The Metabolism of Nitrate and Nitrite in the Sheep

2. HYDROGEN DONATORS IN NITRATE REDUCTION BY RUMEN MICRO-ORGANISMS *IN VITRO*

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Sapiro, Hoflund, Clark & Quin (1949) demonstrated the reduction of nitrate to nitrite by rumen contents *in vitro*, and Lewis (1951) has shown that nitrate is reduced to nitrite and ammonia *in vivo* in the rumen of the sheep. Since many bacteria are known to reduce nitrate to nitrite and ammonia it is reasonable to assume that the appearance of these compounds in the rumen of the sheep after dosing with nitrate is the result of the reducing activity of the rumen micro-organisms.

The reduction of nitrate by pure cultures of bacteria has been studied by a number of workers: Quastel & Stephenson (1925) and Quastel, Stephenson & Whetham (1925) showed that *Escherichia coli* reduces nitrate to nitrite in the presence of such hydrogen donators (H-donators) as lactate, glycerol, succinic acid or malic acid, and Stickland (1931), using washed suspensions, showed that the process was catalysed by an enzyme activating nitrate (nitratase). Aubel (1938) studied the reduction of nitrate to ammonia by *Esch. coli*, with glucose as H-donator. Stephenson & Stickland (1931) demonstrated that organisms possessing both hydrogenase and nitratase reduced nitrate quantitatively to nitrite in the presence of hydrogen. In contrast to these results, Woods (1938) showed that two strains of *Esch. coli* and one strain of *Clostridium welchii* were able, in the presence of hydrogen, to reduce nitrate quantitatively to ammonia. In this reduction there was a transient appearance of nitrite, and Woods provided evidence that both nitrite and hydroxylamine were intermediates in the reaction.

The H-donators in the reduction of nitrate in the rumen are unknown. It is not possible to make a detailed analysis of this process in the rumen of the sheep because of the complexity of the reactions occurring therein. Since the reduction proceeds rapidly even in an animal that has been fasted for 16 hr. it would be difficult to produce any significant change in the rate or extent of the nitrate reduction by the introduction of a potential H-donator into the rumen. However, using washed micro-organisms prepared according to the method of Elsden & Sijpesteijn (1950*a*), it has been possible to study the process under controlled conditions. Suspensions of rumen bacteria prepared in this way reduce nitrate to nitrite and ammonia, in the presence of a number of H-donators, and of those tested hydrogen was the most active.

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EXPERIMENTAL METHODS

Washed cell suspensions. A volume of rumen fluid was withdrawn from the rumen of a sheep fitted with a permanent rumen fistula (Phillipson & Innes, 1939). It was filtered through muslin and the filtrate spun at approx. 1000 rev./min. for 1 min. on the Measuring and Scientific Equipment Ltd. (M.S.E.) minor centrifuge. The deposit was discarded and the supernatant centrifuged at approx. 3000 rev./min. for 30 min. The residue was suspended in 0·05 M phosphate buffer pH 6·5 which had been previously boiled and to which 50% (w/v) $Na_2S.9H_2O$ had been added to a final concentration of 0·02% (w/v). This suspension was again spun at 3000 rev./min. for 30 min., and the residue resuspended in the de-oxygenated buffer.

Manometric methods. The reactions were carried out in Warburg manometer vessels at 37°. The main compartments of the vessels contained 2 ml. of the washed cell suspension in the de-oxygenated buffer, and after thermal equilibrium had been attained the potential H-donator and the nitrate were added from the side bulb. The centre wells contained 0·2 ml. 20% (w/v) KOH, to absorb any CO_2 produced.

At the end of the incubation period of 2 hr. unless otherwise stated, samples of the reaction fluids were analysed for both nitrite and ammonia. When gaseous H_2 was studied as H-donator two series of controls were carried out. The blank NH_3 production was determined when the suspension was incubated in the presence of nitrate but with N_2 as the gas phase, and the blank H_2 consumption was obtained in the absence of nitrate with H_2 as the gas phase. The N_2 was freed from O_2 by passing over Cu turnings heated to 400°. All values of NH_3 formation have been corrected for the small initial NH_3 content of the washed cell suspensions.

Ammonia was estimated on 1 ml. samples by the method of Conway & O'Malley (1942). *Nitrite* was determined by the Griess-Ilosvay method (Association of Official Agricultural Chemists Handbook, 1947): 1 ml. samples from the manometer vessels were clarified by the addition of 3 ml. lead acetate (saturated aqueous solution, diluted 1 in 10), and 1 ml. saturated Na_2PO_4; the mixture was then shaken and filtered, and a sample taken for the colorimetric estimation.

Total nitrogen was determined by the Kjeldahl method as modified by Chibnall, Rees & Williams (1943), and the NH_3 trapped in the boric acid reagent of Conway & O'Malley (1942).

RESULTS

Gaseous hydrogen as a donator

Nitrate reduction. Gaseous hydrogen was first tested as a donator because there was some evidence that rumen micro-organisms activated hydrogen, and also because of the work of Stephenson & Stickland (1931) and Woods (1938) previously referred to. Table 1 shows that in the presence of hydrogen nitrate is reduced quantitatively to ammonia with the consumption of an equivalent amount of hydrogen, according to equation (1).

$$KNO_3 + 4H_2 \rightarrow NH_3 + 2H_2O + KOH. \quad (1)$$

The observed ammonia production (after the initial ammonia of the suspension had been subtracted) was corrected for the ammonia produced in the control experiments carried out in nitrogen. The uptake of hydrogen was corrected for the slight uptake which occurred in the absence of nitrate. After these corrections both the ammonia production and hydrogen uptake were equivalent to 95–100% of the theoretical values for the complete reduction of nitrate to ammonia.

Nitrite and hydroxylamine reduction. The reduction of hydroxylamine and nitrite by hydrogen was next studied (cf. Woods, 1938). For comparison, vessels were included which contained nitrate. The course of the hydrogen uptake in a typical experiment is shown in Fig. 1. The blank uptake of hydrogen by the suspension in the absence of substrate never exceeded 3% of the uptake in the presence of nitrate. Consumption of hydrogen in the presence of nitrate, nitrite and hydroxylamine corresponded to

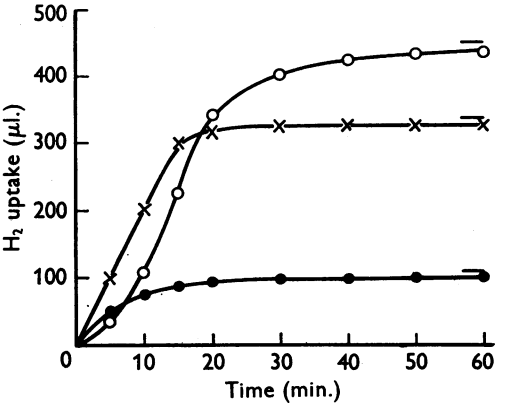

Fig. 1. Hydrogen uptake by washed micro-organisms in the presence of nitrate, nitrite or hydroxylamine. O—O, NO_3; ×—×, NO_2; ●—●, NH_2OH; —, theoretical uptake for reduction to ammonia. Corrections have been applied for the hydrogen uptake in the absence of substrate. 2 ml. washed cell suspension in 0·05 M-phosphate buffer pH 6·5 (10·7 mg. total N/100 ml.). 5 μmol. substrate in 0·2 ml. water.

reduction to ammonia according to equations (1), (2) and (3).

$$NaNO_2 + 3H_2 \rightarrow NH_3 + NaOH + H_2O, \quad (2)$$

$$NH_2OH + H_2 \rightarrow NH_3 + H_2O. \quad (3)$$

Table 1. *Hydrogen uptake and ammonia formation by washed suspensions in the presence of nitrate*

(2 ml. washed cell suspension (9·7 mg. total N/100 ml.); 4 μmol. KNO_3. Theoretical values are for complete reduction of the KNO_3 to NH_3.)

		H_2 uptake		NH_3 production	
Substrate	Gas phase	(μl.)	Percentage theory (corr.)	(μmol.)	Percentage theory (corr.)
KNO_3	H_2	363	97	4·78	97
KNO_3	H_2	360	96	4·83	98
KNO_3	N_2	1	—	0·97	—
KNO_3	N_2	4	—	0·85	—
None	H_2	14	—	0·51	—
None	H_2	16	—	0·35	—

Table 2. *The reduction of nitrate, nitrite and hydroxylamine to ammonia by washed suspensions under hydrogen gas phase*

(2 ml. washed cell suspension (6·7 mg. total N/100 ml. suspension).)

					Molar ratios		
Substrate	(μmol.)	Gas phase	H_2 uptake (μmol.)	NH_3 formation (μmol.)	Substrate added	H_2 taken up (corr.)	NH_3 formed (corr.)
No substrate	—	H_2	0·4	0·4	—	—	—
NH_2OH	16	H_2	14·4	17·7	1	0·9	1·1
NH_2OH	16	N_2	—	0·9	—	—	—
$NaNO_2$	5	H_2	14·3	5·2	1	2·8	0·9
$NaNO_2$	5	N_2	—	0·8	—	—	—
KNO_3	4	H_2	15·6	5·0	1	3·8	1·0
KNO_3	4	N_2	—	0·9	—	—	—

At the completion of the experiments samples of the contents of the manometer vessels were immediately withdrawn for the estimation of nitrite and ammonia. The nitrite concentration accounted for less than 2% of added nitrate or nitrite. After applying the appropriate corrections the results in Table 2 show that nitrate, nitrite and hydroxylamine are all reduced to ammonia in good agreement with the stoicheiometric relationships of equations (1)–(3).

Effect of pH *on the reduction.* The effect of hydrogen ion concentration on the rates of these processes was investigated. The preparation of the washed cell suspension differed in that the rumen micro-organisms were both washed and suspended in a special saline which was prepared as follows: a stock solution of 0·9% (w/v) sodium chloride was boiled immediately before use and cooled rapidly under the tap; to this was added 50% sodium sulphide ($Na_2S.9H_2O$) to a final concentration of 0·02% (w/v) and sufficient N-hydrochloric acid to bring the pH to 6·5. The main compartment of the manometer vessels contained 1 ml. of the suspension and 1 ml. of 0·2 M-phosphate buffer. The buffers covered the range pH 5·4–7·3. A control was set up at each pH containing no substrate. The nitrate, nitrite or hydroxylamine (0·2 ml., 0·05 M) was tipped in from the side arm after thermal equilibrium had been attained. Initial rates of hydrogen uptake, from 3 to 10 min. after tipping in the substrate, were determined and each corrected for the small gas uptake in the controls. The results are presented in Fig. 2. The pH optima for nitrate and nitrite reduction are sharp and at 6·2 and 5·6 respectively, whereas with hydroxylamine there is a broad plateau over the range of 6–7. The optima for the analogous processes catalysed by *Cl. welchii* were 7·5, 6·7 and above 8 respectively (Woods, 1938), i.e. occupying similar relative positions. These findings are of interest in view of the fact that the normal pH of the rumen is around 6·5.

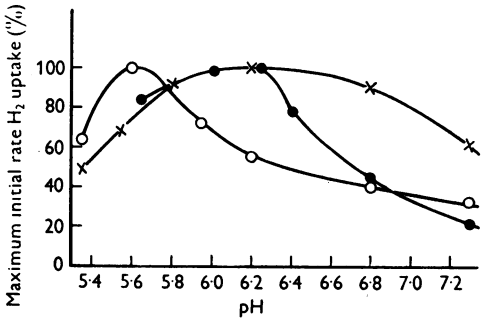

Fig. 2. pH activity curves for the reduction of nitrate, nitrite and hydroxylamine by the washed suspensions in an atmosphere of hydrogen. ●—●, NO_3; ○—○, NO_2; ×—×, NH_2OH. Suspension contained 33·2 mg. total N/100 ml.

Detection of intermediates. These results, like those of Woods (1938), suggest that nitrite and hydroxylamine are intermediates in the reaction, and an attempt was made to ascertain whether nitrite accumulates at any stage during the reduction of nitrate. Nitrite is known to accumulate *in vivo* (Lewis, 1951) and in the reaction catalysed by washed suspension of *Cl. welchii* (Woods, 1938). Nitrate was incubated with the washed cell suspension of rumen bacteria in an atmosphere of hydrogen, and the concentrations of nitrite and ammonia were determined at intervals during the experiment. The reaction was complete in 60 min. For convenience a series of manometers was used, and the reactions were stopped individually when a suitable hydrogen uptake had been recorded. The results are presented in Fig. 3. The total hydrogen uptake and ammonia production again corresponded to complete reduction of the nitrate to ammonia. However, during the first 20 min. there was an accumulation of nitrite. This observation agrees with the *in vivo* findings that when nitrate is introduced into the rumen of the sheep there is a rapid but transient appearance of nitrite (Lewis, 1951).

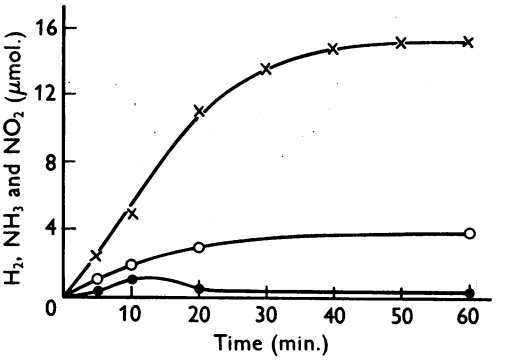

Fig. 3. Hydrogen uptake, ammonia formation and nitrite appearance and disappearance when washed cell suspensions are incubated with nitrate in the presence of hydrogen. ×—×, μmol. H_2 uptake; ○—○, μmol. NH_3 formation; ●—●, μmol. NO_2 formation. 2 ml. washed suspension in 0·05 M-phosphate buffer pH 6·2. (18·8 mg. total N/100 ml.) 4 μmol. KNO_3.

Hydrogen donators other than gaseous hydrogen

Other potential hydrogen donators tested for nitrate reduction by the rumen micro-organisms included substances known to be present in the rumen, e.g. the lower fatty acids, and others possibly present under certain conditions, for example lactate, succinate and glucose. The reactions were carried out in manometer vessels in nitrogen. The substrates were incubated for 2 hr.

with washed rumen bacteria in the presence of nitrate. The ability of the compounds to act as donators was assessed by the amounts of nitrite and ammonia produced over and above that observed in a control without donators. It was found that the fatty acids, acetic, propionic, and *n*-butyric, were completely inactive as hydrogen donators, but succinate or formate gave a considerable production of nitrite and ammonia.

A more comprehensive experiment was carried out to determine the relative efficiency of various substances as hydrogen donators for nitrate reduction. A typical series of results is shown in Table 3. The

Table 3. *The production of nitrite and ammonia from nitrate by washed rumen micro-organisms in presence of various potential* H-*donators*

(2 ml. washed cell suspension in 0·05 M-phosphate buffer pH 6·2 containing 20·3 mg. total N/100 ml. Hydrogen donator (0·4 ml., 0·1 M) and 0·2 ml. 0·1 M-KNO_3 (280 μg. nitrate N) in side bulb. Incubation 2 hr.)

H-donator	Ammonia N formed (μg.)	Nitrite N formed (μg.)
Control	26	3
Hydrogen	174	21
Succinate	94	18
Formate	97	12
DL-Lactate	77	16
Citrate	74	11
D-Glucose	62	22
L(−)-Malate	64	17
D-Mannitol	63	5
Glycerol	52	3
D-Xylose	44	11
Ethanol	32	5

substrates were incubated for 2 hr. with the washed cells in the presence of nitrate. The reduction was not complete in any instance, but the use of more cells led to a proportional increase in the blank production of nitrite and ammonia, whereas if the incubation time were increased the biochemical activity of the suspension would probably undergo considerable change. It will be considered that there was a significant reduction of the nitrate when the production of nitrite and ammonia in the presence of a hydrogen donator was at least double that in the control. On this basis there was a significant reduction of nitrate in the presence of hydrogen, succinate, formate, DL-lactate, citrate, D-glucose, L(−)-malate, and D-mannitol, but not in the presence of glycerol, D-xylose or ethanol.

DISCUSSION

Of the compounds tested as possible hydrogen donators for the reduction of nitrate by rumen micro-organisms, only the lower fatty acids are definitely known to occur in the rumen under the conditions of the *in vivo* investigations (Lewis, 1951; Elsden, 1945): these were, however, totally inactive. Succinate is probably an intermediate in cellulose breakdown (Sijpesteijn, 1949). Lactate and glucose may occur in the rumen under certain conditions (Elsden, 1945; Phillipson, 1942), and Sijpesteijn (1950) has obtained evidence that formic acid is possibly a product of cellulose decomposition by *Ruminococcus flavifaciens*, a cellulose-decomposing organism isolated from the rumen of both sheep and cattle. It is not known whether the other compounds tested as donators occur in the rumen.

Hydrogen, the most active of the substances tested, is known to be a common end product of many bacterial fermentations, and *Veillonella gazogenes*, isolated by Johns (1949) from the rumen of the sheep, is an active hydrogen producer. Elsden & Sijpesteijn (1950*b*) have shown that the rumen organisms possess an active hydrogenase, and a large uptake of hydrogen has been recorded in the presence of carbon dioxide. However, Lugg (1938) states that hydrogen is not a constituent of rumen gases. The situation would be clarified were it possible to show that hydrogen is utilized in the rumen as rapidly as it is produced.

Thus, although several substances have been shown to be active hydrogen donators for the reduction of nitrate in the presence of washed cell suspensions of rumen micro-organisms, it is not as yet possible to state definitely that these account for the process *in vivo*. The transitory appearance of nitrite in the early stages following the addition of nitrate is found both *in vivo* and *in vitro*.

SUMMARY

1. Hydrogen is a very active donator for the reduction of nitrate, nitrite and hydroxylamine to ammonia, in the presence of washed micro-organisms from sheep rumen.

2. Nitrite is an intermediary in the reduction of nitrate, and appears transitorily in the early stages of reduction by hydrogen.

3. The pH optima of nitrate and nitrite reduction in an atmosphere of hydrogen are 6·5 and 5·6 respectively, whereas the optimum for hydroxylamine reduction shows a broad plateau over the range of pH 6–7.

4. Formate, succinate, lactate, citrate, glucose, malate and mannitol are hydrogen donators for nitrate reduction, but at lower rates than hydrogen. Glycerol, xylose and ethanol have little activity and acetate, propionate and *n*-butyrate are inactive.

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Displacement Chromatography on Synthetic Ion-exchange Resins

7. SEPARATIONS USING A STRONGLY BASIC RESIN

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Previous communications (Partridge, 1949, 1950) have reported the use of an ion-exchange resin for the separation of amino-acids from a protein hydrolysate into eight groups, each consisting of no more than three amino-acids. The separation was carried out as a displacement chromatogram, using a strongly acidic cation-exchange resin, and displacing the mixture with ammonia or sodium hydroxide solution. A fractionation of this kind forms a logical starting point for a scheme aiming at the complete separation and isolation of all the component amino-acids in the complex mixture; and the preliminary group separation has thus been termed the 'primary fractionation'. When carried out with a multiple column having a top section packed with sulphonated polystyrene resin (Partridge, Brimley & Pepper, 1950) the primary fractionation results in the immediate isolation of three amino-acids in a pure condition; these are aspartic acid, lysine and arginine. The other amino-acids appear as mixed bands as follows: band II, glutamic acid, serine and threonine; band III, glycine and alanine; band IV, valine and proline; band V, methionine, leucine and isoleucine; band VI, histidine and the leucines.

Various procedures have been described for the further separation of the components of some of these bands, e.g. the separation of glutamic acid from band II by the use of a weakly basic resin (Partridge & Brimley, 1949) or the separation of methionine from leucine by the use of a cation-exchange resin at higher temperatures (Partridge & Brimley, 1951). However, the appearance in commercial production of several new strongly basic resins now offers the possibility of separating other mixed bands and also of bringing all the secondary separations within a generalized scheme.

The separation of a series of amino-acids in displacement chromatograms using strongly acidic resins is determined largely by the order of the values of pK_1 (COOH) for the members of the series (Davies, 1949). Similarly, the order of displacement from strongly basic anion-exchangers is largely determined by the order of pK_2 (NH_3^+) (Davies, Hughes & Partridge, 1950) and thus displacement from an anion-exchanger should give rise, not to a simple reversal of the order found with cation-exchangers, but rather to an entirely new grouping of the amino-acids. The work described in this paper shows that this prediction is borne out in practice and that use may be made of the differences for securing further separations.

EXPERIMENTS AND RESULTS

The ion exchanger used for these experiments was Dowex 2 (obtainable from the Dow Chemical Company, Midland, Michigan). The resin is a cross-