Effects of Carbon Dioxide on Catalase

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In this paper, the authors present the sensitivity of catalase activity to carbon dioxide. The catalatic activity of crystalline enzyme prepared from ox liver is inhibited by half under 13% partial pressure of carbon dioxide in oxygen. On the contrary, the peroxidatic activity of this enzyme is respectably enhanced. This inhibition is completely reversible and independent of pH. The hydrates of carbon dioxide, carbonic acid, carbonate, and bicarbonate, have no inhibitory effects on the enzyme. The probability that the inhibition is due to the action of physically dissolved molecular carbon dioxide, is presented by using carbonic anhydrase.

INTRODUCTION

In 1955, Brestkin and Ivanova[1] reported that catalase is reversibly inhibited 60-90 % when exposed to CO_2. They pointed out that this inhibition is not due to pH change, nor to carbonate or bicarbonate ion. In the present investigation we have studied the mechanism of the inhibition and its reversibility, as well as the effects of CO_2 on the peroxidatic activity of catalase, when pCO_2 is systematically varied in the physiological range.

EXPERIMENTAL

Materials

Catalase, three times recrystallized, was prepared from ox liver by the method of Kitagawa and Shirakawa[2]. The crystalline preparation was suspended in redistilled water and dissolved by adding the minimum volume of 0.1 N sodium hydroxide to make the solution pH 7.5. Enzyme concentration of this stock solution, $2\times10^{-5}M$, was determined by calculation of k_s (assuming it to be 2.7×10^7 litre$\times$mole$^{-1}\times$sec^{-1}.)[3] and determination of haematin contents by the pyridine haemochromogen method[4]. For the assay of catalatic activity and peroxidatic activity, the stock solution was suitably diluted with 0.05 M phosphate buffer pH 6.8.

Partially purified preparations of carbonic anhydrase were obtained from ox erythrocytes by the modified method of Keilin and Mann[5]. The enzyme solution thus obtained was completely devoid of catalatic activity.

Homogenates of fresh animal tissues were obtained from adult male albino rats. After decapitation, the livers and kidneys were removed and chilled without delay and homogenized in cold 0.05 M phosphate buffer, pH 6.8, with the Potter-

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Elvehjem glass homogenizer. One-half ml. of blood was pipetted out from the vein near the heart under light ether anesthesia and then homolyzed by putting it into 1.0 ml. of cold distilled water.

Carbon dioxide and oxygen gas used in these experiments were commercial preparations. The former was washed by bubbling successively into saturated sodium bicarbonate, 10 % cupric sulfate and saturated potassium permanganate made slightly acidic with sulfuric acid, and the latter into 50 % potassium hydroxide and saturated potassium permanganate made slightly acidic with sulfuric acid. Hydrogen peroxide (extra pure, commercial, 30 %) was diluted to 0.5 N with 0.05 M phosphate buffer for catalatic activity assay. Ethanol (pure analytical grade 99.46 v/v %) was diluted to 1.70 M with redistilled water. This solution gave a negative test for aldehydes with Schiff's reagent.

Methods

Catalatic activity was determined by the method of Bonnichsen, Chance and Theorell[6], and by calculating k_0 value from potassium permanganate titration data according to the equation: $k_0 = 2.303/t \cdot \log(x_0/x_t)$, where x_0 is the titer at zero time and x_t is that at t sec.

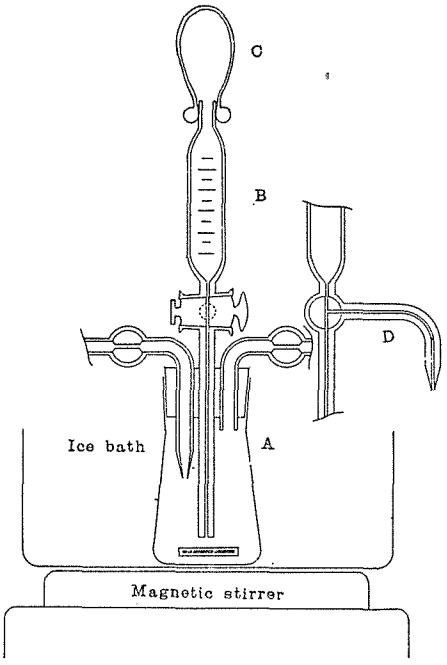

Fig. 1. Sketch of reaction vessel and sampling pippette with three-way stop-cock in position.
A, reaction vessel; B, sampling pippette; C, bulb; D, outlet.

Catalatic activity was tested in the following manner (see Fig. 1): 19.5 ml. of 0.1 M phosphate buffer, pH 6.0 which was previously introduced and saturated thoroughly with gas mixture of a certain pCO_2 in O_2 at 2°C, 1 atm., was added to reaction vessel A, and 0.1 ml. of suitably diluted catalase solution (about 2×

10^{-7} M) was added to it. Then the upper part of the reaction vessel was inserted. The suitable CO_2-O_2 mixture was allowed to flow through the flask, without bubbling, for 5 minutes at 2°C. After temperature equilibration, the reaction was started by adding 0.4 ml. of 0.5 N hydrogen peroxide (pipette B) by squeezing bulb C. At the same time a stop watch was started; B was washed 3 times by sucking back the reaction mixture by manipulating the bulb C quickly. Fifteen seconds after the reaction was started, 2.0 ml. of reaction mixture was sucked into B and then blown out through capillary D into beakers containing 1.0 ml. of 3 N sulfuric acid. Several samples were withdrawn at 15 sec. intervals in this manner. The contents of each micro beaker was titrated with 0.01 N potassium permanganate. The gas mixture was passed through the vessel without break, until the final sample was withdrawn. Reaction mixtures were stirred magnetically.

Carbon dioxide content of each gas mixture prepared in a gas-mixing flask was determined by the Orsat apparatus or modified Haldane's apparatus,* depending upon whether carbon dioxide content was more or less than 30 %, respectively.

The following experiments were carried out to determine quantitatively the carbon dioxide content of the buffered solutions.** Ten to twenty ml. of buffer solution saturated with a gas mixture was quickly transferred to a round flask of 100 ml. capacity. The carbon dioxide gas was driven out by lowering the pH to below 2, with 1 ml. of 3 N sulfuric acid and by distilling the contents of the flask until the volume was reduced to 1-2 ml. Escaping carbon dioxide was trapped in a flask containing 10 ml. of 0.1 N barium hydroxide. After distillation the excess barium hydroxide in the flask was titrated with 0.1 N hydrochloric acid until the color of the indicator, which consisted of thymol blue and cresol red (3 : 1), turned slightly purple. The carbon dioxide content was calculated from the titration figures.

Peroxidatic activity of catalase was tested using the Warburg manometric technique of Laser[8], when ethanol was used as substrate. The reaction vessel contained 1.6 ml. of 0.1 M phosphate buffer, pH 6.0 and 0.2 ml. of catalase solution above the cellophane membrane,*** and 8.5 ml. of the same phosphate buffer in the lower part. Two tenths ml. of 1.70 M ethanol was inserted in the side arm and 0.3 ml. (9 μ moles) of hydrogen peroxide in the cavity of the stopcock. After temperature equilibration at 20°C, the substrate was tipped in, and simultaneously hydrogen peroxide in the cavity was allowed to diffuse to the upper part by turning the stopcock.

In experiments concerning the effect of CO_2 on peroxidatic activity the following operations were carried out: 0.1 M phosphate buffer, which had been thoroughly saturated with a gas mixture of a certain pCO_2 (below 20°C), was added to the flask and then the vessel was flushed with the same gas mixture. After equilibration of temperature and gases, which required 30 minutes or

* Which is modified by Labor Institute of Japan.

** Quantities and forms of it in solution are governed by the final pH of the solution.[7]

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longer, the reaction was started as described previously. The pH of each solution was determined using glass electrodes.

RESULTS

Degree of Inhibition of Catalatic Activity

Fig. 2A shows typical results of the inhibition of the catalatic activity by CO_2. The plot of inhibition versus pCO_2 % in a logarithmic scale resulted in a sigmoidal curve as shown in Fig. 2B. The quantities of the hydrated forms of

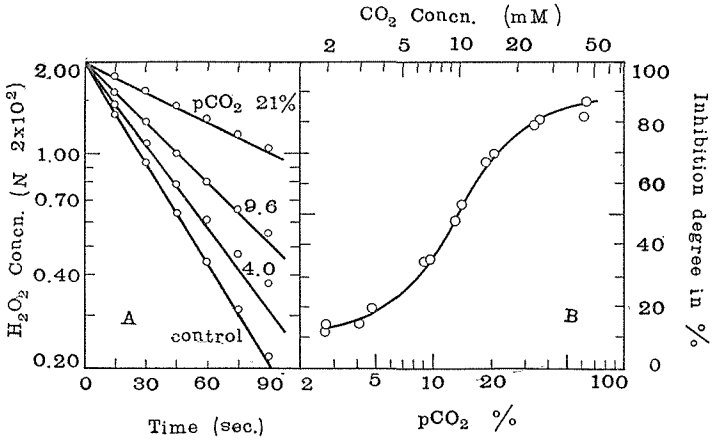

Fig. 2. Effects of carbon dioxide on the catalatic activity.
Enzyme, about $10^{-9}M$; hydrogen peroxide, 0.01N in 0.1M phosphate buffer, pH 6.0 ; under various pCO_2 in O_2 of 1 atm. and at 2°C.

CO_2 are controlled by pH ; at pH 6.0 carbonate is negligible, and 18 % of total CO_2 is in the form of the HCO_3^- ion[7]. Under these conditions, a pCO_2 of 13$\frac{1}{2}$% produces a 50 % inhibition of catalatic activity. These results can be explained on the basis of the law of mass action, between enzyme and inhibitor. The amount of inhibition is unchanged in different buffer media, *i.e.* borax, citrate, *etc.*

Reversibility of Inhibition

To determine whether the inhibition is reversible or irreversible, the follow-

Table 1. Reversibility of inhibition.
Each reaction mixture contains 19.5 ml. buffer, 0.4 ml. of hydrogen peroxide and 0.1 ml. diluted catalase. The method used is the same as that described in Fig. 2, but with the exception that the gases were not passed through continuously.

	No. 1	No. 2	No. 3	No. 4
pCO_2 40% buffer (ml.)	19.5	—	10	5
O_2 only, buffer (ml.)	—	19.5	9.5	14.5
$k_0 \times 10^2$ (sec.$^{-1}$)	0.492	2.46	0.836	1.68
Inhibition degree (%)	80.0	0	66.0	31.6
pCO_2 calculated (%)	39	—	20	10

ing experiments were performed. Phosphate buffer under pCO_2 of 40% was incubated with 0.1 ml. of enzyme solution for 5 min., at 2°C, and then diluted with O_2 to a lower pCO_2. The inhibition decreased to a value corresponding to that expected at the lower pCO_2. The results are shown in Table 1.

pH Dependence

By using carbon dioxide-bicarbonate buffers, the pH dependence of inhibition was studied in order to determine the mechanism of inhibition. The pCO_2 was kept constant at 10%, while the concentration of HCO_3^- ion was varied. The non-inhibited catalatic activity is constant over a wide range of pH from 4-8.5 when rapid method is used[9], and the degree of inhibition is constant at pCO_2 of

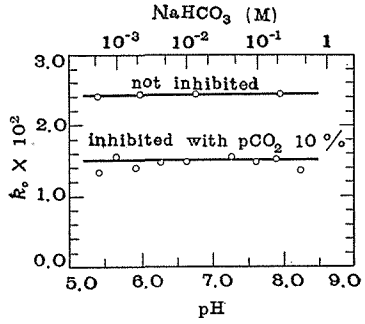

Fig. 3. pH Dependence.
Non-inhibited activity in 0.05*M* phosphate buffer pH 5.4-8.0, inhibited activity in carbon dioxide-bicarbonate buffer, pH 5.4-8.3 under pCO_2 of 10%.

10% (Fig. 3). Under the above-mentioned condition, the quantity of carbon dioxide physically dissolved in the solution is constant. Therefore, the degree of inhibition is independent of the pH of the medium, and inhibition is not caused by bicarbonate or carbonate ions.

Effects of Carbonic Acid

Since bicarbonate and carbonate ions have no influence on the degree of inhibition, the possibility remains that inhibition is due to physically dissolved carbon dioxide or to carbonic acid. When carbon dioxide gas is introduced into water, the following reactions occur :

$$CO_2 \text{ gas} \longrightarrow CO_2 \text{ solution} \quad (1)$$

$$CO_2 + H_2O \longrightarrow H_2CO_3 \quad (2)$$

$$H_2CO_3 \longrightarrow H^+ + HCO_3^- \quad (3)$$

In order to saturate buffer solutions of pH 7 or greater, time is required to reach equilibrium, for the hydration reaction of Eq. (2) becomes rate limiting in this system in absence of carbonic anhydrase. In the upper part of Fig. 4A, changes of pH in the system in presence and absence of carbonic anhydrase are presented, when veronal (0.0167 *M* sodium barbiturate in 0.0167 *M* barbituric acid) is used as buffer and without gas bubbling but simply allowing the buffer to be in contact with air. The catalatic activity at different concentrations of

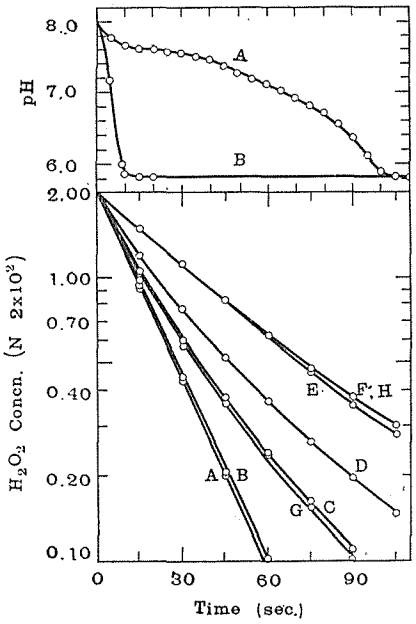

Fig. 4A. Effects of carbon dioxide hydration.

Upper part : change of pH upon the addition of carbon dioxide saturated water to veronal buffer. Curve A, in the absence of carbonic anhydrase (C. A.) ; curve B, in the presence of C. A.

Lower part : catalatic activity. Curve A, in veronal buffer (control run) ; curve B, control run in the presence of C. A. ; curve C, 10 sec. after mixing of buffer the reaction was started by the addition of enzyme in the absence of C. A. ; curve D, mixed buffer was incubated with enzyme for 2 min., and the reaction was started by the addition of hydrogen peroxide in the absence of C. A. ; curve E, same as D but enzyme was incubated for 5 min. ; curve F, same as D but enzyme was incubated for about 15 min. ; curve G. same as C but in the presence of C. A. ; curve H, same as F but in the presence of C. A.

Reaction mixture contained 19.5 ml. of 0.0167 *M* veronal buffer, pH 8.0, or mixed buffer (9.5 ml. of veronal buffer plus 10 ml. of carbon dioxide saturated water), 0.1 ml. of catalase ($4.4\times10^{-7}M$) and 0.4 ml. of 0.5 *N* hydrogen peroxide.

carbonic acid can be determined using this system.*

Experimental results are shown in the lower part of Fig. 4A. If inhibition is due to carbonic acid, the degree of inhibition must be greater in the presence

* In this system, concentration of carbonic acid is calculated as follows ; from Eq. (3),

$$(H_2CO_3)=(H^+)\cdot(HCO_3^-)/K_1' \qquad (4)$$

where K_1' is apparent primary dissociation constant of carbonic acid. Assuming the concentration of bicarbonate ion is nearly equal to the increase of hydrogen ion concentration, following relation is derived from Eq. (4),

$$(H_2CO_3)\simeq(H^+)_t[(H^+)_t-(H^+)]/K_1'$$

where (H^+) and $(H^+)_t$ are hydrogen ion concentration at initial stage and t sec. after mixing of two solution respectively. In case of $(H^+)_t \gg (H^+)$ this relation becomes as,

$$(H_2CO_3)\simeq[(H^+)_t]^2/K_1'$$

or

$$2pH=pK_1'-\log(H_2CO_3) \qquad (5)$$

Eq. (5) shows that carbonic acid concentration increases 100 times by the decrease of one pH unit.

of anhydrase since reaction[2] is rate-limiting in the absence of carbonic anhydrase. When the enzyme was incubated in carbon dioxide-buffer system, the degree of inhibition increases in proportion to the incubation time (curves C, D, E and F). It appears from these results that the inhibiting agent is carbonic acid. However, there is no correlation between degree of enzyme inhibition at various incubation times and carbon dioxide hydration rate. Moreover, the degree of inhibition is the same whether the anhydrase is present or absent in the system, and the degree of inhibition is a function of the time of incubation (curves C and G). Of course, the anhydrase cannot increase or decrease the catalatic activity (curves A and B or F and H).

The results shown in Fig. 4A can be summarized as follows : inhibition is not a function of concentration of carbonic acid, because in the case of curve G, the carbonic acid concentration is about 4000 times greater at the beginning of the reaction than in the case of curve C. Curves C, D, E and F show the necessity of incubation to ensure combination of enzyme and inhibitor.

On the other hand, when 0.033*M* sodium barbiturate, pH 9.25, is used, more alkaline than in case of Fig. 4A, the following results are obtained (Fig. 4B).

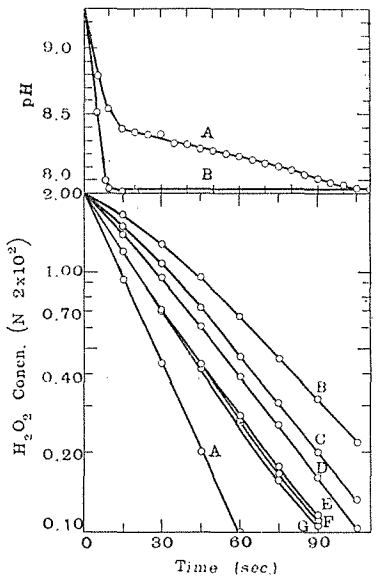

Fig. 4B. Effects of carbon dioxide hydration.

Upper part : change of pH upon the addition of carbon dioxide saturated water to veronal buffer. Curve A, in the absence of C. A. ; curve B, in the presence of C. A.

Lower Part : catalatic activity. Curve A, in veronal buffer (control run) ; curve B, mixed buffer and hydrogen peroxide was incubated for 10 sec., and then reaction was started by the addition of enzyme in the absence of C. A. ; curve C, same as B but incubated for 30 sec. ; curve D, same as B but incubated for 1 min. ; curve E, same as B but incubated for 2 min., curve F, same as B but in the presence of C. A.; curve G, same as E but in the presence of C. A.

Reaction mixture contained 19.5 ml. of 0.033 *M* veronal buffer, pH 9.25, or mixed buffer (9.5 ml. of veronal buffer plus 10 ml. of carbon dioxide saturated water), 0.1 ml. of catalase ($4.4\times10^{-7}M$) and 0.4 ml. of 0.5*N* hydrogen peroxide.

The degree of inhibition decreases with increased incubation time (curve B). However, the inhibition in the earlier stage is nearly equal to that shown in curves E, F and H (Fig. 4A). As the final pH of the media is 7.92, the concentration of carbon dioxide decreases markedly as a result of the carbon dioxide hydration reaction, although there is little increase in the carbonic acid concentration itself. In agreement with this fact, degree of inhibition decreases when incubation time is lengthened (curves C, D and E). The degree of inhibition cannot be increased by increasing the carbonic acid concentration (curve F in contrast to B). In short, the experiments shown in Fig. 4B demonstrate that the inhibition is due to the physically dissolved molecular carbon dioxide.

Returning to the results of Fig. 4A, curve F shows a progressive increase in inhibition with incubation time. This phenomenon is not caused by a decrease in the concentration of active enzyme during the course of the reaction. Since it was expected that hydrogen peroxide competes with the inhibitor for enzyme, the initial reaction velocity was measured. In these experiments the pCO_2 was 10 % and 20 %, and the hydrogen peroxide concentrations from 0.0025 to 0.08 *M*. When the reciprocal of initial velocity and the reciprocal of initial hydrogen peroxide concentration are plotted, the resultant lines intersect near the origin

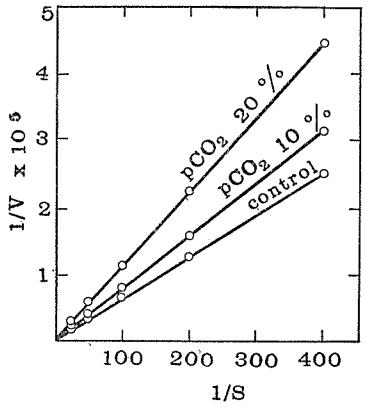

Fig. 5. Influence of hydrogen peroxide concentration on the inhibition.
V, amount of hydrogen peroxide decomposed (moles) at first min.; S, initial hydrogen peroxide concentration (*M*). The conditions are the same as in Fig. 2 but the hydrogen peroxide concentration was variable and the partial pressure of carbon dioxide was constant.

(Fig. 5). Therefore, it is impossible to determine by this method whether this inhibition is competitive or non-competitive,* and to interpret the progressive increase in inhibition as a competition of hydrogen peroxide with the inhibitor for enzyme.

Influence of Carbon Dioxide on Peroxidatic Activity

As shown in Fig. 6, curve A, peroxidatic activity increases with increase of enzyme concentration. Using the following conditions, 9 μ moles hydrogen

* As Km value of catalase and hydrogen peroxide is very large, Reen (10) also could not determine the type of inhibition by 2, 4-dichlorophenol.

peroxide, 0.17 *M* ethanol, in 0.1 *M* phosphate buffer of pH 6.0 and at 20°C, about 2×10^{-6} *M* catalase utilizes half the amount of hydrogen peroxide which diffuses out from the vessel of the lower part, and 3.1×10^{-8} *M* catalase utilizes only 8% for peroxidation of ethanol. However, in the presence of carbon dioxide at partial pressure above 10%, the utilization of hydrogen peroxide for peroxidation remarkably increases. At a pCO_2 of 17%, half of the H_2O_2 is used as shown in Fig. 6, curve B.

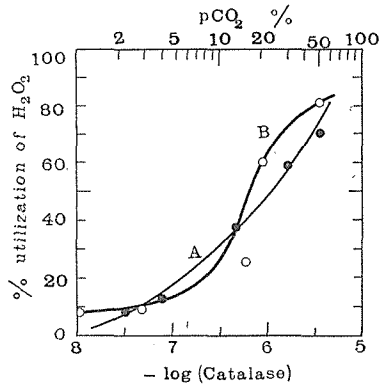

Fig. 6. Carbon dioxide activation of the peroxidatic utilization of hydrogen peroxide.

Determinations were carried out in Laser's apparatus modified for Warburg manometer. Curve A, concentration effect of the enzyme on peroxidatic efficiency using constant quantities of hydrogen peroxide (9 μ moles) and ethanol (340 μ moles); curve B, the carbon dioxide activation with constant quantities of hydrogen peroxide and ethanol (same as in curve A) using low concentrations of catalase ($3.1\times10^{-8}M$).

Absorption Spectrum

An attempt was made to observe the absorption spectrum of carbon dioxide-catalase derivative using a Thunberg type cuvette, containing carbon dioxide-bicarbonate buffer of pH 6.0, at about 5°C, in a Beckman spectrophotometer, model DU. No remarkable differences between free and carbon dioxide-catalase could be detected over the wide wavelength range of 240–700 mμ.

Table 2. Influence of Carbon Dioxide Gas on Catalatic Activity of Fresh Rat Tissue Homogenate.
The conditions of assay are the same as described in Fig. 2.

Tissue	$k_0\times10^2$ (sec.$^{-1}$)		Inhibition (%)
	No inhibitor	pCO_2 of 20%	
Liver[a]	2.99	1.34	55.2
Kidney[b]	2.46	1.04	57.7
Blood[c]	2.55	1,12	50.3

[a] 0.1 ml. of 5 fold diluted homogenate (6.68 g in 23 ml.) was used.
[b] 〃 〃 3 fold 〃 〃 (2.20 g in 5.0 ml.) 〃 〃.
[c] 0.1 ml. of hemolyzed blood (0.5 ml. to 1.5 ml.) was used.

Influence of Carbon Dioxide on Tissue Homogenates

The influence of carbon dioxide on the catalatic activity in fresh tissue homogenates was tested to see whether or not the carbon dioxide-sensitive nature of the enzyme could be due to modifications of catalase during the purification process.

The catalatic activities and its degree of inhibition were measured at a pCO_2 of 20 % in O_2, using the method described in Fig. 2, and the results shown in Table 2.

DISCUSSION

Keilin and Hartree[11] and Theorell[12] stress the importance of the peroxidatic function of catalase, *in vivo*. The present paper supports this view. Although only the peroxidatic activity was considered in this study, oxidatic activity of catalase[13] has been reported recently, and it would be very interesting to test the effects of carbon dioxide on it. The present data show that carbon dioxide does not inhibit formation of complex I of catalase, because the effective concentration for both peroxidatic and catalatic function would be decreased simultaneously. However, this is not observed (Fig. 6). If one accepts the hypothesis that hydrogen peroxide and ethanol compete for complex I, it is difficult to understand the above-mentioned facts.[14]

Recently, George[15] has obtained qualitative evidence indicating that there are analogous complexes of catalase and peroxidase. It appears probable that carbon dioxide enhances the formation of complex II or depresses the dismutation of hydrogen peroxide with complex I, in the presence of ethanol or hydrogen donor. According to such a hypothesis, the enzyme-hydrogen peroxide complex and probable enzyme-hydrogen peroxide-inhibitor complex should be clearly detected spectrophotometrically.

However, it is difficult to test the hypothesis, because high CO_2 pressures would be necessary in the cuvette to achieve concentrations equivalent to inhibitor concentrations of cyanide and azide.[16] On the other hand, the CO_2 may combine with free amino groups of the protein. This has already been demonstrated with hemoglobin,[16] and it is possible that the inhibition of plant cytochrome oxidase by bicarbonate[18] and plant succinic oxidase by high pCO_2,[19] both occur by an analogous mechanism.

SUMMARY

Catalase activity is sensitive to carbon dioxide. The catalatic activity of crystalline enzyme prepared from ox liver is inhibited 50 % under 99 mm pCO_2 in O_2. This inhibition is completely reversible and is probably due to the action of physically dissolved molecular CO_2. The hydrates of CO_2, carbonic acid, carbonate, and bicarbonate, have no inhibitory effects. The inhibition is independent of pH. The peroxidatic activity of catalase is enhanced by CO_2.

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