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Effect of Glutathione and Cysteine on Rat Liver Tryptophan Oxygenase Activity

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Examination of the effect of reductants on hematin, which is a prosthetic group of tryptophan oxygenase, showed that glutathione and cysteine changed the optical absorption of hematin and, therefore, the effect of these reductants on tryptophan oxygenase activity was studied.

Glutathione and cysteine are both SH-compounds, but their effect on tryptophan oxygenase activity is quite different. Glutathione in 0.1—0.2 mM concentration inhibited tryptophan oxygenase activity, but more than 0.1—0.2 mM of glutathione resulted in recovery of inhibition of tryptophan oxygenase activity, which was activated by 5.0 mM glutathione.

On the other hand, cysteine activated tryptophan oxygenase maximally in 0.1—0.2 mM concentration, but more than 0.1—0.2 mM of cysteine rapidly decreased its maximum activity.

The inhibition rate of tryptophan oxygenase activity by these reductants was decreased by the addition of purified rat liver catalase.

Since the conversion of tryptophan to kynurenine *in vivo* was found by Kotake, *et al.*,[2] many enzymic studies for liver tryptophan oxygenase (EC 1. 13. 1. 12) have been made in different animal species and under various experimental conditions.

When partial purification method of tryptophan oxygenase was investigated in our laboratory, it was first found that tryptophan oxygenase was precipitated by the addition of streptomycin in a low concentration of buffer. The tryptophan oxygenase obtained by streptomycin treatment became unstable compared with tryptophan oxygenase in cell sap, and was activated by a low concentration of ascorbic acid, but tryptophan oxygenase in cell sap was activated fully. We clarified that this inhibition of tryptophan oxygenase by high concentration of ascorbic acid was dependent on the binding of ascorbic acid to iron of iron-porphyrin.[3]

On the other hand, examination of the effect of various reductants on hematin, which is a prosthetic group of tryptophan oxygenase, showed that glutathione and cysteine can change the absorption of hematin. It will be assumed that glutathione and cysteine can affect tryptophan oxygenase activity. According to Knox, *et al.*[4] both reductants inhibited *Pseudomonas* tryptophan oxygenase activity, while Feigelson, *et al.*[5] reported that both reductants activated the same enzyme. The results of these two groups are quite different. Therefore, effect of glutathione and cysteine on rat liver tryptophan oxygenase activity was investigated in the present work.

Material and Method

Wistar strain rats were administered with the suspension of 100 mg of L-tryptophan per 100 g of body weight by intraperitoneal injection 4 hr before killing. The livers were used for preparation of tryptophan oxygenase. The rats were killed by decapitation and the livers were removed immediately.

1) Location: *492-36 Mitahora, Gifu, 501-21, Japan.*
2) Y. Kotake and T. Masayama, *Z. Physiol. Chem.*, **243**, 237 (1936).
3) R. Shinohara and I. Ishiguro, *Vitamins* (Kyoto), **41**, 341 (1970).

The freshly removed livers were homogenized in a waring blendor with 4 volumes of ice cold 0.14 M KCl containing 1.0 mM tryptophan. The homogenate was centrifuged at 77000×*g* for 60 min at 0°, and the supernatant fluid was treated with EtOH in the final concentration of 25% at −5°. The precipitate was collected by centrifugation at 13000×*g* for 5 min and dissolved in 10 mM phosphate buffer (pH 7.0). To 180 ml of this solution, 20 ml of a solution containing 0.2 g of streptomycin was added with stirring, and the mixture was stirred for 15 min at 4°. The tryptophan oxygenase was precipitated from the mixture containing the enzyme by the addition of streptomycin to the final concentration of 0.1%. The precipitate was washed once with 0.1% streptomycin solution. The washed precipitate was suspended in 0.2 M KCl–phosphate buffer (9 parts of 0.2 M KCl and 1 part of 0.2 M phosphate buffer pH 7.0) containing 5 mM tryptophan, and then stirred for 30 min. The suspension was then centrifuged at 13000×*g* for 10 min. After the precipitate was removed, the supernatant was fractionated with solid $(NH_4)_2SO_4$. The enzyme protein which precipitated between 0.25 and 0.50 $(NH_4)_2SO_4$ saturation was collected by centrifugation at 10000×*g* for 10 min and dissolved in a minimum volume of 50 mM phosphate buffer (pH 7.0). Then the enzyme solution was passed through a column of Sephadex G-25 which was previously equilibrated with 50 mM phosphate buffer (pH 7.0). The fraction containing tryptophan oxygenase was collected and concentrated, and was used as a partially purified tryptophan oxygenase (represented by SM-TO in the table and graphs).

Activity of the partially purified tryptophan oxygenase was assayed by the method of Knox and Mehler[6] as modified by Greengard and Feigelson by the addition of hematin. The assay system contained 0.5 ml of 0.2 M phosphate buffer (pH 7.0), 0.5 ml of 0.04 M tryptophan, 0.2 ml of 20 μM hematin, enzyme, freshly prepared sodium ascorbate, and water in a total volume of 4.0 ml. The reaction mixture was incubated aerobically for 60 min at 37°, and the reaction was stopped by the addition of 15% metaphosphoric acid, and stood for 10 min, then filtered. The filtrate (3.0 ml) was adjusted to pH 7.0 with 1 N NaOH, and the absorbance was measured by spectrophotometer at 365 nm for kynurenine and 321 nm for formylkynurenine. Amount of the compounds produced was calculated from the respective molar extinction coefficients.

The measurement of catalase (EC 1.11.1.6) activity was carried out by the method of Von Euler and Josephson.[7]

Crystalline dihydrostreptomycin sulfate was used for the enzyme preparation.

Hemin prepared from bovine blood cell was dissolved in diluted NaOH and was used as hematin.

Result

Changes in the Absorption Curve of Hematin by the Addition of Glutathione or Cysteine

Hematin showed its maximum peak at 390 nm. If a small amount of crystal glutathione or cysteine was added to hematin solution, its maximum peak shifted to 365 nm from 390 nm immediately as shown in Fig. 1. It was assumed that hematin would bind glutathione or cysteine because the absorption curve of hematin did not changed by the addition of ascorbic acid.

Effect of Glutathione on Tryptophan Oxygenase Activity

It was assumed from the result shown in Fig. 1 that glutathione and cysteine could affect tryptophan oxygenase activity. The change in tryptophan oxygenase activity in the presence of glutathione is shown in Fig. 2.

At 0.1—0.2 mM of glutathione, 50% of the activity was inhibited, and at the higher concentration the inhibition became weaker. At 5.0 mM glutathione, the enzyme was activated a little. On the other hand, Schimke, *et al.*[8] had stated that the structure of tryptophan oxygenase would be changed by the addition of high concentration of tryptophan because *Km* value of tryptophan, heat stability and rate of inhibition by proteolytic enzyme were changed by the addition of high concentration of tryptophan. Therefore, the inhibition of the enzyme activity by glutathione was studied in the presence of low and high concentration of trypto-

4) T. Tanaka and W. E. Knox, *J. Biol. Chem.*, **234**, 1162 (1959).
5) H. Maeno and P. Feigelson, *J. Biol. Chem.*, **242**, 596 (1967).
6) W. E. Knox and A. H. Mehler, *J. Biol. Chem.*, **187**, 419 (1950).
7) H. V. Euler and K. Josephson, *Ber.*, **56**, 1749 (1923).
8) R. T. Schimke, E. W. Sweeney and C. M. Berlin, *J. Biol. Chem.*, **240**, 322 (1965).

phan. As shown in Fig. 3, the rate of inhibition in the presence of high concentration of tryptophan was stronger than that in the presence of low concentration of tryptophan.

For the purpose of study on the mechanism of inhibition and activation by change in glutathione concentration, the Lineweaver–Burk's plot was examined in the case of 0.1 mM

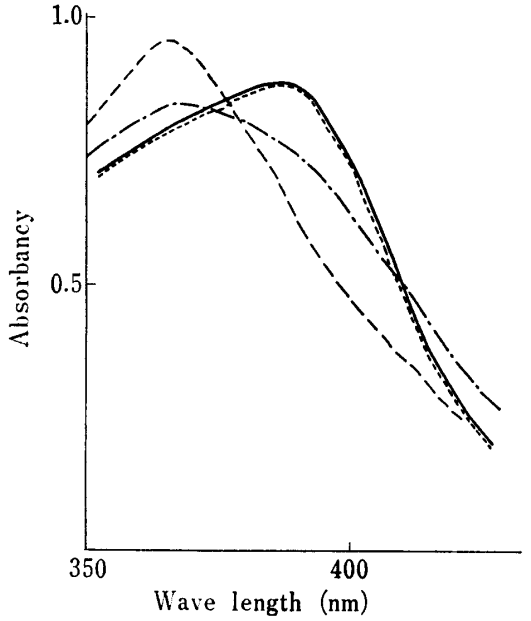

Fig. 1. Visible Absorption Curves of Hematin

———: hematin
----------: hematin+ascorbic acid
– – – –: hematin+cysteine
—·—·—: hematin+glutathione

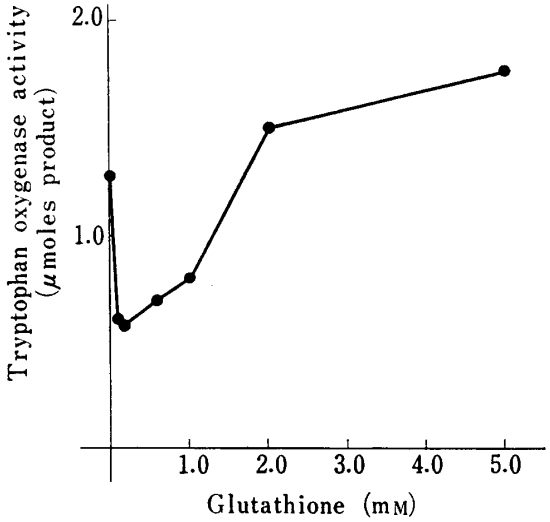

Fig. 2. Effect of Glutathione on the Activity of SM-TO

SM-TO was incubated at 37° for 60 min with glutathione.

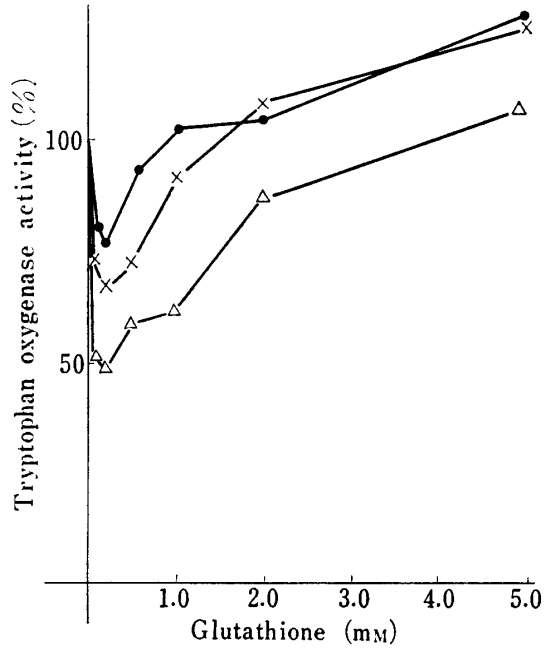

Fig. 3. Effect of Tryptophan Concentration on the Inhibithion of SM-TO by Glutathione

—●—: 0.5 mM tryptophan
—×—: 1.0 mM tryptophan
—△—: 5.0 mM tyrptophan

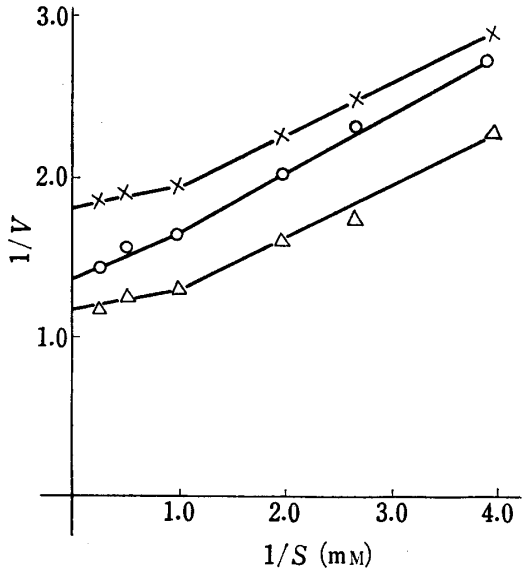

Fig. 4. Double-reciprocal Plot of Reaction Velocity *versus* Substrate Concentration for SM-TO

—●—: without glutathione
—×—: 0.1 mM glutathione
—△—: 5.0 mM glutathione

glutathione, which inhibited tryptophan oxygenase, and 5.0 mM glutathione, which activated it. The results are shown in Fig. 4.

The inhibition of glutathione may be not participated in an active site of the enzyme, but the action of glutathione to the enzyme protein was not certain. Effect of ascorbic acid

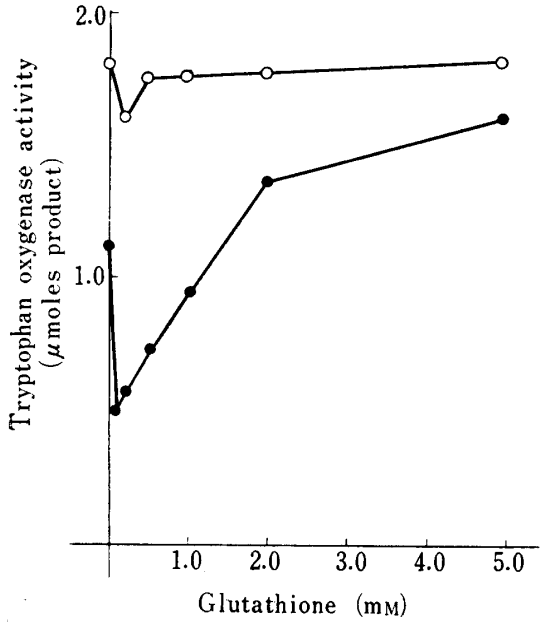

Fig. 5. Effect of Ascorbic Acid on the Inhibition of SM-TO by Glutathione

—●—: without ascorbic acid
—○—: with 5.0 mM of ascorbic acid

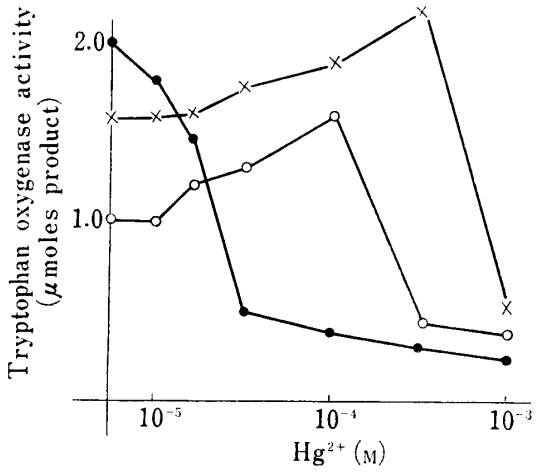

Fig. 6. Recovery of Glutathione Inhibition of SM-TO Activity by Hg^{2+}

After SM-TO was incubated at 37° for 5 min with 0.2 mM and 1.0 mM glutathione, Hg^{2+} was added immediately to each medium and incubated at 37° for 60 min.

—●—: without glutathione
—○—: 0.2 mM glutathione
—×—: 1.0 mM glutathione

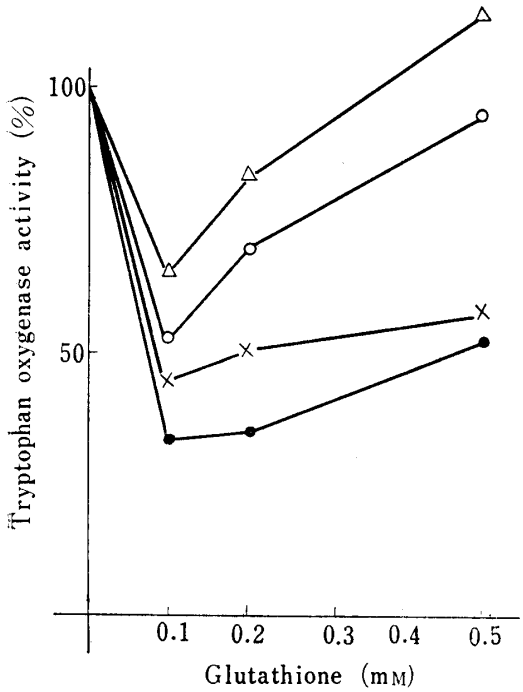

Fig. 7. Effect of Catalase on the Inhibition of SM-TO by Glutathione

SM-TO was incubated at 37° for 60 min in the presence or absence of catalase with glutathione.

—●—: without catalase —×—: catalase 0.38*k*
—○—: catalase 0.85*k* —△—: catalase 1.90*k*

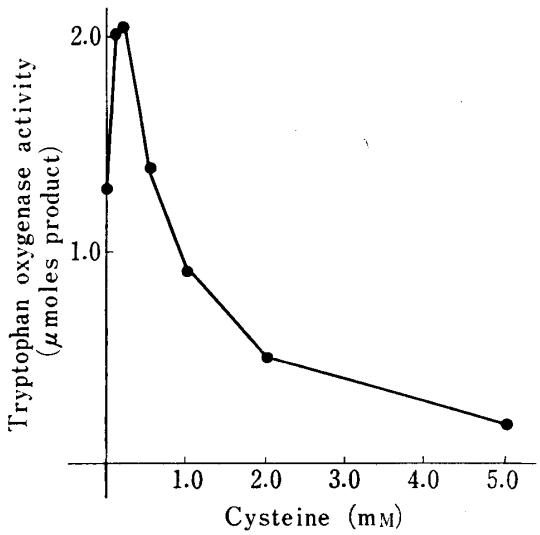

Fig. 8. Effect of Cysteine on SM-TO Activity

SM-TO was incubated at 37° for 60 min with cysteine.

on the inhibition of tryptophan oxygenase activity by glutathione was studied. Fig. 5 shows that there was no inhibition by glutathione at all when ascorbic acid was added to the reaction mixture.

Inhibition of tryptophan oxygenase activity by glutathione was also recovered by the addition of Hg^{2+} as indicated in Fig. 6. That is, in the case of the addition of 0.2 mM glutathione, the inhibition by glutathione was gradually reversed by the addition of Hg^{2+} and was maximally reversed at 0.1 mM Hg^{2+}. One mole of Hg^{2+} will react with two moles of glutathione. Therefore, it was assumed that the inhibition of enzyme activity by 0.2 and 1.0 mM glutathione was maximally reversed by the addition of 0.1 and 0.5 mM Hg^{2+} respectively because 0.1 mM of Hg^{2+} would react th 0.2 mM of glutathione. However, the tryptophan oxygenase activity was rapidly inhibited by Hg^{2+} more than 0.1 and 0.5 mM to the corresponding of 0.2 and 1.0 mM of added glutathione.

As shown in other papers [3,9], *Km* value of tryptophan for tryptophan oxygenase, stability at 0°, rate of inactivation by pre-incubation in the absence of tryptophan, and rate of inhibition by *o*-phenanthroline were changed by the addition of purified catalase prepared from rat liver. From those results, it was assumed that catalase would affect the protein structure of tryptophan oxygenase. Therefore, changes in the enzyme activity by glutathione in the presence of catalase were studied. It was found that, as shown in Fig. 7, inhibition of tryptophan oxygenase activity by glutathione decreased when the amount of catalase added increased.

Influence of Cysteine to Tryptophan Oxygenase Activity

One of the reductants, cysteine, was added to tryptophan oxygenase and the activity was measured. As shown in Fig. 8, tryptophan oxygenase indicated the maximum activity with 0.1—0.2 mM cysteine, but its activity rapidly decreased by the addition of higher concentration of cysteine.

Therefore, the mechanism of activation and inhibition of tryptophan oxygenase by cysteine investigated. When 0.1 mM cysteine, a maximally activating concentration, was added, its Lineweaver–Burk's plot crossed with the plot of control (Fig. 9 B). As shown in Fig. 8, tryptophan oxygenase activity was maximally activated by the addition of 0.1 mM cysteine, but its activity was decreased by high concentration of cysteine more than 0.2 mM. To know the reason why the addition of high concentration of cysteine decrease activity of the enzyme, the Lineweaver–Burk's plot was examined in the presence of 0.1 mM cysteine by which the enzyme was activated maximally and of 2.0 mM cysteine by which the enzyme activity was decreased (Fig. 9 A). It exhibited a competitive inhibition. It was assumed that the decrease of the maximum activity by the addition of high concentration of cysteine might be related to an active site of the enzyme. Because, in the case of the addition of 2.0 mM cysteine, the Lineweaver–Burk's plot was a non linear type and it was assumed that the cysteine will bind the heme of the active site in the presence of low concentration of tryptophan, so there would be any changes in the structure of the enzyme.

As the inhibition of tryptophan oxygenase by glutathione was reversed with ascorbic acid and Hg^{2+} (one-half molar concentration of added glutathione), whether the inhibition of tryptophan oxygenase by cysteine could also be recovered by ascorbic acid and Hg^{2+} was investigated. As shown in Fig. 10, the activity was reversed only slightly when ascorbic acid was added to tryptophan oxygenase containing various concentrations of cysteine.

Hg^{2+} hardly affected the inhibition of tryptophan oxygenase activity by cysteine. Also, as stated before, the inhibition of tryptophan oxygenase activity by glutathione decreased by the addition of catalase and, therefore, this point was also examined with cysteine. As shown in Table I, the concentration of cysteine which induced maximum activity of tryptophan oxygenase tended to become higher according to the amount of catalase present and the inhibition by higher concentration of cysteine was remarkably decreased.

9) I. Ishiguro and R. Shinohara, *J. Biochem.* (Tokyo), submitted.

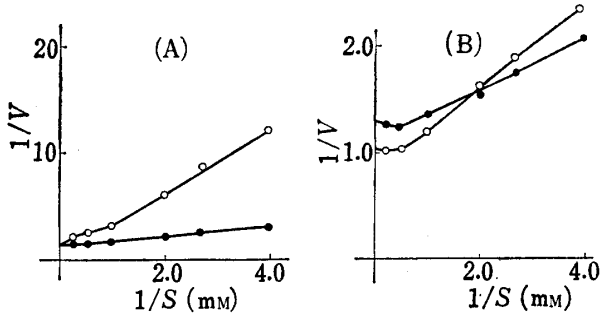

Fig. 9. Double-reciprocal Plot of Reaction Velocity *versus* Substrate Concentration for SM-TO

(A) —●—: 0.1 mM cysteine
—○—: 2.0 mM cysteine
(B) —●—: without cysteine
—○—: 0.1 mM cysteine

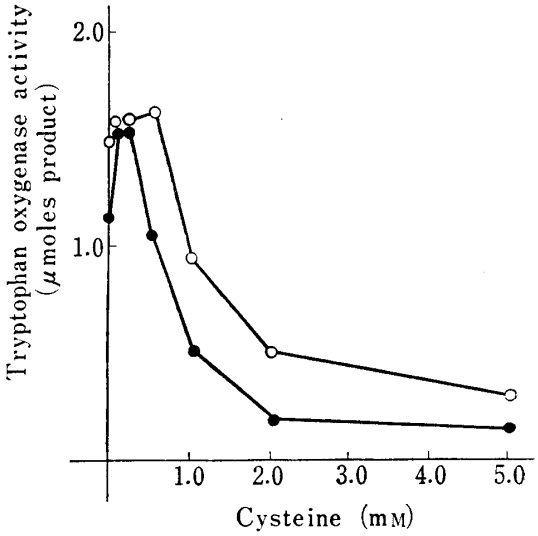

Fig. 10. Effect of Ascorbic Acid on the Inhibition of SM-TO by Cysteine

SM-TO was incubated at 37° for 60 min in the presence or absence of ascorbic acid (5.0 mM) with cysteine.
—●—: without ascorbic acid
—○—: 5.0 mM ascorbic acid

Table I. Effect of Catalase on the Inhibition and Activation of SM-TO by Cysteine

Catalase / Cysteine	0	0.38*k*	0.83*k*	1.90*k*
Nil (control)	100.0%	100.0%	100.0%	100.0%
0.1 mM	185.0	286.1	261.0	335.0
0.2 mM	149.2	304.0	361.0	394.0
0.5 mM	97.0	227.0	294.5	417.0
1.0 mM	63.7	163.8	272.1	365.0
2.0 mM	60.6	109.0	216.5	294.0
5.0 mM	45.5	100.0	155.6	241.0

SM-TO was incubated at 37° for 60 min in the presence or absence of catalase with cysteine. Control is tryptophan oxygenase activity without addition of cysteine. Figures are percentage activity to that of control.

Discussion

The effect of glutathione and cysteine on tryptophan oxygenase activity was investigated. Glutathione inhibited tryptophan oxygenase activity at low concentrations, and its inhibition was reversed by the addition of a higher concentration of glutathione. The inhibition rate of its activity by glutathione increased in proportion to the increased concentration of the substrate tryptophan. This fact might be correlated to a conformational change in tryptophan oxygenase by tryptophan because Schimke, *et al.*[8] had reported that the conformation of tryptophan oxygenase was changed by substrate tryptophan.

On the other hand, tryptophan oxygenase was activated by low concentration of cysteine (0.1—0.2 mM), but its activity was decreased by the addition of a higher concentration of cysteine.

As mentioned above, glutathione and cysteine showed quite different actions to the enzyme. Furthermore, Lineweaver–Burk's plots of glutathione and cysteine for tryptophan oxygenase were also different. Besides, the inhibited activity of tryptophan oxygenase by glutathione was almost reversed by the addition of Hg^{2+} of which concentration was one-half

molar concentration of added glutathione, but the inhibited activity by cysteine could not be reversed. The addition of ascorbic acid could eliminate the inhibition by glutathione, but it could not eliminate the inhibition by cysteine. On the other hand, the inhibition rate of enzyme activity by glutathione and cysteine were both decreased and even there were any activation by the addition of purified catalase which was prepared from rat liver.

As mentioned above, it was clarified that effects of glutathione and cysteine on tryptophan oxygenase activity were quite different although they are both SH-reductants. Further studies on effects both reductants on tryptophan oxygenase activity are in progress.