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Differential Effect of 3-Amino-1,2,4-triazole on Multiple Forms of Rat Liver Catalase[1]

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Injection of 3-amino-1,2,4-triazole into rats resulted in a rapid fall of liver catalase activity which gradually returned to the normal level, but the amount of catalase protein determined by the immunochemical method remained constant without any decrease in parallel with the enzyme activity. Inactivation occurred more rapidly with soluble catalase (or Catalase-II) than with catalase in peroxisomes (Catalase-III). Rate of recovery in enzyme activity from maximum inactivation was also investigated for Catalase-II and -III, and its results indicated that Catalase-II is synthesized more rapidly than Catalase-III.

3-Amino-1,2,4-triazole (AT) was first shown by Heim, *et al.*[3] to produce a rapid fall in catalase activity of the liver and kidney of rats and mice when injected intraperitoneally in a dosage of 1 g/kg body weight. However, under the same experimental conditions the catalase activity in erythrocytes remained constant. Subsequently, incubation of liver and kidney homogenates with AT at 37° was found to produce a similar decrease in the catalase activity of the homogenates,[4,5] but again, in contrast, that of hemolysates did not cause any inhibition of the catalase activity.[5] According to Malgoliash, *et al.*[5] AT produces a complete, irreversible inhibition of crystalline purified preparations of both liver and erythrocyte catalase in the presence of low and constant concentrations of hydrogen peroxide. A similar evidence has been obtained recently by Chang and Schroeder.[6]

Higashi and Shibata[7] have described the heterogeneous nature of rat liver catalase. By chromatography on DEAE-cellulose column they have separated four types of the enzyme among which Catalase-III is contained in peroxisomes and Catalase-II appears to occur in soluble form within the cells. In order to provide further evidence for the multiplicity of rat liver catalase it is of interest to study the inhibitory effect of AT on these Catalase-II and -III both *in vivo* and *in vitro*.

The liver catalase activity, which has been decreased to a minimum level by injection of AT, returns to the normal value during a period of 4—5 days thereafter. This reappearance of enzyme activity has been considered to represent the synthesis of new catalase rather than a reversal of the inhibition.[8] In the present study comparison has been made in the rate of return of the enzyme activity of Catalase-II and -III. It may reveal the difference in turnover of the two types of rat liver catalase.

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2) Location: *1-5-8 Hatanodai, Shinagawa-ku, Tokyo.*
3) W.G. Heim, D. Appleman, and H.T. Pyfrom, *Science*, **122**, 693 (1955); *idem*, *Amer. J. Physiol.*, **186**, 19 (1956).
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8) M. Rechcigl, Jr. and V.E. Price, *Progr. Exp. Tumor Res.*, **10**, 112 (1968).

Experimental

Animals——Male rats weighing 100—200 g were used. Rats were injected intraperitoneally with AT (100 mg/100 g body weight) and the liver catalase was assayed at various times after the treatment.

Total Liver Catalase——A liver was perfused and homogenized in 4 volumes of 0.25M sucrose–1 mM EDTA. To 1 ml of the homogenate (1: 5), 1 ml of 5% sodium deoxycholate, 1 ml of 0.5M Tris buffer, pH 8.0, and 6 ml of distilled water were added. The mixture was centrifuged at 105000 *g* for 60 min to obtain a soluble extract, the catalase in which was assayed as the total liver catalase.

Soluble Liver Catalase——Three milliliters of 1: 5 homogenate was diluted with 6 ml of 0.25M sucrose–1 mM EDTA, and centrifuged at 105000 *g* for 60 min. The resulting supernatant was used for the determination of the soluble catalase.

Separation of Catalase-II and -III——According to the method of Greenfield and Price,[9] the mitochondrial fraction of rat liver was prepared by centrifugation in 8.5% sucrose–10% polyvinylpyrrolidone, pH 7.0, and the catalase was extracted by treatment with ethanol and chloroform in acetate buffer. To this extract was added 1/20 its volume of 0.05M sodium sulfate. The precipitates were separated by centrifugation and dialyzed overnight against 0.01M phosphate buffer, pH 7.0. The dialyzed catalase was subsequently fractionated by chromatography on DEAE-cellulose.[7,10] As reported previously, Catalase-II was eluted at 0.05M NaCl and Catalase-III at 0.1M.

Reaction of AT with Purified Catalase——After the catalase solution had been adjusted to the concentration of 0.5 mg/ml with 50 mM sodium phosphate, pH 7.0, 0.02 ml of 2.0M AT was added to its 1.0 ml to give a final AT concentration of 40 mM. Hydrogen peroxide was dialyzed into the reaction mixture according to Malgoliash, *et al.*[5] in the following manner. One milliliter of catalase solution in dialysis tubing was dialyzed at room temperature against 500 ml of 50 mM sodium phosphate, pH 7.0, containing 40 mM AT and 5 mM H_2O_2. At various intervals, samples were removed for assay of enzyme activity.

Enzymatic Assay of Catalase——Catalase enzymatic activity was assayed by a modification[10] of the spectrophotometric method of Beers and Sizer.[11] The activity unit in 0.1 ml of a sample assayed was expressed as $k_0 \times 3.1$, where k_0 is the first order reaction constant.

Immunochemical Assay of Catalase——Purified rat liver catalase was used as antigen. Antiserum was obtained by immunizing a rabbit with the antigen. The quantitative precipitin reaction was carried out as described in a previous paper.[10] The catalase-anticatalase complex was determined by using bromsulfophthalein.[12] The amount of catalase (antigen) was expressed by the activity unit of purified catalase which would give the same amount of immunological precipitate.

Chemicals——3-Amino-1,2,4-triazole was purchased from Tokyo Kasei Kogyo, Co., Ltd.

Result

Total Liver Catalase after AT Injection

Fig. 1 shows the effect of AT on the total liver catalase, determined both enzymatically and immunochemically. After the injection of AT, the enzyme activity exhibited a rapid fall until a minimum level (1—2% of the control) was reached in about 2 hr. On the other hand, the catalase protein measured by the immunochemical method remained unchanged. It has become evident from this finding that the enzyme-to-antigen ratio, *i.e.*, the ratio of catalase determined enzymatically to the catalase determined immunochemically, indicates a fraction of active catalase in the total (active plus inactivated) catalase.

Starting 2 to 4 hr after the AT injection, the catalase activity increased progressively over a period of 3 days to the normal level. From the rate of this return of catalase activity it was calculated that the rate of catalase synthesis would be 20 μg/hr/g liver.[13]

Soluble Liver Catalase after AT Injection

The effect of AT on the soluble liver catalase is demonstrated in Fig. 2. The enzyme activity decreased to the minimum in 60 min after administration of the drug and returned

9) R.E. Greenfield and V.E. Price, *J. Biol. Chem.*, **217**, 607 (1955).
10) T. Higashi and T. Peters, Jr., *J. Biol. Chem.*, **238**, 3945 (1963).
11) R.F. Beers and I.W. Sizer, *J. Biol. Chem.*, **195**, 133 (1952).
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13) V.E. Price and M. Rechcigl, Jr., *J. Biol. Chem.*, **237**, 3468 (1962).

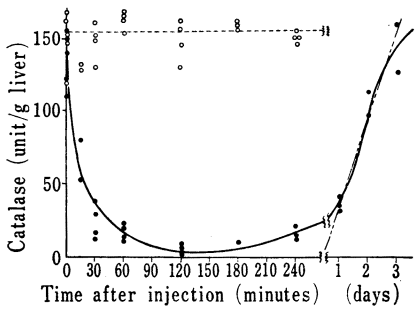

Fig. 1. Total Liver Catalase after Aminotriazole Injection

●: catalase activity enzymatically assayed
○: catalase protein immunochemically determined

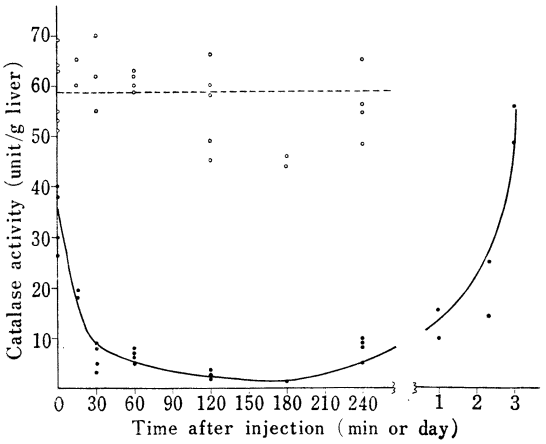

Fig. 2. Soluble Liver Catalase after Aminotriazole Injection

●: catalase activity enzymatically assayed
○: catalase protein immunochemically determined

to the normal level on the third day, while the catalase determined immunochemically remained constant throughout the experimental period.

Soluble/Total Catalase Activity after AT Injection

Table I presents the calculated ratio of the soluble catalase activity to the total catalase activity at each time after AT injection. The ratio decreased with time until 30 min after the injection, indicating that the soluble catalase is more susceptible to AT than the catalase in particles (mainly in peroxisomes). After 60 min, however, both soluble and total catalase activity showed minimum value and the ratio was the same as that before the treatment.

TABLE I. Soluble/Total Liver Catalase Activity after Aminotriazole Injection

Time after injection (min)	Total catalase (units/g liver)	Soluble/total catalase (%)
0	151	30.8
	109	38.4
10—15	98	22.0
	85	23.6
	83	29.0
	81	25.5
	72	26.3
20—30	37	12.5
	29	13.6
60	23	35.0
	20	33.0
120	7.0	34.3
	5.0	39.0
	4.2	35.0
	3.9	38.5
	1.9	31.5

Differential Effect of AT on Multiple Forms of Catalase *in Vivo*

Taking into consideration the fact that the catalase in preoxisomes consists of Catalase-III and that in soluble form within the cells is found to be predominantly Catalase-II,[10] the results

in Table I suggest a differential effect of AT on these two forms of catalase in rat liver. Since Catalase-II is adsorbed on subcellular particles in the presence of polyvinylpyrrolidone,[7,10,14] Catalase-II and -III were isolated by the procedure as described in Experimental, and the effect of AT was examined by determining the enzyme-to-antigen ratio of each catalase. Fig. 3 illustrates a chromatographic separation of Catalase-II and -III in AT-injected rats, and the change in enzyme-to-antigen ratio of both catalases with time after AT injection is shown in Table II. It is evident that Catalase-II was inactivated by AT more easily than Catalase-III.

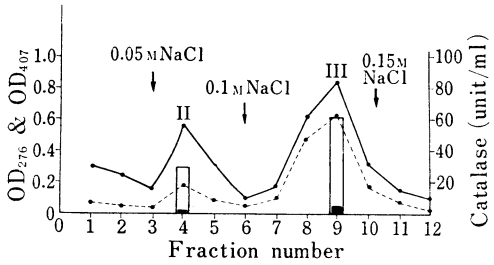

Fig. 3. Separation of Catalase-II and -III by DEAE-cellulose Chromatography

—●—: OD_{276}; ---●---: OD_{407}
■ : catalase activity enzymatically assayed
□ : catalase protein immunochemically determined

Table II. Enzyme-to-Antigen Ratio of Catalase-II and -III after Aminotriazole Injection

Time after injection (min)	Catalase-II (%)	Catalase-III (%)
0	100	100
15	38.5	77.0
30	17.2	39.4
60	5.1	20.5
90	4.0	10.9
120	2.5	5.0

a) Catalase activity enzymatically assayed/catalase protein immunochemically determined

Enzyme-to-antigen ratio was also examined with Catalase-II and -III at the stage of recovery from inactivation by the injected AT. Table III, showing one of such experiments, demonstrates that Catalase-II recovered more rapidly than Catalase-III.

Table III. Enzyme-to-Antigen Ratio of Catalase-III and -III at Recovery from Inactivation by Aminotriazole

Time after injection (hr)	Catalase-II (%)	Catalase-III (%)
2	4.7	10.9
24	8.4	8.1
48	42.0	—
72	51.0	22.0
96	55.0	34.0

Fig. 4 summarizes all the data on the enzyme-to-antigen ratio of Catalase-II and -III after AT injection, including those in Tables II and III. If AT affects Catalase-II and -III in the same manner, the plots should be on each broken line in the graph. This was not the case, and the results indicate that Catalase-II was more rapidly inactivated by AT but recovered more rapidly *in vivo* than Catalase-III.

Reaction of AT with Purified Catalase-II and -III *in Vitro*

In order to compare the susceptibility of Catalase-II and -III to inactivation by AT, the reaction was examined *in vitro* and the results are shown in Fig. 5. Under the specific conditions of the experiments loss in enzyme activity of two types of catalase displayed a similar kinetics. It appears that Catalase-II and -III have no remarkable difference in the sensitivity to AT in the presence of hydrogen peroxide.

14) C. de Duve, R. Wattiaux, and P. Baudhuin, *Adv. Enzymol.*, **24**, 291 (1962).

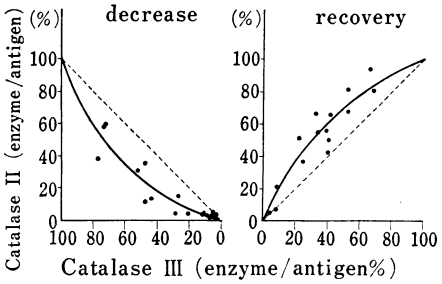

Fig. 4. Relationship between Enzyme-to-Antigen Ratio of Catalase-II and that of Catalase-III after Aminotriazole Injection

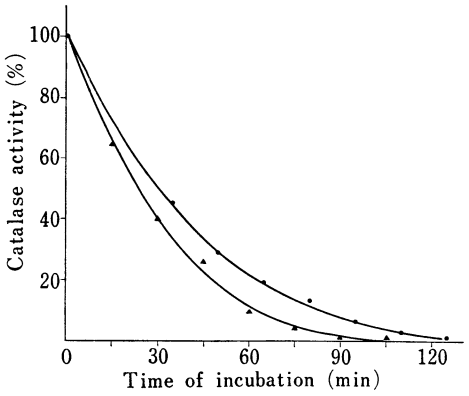

Fig. 5. Inactivation of Catalase-II and -III by Aminotriazole *in Vitro*

▲: Catalase-II, ●: Catalase-III

Discussion

By injecting 3-amino-1,2,4-triazole into rats a rapid fall in liver total and soluble catalase activity was observed until a minimum level of 1—2% was reached in about 2 hr. This residual activity is due to the catalase of contaminating red blood cells, which has been found not to be affected by AT *in vivo*.[3,5,8,15] Kinetics of inactivation of total liver catalase in the present study is the same as that reported by Rechcigl and Price,[8] but, it was found anew that the amount of catalase protein which can be determined immunochemically, remains constant after AT injection. This evidence would indicate that the rate of the synthesis of liver catalase is not influenced by AT administration. The ribosomes and the cell sap prepared from the liver of rats injected with AT, when incubated *in vitro*, incorporated the same amount of a radioactive amino acid into catalase as those from intact liver.[16]

From this new finding it has become possible to express the fraction of enzymatically active catalase in the total (active plus inactivated) catalase by the ratio of enzymatically determined to immunochemically assayed catalase. By investigating this ratio of chromatographically separated Catalase-II and -III at various times after AT injection, differential effect of the chemical on these two types of liver catalase has been revealed. First, Catalase-II is more rapidly inactivated by AT than Catalase-III, and secondly, the rate of recovery is considerably higher in Catalase-II than in Catalase-III. For the explanation of the first evidence we would refer to the difference in intracellular localization of these two types of catalase. Higashi and Shibata[7] have demonstrated that Catalase-III occurs inside the peroxisomes and Catalase-II exists mostly in soluble form within liver cells. Therefore, Catalase-II might be more readily accessible by injected AT than Catalase-III. An alternative possibility that Catalase-II is by nature more sensitive to inactivation by AT, is not sufficient to explain the evidence completely (Fig. 5), although it should be taken into consideration. Recently, Jones and Masters[17] have reported the differential inhibition of the multiple forms of catalase in mouse tissue by injected AT. Their experiments have demonstrated that the more anodic electrophoretic forms are appreciably more susceptible to inhibition than slower migrating species. Their result may not be compatible with those in the present investigations.

15) M. Dogen and T. Higashi, *J. Japan. Biochem. Soc.*, **44**, 959 (1972).
16) K. Kashiwagi, T. Tobe, and T. Higashi, *J. Biochem.*, **70**, 785 (1971).
17) G.L. Jones and C.J. Masters, *FEBS Letters*, **21**, 207 (1972).

Since the return of catalase activity after the initial fall by AT injection was accompanied by a corresponding incorporation of ^{59}Fe into catalase isolated from the liver of experimental rats, Price and Rechcigl[13] concluded that this reappearance of catalase activity represented the synthesis of new catalase rather than a reversal of the inhibition. Based on this conclusion, the results shown in Table III and Fig. 4 can be interpreted to suggest that Catalase-II is synthesized at a higher rate than Catalase-III. The former was more rapidly labeled than latter with injected ^{14}C-leucine in an *in vivo* experiment, the details of which will be reported elsewhere. It is of interest that AT injection has revealed the difference between the two types of rat liver catalase in metabolism as well as in subcellular localization.