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Metabolism of Nitrate and Methemoglobinemia in Ruminants

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反芻家畜における硝酸塩代謝とメトヘモグロビン血症

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Introduction

A disease of cattle as a result of the ingestion of young oat hay or straw, referred to as "oat hay poisoning", was first reported by BRADLEY *et al.* (1939) in America. They experimentally showed that the ingestion of potassium nitrate produced the same symptoms and simultaneously induced severe methemoglobinemia. In an analysis of a number of samples involving toxic hay, nitrate was found present in abnormally high concentrations, 3.2−7.2% of the dry matter. They, therefore, offered this as an explanation for subsequent abortions in pregnant cows, because of asphyxia. Since then, acute nitrate poisoning has been reported in cattle (MORRIS 1958, SIMON *et al.* 1959 and DOLLAHITE *et al.* 1970), sheep (LEWIS 1951, SETCHELL *et al.* 1962 and SINCLAIR *et al.* 1967), turkeys (ADAMS *et al.* 1969), turkeys and chickens (MARRET *et al.* 1968), swine (LONDON *et al.* 1967), and rabbits (KILGOER *et al.* 1959).

On the other hand, the effects of prolonged daily nitrate or nitrite intake are not so unequivocal. With rapid progress in modern agriculture, the use of nitrogen fertilizer has been tremendously increased lately. In 1970, waste water from a potato-starch factory in the eastern part of Tokachi district was sprinkled into a pasture as fertilizer. The waste water contained excessive nitrogen and potassium so that the danger of disease from these agents was high. Therefore, blood samples were taken from cattle fed the affected grass, and levels of nitrate and nitrite with other substances were measured. The results showed an apparent higher concentration of nitrate but a normal level of nitrite. Nevertheless no symptoms of nitrate poisoning were observed in the district. In addition, incidences of this toxicosis have been reported continuously, but efforts to duplicate the symptoms with natural feeds in the laboratory are usually unsuccessful. So, the etiology of nitrate toxicity in ruminants still remains a very elusive problem.

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LEWIS (1951) found that rumen micro-organisms reduce nitrate to ammonia with nitrite as an intermediate product. WANG *et al.* (1961) using N^{15}-labelled nitrate confirmed these results in sheep and found that nitrate, nitrite and ammonia were absorbed in considerable amounts directly from the rumen into the blood. Using an artificial rumen, it was shown that the reduction rate of nitrate to nitrite depends upon the substrate existing in rumen liquor and that, in particular, glucose promoted this reduction (BARNETT *et al.* 1957).

Nitrate in the rumen is usually reduced to ammonia in a series of steps (DOLLAHITE, 1970) ;

Nitrate Nitrite Hydroxylamine Ammonia

$NO_3 \longrightarrow NO_2 \longrightarrow NH_2OH \longrightarrow NH_3$

Nitrate and its metabolites are recognized as potentially noxious substances. Especially, nitrite which is well-known as a strong oxidant oxidizes hemoglobin to mthemoglobin.

The present study was designed to provide information concerning ; (1) the reduction of nitrate to nitrite or ammonia in the rumen, subsequent changes of these elements in the blood, and the methemoglobin formation rate. (2) the degree of produced methemoglobinemia and nitrite conversion to nitrate after intravenous injection of nitrate and/or nitrite.

Materials and Methods

Two young female goats (25kg) and one heifer (180kg) were used in the present experiment. One goat was fitted with a permanent rumen fistula for direct administration of nitrate to the rumen, and the other was intact for single or combined intravenous administration of nitrate and/or nitrite.

Experiment I : The heifer was given 100gm sodium nitrate (0.55gm/kg) with a stomach tube. The goat was given three different amounts of sodium nitrate, 5 gm (0.2 gm/kg), 10gm (0.4gm/kg), and 20gm (0.8gm/kg) through the fistula. In each experiment, rumen liquor and blood were taken at one hour intervals. In order to estimate the excretion rate of given nitrate in urine during a 24 hour period as nitrate, nitrite and ammonia, the goat given sodium nitrate 10gm (0.4gm/kg) was kept in a metabolic cage. At the same time serum electrolytes such as inorganic phoshorus, sodium, calcium and magnesium were determined by atomic absorption spectroscopy.

Blood samples were taken from the jugular vein and centrifuged in less than 5 minutes to prevent oxidation of nitrite to nitrate, and clotting was prevented by heparin sodium salt. Rumen liquor was filtered with gauze within 5 minutes to remove floating matters.

Experiment II : Sodium nitrate or nitrite was intravenously administrated as single or mixed aqueous solution to the jugular vein of the intact goat. Injection forms were composed of single administrations of sodium nitrate 0.05 gm/kg or sodium nitrite 0.035 gm/kg and mixed forms of sodium nitrate 0.05gm/kg plus sodium nitrite 0.035gm/kg or

sodium nitrate 0.25gm/kg plus sodium nitrite 0.035gm/kg. Blood samples for nitrate, nitrite, ammonia and methemoglobin determination were collected at 15, 30, 60, 120, 240 and 360 minutes after injectino from the other side of the jugular vein.

Analytical methods: Nitrate and nitrite were determined by colorimetric analysis (SCHNEIDER *et al.* 1973). Ammonia was estimated by the Indo-phenol method. Methemoglobin and total hemoglobin were determined by the EVELYN and MALLOY (1938) method. Rumen liquor and urine were decolorized as soon as possible using saturated lead acetate, charcoal and saturated sodium sulfate (LEWIS 1951). Subsequent analytical methods were the same as for blood.

Results

Experiment I: The changes of nitrate, nitrite and ammonia in rumen liquor and in blood and methemoglobin were followed with a lapse of time in the heifer given sodium nitrate 100gm (0.55gm/kg) through a stomach tube (Fig. 1). The nitrate decreased rapidly within 2 hours and was restored subsequently to its original state. Whereas levels of nitrite and ammonia increased progressively for 3 hours, following that time they decreased quickly. There was an intimate relationship between nitrate degradation and its metabolite formation in the rumen. Nitrate appearance in the blood was observed immediately after

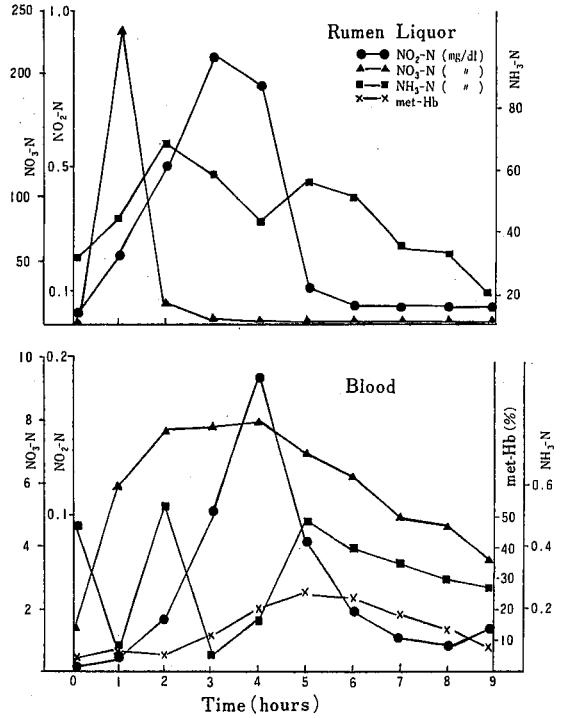

Fig. 1. Intraruminal Administration of Sodium Nitrate (0.55gm/kg) to Heifer

intra-ruminal administration and remained at a high level throughout the experiment, notwithstanding low concentrations in the rumen. Blool nitrite reached its peak at 4 hours which was an hour later than that of the rumen. Methemoglobin formation reached its maximum level 5 hours after treatment. There was no definite rising tendency for blood ammonia. Nitrite excretion in urine was detected in 3 hours, but total amounts were not determined.

To make a comparative study of nitrate doses, the same investigation was carried out on the goat with the rumen fistula. The results using three different amounts of sodium nitrate are shown in Fig. 2 to Fig. 4. Fig. 2 shows generally the same tendencies as in the heifer, excluding the marked increase of nitrite concentration within an hour. While the ruminal nitrate level fell rapidly, there was still an appreciable amount present 2 hours after dosing. A pronounced rise of ammonia was observed in the rumen. In the blood, the nitrite level peak appeared an hour before that of nitrate, and ammonia decreased slightly at an earlier stage. In the following two experiments, sodium nitrate 0.4gm/kg (Fig. 3) and 0.8gm/kg (Fig. 4), nitrate and nitrite in the rumen did not decline sharply. These prolonged disappearances of nitrate and nitrite should bring about high levels of these elements in the blood. Therefore, the extent of methemoglobin formation gradually became greater, and the maximum level attained at a later time (cf. 9 hours after 0.8gm/kg dosing). In these experiments, blood ammonia was very changeable and did not showed any constant

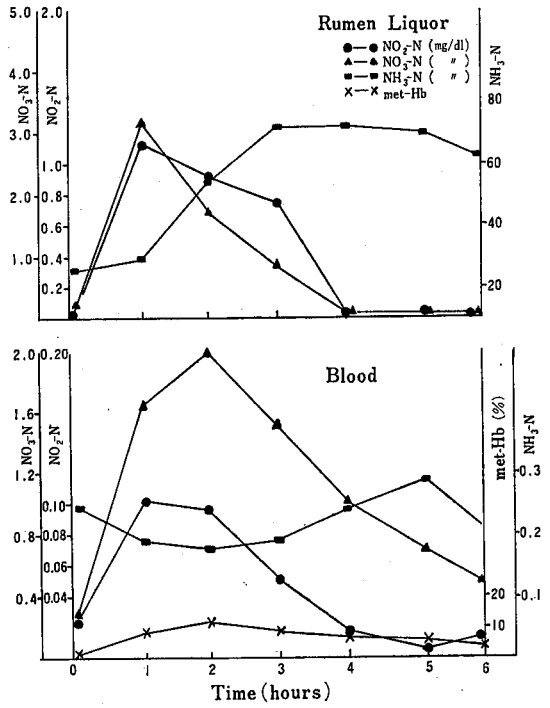

Fig. 2. Intraruminal Administration of Sodium Nitrate (0.2gm/kg) to Goat

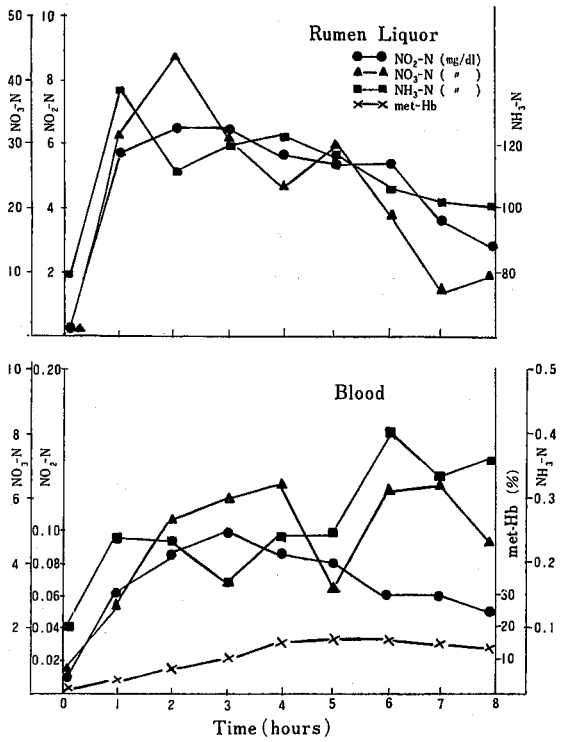

Fig. 3. Intraruminal Administration of Sodium Nitrate (0.4gm/kg) to Goat

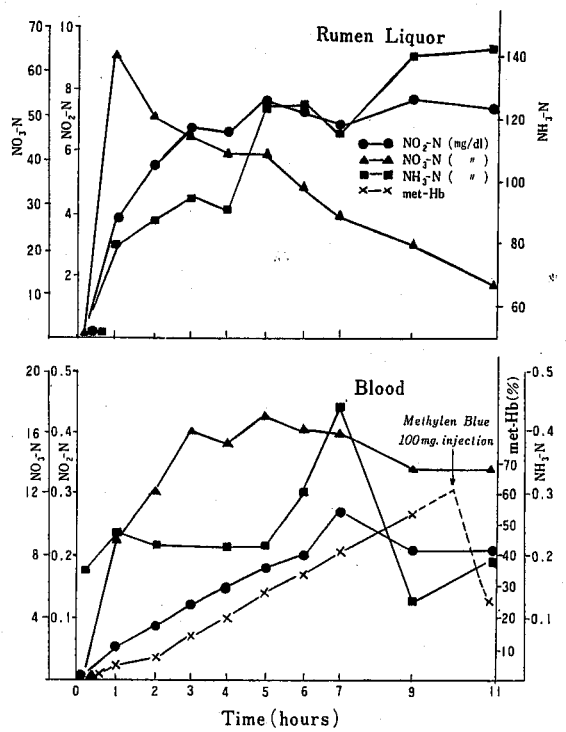

Fig. 4. Intraruminal Administration of Sodium Nitrate (0.8gm/kg) to Goat

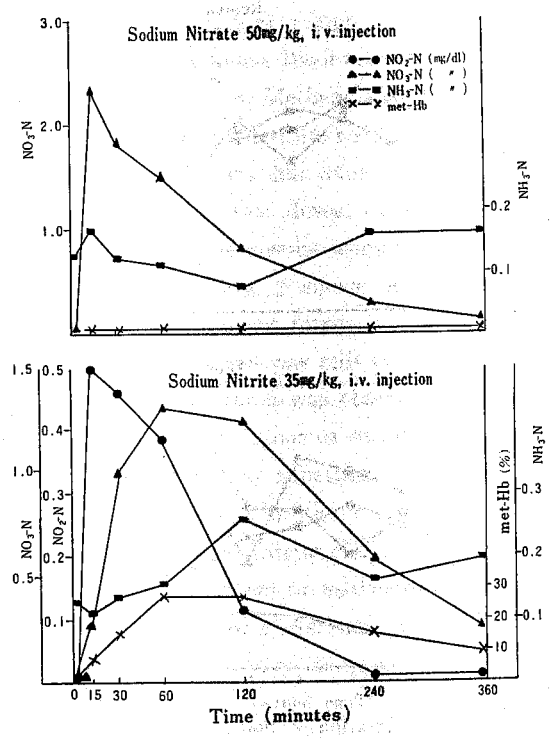

Fig. 5. Intravenous Administration of Sodium Nitrate or Sodium Nitrite to Goat

relationship. The urine excretion rate, as nitrate, nitrie and ammonia in 24 hours totaled 59%. During the experimental period, serum inorganic phosphorus, sodium, potassium, calcium and magnesium were within the normal range (not indicated in this paper). In Fig. 4 100mg of methylene blue was injected into the jugular vein because of severe methemoglobinemia with uneasiness, violent respiratory effort and progressive cyanosis. The methemoglobinemia was decreased promptly, and these conditions were improved within half an hour.

Experiment II : In this experimental series, sodium nitrite and/or nitrate was injected into the jugular vein in order to observe the level of methemoglobin and the prospective oxidation of nitrite to nitrate in the blood of the goat. The changes in blood following the administration of sodium nitrate 50mg/kg or sodium nitrite 35mg/kg are shown in Fig. 5. In the case of nitrate dosing, nitrate almost disappeared from the blood in 6 hours, and there was scarcely any nitrite or methemoglobin throughout the experiment except for normal ammonia levels. The maximum level of methemoglobin (26%) was observed within 60 minutes after nitrite dosing and associated with nitrate subsequently decreased slowly. There was a slight increase of ammonia. It was very interesting to find in the nitrite-only experiment that a large amount of nitrate appeared in spite of nitrite injection and administrated nitrite vanished within 4 hours, but generated nitrate remained at a fairly high level even after 6 hours. The next experiment was the administration of sodium nitrite 35 mg/kg plus sodium

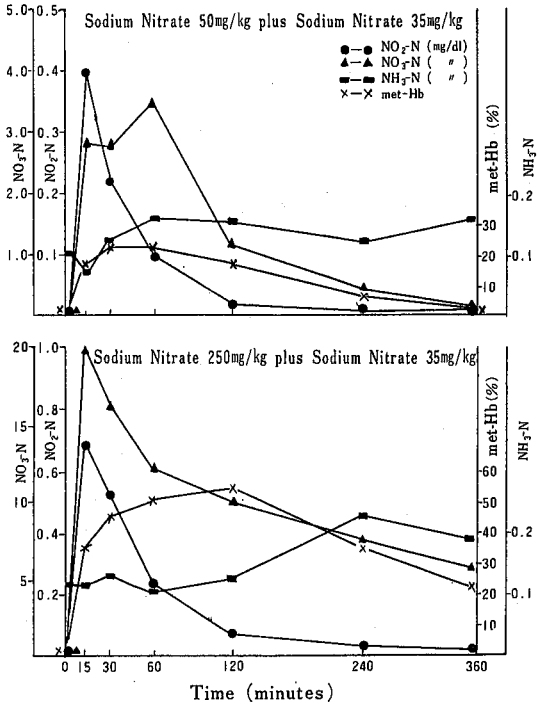

Fig. 6. Intravenous Administration of Sodium Nitrite Plus Sodium Nitrite to Goat

nitrate 50mg/kg (Fig. 6). This result also showed an apparent peak of nitrate within 60 minutes after dosing. The greater dose of sodium nitrate (25mg/kg) with nitrite (35mg/kg) induced the high level of methemoglobin (54%) which was about twice as much as the lower nitrate dose. When nitrate and nitrite were injected simultaneously, an identical pattern with that of nitrite alone was obtained with the exception of the nitrate concentration which had an additional level and two peaks (Fig. 6). If an excessive amount of nitrate was associated with the defined nitrite in the blood, it may be suggested that the nitrate sustained a high level and accelerated the methemoglobin formation.

Discussion

It is evident that nitrate is reduced to ammonia by the micro-organisms in the rumen and nitrite as an intermediate in these reduction process participates in methemoglobin formation in the blood. Present studies emphasize the very rapid conversion of nitrate into nitrite and ammonia in the rumen. The dense microbial flora maintain reducing conditions and can reduce added nitrate without delay. Fig. 1 showed the rapid disappearance of nitrate from the rumen in the heifer which was accompanied by a maximum concentration of ammonia and nitrite within 3 hours. There is no question that this conversion rate was controlled by following factors : nitrate concentration, population and variety of microbial

flora and ruminal conditions, etc. WANG *et al.* (1961) estimated that about 14% of the added nitrate was converted into nitrite in cattle, but in this experiment data are not sufficient to account accurately for the amount of nitrite formed in the rumen. Further, the concentration of nitrate persisted in the blood beyond the time of precipitous decrease in rumen nitrate. The maximum concentration of nitrite in the rumen appeared about 3 hours after the addition of nitrate, and the maximum conversion of hemoglobin to methemoglobin occured at almost the same time. However, very little delay on methemoglobin formation would be expected once the nitrite reached the blood stream. The data supported the idea that the bulk of the nitrite is absorbed directly from the rumen rather than from lower sections of the digestive tract. The virtual coincidence of attaining maximum nitrite concentration in the rumen and a maximum methemoglobin level in the blood indicates that the absorption is very rapid. The maximum concentration of blood nitrite-nitrogen was 0.19mg/dl at 4 hours which was 22% of the rumen concentrations. This absorption rate from the rumen substantially coincides with the report by LEWIS (1951).

The results of three different amouts in sodium nitrate administration through the rumen fistula to the goat (Fig. 2-4) showed about the same tendency as the heifer. Increasing the dose of nitrate led to a marked accumulation of nitrite in the rumen and a sharp increase in the extent of methemoglobinemia. Fig. 3 and Fig. 4 showed the existence of nitrite for a longer period and a large amount of ammonia in the rumen. This indicates that the reduction of nitrite to ammonia may be a rate limiting factor involving nitrate reduction. It is possible that most of the ammonia produced later would possibly be less utilized owing to saturation of this mechanism and the rise in concentration would be greater in proportion to the disappearance of nitrate, on the whole fitting with the observed steady rise in ammonia over several hours.

Since the blood nitrite level correlates intimately with the methemoglobin rate (Fig.7), eventually methemoglobinemia will depend on the potentiality of ruminal micro-organisms reducing nitrate to nitrite. It can't be considered negligible, however, that those blood levels are also reflected on the excretion velocity into urine. The urine excretion rate of the goat for 24 hours totalled about 59.3% together with nitrate, nitrite and ammonia (49.3, 0.0008 and 0.032% respectively). This result almost agrees with the report by KAMEOKA *et al.* (1935) who found that when nitrate was fed to rabbits as 2% of their diet about 50% of the added amount of nitrate was excreted into urine. Similarly, KILGORE (1959) reported that it was 39-44% in rabbits. Yet, LEWIS (1951) administrating nearly the same dose to sheep reported only 4-8% of the nitrate lost from urine an 11 hour period. Surely, there is a large difference between non-ruminants and ruminants depending upon the microbial conditions. There is need of further investigation on this problem. Although the nitrite excretion rate is very low compared with nitrate, a large part of it may be converted into nitrate in the blood as discussed below.

The effectiveness of methylene blue was confirmed when the highest amount of

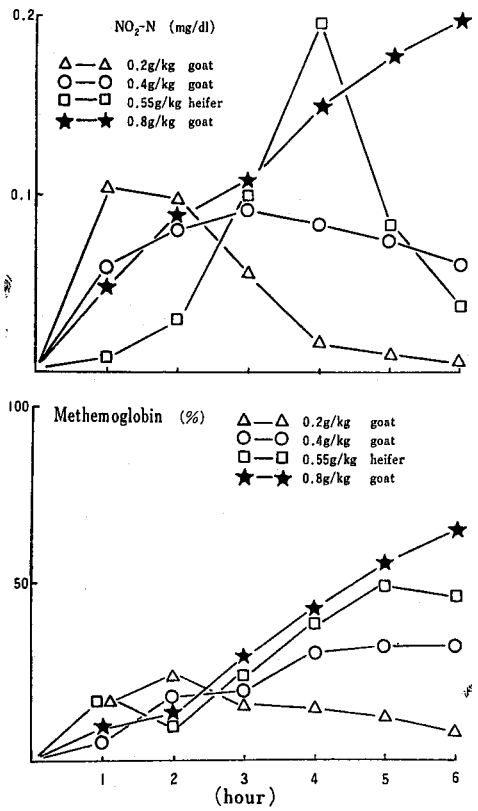

Fig. 7. The Relation between Plasma Nitrite and Methemoglobin by Intraruminal Administration of Sodium Nitrate.

nitrate was added to the goat (Fig. 5). At this time, the methemoglobin level decreased quickly but nitrite and nitrate concentrations in blood were not affected. Accordingly it may be suggested that methylene blue works on the reduction of methemoglobin specifically.

When nitrate was administered intravenously to the goat (Fig. 6), neither nitrite nor methemoglobin was found. This result shows added nitrate did not change to nitrite in the blood. On the other hand, administration of nitrite showed the coincidence of nitrite decline with nitrate appearance, and this seems to couple with methemoglobin formation. Therefore, nitrate itself is an innoxious or inert substance in blood, but nitrite having active chemical properties is very harmful to animals. It is obscure whether methemoglobin protects from its very tsrong oxidative action. Under the higher concentrations of nitrate, this nitrite oxidation was manifestly prevented by it, accounting for severe methemoglobinemia. It is easily conceivable that there is some enzymatical equilibrium among them.

Summary

Nitrate placed in the rumen was rapidly reduced to nitrite and ammonia. They passed

directly from the rumen to the blood stream. The rate of methemoglobin formation depends upon the added amount of nitrate and was closely proportional to the blood nitrite level. The excretion rate of administrated nitrate in urine totalled 59% in the goat over a 24 hour period.

Nitrite injected into the blood was immediately converted into nitrate, but the reverse reaction was not found. If higher concentrations of nitrate exist in blood, this nitrite oxidation was certainly prevented by it, accounting for severe methemoglobinemia.

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摘　　要

反芻家畜における硝酸塩の代謝とメトヘモグロビン血症の関係を明らかにするために，未経産牛と山羊を用い，硝酸塩の第一胃内投与ならびに硝酸塩・亜硝酸塩の静脈内投与を試み，以下の成績を得た。

ルーメン内に投与した硝酸塩はミクロフロラにより亜硝酸塩とアンモニアに還元された。また，投与量の増加に伴い，ルーメン内・血中ともに両者の増量とメトヘモグロビンの増加傾向が認められた。投与後におけるルーメン内亜硝酸塩のピークは血液内と一致し，亜硝酸塩はルーメンより速やかに血液中に吸収されるものと推測される。この実験で，投与した硝酸塩の59%が24時間尿に排泄された。

硝酸塩の単独静脈内投与で，亜硝酸およびメトヘモグロビンは全く形成されない。したがって血液内において，硝酸塩は安定な物質と思われる。一方，亜硝酸塩投与の場合，明らかに硝酸塩への酸化が認められた。この時，高濃度の硝酸塩が共存すると，この亜硝酸塩の酸化は抑制される。

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