffer, final concentrations of hydrogen peroxide, and 3-NPA were 0.12 M, and 0.0041 M, respectively, and catalase unit was 438/reaction mixture, at 20°C.

al of hydrogen peroxide solution and 0.05 ml of dilutor solution (or dist. water) was poured into cell in the apparatus, and then 0.9 ml of enzyme solution was added to the mixture. Then, the mixture was quickly mixed up with a small plastic rod, the optical density was read at 240 m μ with a Perkin-Elmer 139 UV-VIS spectrophotometer. The reaction temperature was 20°C.

Inhibitor Concentration. All experiments were done at the concentration of hydrogen peroxide below the K_m of catalase (1.1 M^8)

RESULTS AND DISCUSSION

Effects of pH on the Inhibition of Catalase by 3-NPA

As shown in Fig. 1, 3-NPA inhibited the catalase reaction at every pH tested, and the degree of inhibition was markedly influenced by pH, that is, the degradation rate of hydrogen peroxide was much slower at pH higher than 7. As the control reactions were not affected with pH, it is thought that this inhibition closely relates to the dissociation of 3-NPA as an ampholyte, and the pK (9.1) of the amino group in this compound rather than to the pK (3.8)⁹ of carboxylic group in it.

Inhibitory Effects of 3-NPA, 1-Nitropropane, and Propionate

Fig. 2 showed that 3-NPA was the more

powerful inhibitor than 1-nitropropane at every pH tested, but propionate had not any inhibitory effects at all. This seems that catalase is not inhibited by carboxylic group alone, but essentially by nitro group, and that, when both carboxylic and nitro groups are present in the same molecule like 3-NPA, the former enhances the inhibitory action of the latter.

On the other hand, the inhibition by 1-nitropropane was clearly increased in accordance with pH changes from 6.0 to 8.0. Such influences of pH, as described in the preceding section, can be interpreted by the pre-

FIG. 2. Comparison between Inhibitions by 3-NPA, 1-Nitropropane, and Propionate at Different pH Values.

—, no inhibitor; ----, +3-NPA; — · —, +1-nitropropane; · · · ·, +propionate.

Experimental conditions, the same as those in Fig. 1. Final concentrations of these substances are 0.004 M.

⁸ Y. Ogura, *Arch. Biochem. Biophys.*, **57**, 288 (1955).
⁹ M. T. Bush, O. Touster and J. E. Brockman, *J. Biol. Chem.*, **188**, 685 (1951).

sumption that anion form derived from nitro group participates in the inhibition, because the equilibrium in nitro—aci-nitro tautomerism¹⁰⁾ of 1-nitropropane is transformed into anion form by addition of alkali. Thus the inhibition by 3-NPA may be produced not only by the inhibitory action of anion form of nitro group, but also by the additional effect of carboxylic group accelerating the inhibition.

3. Change of Absorption Spectra

Fig. 3 showed that the maximum absorption spectrum of free catalase in the Soret region was 405 m μ . Many investigators have shown that the maximum absorption of free catalase is shifted to a longer wavelength with an addition of potassium cyanide and sodium azide,¹¹⁾ and that at the same time its absorption intensity is decreased in both cases.

In the present experiment, the maximum absorption band of free catalase did not clearly shift by an addition of 3-NPA, but the intensity of which decreased.

This decreased value looked somewhat like the value obtained with sodium azide. Therefore, the inhibition by 3-NPA is thought to

FIG. 3. Spectra of Free Catalase, its Complexes with 3-NPA, Potassium Cyanide, and Sodium Azide.

Experimental conditions: 0.25 M phosphate buffer pH (6.0), final concentrations of 3-NPA, cyanide, and azide were 3.7 M, 2×10^{-2} M and 2×10^{-2} M, respectively, and catalase unit was 2625/2.5 ml of the final volume.

be due to structural changes of hematin-prosthetic group by the reaction with 3-NPA, presumably with nitro and carboxylic group of this substance.

4. Inhibition Type of 3-NPA

Progressive inhibition ($\alpha \rightarrow \beta$ transition) has

FIG. 4. Inhibition Patterns by Different Inhibitors.

Both 3-NPA and cyanide do not show progressive inhibition ($\alpha \rightarrow \beta$ transition), but both phenol and hydroxylamine do: 0.022 M hydrogen peroxide, 15 units catalase, 0.1 M phosphate buffer (pH 7.0). The concentrations of inhibitors are shown as the final concentration.

10) G. W. Wheland, "Advanced Organic Chemistry", John Wiley & Sons, Inc., New York, 1949, p. 629.

11) P. Nicholls and G. R. Schonbaum, in P. D. Boyer, H. Lardy and K. Myrback (editors), "The Enzymes", Vol. 8, Academic Press Inc., New York, 1963, p. 147.

been observed with catalase inhibitors, except cyanide and formate.

As shown in Fig. 4, 3-NPA did not show this kind of inhibition. 3-NPA can consequently be thought to react with catalase in a similar fashion to that of cyanide or formate.

More detailed experiments on this respect

have been made by following the Ogura's method.¹²⁾ The results obtained were shown in Fig. 5, 6, 7, and 8. In these Figs., H (ordinate) is defined as the degree of inhibition, which is expressed by the next equation.

$$H = (V - V_i) / V,$$

FIG. 5. Effect of 3-NPA on the H — $\log [I]$ —curve of Sodium cyanide.

Experimental conditions: 0.022 M hydrogen peroxide, 38 units catalase, 0.1 M phosphate buffer (pH 7.0), 20°C. —○—, corresponding H values of cyanide. —△—, corresponding H values of cyanide in the simultaneous presence of 1.06×10^{-3} M 3-NPA. —, calculated on the assumption of competitive interaction. ϕ of cyanide = 1.2×10^{-5} M/l.

FIG. 6. Effect of 3-NPA on the H — $\log [I]$ —Curve of Phenol.

Experimental conditions, the same as those in Fig. 5. —○—, corresponding H values of phenol. —△—, corresponding H values of phenol in the presence of 1.06×10^{-3} M 3-NPA. —, calculated on the assumption of competitive interaction. ϕ of phenol = 1.0×10^{-3} M/l.

FIG. 7. Effect of Phenol on the H — $\log [I]$ —Curve of 3-NPA.

Experimental conditions, the same as those in Fig. 5. —○—, corresponding H values of 3-NPA. —△—, corresponding H values of 3-NPA in the presence of 1.2×10^{-3} M phenol. —, calculated on the assumption of competitive interaction. ϕ of 3-NPA = 8.7×10^{-4} M/l.

FIG. 8. Effect of 3-NPA on the H — $\log [I]$ —Curve of Hydroxylamine.

Experimental conditions, the same as those in Fig. 5. —○—, corresponding H values of hydroxylamine. —△—, corresponding H values of hydroxylamine in the presence of 1.06×10^{-3} M 3-NPA. —, calculated on the assumption of competitive interaction. ϕ of hydroxylamine = 4.5×10^{-6} M/l.

12) Y. Ogura, Y. Tonomura, S. Hino and H. Tamiya, *J. Biochem. (Tokyo)*, **37**, 179 (1950).

where V and V_i are the degradation rates of hydrogen peroxide in the absence and presence of inhibitor, respectively.

Representing the concentration of inhibitor causing 50% inhibition as ϕ , Ogura has theoretically decided following relationships between two different inhibitors (G_1 and G_2).

1. If G_1 and G_2 reacted competitively with the same active center of catalase, the following equation can be established.

$$\phi_{i,2} = K_{G_2}(1 + G_1/K_{G_1})$$

2. If G_1 and G_2 combined with different active centers of enzyme, exerting "repulsive" influence upon each other, the following relation can be acceptable.

$$K_{G_2} < \phi_{i,2} < K_{G_2}(1 + G_1/K_{G_1})$$

where $\phi_{i,2}$ is the concentration of inhibitors causing 50% inhibition in the presence of the constant amount of G_1 and of variable amounts of G_2 and K_{G_1} and K_{G_2} are the dissociation constants of catalase- G_1 and catalase- G_2 complex, respectively. Assuming cyanide as G_2 and 3-NPA as G_1 , we found that the value $\phi_{i,2}$ is $10^{-4.54}$ M at $G_1 = 1.06 \times 10^{-3}$ M, in Fig. 5.

If cyanide and 3-NPA are inhibitors belonging to the same type, both will competitively react with catalase. In such a case, the theoretical value $\phi_{i,2}$ will be identical with the observed value $\phi_{i,2}$, $10^{-4.54}$ M. Now $K_{G_2} = 10^{-4.93}$ M (Fig. 5) and $K_{G_1} = 10^{-3.06}$ M (Fig. 7). Then the theoretical value $\phi_{i,2} = 10^{-4.58}$ M. The excellent agreement between the calculated value and the observed one may be taken as an evidence that both cyanide and 3-NPA act competitively with each other, in other words, 3-NPA is the inhibitor of the cyanide type.

On the interactions between 3-NPA and phenol (the phenol type inhibitor) and also between 3-NPA and hydroxylamine (the azide type inhibitor), both cases were in repulsive relations towards enzyme as shown in Fig. 6, 7, and 8.

That is, in Fig. 6 (the presence of the con-

stant amount of 3-NPA to phenol),

$K_{G_2} = 10^{-2.98} < \text{the observed } \phi_{i,2} = 10^{-2.64} < K_{G_2}(1 + G_1/K_{G_1}) = 10^{-2.56}$ in Fig. 7 (the presence of the constant amount of phenol to 3-NPA),

$K_{G_2} = 10^{-3.06} < \text{the observed } \phi_{i,2} = 10^{-2.81} < K_{G_2}(1 + G_1/K_{G_1}) = 10^{-2.77}$ and in Fig. 8 (the presence of the constant amount of 3-NPA to hydroxylamine),

$K_{G_2} = 10^{-5.35} < \text{the observed } \phi_{i,2} = 10^{-5.02} < K_{G_2}(1 + G_1/K_{G_1}) = 10^{-4.92}$.

These results were summed up in Table I.

TABLE I. OGURA'S CLASSIFICATION AND INHIBITION TYPE OF 3-NPA

G_1 and G_2 represent inhibitors. C, competitive interaction between inhibitors. R, repulsive interaction.

G_1	G_2 Ogura's Classification			Data obtained 3-NPA
	Cyanide T.	Azide T.	Phenol T.	
Cyanide Type	C	R	R	C
Azide Type	R	C	R	R or C
Phenol Type	R	R	C	R or C

Although 3-NPA was shown as the repulsive inhibitor to phenol and hydroxylamine in Table I, it is doubtful that 3-NPA and phenol or 3-NPA and hydroxylamine react repulsively with different sites of enzyme, because significant differences were not obtained between the observed and calculated values as shown in Fig. 6, 7, and 8. Therefore, it seems likely that both 3-NPA and phenol or hydroxylamine have competitive influences rather than repulsive ones upon enzyme. In fact, both phenol and hydroxylamine have been classified into one category by Nicholls and Schonbaum,¹¹⁾ who have pointed out that the inhibition by cyanide is caused by "covalent ligand" in character and that both phenol and hydroxylamine (phenol and azide type) inhibition are caused by "ionic" in character. In consideration of these matters mentioned above, the authors tentatively think that the 3-NPA inhibition may be mixed up with following

actions: one is the inhibition as "covalent" character which is the cyanide type produced by anion form of nitro group and the other is the inhibition as "ionic" in character which is the phenol or azide type produced by dissociated carboxylic group.

5. ϕ of 3-NPA

Kinetic studies on the inhibition of catalase are very complicated. For example, Ogura⁸⁾ is reported on the inhibition by cyanide at, if the concentration of hydrogen peroxide is smaller than the K_m value, ϕ will be equal to the K_i (the dissociation constant of enzyme-inhibitor complex).

On the other hand, Wolfe et al.¹³⁾ have shown that ϕ is equal to the $1.3 \times K_i$ at the inhibition by cyanide.

In the present investigations, where the substrate concentrations were below the K_m and every reaction followed the first order reaction

(Fig. 1 and 2), we could not detect whether $\phi_{3\text{-NPA}}$ is equal to the $K_{i3\text{-NPA}}$ or not, because the concentrations of enzyme-substrate and enzyme-inhibitor complex were not determined. However, the inhibitory powers of toxin to enzyme can be compared by ϕ values under these conditions.

$\phi_{3\text{-NPA}}$, $\phi_{\text{potassium cyanide}}$, $\phi_{\text{hydroxylamine}}$, and ϕ_{phenol} were found to be 8.7×10^{-4} , 1.2×10^{-5} , 4.5×10^{-6} , and 1.0×10^{-3} M, respectively, from Fig. 5, 6, 7, and 8.

Thus 3-NPA showed the similar inhibitory effect on catalase as phenol did, but not so powerfully as potassium cyanide or hydroxylamine. Subsequently, the catalase inhibition by 3-NPA can be expected as one of the toxic causes upon animals.

However, it is uncertain whether or not this kind of expectation can directly be connected with the lethal factor in vivo, because, the biological significance of catalase has not been clarified.

13) A. D. Wolfe, R. F. Beers and I. W. Sizer, *Arch. Biochem. Biophys.*, **72**, 353 (1957).