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Nitrate poisoning in cattle. 2. Changes in nitrite in rumen fluid and methemoglobin formation in blood after high nitrate intake

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Summary

Experiments have shown that the daily supply of equal doses of nitrate to cows, as hay with a high nitrate content or as potassium nitrate, will induce higher nitrite contents in the rumen fluid and a higher percentage of methemoglobin in the blood during the first days, after which they remain on this higher level. These increases are probably due to a change in the activity of the reducing micro-organisms in the rumen. The changes also partly explain the controversial data in the literature on the acceptable dosages of nitrate to be supplied to ruminants. This may have led to the mis-interpretation that ruminants should tolerate daily intakes up to 90 g of NO_3^- per 100 kg body weight.

Introduction

In the Netherlands the incidence of nitrate poisoning in cattle is increasing. It occurs especially in cattle on rations mainly consisting of turnips, hay or wilted silage, which may have nitrate contents up to 6 % NO_3^- in the dry matter and over (Kemp et al., 1976). In the rumen micro-organisms can reduce nitrate to ammonia, and nitrite is an intermediate in this reaction. The nitrite is partly absorbed from the rumen and converts hemoglobin in the blood into methemoglobin, reducing oxygen transportation.

Data in the literature on the acceptable quantities of nitrate to be supplied to cattle vary widely. For instance, some authors mention 10 g NO_3^- per 100 kg body weight as a maximum daily nitrate intake; while others consider a daily intake of 90 g of nitrate possible (Crawford, 1960). Besides the various authors proceeding from diverse criteria, there are other reasons for this discrepancy. Up till now little attention was paid to the effect on the formation of methemoglobin of the rate at which the nitrate-containing rations are consumed by the animals. There is also evidence that equal dosages of nitrate may lead to diversity in the methemoglobin levels, possibly brought about by the effect of the rations on the pH value of the rumen content. Another cause for differences in the maximum acceptable nitrate dose may be related to too few blood samples having been taken during the experiments to establish reliable changes in the methemoglobin level. Differences in the method of preserving and analysing the samples may also have been of influence.

In this paper it will be demonstrated that the length of the period in which equal dosages of nitrate were supplied to the animals may have a considerable effect on the methemoglobin contents in the blood. The results of the three experiments described show that after the first dose of nitrate appreciably less nitrite was formed in the rumen than after supplying nitrate in equal dosages during several successive days. Also the blood methemoglobin level on the first day after application was considerably lower than on the subsequent days, when a change had taken place in the microbial activity in the rumen.

Experiments

Three experiments were carried out, viz N 20, N 27 and N 35 with 4, 4 and 6 non-lactating Friesian cows, respectively, in the age of 3-8 years and with a body weight of 415-669 kg. In N 20 and N 27 a few animals were fitted with a rumen fistula. All the animals had a catheter in the jugular vein during the experiments. The rations consisted of hay and concentrates. The intake of feed and water were recorded continuously and automatically.

Details of the rations fed

Exp. N 20 lasted 18 days. Two times a day, viz at 07h30 and at 19h30 the cows were supplied with concentrates. At 07h45 and at 19h45 the cows were provided with hay low in nitrate. The hay feeds were consumed in 75 minutes at a maximum. From the second day up to the eighteenth all the animals received once daily potassium nitrate at 08h30. Data on the intake of dry matter and on the dosages of nitrate as potassium nitrate are mentioned in Table 1.

Exp. N 27 also lasted 18 days, the rations consisting of hay and concentrates as well. However, from the second day at 08h15 nitrate-rich hay was supplied and at 17h15 hay low in nitrate. All the animals received concentrates at 08h00 and at 17h00. In the morning the hay was consumed within 45 minutes.

Exp. N 35 lasted 6 days, the rations consisting of hay low in nitrate and concentrates. The concentrates were administered at 07h30 and at 17h15. Cows 1 and 2 were provided once daily with potassium nitrite from the second up to the sixth day of the experimental period. Potassium nitrite was supplied to cows 3, 5 and 6 from the second up to the fourth day; cow 4 received the same from the second up to the fifth day.

Table 1. Average daily intake of dry matter and of nitrate and nitrite per meal.

Exp. No	Cow No	Body weight (kg)	Concentrates (kg dry matter per 100 kg body weight per day)	Hay (kg dry matter per 100 kg body weight per day)	NO_3^- intake (g per 100 kg body weight per meal)	NO_2^- intake (g per 100 kg body weight per meal)
N20	1	655	0.3	0.8	16 } 15 g	
	2	669	0.3	1.0	16 } in the	
	3	577	0.3	1.0	16 } form of	
	4	549	0.3	1.0	16 } KNO_3	
	1	576	0.25	1.0	12.4 } in the	
	3	510	0.25	1.0	15.4 } form	
	4	550	0.25	1.1	14.8 } of	
	6	465	0.25	1.1	15.2 } hay	
N35	1	525	0.5	1.3		2 }
	2	565	0.5	1.3		2 } in the
	3	515	0.5	1.3		3 } form
	4	475	0.5	1.3		3 } of
	5	460	0.5	1.3		3 } KNO_2
	6	415	0.5	1.3		3 }

Experimental routine and sampling

From two weeks before until the first day of the experimental period the rations in the three experiments consisted of hay low in nitrate and concentrates. With the hay low in nitrate and the concentrates a maximum of 1 g of NO_3^- per 100 kg body weight was supplied per animal per meal. During Exp. N 20 and N 35 the animals received the similar hay as during the pre-periods. In Exp. N 27 only the hay administered at 08h15 was replaced by nitrate-rich hay. Before starting the experiments the hay and the concentrates required were thoroughly mixed, sampled and weighed out in separate portions per experimental cow per feed. The doses KNO_3 and KNO_2 (experiment N 20 and N 35, respectively) mentioned in Table 1 were supplied to the cows fitted with a rumen fistula at the indicated times via this fistula, after solving in 2.5 litres of water. An esophagus probe was used to bring the solution into the rumen of the animals without fistulas.

During some days of the experiments N 20 and N 27 the rumen fluid was sampled very frequently. This took place every 15 minutes, when rapid changes in the nitrate and nitrite contents were to be expected. The samples were taken by means of a perforated plastic pipe in the ventral rumen sack, connected by a tube via the fistula with a pump mounted next to the animal. A return tube leading from the pump to within the fistula enabled some rumen fluid to be circulated before tapping 40 ml of it through a tap in the circuit. This sampling technique, which did not disturb the animals, took one minute per sample at most.

A few days before the experimental periods all the animals were fitted with a catheter in the jugular vein. During the experiments blood samples were taken very frequently in order to establish the changes in the methemoglobin contents accurately. With those animals sampled for rumen liquid as well as blood this took place at the same time. To prevent blockage of the catheters, about 2 ml of heparin solution (1 %) were injected into the catheters after sampling.

Per sampling a maximum of 5 to 6 ml of blood was taken; the first blood contaminated with heparin was not analysed.

When great changes were to be expected in the methemoglobin content in the blood, samples were taken every 15 minutes. With decreasing methemoglobin contents in the blood samples were taken every hour.

Chemical procedures

Ration. During 30 minutes 1 g of dried sample was extracted at room temperature in about 70 ml of water in a shaking machine.

The volume was made up to 100 ml. The nitrate content of the extract was determined with an Orion 92-07 ion selective nitrate electrode and reference electrode. A calibration curve was constructed with 0.2-20 m*M* nitrate solutions.

Rumen fluid. Immediately after taking the rumen liquid samples the pH was determined. Then 40 ml of rumen fluid were added to 10 ml of a saturated lead acetate solution (about 30 %). The samples were kept at 3 °C and at the soonest possible moment analysed for nitrate, nitrite and ammonium. A previous investigation had shown that samples preserved in this way could be stored for at least some weeks without the contents changing.

After removal of the excess lead acetate with a measured amount of a 10 % sodium sulphate solution, the nitrite content was determined in an automatic analyser with sulphanilamide and naphtylethylenediamine as reagents.

Nitrate was determined in a similar way after reduction of the nitrate with hydrazine to nitrite and correction for the nitrite content of the sample.

Ammonium was determined in an automatic analyser with salicylic acid and dichloroisocyanurate as reagents to form a yellow indophenol compound (Bietz, 1974).

Blood. The blood hemoglobin content was determined according to van Kampen & Zijlstra (1961). Hemoglobin and methemoglobin are converted to methemoglobin cyanide by the addition of 5 ml of a solution of potassium cyanide and potassium hexacyanoferrate to 0.2 ml of blood. Extinction was measured at 540 nm, the molar extinction was 11.0 cm^2 per μmol.

The methemoglobin content of the blood was determined directly after sampling with the method originally described by Evelyn & Malloy (1938). To 0.3 ml of blood 20 ml of a 1/15 *M* phosphate buffer of pH 6.9 was added. Hemolysis was accelerated by the addition of one drop of a 10 % saponine solution. The extinction at 630 nm was measured before and after addition of one drop of a 10 % KCN

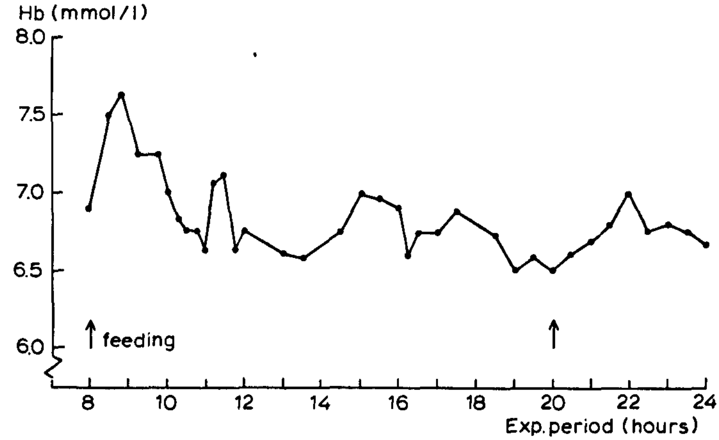

Fig. 1. Changes in the hemoglobin content of a cow during the day.

solution. Methemoglobin is calculated from the extinction difference at 630 nm, the extinction difference proved to be 3.25 cm^2 per μmol. Methemoglobin was expressed in percentages of the hemoglobin content in the same sample. The hemoglobin content was determined in all the blood samples, because in the same animal relatively great changes in the hemoglobin content may occur within one day. An example of this is given in Fig. 1.

Results and discussion

Fig. 2 shows the trend in the nitrate and nitrite contents of the rumen liquid as well as the percentages of methemoglobin in the blood of one of the experimental animals after consuming hay rich in nitrate. Data on the other experimental cows correspond. The great number of analyses enabled the shape of the curve and the maximum value to be determined accurately. The graph clearly indicates that samples should be taken every 15 minutes, when changes in the composition of the rumen fluid and of the blood are brought about so rapidly. With an intake of 12.5 g of NO_3^- per 100 kg body weight, consumed with the hay in 45 minutes, the maximum nitrate content of almost 9 mmol of NO_3^- per litre of rumen fluid occurred some 30 minutes from the outset of feeding. The highest value of nitrite was 2.5 mmol/litre and occurred one hour after the highest value of nitrate was attained. When the same dosage of nitrate, as potassium nitrate solution, was supplied all in at the same time through the rumen fistula, the nitrate concentration in the rumen liquid was somewhat more irregular at the beginning, because mixing was still insufficient. The nitrite concentration, however, corresponded to that after the intake of nitrate-rich hay.

The methemoglobin content in Fig. 2 shows that at this nitrate intake the maximum methemoglobin percentage was about 18, which is 1.3 mmol/litre, attained

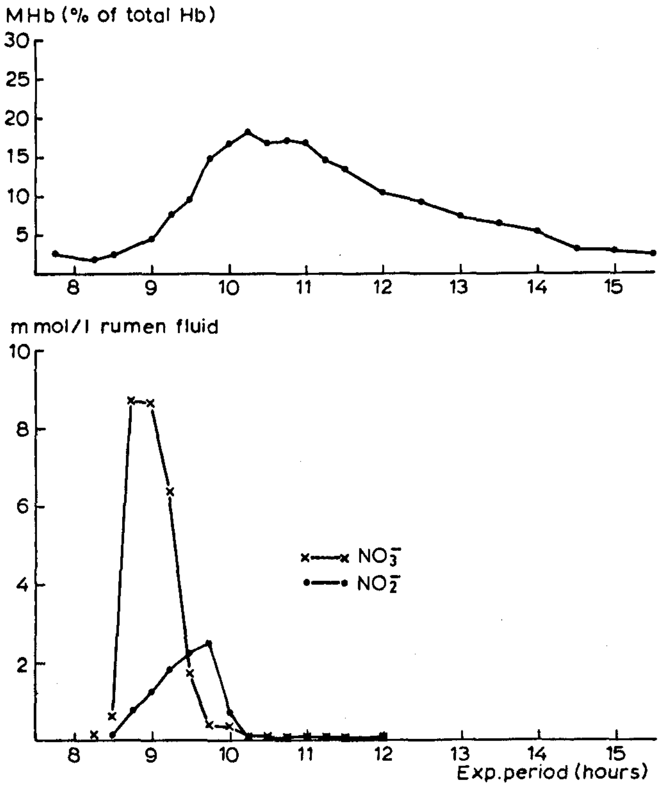

Fig. 2. Changes in the contents of nitrate and nitrite in the rumen fluid and in methemoglobin content of a cow after feeding nitrate-rich hay (Exp. N27, cow 1, day 4).

some two hours from the outset of consuming the nitrate-rich hay. Data on a great number of feeding experiments, which will be published later on, give more details on the relation between the nitrate intake and the methemoglobin formation and also at the moment when the highest values will occur. The highest methemoglobin percentage was always attained when the nitrite concentration in the rumen fluid had decreased to almost zero.

Fig. 3 shows the highest contents of methemoglobin, measured in four experimental animals during 18 days in Exp. N 20. These cows were supplied from the second day with 16 g NO_3^- per 100 kg body weight, of which 15 g were given as potassium nitrate. Of two of these animals the graph also indicates the highest contents of nitrite in the rumen fluid on five different days during the experimental period. All these highest values were determined in the way demonstrated in Fig. 2. On the first day on which no additional nitrate was supplied, normal contents were found in the rumen liquid as well as in the blood: in the rumen liquid 0.25 mmol of NO_2^- per litre and in the blood 2 to 3 % methemoglobin. During the first four experimental days the highest contents of methemoglobin in all four experimental cows

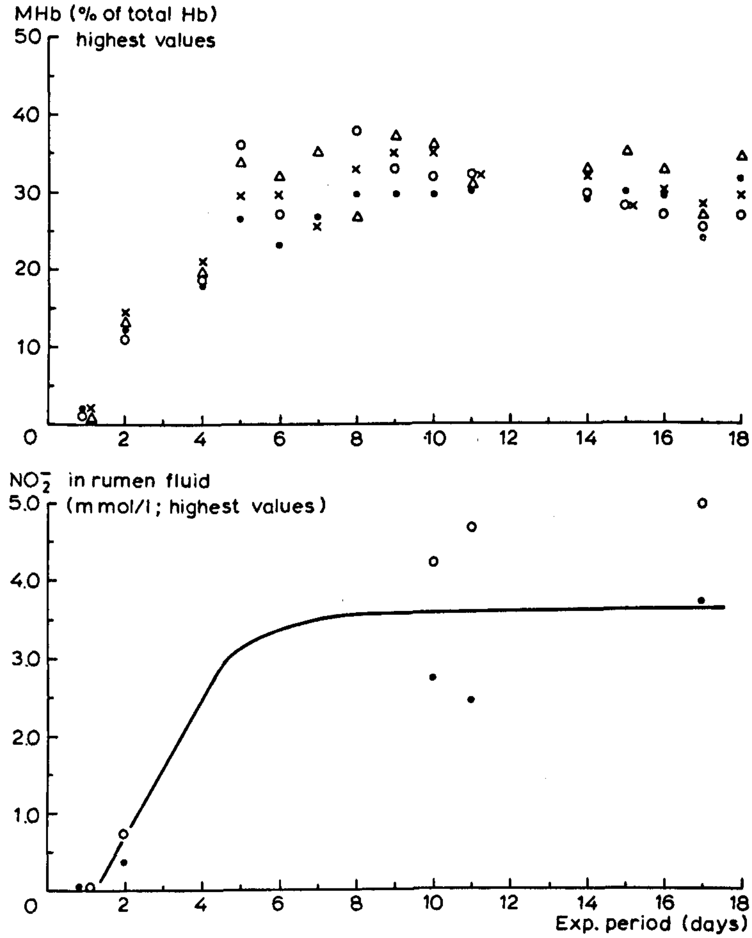

Fig. 3. Changes in the content of nitrite in the rumen fluid and in methemoglobin content after daily supplies of equal dosages of potassium nitrate (Exp. N20).
● cow 1; ○ cow 2; × cow 3; △ cow 4.

was found to increase appreciably from day to day. This increase can be explained by an increase in the maximum nitrite contents in the rumen fluid. After the first four days the maximum methemoglobin percentages were maintained at a level of about 30; the nitrite contents in the rumen liquid neither changing distinctly after this.

As a confirmation of the results of experiment N 20, Fig. 4 shows the changes in methemoglobin contents during the first days after administering the nitrate-rich hay (Exp. N 27). In this experiment the maximum methemoglobin contents in the blood were considerably raised during the first days and remained at some 23 % after two days of administering the nitrate-rich hay. The higher methemoglobin

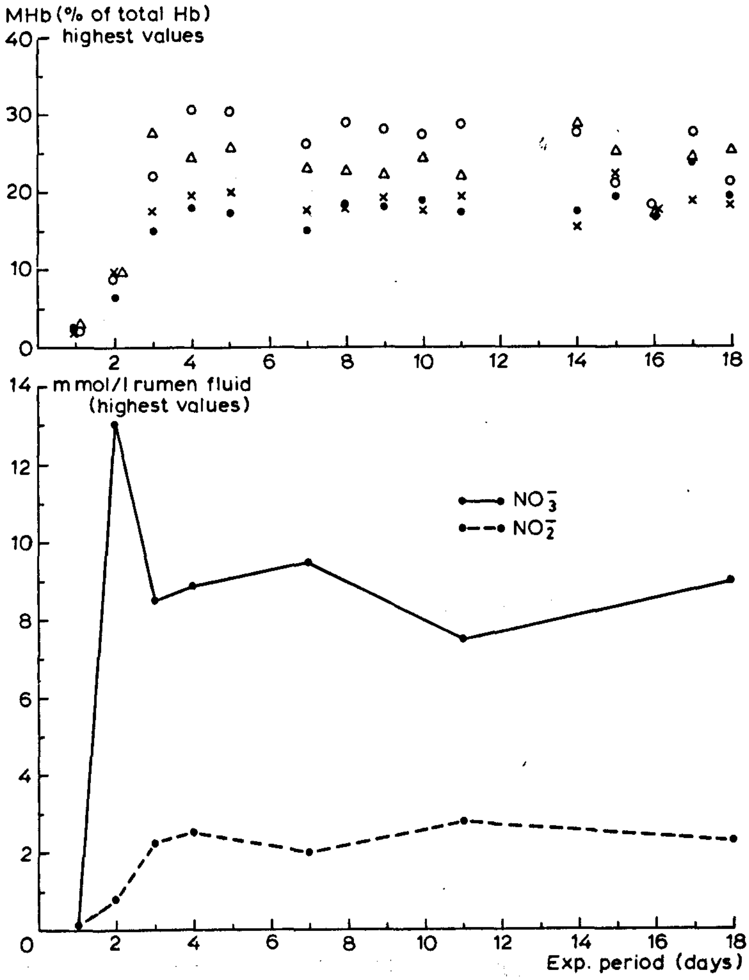

Fig. 4. Changes in the contents of nitrate and nitrite in the rumen fluid of cow 1 (bottom) and in methemoglobin content (top) after daily supplies of almost equal amounts of nitrate-rich hay (Exp. N27).
● cow 1; ○ cow 3; × cow 4; △ cow 6.

contents in the blood during the first days are associated with the also rising maximum nitrite contents in the rumen fluid. The maximum nitrate concentrations decrease. Besides between-animal differences, the variable nitrate intake with the hay, as shown in Table 2, is responsible for the variation in methemoglobin contents. Thus, the lowest maximum methemoglobin contents in cows 1 and 4 were found to be associated with the lowest nitrate intakes.

Comparison of the data in Fig. 3 and 4 points out that the differences in methemoglobin contents between these two experiments might also be explained by the variations in r'trate intake. In experiment N 20 this intake was somewhat greater

Table 2. Daily intake of NO_3- in g/100 kg of body weight (Exp. N27).

Day	Cow 1	Cow 3	Cow 4	Cow 6	Day	Cow 1	Cow 3	Cow 4	Cow 6
1	1.0	1.0	1.0	1.3	10	12.5	15.7	14.9	14.8
2	12.1	14.0	15.0	16.1	11	12.3	15.9	14.7	14.9
3	12.3	15.9	14.8	15.8	12	12.6	16.2	15.0	16.0
4	12.5	15.8	14.9	14.8	13	12.6	16.2	15.0	16.0
5	12.6	16.0	14.5	15.0	14	12.6	15.7	14.7	15.3
6	12.6	15.1	14.8	15.3	15	12.5	14.2	14.8	14.1
7	12.6	14.9	14.6	15.8	16	12.5	14.5	14.8	14.9
8	12.6	15.9	14.9	15.0	17	11.9	15.8	14.5	14.8
9	12.6	16.2	15.0	15.1	18	11.9	14.9	14.6	15.2

than in Exp. N 27. The rising maximum nitrite concentration in the rumen fluid is more evident in Fig. 4 than in Fig. 3, because during Exp. N 20 the rumen liquid was sampled on too few days. The changes occurring in the maximum nitrite contents are also represented by a line in Fig. 3; this is based on the data in Fig. 4 and also on the relation between the maximum contents of nitrite in the rumen fluid and the blood methemoglobin, to be discussed hereafter. In this respect we state that in both experiments the highest ammonium contents in the rumen fluid did not change distinctly throughout the experimental period. In the light of more detailed data this aspect will be studied more closely in a later stage. In the experiments described here, the pH of the rumen liquid varied between 6.3 and 7.0, significant differences could not be demonstrated between the two experiments.

Striking is that the differences between the maximum methemoglobin contents after dosing of potassium nitrate at the same time and after the considerably slower consumption of the nitrate-rich hay do not show a much greater difference. These variations would have been larger if the rate of hay intake had been much lower. The methemoglobin formation will only be affected when equal amounts of nitrate are consumed at largely different rates of intake. More detailed data on this aspect will be reported later. The relations between the maximum nitrite contents in the

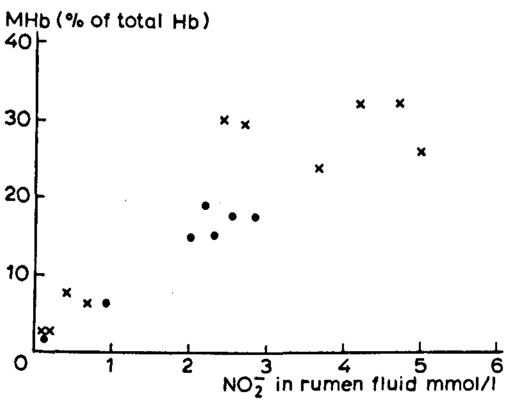

Fig. 5. Relation between the highest values of nitrite in the rumen fluid and of methemoglobin in the blood (Exp. N20 and N27).

rumen fluid and, in the same animals, the highest methemoglobin values in the blood established at the same time, are shown in Fig. 5. With the rising nitrite contents in the rumen fluid higher methemoglobin contents are to be expected in the blood.

Fig. 6 gives data on 32 feeding experiments carried out up till now, with lactating and non-lactating cows on rations consisting of hay ranging from 0.2 % to over 6 % of NO_3^- in the dry matter; hay + concentrates; hay + KNO_3; fresh turnips; maize silage + KNO_3, and freshly mown grass. When hay was supplied, the experimental animals were allowed to consume hay for up to one hour per meal, for the other rations for up to two hours per meal.

In the relation shown in Fig. 6 between the highest contents of nitrite in the rumen liquid per animal per day and the highest percentages of methemoglobin in the blood occurring in the same animals on the same day, all the highest values were analysed in the same way as described in Fig. 2. The correlation coefficient (r) was 0.92.

In view of the high correlation coefficient of this relation, it is unlikely that factors other than the nitrite concentration in the rumen fluid can be of much interest in the interpretation of variations in the methemoglobin contents. Nevertheless, with a given nitrite content in the rumen liquid the maximum methemoglobin percentages can still vary widely. However, the present methodology is incomplete.

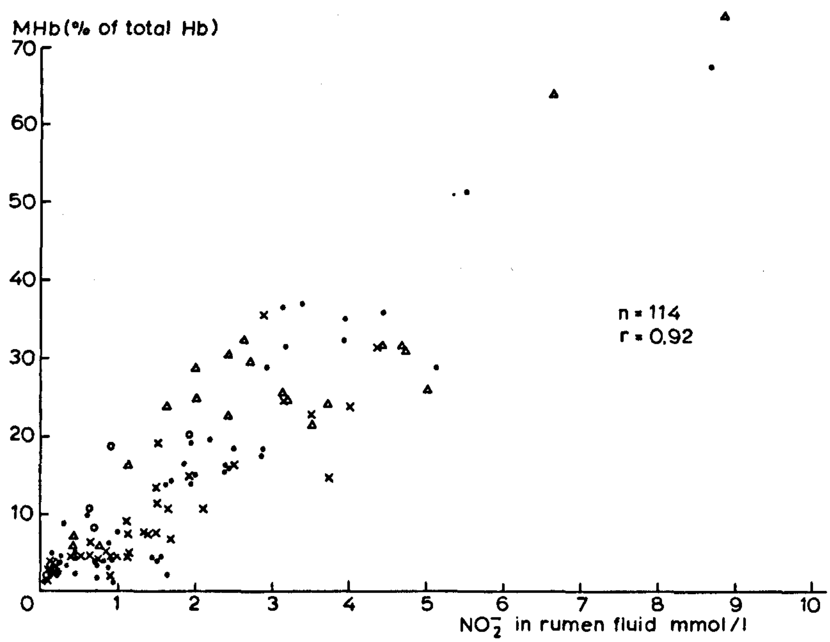

Fig. 6. Relation between the highest values of nitrite in the rumen fluid and of methemoglobin in the blood of cows fed with various rations.
● hay; × turnips; △ KNO_3; ○ freshly mown grass.

Cow						
I	0	2	2	2	2	2
II	0	2	2	2	2	2
III	0	3	3	3		
IV	0	3	3	3	3	
V	0	3	3	3		
VI	0	3	3	3		

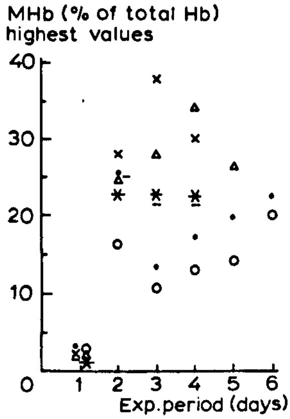

Fig. 7. Daily intake of NO_2^- in g/100 kg body weight (top) and the effect of it on the methemoglobin content in the blood of cows (bottom) (Exp. N35).
● cow 1; ○ cow 2; × cow 3; △ cow 4; – cow 5; * cow 6.

For instance, not only the height of the maximum nitrite concentration in the rumen fluid will determine the amount of nitrite passing through the rumen wall; the time during which these increased nitrite contents occur in the rumen liquid is also important. However, a part of the variation in the maximum methemoglobin contents may also be due to between-animal differences in the blood hemoglobin contents, but also to within-animal fluctuations in this content (see Fig. 1). Moreover, based on various considerations, it is likely that the relations shown in Fig. 5 and 6 are not linear.

These differences in the effects of nitrate dosages on the formation of methemoglobin in the blood during the first days, evidently do not occur when an equal dose of nitrite is supplied during a few days (Exp. N 35). In Fig. 7 (bottom) it is demonstrated that there were no significant changes in the level of the maximum contents of methemoglobin from the first dose of potassium nitrite during the experimental period. The day-to-day differences at equal doses of nitrite are sometimes rather great. The dosages of nitrite mentioned in Fig. 7 (top) can partly account for the between-animal differences. Since the rising methemoglobin percentages in the blood do not occur with equal doses of nitrite supplied during successive days, while changes do occur when nitrate is supplied, this argues in favour of changes in the activity of the rumen micro-organisms.

Data in the literature on the changes occurring in the formation of methemoglobin after supplying additional nitrate on subsequent days, are rather few. In the work of Jamieson (1959), Simon et al. (1959), Davison et al. (1962), McIlwain et al. (1963), Winter et al. (1964) and Davison et al. (1965), sometimes done with another object in view, the sampling of rumen fluid and blood were mostly carried out at intervals of one or more weeks. Nakamura et al. (1976) record data on changes occurring in the methemoglobin content in the blood of sheep during the first week after dosing nitrate. These results from sheep correspond closely to our findings in cows.

The controversial data in the literature on the acceptable nitrate dosages to be supplied to ruminants can partly be explained from conclusions which have been drawn from one-day experiments. This may have led to the mis-interpretation that intakes up to 90 g NO_3^- per 100 kg body weight per day should still be acceptable. To draw practical implications from the relation between intake of nitrate and formation of methemoglobin in the blood, it is necessary to base this on data obtained from adapted animals. Moreover, the acceptable dosage of nitrate to be supplied should be expressed per feed rather than per day.

Besides, in studying these relations it is essential to sample the rumen liquid and the blood at short intervals, since these processes take place so rapidly. A great number of data from many samples will give more reliable information on the effect of a treatment applied.

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