STUDIES ON NITROGLYCOL POISONING DECOMPOSITION MECHANISM OF NITROGLYCOL BY NITRITE FORMING ENZYME

Hiroshi TSURUTA and Hiromichi HASEGAWA

National Institute of Industrial Health, Kizuki-Sumiyoshi, Kawasaki

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We have found a new enzyme named nitrate forming enzyme which catalyzed the production of inorganic nitrate from nitroglycol or nitroglycerin in the blood and in the liver. On the other hand, the presence of nitrite forming enzyme in the liver has been known, though the mechanism of enzyme reaction was obscure. In this report, we investigated kinetically the reaction mechanism of nitrite forming enzyme by using nitroglycol, nitroglycerin and other nitrite or nitrate esters as substrate.

Clark and Litchfields' scheme for explaining the decomposition of nitroglycol, which was, in fact, proposed as the metabolic path of nitroglycol in the body, was found to be unsuitable, because the main compound in their scheme, nitrite ester, was clarified to be not formed during the decomposition of nitroglycol. Instead of their scheme, we have proposed a new scheme.

It has been clarified in the previous papers^{1,2,3)} that nitroglycol was decomposed by nitrate forming enzyme and nitrite forming enzyme, independently.

Nitrate forming enzyme in the liver was partially purified by us. By using the purified enzyme, the properties of the enzyme were studied kinetically, and the mechanism of nitroglycol decomposition has been proposed in the previous paper³⁾.

Nitrite forming enzyme was already reported to be purified from the hog liver acetone powder by ammonium sulfate fractionation⁴⁾. The mechanism of the enzyme reaction was studied by many investigators^{4,5)} using nitroglycerin, erythrityl tetranitrate and mannitol hexanitrate as substrate.

Recently, Clark and Litchfield⁶⁾ have proposed a following metabolic scheme of nitroglycol in the liver.

In the scheme, nitroglycol is supposed to be converted into nitrite ester in the

presence of reduced glutathione (GSH) and nitrite forming enzyme. Then, the nitrite ester is hydrolyzed nonenzymatically into inorganic nitrite and ethylene glycol mononitrate (EGMN). EGMN is further metabolized into inorganic nitrite and ethylene glycol through the similar way as nitroglycol by the action of nitrite forming enzyme. Ethylene glycol and nitrate, which is the oxidation product of nitrite, are finally excreted into the urine. This scheme might be supposed to explain well the formation of inorganic nitrite, nitrate and EGMN in the body of animals injected with nitroglycol. More recently, we have found a new enzyme, nitrate forming enzyme, which acted on nitroglycol producing inorganic nitrate and ethylene glycol³⁾. At present, it may be therefore supposed that the metabolism of nitroglycol in the body proceeds by both enzymes.

At any rate, it is certain that the nitrite forming reaction has a significant role on the metabolism of nitroglycol as well as the nitrate forming reaction. However, there appeared no scheme, even the above scheme, with conclusive proofs.

Some investigators^{7,8)} assumed a similar reaction as organic nitrate-hydrazine reaction about organic nitrate-mercaptans. This suggestion has led us to the following scheme:

This paper deals with the kinetic constants of nitrite formation from nitroglycol by nitrite forming enzyme and its reaction mechanism in consideration with the above two schemes.

METHODS

Nitrite forming enzyme

Nitrite forming enzyme was prepared from rabbit liver acetone powder according to the method of Heppel *et al.*⁹⁾. Twenty grams of rabbit liver acetone powder in 200 ml of 0.067 M phosphate buffer (pH 7.4) were shaken for 2 hr at 0°C, and the insoluble particles were centrifuged off at 13000×g. To the supernatant fraction, solid ammonium sulfate was added to obtain a precipitate between 50 and 80% saturation. The precipitate was dissolved in 0.067 M phosphate buffer (pH 7.4) and dialyzed for 24 hr against deionized water at 0°C. Insoluble parts were centrifuged off. The value of pH of the clear supernatant fraction was adjusted to pH 5.1 with 1 N acetic acid, and the precipitate was removed off by centrifuging. The supernatant diluted to 100 ml with deionized water was further fractionated by ethyl alcohol at -10°C. The precipitate fraction obtained between 50 and 65% of alcohol was collected. The precipitate dissolved in 0.067 M phosphate buffer (pH 7.4) was used as an enzyme solution. The enzyme concentration in an enzyme sample was expressed as mg of dry weight/ml of the sample.

Substrates for nitrite forming enzyme

1) *Nitroglycol*. Nitroglycol was synthesized according to the method described in

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previous papers^{2,3}).

2) *Nitroglycerin*⁹). Nitroglycerin was synthesized according to the following procedure. Glycerin was slowly dropped into 7 folds volumes of a mixed acid solution (conc. HNO_3 : conc. $\text{H}_2\text{SO}_4=1:2$) at below 10°C . After one hour of the titration, the mixed solution was poured into ice-water and then neutralized with 10% Na_2CO_3 solution. A heavy oily solution thus obtained at the bottom of a flask was purified by washing with water. In the experiments, 7 mM nitroglycerin solution prepared by diluting with water were used.

3) *Ethylene glycol mononitrate (EGMN)*. EGMN was synthesized by the method of Ferris *et al.*¹⁰). To a solution of 40 g of silver nitrate in 200 ml of dry, distilled acetonitrile was added a solution of 25 g of ethylene bromohydrin in 50 ml of acetonitrile. After three days at room temperature (17°C), silver bromide was rejected by filtration. The filtrate was poured into a liter of ice-water, and oily substance was collected. EGMN was extracted into 50 ml of methylene chloride from the oil solution. After drying, the solvent was removed by distillation at atmospheric pressure, and the residue was distilled at a reduced pressure (4 mmHg) to yield a colorless liquide, EGMN, bp. $70-71^\circ\text{C}$. The infrared spectrum of this liquide showed the characteristic pattern of organic nitrate esters at 1635 and 1280 cm^{-1} and a band at 3640 cm^{-1} due to OH stretching. This spectrum agreed with the infrared spectrum of EGMN reported by Rossmly¹¹).

EGMN was used as an aqueous solution diluted with water just before the experiments.

4) *Isoamyl nitrate, ethyl nitrate and isoamyl nitrite*. These organic nitrate esters and nitrite ester were purchased from Tokyo Kasei Industry Co.. These esters were used as ethanol solution.

Assay of the nitrite forming reaction

A mixture of 0.5 ml of 0.5 M phosphate buffer (pH 7.4) and 0.1 ml of 240 mM reduced glutathione was adjusted to pH 7.4 with a diluted sodium hydroxied solution, and made up to 2 ml by adding water. Then, 0.5 ml of a diluted enzyme solution were added. After 3 min, the reaction was started by adding 0.5 ml of 30 mM nitroglycol at 37°C . At adequate intervals of time, 1 ml of the reaction mixture was taken out. Then, it was centrifuged after the addition of deproteinizing agent, 1 ml of 5% HgCl_2 . The amount of inorganic nitrite in the supernatant was determined by Shinn's method¹²). The enzyme activity was expressed as μM of NO_2^- produced/min/mg of dry weight of the enzyme sample.

When substrates such as iosamyl-, ethyl-nitrate or isoamyl nitrite, which were dissolved in ethyl alcohol, were used, the volume of the substrate solution added was below 0.1 ml, because the velocity of the enzyme reaction has been known to be generally influenced by the dielectric constant of the reaction system.

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When isoamyl nitrite was used as the substrate, the velocity of the enzyme reaction could not be measured by determining the amount of inorganic nitrite, because both isoamyl nitrite and inorganic nitrite were colored by Shinn's method¹²⁾. In the case, therefore, the reaction velocity was measured from the change of optical density at 335 μ m which was an absorption maximum of the reaction product, GSNO.

Measurement of nitroglycol content in the reaction mixture

Nitroglycol was determined by the method described in the previous paper³⁾.

Determination of reduced glutathione (GSH)

The amount of reduced glutathione was determined by alloxan "305" method¹³⁾.

RESULTS

Michaelis constant of the decomposing reaction of nitroglycol by nitrite forming enzyme

As reported in the previous paper³⁾, a study on nitrate forming enzyme, when nitroglycol was incubated with the liver slice or homogenate, inorganic nitrate and nitrite were produced from nitroglycol by the action of nitrate forming enzyme and nitrite forming enzyme, respectively. Therefore, it has become clear that

Fig. 1. Effect of GSH concentration on enzyme activity.

Reaction mixture consisted of 0.5 ml of 0.5 M phosphate buffer (pH 7.4), 0.1 ml of various concentrations of GSH, 0.5 ml of enzyme solution (final concentration: 1.28 mg/ml), 0.5 ml of 30 mM nitroglycol and 1.4 ml of water. Experimental condition: 37 °C.

The reaction velocity was calculated by measuring the amount of inorganic nitrite produced for 10 min.

- : In the presence of GSH and the enzyme,
- : In the presence of only GSH.

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nitrite forming enzyme catalyzed the decomposition of nitroglycol producing inorganic nitrite as well as other organic nitrate esters. Then, at first, kinetic constants about nitrite forming enzyme were measured by using an enzyme sample extracted and purified from the rabbit liver acetone powder. It has been known that reduced glutathione was necessary for nitrite forming enzyme⁴). In Fig. 1, the effect of reduced glutathione on the enzyme activity was examined at a constant concentration of nitroglycol, 5 mM. As shown in the figure, the enzyme activity increased with the concentration of GSH and attained to the maximum at 8 mM GSH. On the other hand, nonenzymatic formation of inorganic nitrite from nitroglycol was also observed in the presence of GSH, and its velocity showed a linear increase with the increase of the concentration of GSH. From the above results, enzymatic reaction was carried out in the presence of 8 mM GSH except the cases indicated especially. Fig. 2 showed the reaction curves expressed as the formation of inorganic nitrite in the presence and absence of the enzyme. In both cases, the reaction curves were linear.

Fig. 3 shows the reaction velocity corresponding to the enzyme concentration. In the figure, the velocity was shown by subtracting the amount of inorganic nitrite formed per min in the absence of the enzyme from that in the presence of the enzyme. As indicated in this figure, the reaction velocity was linearly propor-

Fig. 2. Formation of NO_2^- from nitroglycol by nitrite forming enzyme.

The reaction was carried out at 37°C by adding 0.5 ml of 30 mM nitroglycol into a reaction medium, which contained 0.5 ml of 0.5 M phosphate buffer (pH 7.4), 0.1 ml of 240 mM GSH, 0.5 ml of enzyme solution (final concentration: 1.28 mg/ml) and 1.4 ml of water.

- : With the enzyme,
- : Without the enzyme.

Fig. 3. Relationship between the enzyme concentration and the reaction velocity.

Contents of the reaction mixture: 0.5 ml of 0.5 M phosphate buffer (pH 7.4), 0.1 ml of 240 mM GSH, 0.5 ml of enzyme solution, 0.5 ml of 30 mM nitroglycol, and 1.4 ml of water. The reaction was carried out at 37°C. The reaction velocity was expressed as μM of NO_2^- /min after subtracting the amount of inorganic nitrite formed nonenzymatically.

Fig. 4. Effect of pH on enzyme activity.

Contents of the reaction mixture: 0.5 ml of 0.5 M phosphate buffer of various pH values, 0.1 ml of 240 mM GSH, 0.5 ml of enzyme solution (final concentration: 1.28 mg/ml), 0.5 ml of 30 mM nitroglycol, and 1.4 ml of water. The reaction was carried out at 37°C for 2 min, and the amount of inorganic nitrite produced was determined.

- : With the enzyme,
- : Without the enzyme.

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tional to the enzyme concentration.

In Fig. 4, the effect of pH on the reaction velocity was examined. The velocity of nitrite formation in the absence of the enzyme showed a rapid increase at over pH 7.0. The velocity in the presence of the enzyme, accordingly, was difficult to be measured at over pH 8.0, because the reaction velocity in the absence of the enzyme became too large. The value of optimum pH for the enzyme reaction was found to be pH 7.5–8.5, which was nearly the same as that reported by Heppel *et al.*⁴⁾, who used nitroglycerin as substrate.

As clarified from the above results, nitroglycol was decomposed in the presence of GSH producing inorganic nitrite, and this reaction was accelerated by nitrite forming enzyme. Then, for further clarification of the reaction mechanism, the enzymatic decomposition of nitroglycol must be studied apart the nonenzymatic decomposition, because the both reactions may not proceed according to a similar mechanism, although inorganic nitrite was found to be a final product. Most simple technique for separating the enzyme reaction from the nonenzymatic one will be as follows; (1) large concentration of the enzyme, and (2) short reaction time. At above conditions, the nonenzymatic formation of inorganic nitrite will be disregarded in comparing with the enzymatic one. Following experiments for the assay of enzyme reaction were performed at above conditions.

Then, at the conditions, the reaction velocities (v) at various concentrations of

Fig. 5. Relationship between the concentration of nitroglycol and the reaction velocity.

Contents of the reaction mixture: 0.5 ml of 0.5 M phosphate buffer (pH 7.4), 0.1 ml of 240 mM GSH, 1 ml of enzyme solution (final concentration: 5.75 mg/ml), 0.5 ml of various concentrations of nitroglycol, and 0.9 ml of water. The reaction velocity (v) was calculated by determining the amount of NO_2^- produced for 2 min at 37°C, and was expressed as mM of nitroglycol decomposed/mg of enzyme/min.

nitroglycol were measured, and the results were shown in Fig. 5. indicating the relationship between $1/v$ and $1/[\text{nitroglycol}]$. From the tangent of the stright line in the figure, the value of Michaelis constant at pH 7.4 and 37°C was calculated to be 10 mM. Though the relationship at optimum pH, pH 7.78, was not indicated in the figure, the value of Michaelis constant calculated was 11.5 mM.

Mechanism of nitrite formation from nitroglycol by the enzyme in the presence of reduced glutathione

1) Examination of a following scheme proposed by Clark and Litchfield⁶⁾;

As described already, this scheme has been proposed by Clark and Litchfield⁶⁾ as a mechanism of nitroglycol decomposition in the body, although the decomposition of nitroglycol in the body has been clarified in the previous paper³⁾ by us to be performed by both nitrate forming enzyme and nitrite forming enzyme. In this paper, accordingly, the above scheme was taken up as a mechanism of nitroglycol decomposition by nitrite forming enzyme, and not as a metabolic path of nitroglycol in the body. In the scheme, nitroglycol was first converted to nitrite

Fig. 6. Light absorption spectra of GSNO and inorganic nitrite.

Absorption spectra of GSNO were measured by adding various concentrations of NO_2^- into 16 mM GSH at pH 2.5–3.0.

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ester in the presence of GSH by the enzyme, and then, the nitrite ester was hydrolyzed nonenzymatically into HNO_2 and EGMN. Inorganic nitrite and EGMN were already found in the body of animals injected with nitroglycol, but the nitrite ester, which might be the most important product in the scheme, has not yet been detected in the body. We have examined the properties of nitrite ester as described below.

It has been well known that nitrite esters were generally labile resulting the production of inorganic nitrite. Inorganic nitrite shows its light absorption maximum at $360\text{ m}\mu$ as shown in Fig. 6. Addition of reduced glutathione into an inorganic nitrite solution produces a new band at $335\text{ m}\mu$. In Fig. 6, 16 mM of reduced glutathione were added into inorganic nitrite solution of various concentrations, and when the change of spectrum finished after several minutes, the absorption spectrum was measured. Furthermore, one molecule of GSH was found to react with one molecule of inorganic nitrite producing a compound, GSNO. Accordingly, the reaction between GSH and HNO_2 may be expressed as

Thus, the new band at $335\text{ m}\mu$ may correspond to the reaction product, GSNO. In Fig. 7, the relationship between the optical density at $335\text{ m}\mu$ and the concentration of GSNO was shown. As seen in the figure, the optical density at $335\text{ m}\mu$ increased linearly with the concentration of GSNO.

The reactions indicated in Fig. 6 and 7 were carried out in acid pH, because

Fig. 7. Relationship between the concentration of GSNO and the optical density at $335\text{ m}\mu$.

GSNO solution was prepared by standing a mixture of 0.2 ml of 240 mM GSH and 3 ml of various concentrations of NO_2^- solution at pH 2.5–3.0 and 17°C for 2 hr.

the formation of GSNO from reduced glutathione and inorganic nitrite proceeded only in acid pH.

In Fig. 8, the effect of pH on the reaction was indicated. The results in the figure were obtained by tracing the change of optical density after mixing 1 mM HNO_2 and 8 mM GSH at various pH values and 18°C. As shown in the figure, the formation of GSNO was observed only in acid pH, and the reaction proceeded scarcely at pH 7.0 even after standing for one hour at 37°C.

As described already, nitrite ester in water is very labile, and decomposes into inorganic nitrite. The specific absorption spectrum of nitrite ester in ethyl alcohol as shown in Fig. 9 changes slowly by introducing into water, and reaches finally to the spectrum of inorganic nitrite. It may be reasonable to consider that ethylene glycol nitrite ester in water shows a similar change as isoamyl nitrite, although the decomposition of ethylene glycol nitrite ester was not yet examined. So far as the above experiments indicate, the scheme proposed by Clark and Litchfield⁶⁾ that nitrite ester is hydrolyzed into inorganic nitrite seems to be correct.

On the other hand, however, it must be considered that nitrite ester is supposed to be formed in the presence of nitrite forming enzyme and reduced glutathione, and therefore, that the enzyme and reduced glutathione are present with the nitrite ester produced in the reaction mixture. Accordingly, the effect of GSH or the enzyme on the decomposition of nitrite ester in water must be examined. By adding reduced glutathione into an isoamyl nitrite solution, the specific absorption spectrum of isoamyl nitrite observed at the wave length of 300–400 $\text{m}\mu$ diminished, and

Fig. 8. Effect of pH on the formation of GSNO from GSH and inorganic nitrite.

The reaction mixture contained 1 ml of 3 mM NO_2^- solution, 0.1 ml of 240 mM GSH, 0.5 ml of phosphate buffer solution and 1.4 ml of water. The reaction was performed at 18°C.

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Fig. 9. Spontaneous decomposition of isoamyl nitrite in water.

The assay was carried out by introducing 0.1 ml of 150 mM isoamyl nitrite in ethanol at 26°C into a mixture which contained 0.5 ml of 0.5M phosphate buffer (pH 7.8), and 2.4 ml of water.

-----: 5 mM isoamyl nitrite in ethanol,

(1) : Immediately after the addition of isoamyl nitrite,

(2) : 15 min after the addition of isoamyl nitrite,

(3) : 30 min after the addition of isoamyl nitrite,

(4) : 24 hr after the addition of isoamyl nitrite.

a new spectrum, which has its absorption maximum at 335 mμ, appeared. This new spectrum seems to indicate the formation of GSNO, that is, the reaction between isoamyl nitrite and reduced glutathione may be represented as follows:

The above reaction could proceed in a neutral medium, though the reaction between inorganic nitrite and reduced glutathione took place only at acid pH as described already. Moreover, the formation of GSNO from isoamyl nitrite was found to be accelerated by nitrite forming enzyme as shown in Fig. 10. The figure showed the results obtained by adding various concentrations of the enzyme into isoamyl nitrite and reduced glutathione at pH 7.4. As indicated in the figure, the velocity of the formation of GSNO was proportional to the concentration of the enzyme. It was therefore concluded that isoamyl nitrite, probably also ethylene glycol nitrite ester, was decomposed by reduced glutathione and nitrite forming enzyme

Fig. 10. Decomposition of isoamyl nitrite by nitrite forming enzyme in the presence of GSH.

The assay was carried out by introducing 0.1 ml of 150 mM isoamyl nitrite in ethanol at 15°C into a mixture which contained 0.5 ml of 0.5 M phosphate buffer (pH 7.4), 0.1 ml of 240 mM GSH, 0.1 ml of enzyme solution (contained 99 or 594 μ g of enzyme) and 2.2 ml of water. The amount of GSNO formed was calculated by measuring the increase of optical density at 335 $m\mu$.

resulting the formation of GSNO*. Moreover, the decomposition of isoamyl nitrite was about 5000 times faster than that of nitroglycol at the same conditions in respect of the substrate concentration, the reduced glutathione concentration, the enzyme concentration and pH.

From these results, if ethylene glycol nitrite ester were produced as proposed by Clark and Litchfield⁶⁾ in the process of the decomposition of nitroglycol, the formation of GSNO would be observed as the result of the decomposition of nitrite ester by GSH and the enzyme. Then, nitroglycol or nitroglycerin as substrate was incubated with both GSH and the enzyme at pH 7.4 for about one hour, and it was examined whether or not the light absorption spectrum caused by the formation of GSNO appeared. The results were shown in Figs. 11 and 12. For both substrates, as seen in the figures, the final spectra were similar, and their maxima were found at about 360 $m\mu$. These spectra seem to indicate that inorganic nitrite is the final product, but GSNO is not. The amount of inorganic nitrite calculated by using the relationship between the optical density and the amount of inorganic nitrite shown in Fig. 6 was 1.3 mM at 100 min for nitroglycol and 1.6 mM at 120

* The compound, GSNO, was decomposed into inorganic nitrite by a deproteinizing agent, $HgCl_2$. Thus, the amount of GSNO could be determined by Shinn's method¹²⁾ which has been usually used for the determination of inorganic nitrite.

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Fig. 11. The absorption spectra of the product produced from nitroglycol by the enzyme.

Contents of the reaction mixture: 0.5 ml of 0.5 M phosphate buffer (pH 7.4), 0.1 ml of 240 mM GSH, 1.0 ml of enzyme solution (final concentration: 2.3 mg/ml), 0.5 ml of 30 mM nitroglycol and 1.4 ml of water. The reaction was carried out at 18°C.

Fig. 12. The absorption spectra of the product produced from nitroglycerin by the enzyme.

Contents of the reaction mixture: 0.5 ml of 0.5 M phosphate buffer (pH 7.4), 0.1 ml of 240 mM GSH, 0.5 ml of enzyme solution (final concentration: 1.15 mg/ml), 1.0 ml of 7 mM nitroglycerin and 1.4 ml of water. The reaction was carried out at 18°C.

min for nitroglycerin. Simultaneously, the values determined by Shinn's method¹²⁾ were found to be 0.86 and 1.00 mM, respectively. The amounts of inorganic nitrite determined by both methods may be regarded to be the same in consideration of the low optical densities of inorganic nitrite at 360 m μ . From these facts, inorganic nitrite may be concluded to be the final product from nitroglycol or nitroglycerin, but GSNO is not produced. Moreover, this conclusion was further ascertained from the fact that if GSNO of about 1 mM had been formed, its absorption spectrum would have had the maximum of $\log I_0/I=1.0$ at nearly 335 m μ . In the above reactions, the formation of GSNO from inorganic nitrite and GSH may be disregarded, because the reaction does not proceed at pH 7.4 as indicated in Fig. 8.

Thus, the formation of nitrite ester in the scheme proposed by Clark and Litchfield⁶⁾ was denied, that is, the decomposition of nitroglycol or nitroglycerin did not proceed passing through nitrite ester.

2) Formation of ethylene glycol mononitrate and inorganic nitrite.

In the blood of animals injected with nitroglycol, EGMN was found by Clark and Litchfield⁶⁾. The formation of EGMN and fairly rapid decomposition of EGMN by nitrate forming enzyme were already assumed in the previous paper³⁾. The accumulation of EGMN in the blood might be impossible if only nitrate forming enzyme acted on nitroglycol to form EGMN. The formation and the accumulation of EGMN was, therefore, assumed to be caused by nitrite forming enzyme, though the mechanism has not yet been clarified. In this report, the formation of EGMN was attended to investigate kinetically. Then, the decomposition rate of nitroglycol and EGMN by the enzyme were compared by determining the amount of inorganic nitrite formed per unit reaction time, because if the decomposition of EGMN proceeded more slowly than that of nitroglycol, it might be considered that the accumulation of EGMN formed from nitroglycol took place in the process of nitroglycol decomposition.

Fig. 13 shows the results obtained by examining the decomposition of nitroglycol, EGMN and other nitrate esters. The velocity of EGMN decomposition calculated from the results shown in the figure was found to be about one-thirtieth of that of nitroglycol. Accordingly, if nitroglycol were decomposed into EGMN and inorganic nitrite as expressed below

the product, EGMN, might be considered to be accumulated in the reaction system. In Fig. 13, for conveniently, various nitrate esters were examined. The reaction velocities of these compounds were 40 for nitroglycerin, 1/15 for isoamyl nitrate, 1/30 for EGMN and 1/60 for ethyl nitrate, where the velocity for nitroglycol was expressed as 1. If nitroglycol were decomposed according to the above reaction

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Fig. 13. Decomposition of several organic nitrate esters into NO_2^- by nitrite forming enzyme.

Contents of the reaction mixture: 0.5 ml of 0.5 M phosphate buffer (pH 7.4), 0.1 ml of 240 mM GSH, 1.5 ml of enzyme solution (final concentration: 7.84 mg/ml), 0.1 ml of 150 mM various organic nitrate esters and 0.8 ml of water. The reaction was carried out at 37°C.

—○—: Nitroglycerin, —●—: Nitroglycol, —▲—: EGMN,
—△—: Isoamyl nitrate, —□—: Ethyl nitrate.

Fig. 14. Relationship between the amount of nitroglycol consumed and that of inorganic nitrite produced.

Contents of the reaction mixture: 0.5 ml of 0.5 M phosphate buffer (pH 7.4), 0.1 ml of 240 mM GSH, 1.5 ml of enzyme solution (final concentration: 7.98 mg/ml), 0.5 ml of 30 mM nitroglycol and 0.4 ml of water. The reaction was carried out at 37°C.

formula, and if EGMN were scarcely decomposed, the decreased amount of nitroglycol should be equal to the amount of inorganic nitrite formed. For the determination of nitroglycol, nitroglycol in ether was decomposed into inorganic nitrite by adding potassium hydroxide, and inorganic nitrite was determined by Shinn's method^{13,13)}. By this determination procedure, one mole of nitroglycol is determined as one mole of inorganic nitrite, that is, only one mole of inorganic nitrite is gotten from one mole of nitroglycol by the decomposition procedure. EGMN was ascertained to be not decomposed by the above procedure⁶⁾. In Fig. 14, the amount of inorganic nitrite produced by the action of the enzyme was compared with that of nitroglycol consumed. As seen clearly from the figure, the amount of inorganic nitrite formed and nitroglycol consumed showed one to one correspondence.

It may be therefore concluded that nitroglycol is decomposed into EGMN and inorganic nitrite in the presence of reduced glutathione by nitrite forming enzyme.

3) *Consumed amount of reduced glutathione for the production of inorganic nitrite from nitroglycol.*

As described already, reduced glutathione is necessary for the decomposition of nitroglycol by the enzyme. The amount of reduced glutathione consumed for producing one mole of inorganic nitrite from nitroglycol by the action of the enzyme was determined in two cases, low and large amount of reduced glutathione.

Fig. 15 shows the relation between the amount of inorganic nitrite produced

Fig. 15. Relationship between the amount of NO_2^- produced and that of GSH consumed in the presence of a high concentration of GSH.

Contents of the reaction mixture: 0.5 ml of 0.5 M phosphate buffer (pH 7.4), 0.1 ml of 120 mM GSH, 1.5 ml of enzyme solution (final concentration: 7.97 mg/ml), 0.5 ml of 30 mM nitroglycol and 0.4 ml of water. The reaction was carried out at 37°C.

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and that of reduced glutathione consumed at a fairly large concentration of reduced glutathione, 4.0 mM. As shown in the figure, the amount of inorganic nitrite produced from nitroglycol was less than 0.1 mM, and the consumed amount of reduced glutathione for nitrite formation was less than 0.3 mM. Therefore, most reduced glutathione added in the reaction system remained in unreacted state, that is, the amount of reduced glutathione added was sufficiently enough. At this condition, the amount of reduced glutathione consumed for producing one mole of inorganic nitrite was found to be 3.5 moles from the results shown in the figure. Although the result carried out without the enzyme was not indicated in the figure, the nonenzymatic formation of inorganic nitrite from nitroglycol was performed by consuming 3 moles reduced glutathione for producing one mole of nitrite.

Fig. 16 shows the results obtained in the addition of 0.45 mM reduced glutathione, which is about one-tenth of that used in the experiment shown in Fig. 15. In this experiment, the concentration of the enzyme was enough for completing the reaction within 10 min, at which all of reduced glutathione added was consumed and nonenzymatic formation of inorganic nitrite was not observed. As shown in the figure, the formation of inorganic nitrite or the decomposition of nitroglycol finished within 10 min after the beginning of the reaction, and simultaneously, reduced glutathione added was all consumed. At the time, the amount of inorganic nitrite

Fig. 16. Relationship between the amount of NO_2^- produced and that of GSH consumed in the presence of a low concentration of GSH.

Contents of the reaction mixture: 0.5 ml of 0.5 M phosphate buffer (pH 7.4), 0.2 ml of 6.75 mM GSH, 1.5 ml of enzyme solution (final concentration: 7.88 mg/ml), 0.5 ml of 30 mM nitroglycol and 0.3 ml of water. The reaction was carried out at 37°C.

produced was a half of that reduced glutathione added or consumed, that is, 2 moles of reduced glutathione were consumed for producing one mole of inorganic nitrite.

The difference of necessary amount of reduced glutathione for producing inorganic nitrite in both cases may be probably explained as follows; in the process of the determination of remaining reduced glutathione, the pH value of the sample decreases by the addition of metaphosphoric acid as deproteinizing agent, and at the pH, inorganic nitrite in the sample reacts with remaining reduced glutathione resulting the formation of GSNO as examined already. Since unreacted reduced glutathione in the experiment shown in Fig. 15 is consumed for the formation of GSNO, the amount of reduced glutathione consumed for the production of one mole of inorganic nitrite may be considered to be 2 moles by subtracting one mole of reduced glutathione consumed for the formation of GSNO from 3 moles. In the experiment shown in Fig. 16, of course, the formation of GSNO from inorganic nitrite and reduced glutathione does not take place, because all of reduced glutathione is already consumed before the determination of reduced glutathione.

After all, it was concluded that 2 moles of reduced glutathione were necessary for the production of one mole of inorganic nitrite from nitroglycol.

4) *Measurement of activation energy of the reaction;*

Fig. 17. Relationship between the reaction temperature and the velocity of NO_2^- formation in the presence of a high concentration of GSH.

Contents of the reaction mixture: 0.5 M phosphate buffer (pH 7.4), 0.1 ml of 240 mM GSH, 0.5 ml of enzyme solution (final concentration: 0.587 mg/ml), 0.5 ml of 30 mM nitroglycol and 1.4 ml of water. The reaction velocity (v) was expressed as $\mu\text{M NO}_2^-$ produced/mg of enzyme/min.

—○—: With the enzyme,
—△—: Without the enzyme.

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Activation energy for the decomposition of nitroglycol was measured in the following four cases; (a) in the presence of the enzyme and a large amount of GSH, (b) in the presence of the enzyme and a small amount of GSH, (c) in the presence of a large amount of GSH without the addition of the enzyme, and (d) in the presence of a small amount of GSH without the enzyme. The assay of enzyme reaction, as mentioned already, was carried out at the condition, at which nonenzymatic decomposition of nitroglycol proceeded scarcely.

Fig. 17 shows the results obtained in the presence of a large amount of GSH (8 mM) with and without the addition of the enzyme. In the figure, the ordinate shows the logarithmus of the reaction velocity ($\log v$), and the abscissa shows the reciprocal of absolute temperature experimented ($1/T$). As seen from the figure, the relationship between $\log v$ and $1/T$ was linear in both cases, and the values of activation energy were found to be the same, 17000 cal/mole.

The results shown in Fig. 18 were obtained in the presence of a small amount of GSH (0.2 mM) with and without the addition of the enzyme. From the tangent of straight lines shown in the figure, the values of activation energy were calculated as 2700 cal/mole for enzymatic reaction and 7100 cal/mole for nonenzymatic one.

These facts seem to suggest that the decomposition of nitroglycol consists of two reactions, at least. One is the reaction catalysed by the enzyme and another

Fig. 18. Relationship between the reaction temperature and the velocity of NO_2^- formation in the presence of a low concentration of GSH.

Contents of the reaction mixture: 0.5 ml of 0.5 M phosphate buffer (pH 7.4), 0.1 ml of 6 mM GSH, 1.5 ml of enzyme solution (final concentration: 5.94 mg/ml), 0.5 ml of 30 mM nitroglycol and 0.4 ml of water. The reaction velocity (v) was expressed as $\mu\text{M NO}_2^-$ produced/mg of enzyme/min.

—○—○—: with the enzyme, —△—△—: without the enzyme.

proceeds without the enzyme. The latter reaction may be the rate determining one in the presence of a large amount of GSH. On the other hand, the former shows its important property only in the presence of a small amount of GSH. Probably, the latter reaction might be more significant than the former in the body, because the amount of GSH in the liver cells has been found to be about 1.68 mg per g of the liver, which was supposed to correspond to about 5.6 mM. The value of Michaelis constant in the presence of a small amount of GSH at pH 7.4 was 6.5 mM, which was about a half of that in the presence of a large amount of GSH. The differences observed on the values of activation energy and on Michaelis constants in the small and large amount of GSH indicate the need of further investigations about the reaction mechanism, especially about the intermediary compounds.

5) *Michaelis constant of the following reaction;*

Heppel and Hilmore⁴⁾ have reported that the decomposition of nitroglycerin into inorganic nitrite by nitrite forming enzyme took place its maximum velocity in the presence of 8 mM GSH. This fact was ascertained by us. Namely, in the presence of 8 mM GSH, the relation between the reaction velocity and the concen-

Fig. 19. Relationship between the concentration of nitroglycerin and the velocity of NO_2^- formation.

Contents of the reaction mixture: 0.5 ml of 0.5 M phosphate buffer (pH 7.4), 0.1 ml of 240 mM GSH, 0.5 ml of enzyme solution (final concentration: 2.88 mg/ml), 0.5 ml of various concentrations of nitroglycerin and 1.4 ml of water. The reaction was carried out at 37°C for 2 min and the amount of NO_2^- produced was determined. The reaction velocity (v) was expressed as mM of nitroglycerin decomposed/mg of enzyme/min.

NITRITE FORMATION FROM NITROGLYCOL

tration of nitroglycerin was examined at pH 7.4. The results were shown in Fig. 19. In the experiments, as mentioned already, the nonenzymatic decomposition of nitroglycerin by GSH proceeded scarcely by adding a large concentration of the enzyme and by shortening the reaction time. From the linear relationship shown in the figure, Michaelis constant was calculated as 2.5 mM. The value of Michaelis constant at optimum pH, pH 7.78, was found to be 3.8 mM. In the presence of a small amount of GSH, 0.4 mM, the value at pH 7.4 was 1.1 mM.

DISCUSSION

Our study was performed to clarify the decomposing path of nitroglycol by nitrite forming enzyme.

At first, the scheme proposed by Clark and Litchfield⁶⁾,

was examined, and the following facts were found out.

a) The intermediary compound in the scheme, nitrite ester, if it were formed, was decomposed by the enzyme in the presence of GSH according to the following equation:

b) GSNO in the above reaction should be detected by measuring its absorption spectrum, if nitrite ester were formed in the decomposing path of nitroglycol. The special spectrum of GSNO could not however observed.

c) The final product observed in the decomposition of nitroglycol or nitroglycerin was inorganic nitrite.

d) Inorganic nitrite can produce GSNO by reacting with GSH at acid side;

where the reaction proceeds scarcely at pH 7 or alkaline side.

From these facts, Clark and Litchfields' scheme, in which nitrite ester was given the most significant role, was clarified to be unsuitable.

Our study on the reaction mechanism of nitroglycol has further obtained the following facts.

e) EGMN decomposes by the action of nitrite forming enzyme, but its velocity is one-thirtyth of nitroglycol at the same conditions. Accordingly, at the experimental conditions described in this paper, the decomposition of EGMN proceeds scarcely.

f) The ratio of the amount of inorganic nitrite produced to that of nitroglycol consumed is one to one.

g) The ratio of the amount of inorganic nitrite produced to that of reduced glutathione consumed is one to two.

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These facts indicate that one molecule of nitroglycol is decomposed into one molecule of EGMN and one molecule of inorganic nitrite in the presence of two molecules of reduced glutathione by the enzyme. Furthermore, we have found out that the decomposition of nitroglycol consisted of, at least, two reactions, which were the enzyme reaction and the nonenzyme one.

From the above results, we have assumed the following scheme;

This scheme was suggested from the reaction between organic nitrate ester and hydrazine⁷⁾;

In the above scheme, GSNO₂ has not yet been detected, because the method for the determination of GSNO₂ has not been found out. Although the presence of GSNO₂ has not yet been reported, the presence of S-alkyl-thiosulfate¹⁴⁾, RSSO₃H, might support the above scheme.

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