EXPERIMENTAL STUDIES ON THE NITROGLYCOL POISONING FREE NITROGLYCOL AND NITRATE IN THE BLOOD OF RABBIT

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In the previous paper¹⁾, we have assumed that nitrite ion was not produced from nitroglycol *in vivo*, and that methemoglobin found in the blood of rabbits injected with nitroglycol was formed by the action of nitroglycol, but not by nitrite. In this paper, this assumption was ascertained. When rabbit received nitroglycol subcutaneously, a large amount of free nitroglycol was found in the blood immediately after the injection. The amount of methemoglobin found in the blood was in parallel with the amount of free nitroglycol in the blood. As a metabolic product of nitroglycol, nitrate ion was detected in the blood, but nitrite was not. When nitroglycol was injected daily, the rate of nitrate production increased day after day. This phenomenon was interpreted as being due to the increment of nitroglycol hydrolyzing enzyme.

In the previous paper¹⁾, it has been reported that nitroglycol is a strong methemoglobin former. Heppel and Hilmore²⁾ found out that nitroglycerine, which had an analogous structure with nitroglycol, was decomposed enzymatically into nitrite. Methemoglobin formed by nitrite *in vitro* shows its light absorption maxima at 540 and 630 m μ at pH 6.8¹⁾, whereas that formed by nitroglycol *in vivo* and *in vitro* at 500 and 630 m μ at the same conditions. From these facts, we have assumed that nitrite was not produced from nitroglycol.

This paper deals with the metabolic products of nitroglycol and with the relationship between the amount of free nitroglycol in the blood and that of methemoglobin formed by the action of nitroglycol.

MATERIALS AND METHODS

Reagents: Brucine was purchased from Sigma Chem. Co. Chloroform of Wako Pure Chemical Co. was used after distillation. Other reagents were these of the special grade of Wako Pure Chem. Co.

Methods: Rabbits received 0.2 g of nitroglycol per kg of body weight daily. The blood (2~3 ml) was punctured from the heart with an injection syringe (capacity: 5 ml)

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containing heparin. After adding 1.5 ml of deionized water into 0.5 ml of the blood, 5 ml of ethylether was added and nitroglycol in the blood was transferred perfectly into ethereal layer by shaking vigorously. The ethereal layer was used for the determination of free nitroglycol and the residual for the determination of NO_3 .

*Determination of Free Nitroglycol in the Blood*³⁾: Seven ml of ethylalcohol, 1 ml of deionized water and 1 ml of 2% NaOH solution were added into the ethereal solution. This mixture was kept for 60 minutes in a water bath of 40°C to decompose nitroglycol into alcohol and NO_2 . Then ether was evaporated off till 10 ml at 50~60°C. 1 ml of 6 N HCl solution and 1 ml of 1% sulfanylamide solution were added, and after 3 minutes 1 ml of 0.5% ammonium sulfamate was added. Two minutes after, 1 ml of 1% N-(1-naphtyl)-ethlenediamine solution was added. After standing for 10 minutes, the optical density was measured at 530 m μ by using a Hitachi spectrophotometer EPU-2. And nitroglycol content ($\mu\text{g}/\text{ml}$ of the blood) was computed using the calibration curve.

*Determination of Nitrate in the Blood*³⁾: Three ml of deionized water and then 2 ml of 8% trichloroacetic acid (TCA) solution were added into the aqueous residue treated with ether as described above. After the resulting precipitate was filtered off, 2 ml of 4% brucine dissolved in chloroform were added into 5 ml of the filtrate. It was kept at 0°C for 5 minutes. Then 5 ml of conc. H_2SO_4 were added slowly. This procedure was carried out using a magnetic stirrer at 0°C. Then chloroform was evaporated off by heating at 90°C for 25 minutes. After cooling with tap water, the color intensity was measured at 410 m μ using a Hitachi spectrophotometer EPU-2 and the nitrate content was calculated from the calibration curve.

Methemoglobin: Methemoglobin content in the blood was determined according to the Horecker and Brackett's method⁴⁾.

RESULTS

Nitroglycol in the blood—The amount of nitroglycol in the blood of rabbit received 0.2 g of nitroglycol per kg of body weight daily was plotted with time after the injection. The results were shown in Figs. 1 and 2*.

As seen in the figures, the nitroglycol content in the blood increased markedly as soon as nitroglycol was injected subcutaneously into rabbits. The increasing rate of nitroglycol content in the blood was seemed to be independent of the frequency of the injection, but in every case, the nitroglycol content reached to the maximum at 1~2 hours after the injection. Thereafter, it began to decrease, and at 24 hours after the injection no nitroglycol was detected in the blood.

Methemoglobin Content in the Blood—Together with the estimation of nitroglycol, the amount of methemoglobin was measured. The results were shown in Figs. 3 and 4.

* The data obtained by using the rabbit of No. 1 were shown in Figs. 1, 3 and 6, and those obtained by using the rabbit of No. 2 in Figs. 2, 4 and 7.

Fig. 1. Nitroglycol content in the blood of rabbit injected with nitroglycol.

The rabbit of No. 1 (2 kg) was injected with 0.2 g of nitroglycol per kg of the body weight daily and the amount of nitroglycol in 1 ml of the blood was determined. The number of times of the injection was indicated in the figure. Ordinate: the amount of nitroglycol in the blood ($\mu\text{g}/1\text{ ml}$ of the blood). Abscissa: time after the injection.

Fig. 2. Nitroglycol content in the blood of rabbit injected with nitroglycol.

The rabbit of No. 2 (3 kg) was injected with 0.2 g of nitroglycol per kg of the body weight daily and the amount of nitroglycol in 1 ml of the blood was determined.

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Fig. 3. The change of methemoglobin content in blood of the rabbit injected with nitroglycol.

Rabbit (No. 1) received 0.2 g of nitroglycol per kg of the body weight every day. The amount of methemoglobin was expressed as the ratio, $[\text{Methemoglobin}]/[\text{Totalhemoglobin}]$. Ordinate: percentage of the amount of methemoglobin. Abscissa: time after the injection.

Fig. 4. The change of methemoglobin content in blood of the rabbit injected with nitroglycol.

Rabbit (No. 2) received 0.2 g of nitroglycol per kg of the body weight every day.

The increasing rate of methemoglobin content was found to be very large. In Fig. 3, the amount of methemoglobin at the third injection reached to about 80 per cent after 3 hours, at which the rabbit died in the course of severe convulsions. In Fig. 5, the maximum methemoglobin content was plotted against the maximum amount of free nitroglycol.

Fig. 5. The maximum amount of methemoglobin versus the maximum amount of nitroglycol in the blood.

The maximum amount of methemoglobin and of nitroglycol in the blood were obtained from the data indicated in Figs. 1, 2, 3 and 4. ○: rabbit of No. 1, △: rabbit of No. 2.

As shown in Fig. 5, the relationship between the amount of methemoglobin formed in the blood and the maximum amount of free nitroglycol was linear. This fact accords with the results reported in the previous paper¹⁾, in which it has been clarified that nitroglycol was a strong methemoglobin former in vivo and in vitro.

Nitrate in the Blood—Prior to the injection with nitroglycol, the amount of nitrate in 1 ml of the blood was measured and was found to be 28.8 μg for the rabbit of No. 1 and 32.6 μg for the rabbit of No. 2. After the injection, nitrate increased rapidly as shown in Figs. 6 and 7. In Fig. 8, the initial rate of NO₃ increment (μg of NO₃/ml/hour) was plotted with the frequency of injection. As seen in Fig. 8, the rate increased by repeating the injection with nitroglycol.

The large rate of NO₃ increment seems to suggest the rapid decrease of nitroglycol but the results in Fig. 8 did not correspond to the results in Figs. 1 and 2. If smaller amount of nitroglycol than that used in these experiments is given, the rate of nitrate formation may be inversely proportional to the amount of nitroglycol in the blood.

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Fig. 6. The change of nitrate content in the blood of rabbit injected with nitroglycol.
Rabbit (No. 1) received 0.2 g of nitroglycol per kg of the body weight daily. Ordinate: the increased amount of nitrate ion in 1 ml of the blood ($\mu\text{g}/1\text{ ml}$ of the blood). Abscissa: time after the injection.

Fig. 7. The change of nitrate content in the blood of rabbit injected with nitroglycol.
Rabbit (No. 2) received 0.2 g of nitroglycol per kg of the body weight daily.

Fig. 8. The increased amount of nitrate in the blood per hour versus the frequency of the injection.

The amount of nitrate increased in the blood per hour after the injection was calculated from the data indicated in Figs. 6 and 7. Ordinate: the amount of nitrate increased in the blood per hour ($\mu\text{g}/\text{ml}\cdot\text{hour}$). Abscissa: number of times of the injection. $\circ$: rabbit of No. 1, $\bullet$: rabbit of No. 2.

DISCUSSION

The most significant finding in the present experiments was that when nitroglycerol was injected daily, the rate of nitrate formation increased day after day. This fact suggests that the amount of enzyme which decomposes nitroglycerol into nitrate and alcohol, or the prosthetic group of this enzyme which may be required for the action of the enzyme increased. It has been reported by Heppel and Hilmore that nitroglycerine was decomposed into nitrite and alcohol by enzyme action, which was extracted from the liver and purified, and that the reduced form of glutathione was necessary for the enzyme action as its prosthetic group. But it is not yet clear whether or not the prosthetic group is necessary for the action of nitroglycerol hydrolyzing enzyme.

In the present experiments, a large amount of nitrate was detected in the blood of rabbits injected with nitroglycerol, but no nitrite. Nitrite ion, if it is present in the blood, is detectable by the same method as that used in the determination of nitroglycerol, because nitroglycerol is determined as nitrite after hydrolyzing. But no trace of nitrite was detected in the aqueous residue obtained after nitroglycerol was removed

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into ether. The fact that nitrite was not produced from nitroglycol in the body has been already assumed in the previous paper¹⁾ from the fact that methemoglobin found in poisoned rabbits was not NO₂-methemoglobin. And, as will be reported in the next paper nitroglycol is decomposed into nitrate by incubating with the blood, but not into nitrite. So, it may be impossible that the enzyme which decomposes nitroglycol into nitrate and alcohol acts upon nitroglycol on the same mode as the enzyme which decomposes nitroglycerine into nitrite and alcohol. The details of the nitroglycol hydrolyzing enzyme will be described in the next paper.

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