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Metabolism of Nitrate by Cattle

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(Received 21 April 1961)

Nitrate toxicity has been observed frequently in ruminants fed on a variety of plant materials high in nitrate (Bradley, Beath & Eppson, 1939; Grisby & Farwell, 1950; Newson, Stout, Thorp, Barber & Groth, 1937; Savage, 1949; Whitehead, Viets & Moxon, 1948; Whitehead & Moxon, 1952). Davidson, Doughty & Bolton (1941) described the toxicity symptoms in nitrate poisoning in detail. Bradley *et al.* (1939) indicated that the symptoms of nitrate toxicity are associated with the high levels of methaemoglobin and hence the low oxygen-carrying capacity of the blood of affected animals. Lewis (1951) demonstrated that micro-organisms in the rumen of sheep reduce nitrate to ammonium ions with nitrite as an intermediate; enough nitrite was transferred to the blood to convert a considerable fraction of the haemoglobin into methaemoglobin. Sund, Wright & Simon (1957) reported that a non-contagious abortion observed among cattle pastured on marsh land in central Wisconsin apparently was a manifestation of nitrate toxicity.

The work presented here was designed to study the time-course of nitrate reduction in the rumen, the changes induced in the blood by metabolites of nitrate, and the disposition of [^{15}N]nitrate by the animal.

MATERIALS AND METHODS

The experimental animals were 700–800 lb. Hereford cows with permanent rumen fistulas which were fitted with a plug. A known amount of KNO_3 in 500 ml. of water was

added through the rumen fistula each morning before the cow was offered hay, concentrates and water *ad lib.* The conversion of haemoglobin into methaemoglobin in the blood was determined daily about 3 hr. after the administration of KNO_3 . In the experiments with ^{15}N , a solution of K^{15}NO_3 was added to the rumen through the fistula in a cow which showed about 15% methaemoglobin in its daily samples of blood. Throughout the day that K^{15}NO_3 was added, periodic samples were taken of rumen liquor, blood and urine. Blood samples were removed from the jugular vein. Analysis for methaemoglobin was performed immediately on freshly drawn blood, and the remaining blood was treated with sodium oxalate to prevent clotting. Urine samples obtained with a catheter were preserved with toluene in a refrigerator until analysed. The rumen liquor withdrawn through the fistula was strained through 2 layers of cheese cloth; it then was clarified by the method of Lewis (1951), and the clear supernatant solution was used for analysis.

Analyses for nitrate, nitrite, urea and ammonia in the rumen liquor, blood and urine were made by the methods described in the A.O.A.C. handbook (Lepper, 1945). ^{15}N was determined with a Consolidated-Nier isotope ratio mass spectrometer. Methaemoglobin and total haemoglobin were determined by the method of Evelyn & Malloy (1938). The blood sample treated with oxalate was centrifuged, and the cells were washed twice by centrifuging from 0.85% NaCl and then were refrigerated until used. Samples (10 ml.) of the supernatant fluid were stirred while to each were added 3 ml. of 0.5 M- $\text{Ba}(\text{OH})_2$ and 3 ml. of 0.5 M- ZnSO_4 to precipitate the proteins. The precipitate was removed by centrifuging, and the clear supernatant fluid was used for analysis of ammonium, nitrite and nitrate ions.

Haemin and globin were separated and purified essentially as described by London, Morell & Kassenaar (1958).

The washed red cells were lysed in cold deionized water, and the lysed suspension was filtered with stirring into 12 vol. of chilled acetone containing 1.2 ml. of 12N-HCl/100 ml. The precipitated globin was filtered and then washed with chilled acetone. The washings were added to the filtrate carrying the haemin in solution. The globin was purified by dissolving the crude precipitate in water, adding 14% trichloroacetic acid to complete precipitation, and washing the new precipitate twice with 7% trichloroacetic acid and once with distilled water. The globin was dissolved in N-NaOH, and the precipitation and washings were repeated. The precipitate finally was washed once with a mixture of equal volumes of acetone and ether, twice with acetone and once with ethanol before drying. The dried white powder was hygroscopic.

The haemin was recovered from the acetone solution by addition of one-third volume of deionized water followed by vacuum evaporation, filtration and washing with two portions of cold water. It was purified further by dissolving it in acetone and repeating the precipitation and washings before drying it over CaCl_2 in a vacuum.

RESULTS

Metabolism of nitrate added directly to the rumen

The concentrations of ammonium, nitrite and nitrate ions in the rumen liquor were followed periodically in a cow given 70 g. of KNO_3 through a rumen fistula. The nitrate concentration decreased rapidly whereas ammonium and nitrite increased (Fig. 1). Ammonium and nitrite ions were at their maximum concentrations about 3 hr. after nitrate was administered.

Although rather high concentrations of nitrite were produced in the rumen, very little methaemoglobin was detected in the blood, and appreciable concentrations appeared only after several days of treatment (Table 1). After 16 days, the daily dose of KNO_3 was increased to 100 g. As indicated in Table 1, the percentage conversion into methaemoglobin approached 15% after a few days on 100 g. of KNO_3 a day. In later experiments, the animals

were 'conditioned' by giving them 100 g. of KNO_3 daily until their blood carried about 15% methaemoglobin 3 hr. after treatment.

Experiments were performed with K^{15}NO_3 , so that the nitrate nitrogen and products derived from it could be traced. A solution of 120 g. of K^{15}NO_3 , 1.7 atom % ^{15}N excess, was introduced into the rumen through the fistula of a 'conditioned' cow in the morning before feeding. Analyses from the first experiment are recorded in Figs. 2 and 3. Fig. 2 indicates the accumulation of methaemoglobin in the blood of the treated cow and the ^{15}N concentrations of the nitrate plus nitrite fraction and the ammonia fraction in the rumen liquor. A peak value of 16% for blood methaemoglobin was recorded 3 hr. after labelled nitrate was supplied; this peak appeared about an hour after the peak concentration of ^{15}N in the ammonium

Fig. 1. Reduction of nitrates in the rumen of a cow receiving 70 g. of KNO_3 via rumen fistula. O, Nitrate nitrogen; ∇ , nitrite nitrogen; $\square$, ammonium nitrogen.

Table 1. *Induction of methaemoglobin formation in the blood of cattle by repeated dosing with potassium nitrate*

Days of treatment*	Time after dosing (hr.)	Percentage conversion into methaemoglobin	Days of treatment†	Time after dosing (hr.)	Percentage conversion into methaemoglobin
0	0	0.26	16	6	0.62
1	0	0.25	19	6	2.44
1	4	0.20	20	4	11.00
3	4	1.20	20	6	1.70
4	6	0.00	23	3.5	10.50
5	2	0.00	23	5	7.80
5	4	1.20	23	6	6.40
5	6	0.30	25	3	14.30
9	4	4.65	27	3	14.90
12	5	1.87	28	4	14.20
12	6	1.68	—	—	—

* Animal received 70 g. of KNO_3 daily.

† Animal was shifted to 100 g. of KNO_3 on day 16 and received this amount daily thereafter.

ions of the rumen was reached. The decrease in ^{15}N concentration in the nitrate plus nitrite fraction followed a more precipitous curve than that observed earlier for the disappearance of unlabelled nitrate from the rumen (Fig. 1).

Fig. 3 indicates the appearance of ^{15}N in the fractions recovered from urine; the curve for methaemoglobin formation in the blood is included

Fig. 2. Reduction of nitrate in a cow's rumen and formation of methaemoglobin in the blood. 120 g. of K^{15}NO_3 , 1.7 atom % ^{15}N excess, was added to the rumen. Expt. 1: $\circ$, nitrate nitrogen plus nitrite nitrogen; $\square$, ammonium nitrogen; $\bullet$, percentage conversion into methaemoglobin.

Fig. 3. Formation of methaemoglobin in the blood and accumulation of ^{15}N in the nitrogenous compounds of the urine of a cow receiving 120 g. of K^{15}NO_3 , 1.7 atom % ^{15}N excess, through a rumen fistula. Expt. 1: $\square$, ammonium nitrogen; $\blacktriangledown$, urea nitrogen; $\blacksquare$, total nitrogen; $\bullet$, percentage conversion into methaemoglobin.

for reference. The total nitrogen carried a higher concentration of ^{15}N and reached a peak concentration of ^{15}N sooner than urea or ammonium nitrogen. Urea and ammonium ions reached their highest concentrations of ^{15}N at almost the same time; the urea had the higher concentration of ^{15}N .

The blood samples in the first experiment were too small to provide sufficient nitrogen for proper mass analysis of fractions; the protein nitrogen diluted the ^{15}N excessively in the total blood nitrogen. Adequate samples of blood (125 ml.) for mass analysis on fractions were taken in the second experiment. Fig. 4 shows the ^{15}N concentrations in various nitrogenous fractions from the blood of a cow that received K^{15}NO_3 in Expt. 2. ^{15}N accumulated quickly in the nitrate plus nitrite fraction of the blood and remained high during the 8 hr. period of sampling (note that these values in Fig. 4 are divided by 10). The methaemoglobin level approached a maximum of 22 % at 3 hr. The highest concentration of ^{15}N in the ammonium ions in the blood also was observed near 3 hr., when its atom % ^{15}N excess was about a twelfth of that in the nitrate plus nitrite fraction. The ^{15}N in haemin reached a peak concentration more rapidly than did the ^{15}N of the ammonium ion. The globin fraction showed no detectable increase in ^{15}N above its normal abundance, although the haemin

Fig. 4. Formation of methaemoglobin in the blood, and accumulation of ^{15}N in the nitrogenous fractions of the blood of a cow receiving 120 g. of K^{15}NO_3 , 1.7 atom % ^{15}N excess, through a rumen fistula. Expt. 2: $\circ$, atom % ^{15}N excess $\times 10^{-1}$ of nitrate plus nitrite nitrogen in plasma; $\square$, ammonium nitrogen in plasma; $\triangle$, haemin; $\bullet$, percentage conversion into methaemoglobin.

retained its label despite the vigorous treatment involved in its separation and purification.

The results of urine analysis are presented in Fig. 5. Again the ^{15}N in the total nitrogen (which includes nitrate and nitrite) rose more rapidly and to a higher level than in urea or ammonium nitrogen. The concentrations of ^{15}N in the total, urea and ammonium ion fractions were highest near 4 hr. and declined to only about half their peak concentrations by 8 hr. The maximum conversion of haemoglobin into methaemoglobin in the blood occurred shortly before the maximum concentration of ^{15}N was reached in the urine nitrate.

Reactions of nitrate and nitrite with blood

As the haemin from blood of cows given K^{15}NO_3 accumulated ^{15}N , it appeared worth while to study the binding of nitrite *in vitro*. To 5 ml. of fresh blood plus oxalate was added 750 μg . of $^{15}\text{NO}_2^-$, 3.5 atom % ^{15}N excess, and the mixture was refrigerated overnight. The methaemoglobin concentration was determined, and the cells were separated and washed by centrifuging until the washings gave a negative test for nitrite. The globin and haemin were separated as described, samples of each were digested and their ^{15}N concentrations were determined. The blood containing 51 % methaemoglobin showed no detectable ^{15}N in its globin, but the haemin had 0.023 atom % ^{15}N excess. This demonstrated that the nitrogen from

nitrite was bound firmly enough to the haemin of methaemoglobin to withstand displacement by acetone containing 1.2 ml. of 12N-HCl/100 ml. The nature of this binding is not known, and no attempt was made to determine whether it was accompanied by an alteration in the composition of the haemin molecule.

Whole blood, cells suspended in 0.85 % NaCl, and lysed cells, each containing about 50 % methaemoglobin, were shaken overnight in pure O_2 in an effort to displace nitrite. Although the percentage of methaemoglobin decreased in each case, no detectable nitrite or NH_3 was released into the medium. Treatment of other samples of the same materials with 2.5 % neutralized NaCN was followed immediately by disappearance of methaemoglobin, but no significant quantities of nitrite or NH_3 appeared in the medium.

DISCUSSION

It is generally recognized that the toxicity of nitrate for ruminants depends upon its reduction to nitrite in the rumen and the combination of the nitrite with haemoglobin to form methaemoglobin in the blood. In addition to the acute symptoms that accompany nitrite poisoning, other conditions, such as a non-contagious abortion in cattle (Sund *et al.* 1957), have been attributed to ingestion of materials high in nitrates. The current studies re-emphasize the very rapid conversion of nitrate into nitrite and ammonium ions in the rumen. The dense microbial flora of the rumen maintains reducing conditions and can reduce added nitrate without delay. Fig. 1 shows the rapid disappearance of nitrate added directly into the rumen of a cow through a fistula, a disappearance which was accompanied by appearance of a maximum concentration of ammonia and nitrite within 3 hr. Our approximations indicated that at this time about 14 % of the added nitrate had been converted into nitrite, but such calculations are subject to substantial corrections for normal pools and absorption of nitrate, nitrite and ammonia.

Despite the fact that nitrite was formed quickly in the rumen with no evident need for adaptation of the microflora, toxicity to the animal was delayed and very little methaemoglobin appeared in the blood for several days after daily additions of nitrate to the rumen were initiated. The nature of this adaptation is not clear. Considerable quantities of nitrate appeared in the blood (Fig. 4), but it seems questionable that substantial reduction of nitrate is accomplished in the blood. One cannot ignore the fact that the time of highest conversion of nitrate into nitrite in the rumen (Fig. 1) very nearly coincided with the time of maximum methaemoglobin formation in the blood

Fig. 5. Formation of methaemoglobin in the blood, and accumulation of ^{15}N in the nitrogenous fractions of urine, from a cow receiving 120 g. of K^{15}NO_3 , 1.7 atom % ^{15}N excess, through a rumen fistula. Expt. 2: $\circ$, nitrate nitrogen; $\square$, ammonium nitrogen; $\blacktriangledown$, urea nitrogen; $\bullet$, total nitrogen; $\bullet$, percentage conversion into methaemoglobin.

(Fig. 2). Further, the concentration of nitrate persisted in the blood (Fig. 4) beyond the time of a precipitous decrease in percentage of methaemoglobin and the similar decrease in nitrite concentration in the rumen. Data still are insufficient to account accurately for the amount of nitrite formed in the rumen and in the blood, but Pfander, Garner, Ellis & Muhrer (1957) found very little toxicity from nitrate added intravenously, an observation that supports the primary formation of nitrite in the rumen rather than in the blood.

[^{15}N]Nitrate was added directly into the rumen to yield more detailed and specific information on nitrate reduction in the rumen, the transfer of nitrate and its products to the blood stream, and their elimination in the urine. The ^{15}N concentration of the nitrate plus nitrite fraction decreased rapidly (Fig. 2). The ^{15}N of the ammonium fraction increased as that of the nitrate plus nitrite decreased. After about 2.5 hr., the ^{15}N concentration of the ammonium ions remained higher than that of the nitrate plus nitrite ions. This response likely arose from a differential dilution of the ions; the ingested feed carried much more nitrate than it did nitrite or ammonia, so that dilution of the ^{15}N in ammonia occurred more slowly than the dilution in the nitrate plus nitrite.

The maximum concentration of nitrite in the rumen appeared about 3 hr. after nitrate was added to the rumen (Fig. 1), and the maximum conversion of haemoglobin into methaemoglobin occurred at almost the same time (Fig. 2). One would expect very little delay in methaemoglobin formation once the nitrite reached the blood stream. The data support the idea that the bulk of the nitrite (and by implication ammonia and nitrate) is absorbed directly into the blood from the rumen rather than from lower sections of the digestive tract. The virtual coincidence of attaining a maximum nitrite concentration in the rumen and a maximum methaemoglobin level in the blood indicates that the absorption is very rapid.

In relation to the rapid drop in total nitrogen in the rumen (Fig. 1) and ^{15}N in the nitrate plus nitrite of the rumen (Fig. 2) it is interesting to observe (Fig. 4) the persistence of a high concentration of ^{15}N in the nitrate plus nitrite fraction in the blood plasma 4–8 hr. after the administration of potassium [^{15}N]nitrate. The rapid decrease in the percentage methaemoglobin after 3–3.5 hr. (Figs. 2–4) suggests that the nitrate plus nitrite fraction of the plasma consisted mostly of nitrate, and that the conversion of nitrate into nitrite in the blood probably was negligible.

The ^{15}N concentration of the ammonium ions always was far less than that of the nitrate plus nitrite of the plasma (Fig. 4), although they were approximately equivalent in the rumen after 2.5 hr.

(Fig. 2). More favourable absorption of nitrate plus nitrite than of ammonium ion from the rumen into the blood, or more extensive isotopic dilution of ammonium than of nitrate plus nitrite in the blood, could explain the observations.

The peak concentration of ^{15}N in the haemin appeared before the maximum total conversion into methaemoglobin (Fig. 4). This suggests that the ^{15}N concentration of the nitrite in the rumen was at a maximum before the absolute concentration of nitrite in the rumen reached a maximum under our experimental conditions. It again emphasizes the rapidity with which nitrite is absorbed from the rumen into the blood. Land & Virtanen (1959) reported incorporation of ^{15}N from ammonium nitrate into milk protein an hour after feeding with the labelled compound.

The appearance of maximum concentrations of ^{15}N in the total nitrogen fraction of the urine was earlier than for the urea and ammonium nitrogen (Fig. 3) probably because the total nitrogen included nitrate. The concentrations of ^{15}N in urea and ammonium ions rose and fell together and reached peak values at approximately the same time (5–6 hr. after administering potassium [^{15}N]nitrate). Fig. 5 emphasizes that the highest concentration of ^{15}N in nitrate appeared near the time of greatest conversion of haemoglobin into methaemoglobin. Again these appeared before the highest concentrations of ^{15}N appeared in the total nitrogen, urea nitrogen and ammonium nitrogen. Comparison of Figs. 3–5 suggests that there was a lag of as much as an hour between the times the highest concentrations of ^{15}N were observed in the urine compared with the blood. In part, this may reflect inherent delay in sampling the urine rather than a true lag in equilibration between the two fluids.

SUMMARY

1. A cow receiving a direct addition of 70–100 g. of potassium nitrate a day into the rumen through a fistula showed no methaemoglobin in the blood for several days. This delay in methaemoglobin formation could not be attributed to failure of the micro-organisms of the rumen to form nitrite, for they immediately showed vigorous nitrate reduction.

2. Potassium [^{15}N]nitrate was added to the rumen to trace the formation, absorption and elimination of nitrate and the compounds formed from it. Nitrate, nitrite and ammonium ions were absorbed in considerable quantities directly from the rumen into the blood. The highest concentrations of ^{15}N in the ammonium ion in the blood appeared 3–4 hr. after potassium [^{15}N]nitrate was added to the rumen, whereas the highest ^{15}N concentration in the nitrate plus nitrite fraction

appeared in the blood after 5–6 hr., and the high concentration persisted.

3. The curves for excretion of the ^{15}N -labelled total, nitrate, urea and ammonium nitrogen fractions in the urine lagged behind their corresponding curves for appearance and disappearance of these components in the blood by about an hour.

4. The formation of methaemoglobin in the blood followed the time course of nitrite formation in the rumen rather closely, suggesting that nitrite was passed rapidly and directly from the rumen to the blood. There was no direct evidence for nitrite formation from nitrate in the blood.

5. Nitrite was bound tightly to the haem of methaemoglobin, for it could be recovered in haem that had been subjected to treatment with acetone containing 1.2 ml. of 12N-hydrochloric acid/100 ml.

This paper is published with the approval of the Director of the Wisconsin Agricultural Experiment Station. The work was supported in part by research grant E-848 from the Division of Research Grants, National Institutes of Health. We are indebted to R. E. Nichols and T. Kowalczyk for providing the fistulated cattle used in these experiments.

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Plant Polyphenols

4. HYDROXYCINNAMIC ACID-SUGAR DERIVATIVES*

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(Received 16 February 1961)

Hydroxycinnamic acids, which occur in most higher plants, are almost always present in bound form and are liberated from plant extracts by hydrolysis with acid or alkali. Of the organic substances known to occur in combination with hydroxycinnamic acids, quinic acid is the one most frequently mentioned in the literature: thus chlorogenic acid (a quinic ester of caffeic acid) appears to be almost ubiquitous and at least four other caffeoyl quinic acids are known. *p*-Coumaroylquinic acid is also reported to occur with some frequency (Cartwright, Roberts, Flood & Williams, 1955; Griffiths, 1959). In contrast, few simple sugar derivatives have been described (Karrer, 1958) and, with the exception of *o*-coumaroylglucoside (melilotoside) (Charaux, 1925), none has been fully characterized.

In view of current interest in the biosynthesis (McCalla & Neish, 1959), natural distribution (Bate-Smith, 1954; Herrmann, 1958) and biological function (Lee & Le Tourneau, 1958; Rabin & Klein, 1957) of the hydroxycinnamic acids, a study was made of their derivatives occurring in the potato and a number of other higher plants. Besides the *p*-coumaroylglucose and caffeic acid 3- β -glucoside found in potato berries (Corner & Harborne, 1960), seven other naturally occurring cinnamic acid-glucose derivatives have been found. Further, simple glucose esters have been identified in nearly a hundred plant species.

The finding that glucose esters are of wide occurrence suggests that they may be formed during the metabolism of free hydroxycinnamic acids in plant tissues. Previous work has only indicated that phenolic glucosides (Kosuge & Conn, 1959) and quinic esters (Levy & Zucker, 1960) are

* Part 3: Harborne & Sherratt (1961).