***N*-Carbamoyl- β -D(+)-glucopyranosylamine Metabolism by Rumen Microbes**

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(Received 13 April 1979; revised 13 August 1979)

Degradation of *N*-carbamoyl- β -D(+)-glucopyranosylamine (NCG) by rumen microorganisms *in vitro* required a viable population as it did not occur if the microbial preparation had been sterilized. Production of CO₂ from glucose, or the glucose portion of NCG, was not affected by acetohydroxamic acid (AHA), but urea hydrolysis was inhibited by 79%. With *N*-[¹⁴C]carbamoyl- β -D(+)-glucopyranosylamine, production of ¹⁴CO₂ decreased and [¹⁴C]urea accumulated when AHA was included in the medium. Cell-free rumen fluid did not degrade NCG. These observations support the hypothesis that the first nitrogenous component formed from the degradation of NCG is urea.

INTRODUCTION

Recently, *N*-carbamoyl- β -D(+)-glucopyranosylamine (NCG; Fig. 1), a condensation product of glucose and urea, has been examined as a potential source of dietary non-protein nitrogen for ruminants (Milligan *et al.*, 1972; McAllan *et al.*, 1975; Smith *et al.*, 1975). It was found (Milligan *et al.*, 1972) to yield ammonia in the rumen at a rate more likely to be compatible with the rate of microbial incorporation of ammonia into protein than was the exceedingly rapid breakdown of urea which is the major non-protein nitrogen source used at present (Chalupa, 1968). In addition, results from animal feeding trials have shown that NCG will support a portion of a ruminant's nitrogen requirement (Martin, 1976).

This investigation was undertaken to determine whether the production of ammonia from NCG by the mixed rumen population is, in fact, a biological process rather than simply a reversal of the condensation reaction used in the synthesis of the compound. It was also intended to gain information on the pathway of breakdown by identifying the initial nitrogenous components produced from the degradation by anaerobic rumen microbes.

METHODS

Chemical synthesis and physical methods. *N*-Carbamoyl- β -D(+)-glucopyranosylamine (NCG) was synthesized and purified by the method of Benn & Jones (1960). *N*-[¹⁴C]Carbamoyl- β -D(+)-glucopyranosylamine and *N*-carbamoyl- β -D(+)-[U-¹⁴C]glucopyranosylamine were prepared using, respectively, [¹⁴C]urea or [U-¹⁴C]glucose. The purity of NCG was assessed by (i) descending paper chromatography using butan-1-ol/pyridine/water (9:5:4, by vol.) and visualizing spots by the AgNO₃ technique of Trevelyan *et al.* (1950) and (ii) chromatography through Sephadex G-10 (100 × 2.5 cm) using 10 mM-KCl as solvent, collecting fractions of 5.3 ml and assaying them by the paper chromatographic technique. All NCG preparations used for incubation were >99.7% pure.

Gases used in this study were rendered O₂-free using the technique of Hungate (1968). To the sterile complex medium (Caldwell & Bryant, 1966) was added either filter-sterilized NCG (4.5 to 90.1 mM), [¹⁴C]urea (8.3 mM) or [U-¹⁴C]glucose (2.8 mM). Homogenized rumen contents (HRC) were added and the

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Fig. 1. A proposed structure for *N*-carbamoyl- β -D-(+)-glucopyranosylamine (NCG).

mixture was dispensed in 10 ml amounts to sterile Hungate tubes; these were sealed under 100 % CO_2 and incubated at 39 °C. Homogenized rumen fluid was prepared by removing the rumen contents from a sheep that had been fed on 25 g NCG and 600 g brome grass (*Bromus inermis*) hay twice daily, for at least 3 weeks. The contents were transported to the laboratory in a Dewar flask, flushed with CO_2 within 30 min of collection and then stored until needed. The rumen contents were strained through one layer of cheesecloth to remove large particles and transferred to an Omnimixer vessel (Ivan Sorvall, Conn., U.S.A.), the head space was flushed with 100 % CO_2 and the vessel was sealed, placed in an ice/water mixture and attached to the drive motor which was operated at full speed for 2 min. This preparation was designated HRC.

To measure the degradation of NCG, 1 ml medium was chromatographed as a band on Whatman no. 1 chromatography paper (23 $\times$ 57 cm) using NCG, glucose and urea as standards. After development, the origin and areas corresponding to urea and NCG were cut out, placed in 1 ml water, shaken, mixed with 10 ml Aquasol 2 (New England Nuclear) and then counted by liquid scintillation. The area corresponding to urea in a second chromatogram was cut out, placed in a sealed Hungate tube and exposed to Jackbean urease (Worthington Biochemical Corp., N.J., U.S.A.) as outlined by Fawcett & Scott (1960). The resulting CO_2 was flushed from the tube and collected. The collection system consisted of a water trap, followed by an H_2S trap (cadmium acetate, 15.25 g; acetic acid, 62.5 ml; water, 437.5 ml) and then a CO_2 trap (10 ml of the following solution: toluene, 500 ml; methanol, 300 ml; ethanolamine, 200 ml; 2,5-diphenyloxazole, 5.0 g; 1,4-bis[2-(5-phenyloxazolyl)]benzene, 0.2 g). A 22 gauge needle connected to the trapping system was forced through the Hungate tube stopper, 0.3 ml conc. H_2SO_4 was injected via a second needle into the medium, the medium was heated to boiling and flushed with N_2 for 2 min. The CO_2 trapping scintillation solution was poured into a scintillation vial, along with methanol washings from all inner surfaces of the CO_2 collector, and counted.

When required, filter-sterilized acetohydroxamic acid (AHA) was added to the medium through the Hungate tube stopper with a syringe and needle. Depending on the experiment, AHA was maintained at either 10.7 or 26.7 mM by extra additions throughout the course of incubation according to AHA decay in rumen fluid as described by Jones (1968).

Biological nature of NCG degradation. Rumen contents from a sheep partially adapted to feeding on NCG were homogenized and one half was sterilized at 121 °C under 100 % CO_2 . Complex medium was prepared without rumen fluid and sterilized at 121 °C; to one half was added filter-sterilized [carbamoyl- ^{14}C] NCG to give 4.5 mM and to the other was added filter-sterilized [^{14}C]urea to give 8.3 mM. Each half of the medium was divided into four equal portions. Two portions from each half were inoculated with 40 % (v/v) unsterilized HRC and the other two were inoculated with 40 % (v/v) sterile HRC. After all portions had been dispensed in 10 ml amounts to sterile Hungate tubes and sealed under 100 % CO_2 , Jackbean urease [0.3 mg per tube, 141 Sumner Units (SU) mg^{-1}] was added to half the tubes of each inoculum treatment. All tubes were incubated at 39 °C. They were then acidified and the CO_2 was trapped and counted.

Non-specific acetohydroxamic acid effects. Complex medium without HRC was dispensed into eight separate flasks and sterilized. Filter-sterilized [carbamoyl- ^{14}C]NCG, [glucosamine- ^{14}C]NCG, [^{14}C]urea and [^{14}C]glucose were added to separate duplicate flasks of medium to give 4.5 mM, 4.5 mM, 8.3 mM and 2.8 mM, respectively. To one flask from each substrate group, filter-sterilized AHA was added to give 10.7 mM; the other served as a control. To all flasks, 40 % (v/v) HRC was added, and the contents were mixed and dispensed anaerobically and aseptically in 10 ml lots to sterile Hungate tubes; these were sealed under 100 % CO_2 and incubated at 39 °C. After incubation, the tubes were acidified, and the CO_2 was collected and counted.

Detection of urea production. Complex medium containing 90.1 mM-[carbamoyl- ^{14}C]NCG (5.55×10^4 d.p.m. mol^{-1}), with or without 26.7 mM-AHA, was inoculated with 40 % (v/v) HRC and dispensed anaerobically and aseptically in 10 ml quantities to sterile Hungate tubes; these were sealed under 100 % CO_2 and incubated at 39 °C. At intervals, two tubes were removed from each group (with or without AHA) and 1 ml of the medium was chromatographed as a band on each of two separate sheets of Whatman no. 1

Table 1. *Biological nature of the degradation of N-[¹⁴C]carbamoyl-β-D(+)-glucopyranosylamine by mixed rumen microbes*

Complex medium containing [¹⁴C]urea (8.3 mM) or [*carbamoyl*-¹⁴C]NCG (4.5 mM) was inoculated with 40 % (v/v) sterilized or unsterilized homogenized rumen contents from a 'partially adapted' sheep fed 50 g NCG daily for only 9 d. Where indicated, Jackbean urease (42.3 Sumner Units per 10 ml) was also added before incubation at 39 °C. The values for production of ¹⁴CO₂ are for single analyses or means of duplicate analyses; the range about the mean was less than 2 %.

Substrate	Urease	Inoculum	Incubation time (h)	¹⁴ CO ₂ produced (% of added ¹⁴ C)	
Urea	+	Sterile	0	14.5	
			12	84.7	
	+	Viable	0	11.8	
			1	86.8	
	—	Sterile	0	0.1	
			12	1.6	
	—	Viable	0	7.7	
			1	74.7	
	NCG	+	Sterile	0	0.4
				12	0.5
48				0.4	
+		Viable	0	0.5	
			12	4.7	
			48	10.9	
—		Sterile	0	0.1	
			12	0.1	
			48	0.2	
—		Viable	0	0.5	
			12	4.1	
			48	11.8	

paper. NCG, urea and glucose were included as standards. After development, the standards strip was cut from the chromatogram and visualized with the AgNO₃ technique. The strips at the origin and those corresponding to NCG and urea were cut out and counted by liquid scintillation as described above. The area corresponding to urea was cut out from the second chromatogram, treated with 0.3 mg Jackbean urease, and the CO₂ produced was collected and counted as previously outlined. After acidification, all free CO₂ present in the Hungate tube was collected and counted as previously outlined; the removal of 2 ml of medium for chromatography was taken into account.

In triplicate analyses, CO₂ collection was found to be 99 % complete. The yield of CO₂ from [¹⁴C]urea on filter paper treated with Jackbean urease was 58 % of the theoretical yield in duplicate analyses. The inefficiency may have been due to binding of the urease or urea to the paper causing unavailability of one to the other (Lodish & Zinder, 1965). The efficiency of recovery of NCG from chromatograms was 76.9 % based on duplicate analyses.

Location of NCG degradation activity in rumen contents. Using an anaerobic glove box (Aranki & Freter, 1972), whole rumen contents from a fully adapted sheep were put sequentially through a series of sterilized filters (four layers of cheesecloth, Whatman no. 50 paper, and membrane filters of 8, 1.2 and 0.2 μm pore sizes) to yield a cell-free filtrate which was then mixed with 0.1 ml sterile [*carbamoyl*-¹⁴C]NCG (0.045 mmol, 1.98 × 10³ d.p.m.) solution and either 1 ml sterile Jackbean urease solution (0.3 mg in 0.6 mM-NaH₂PO₄/Na₂HPO₄ buffer, pH 6.6) or 1 ml sterile buffer. After incubation at 39 °C for 4 d, the mixture was subjected to chromatography and the CO₂ produced was collected.

RESULTS AND DISCUSSION

Effect of using Jackbean urease and sterilized homogenized rumen contents on the hydrolysis of urea and the degradation of NCG

¹⁴CO₂ was produced rapidly from [¹⁴C]urea and at a lesser rate from [*carbamoyl*-¹⁴C]-NCG by HRC (Table 1). Jackbean urease did not produce ¹⁴CO₂ from NCG in the presence of sterilized HRC but was very effective in hydrolysing urea under similar conditions. A

Table 2. *Effect of acetohydroxamic acid on the production of CO₂ from glucose, urea and N-carbamoyl-β-D(+)-glucopyranosylamine (NCG) by mixed rumen microbes*

Complex medium containing [U-¹⁴C]glucose (2.8 mM), [¹⁴C]urea (8.3 mM), [carbamoyl-¹⁴C]NCG (4.5 mM) or [glucosamine-U-¹⁴C]NCG (4.5 mM), and with or without acetohydroxamic acid (AHA; maintained at 10.7 mM), was inoculated with 40 % (v/v) homogenized rumen contents from a sheep fully adapted to feeding on NCG. The values for the production of ¹⁴CO₂ are means of duplicate and triplicate analyses; the range about the mean was less than 4 %.

Incubation time (h)	Recovery of ¹⁴ CO ₂ from substrate (% of added ¹⁴ C)							
	[U- ¹⁴ C]Glucose		[¹⁴ C]Urea		[carbamoyl- ¹⁴ C]NCG		[glucosamine-U- ¹⁴ C]NCG	
	-AHA	+AHA	-AHA	+AHA	-AHA	+AHA	-AHA	+AHA
0	0.2	0.2	1.2	0.2	0.9	0.8	0.3	0.4
4	3.4	4.6	73.4	11.5	12.0	7.9	ND	ND
7	9.2	14.5	98.1	19.0	21.5	8.2	ND	ND
18	14.8	13.1	ND	20.1	41.1	ND	ND	ND
30	32.3	31.6	ND	18.9	98.5	65.2	10.7	6.8
46	36.8	36.8	ND	22.7	ND	ND	ND	ND
48	ND	ND	ND	ND	ND	ND	26.5	24.3

ND, Not determined.

viable inoculum plus Jackbean urease did not yield a greater amount of ¹⁴CO₂ from [carbamoyl-¹⁴C]NCG than the viable inoculum alone indicating that the inoculum at 40 % (v/v) either supplied adequate amounts of bacterial urease or that urease is not required for the production of ammonia. Autoclaved HRC produced negligible ¹⁴CO₂ from both substrates; thus the degradation of NCG, like urea, required a viable biological system.

Acetohydroxamic acid effect on CO₂ production from glucose, urea and NCG

There was 79 % inhibition of hydrolysis of urea by HRC when AHA was added to the medium (Table 2). This is comparable to the results of Jones (1968). However, AHA did not influence the production of ¹⁴CO₂ from [U-¹⁴C]glucose. Production of ¹⁴CO₂ from [carbamoyl-¹⁴C]NCG in the presence of AHA was decreased whereas little effect was seen with [glucosamine-¹⁴C]NCG as substrate. More ¹⁴C from [U-¹⁴C]glucose was recovered in CO₂ (37 % at 46 h) than would be expected on the basis of fermentation pathways in the rumen (Hungate, 1966) but this may have been due to the inadvertent recovery of labelled volatile organic acids with the CO₂. In subsequent experiments, a liquid trap was inserted in the CO₂ collection apparatus.

Production of urea from NCG

The mean total recovery of ¹⁴C on incubation of [carbamoyl-¹⁴C]NCG with HRC in the presence of AHA was 95.8 % compared with 93.8 % recovery for the control (Table 3). Although not shown in Table 3, the amount of ¹⁴C measured by counting the urea area of chromatograms was consistently 2 to 3 % less than the amount of ¹⁴CO₂ produced by the action of urease on a similar urea-containing area from a comparable chromatogram; this may have been due to interference of the paper in the scintillation vials.

Clearly, when the urea-containing areas of the paper chromatograms of complex medium incubated with [carbamoyl-¹⁴C]NCG were exposed to Jackbean urease, ¹⁴CO₂ was produced. The total ¹⁴C recovered in urea plus CO₂ was similar in the presence and absence of AHA. However, there was much more ¹⁴C in urea in the cultures containing AHA than in the absence of AHA at all times after initiation of incubation; urea accumulated when urease was inhibited. The detection of urea on the chromatogram of AHA-containing medium incubated for 45 h with [carbamoyl-¹⁴C]NCG as the labelled substrate provided

Table 3. *Effect of acetohydroxamic acid on the relative distribution of ^{14}C derived from $\text{N}-[^{14}\text{C}]\text{carbamoyl-}\beta\text{-D}(+)\text{-glucopyranosylamine}$ in cultures of mixed rumen microbes*

Complex medium containing [*carbamoyl- ^{14}C*]NCG (90.1 mM; 5.55×10^4 d.p.m. mol^{-1}), with or without acetohydroxamic acid (AHA; maintained at 26.7 mM), was inoculated with homogenized rumen contents from a sheep fully adapted to feeding on NCG. To determine the ^{14}C present in urea, the urea-containing band was cut from the chromatogram, treated with Jackbean urease and the $^{14}\text{CO}_2$ produced was trapped and counted. The values for ^{14}C distribution are from single analyses or means of duplicate analyses; the range about the mean was less than 6%.

Incubation time (h)	AHA	Distribution of ^{14}C (% of added ^{14}C)				Total recovery*
		Urea CO_2	Culture CO_2	NCG	Origin of chromatogram	
0	—	0.3	0.1	100.7	0.2	101.3
0	+	0.7	0.1	101.9	0.2	102.9
19	—	0.1	12.5	75.5	0.5	88.6
19	+	2.2	8.3	80.2	0.8	91.5
28	—	0.1	16.4	73.4	0.5	90.4
28	+	4.0	15.1	75.8	1.6	96.5
45	—	0.8	22.2	73.6	0.8	97.4
45	+	7.4	16.9	63.5	1.5	89.3
65	—	0.1	30.2	62.8	0.4	93.5
65	+	11.2	23.2	59.7	0.9	95.0
89	—	0.1	36.2	57.1	0.7	94.1
89	+	16.9	22.9	51.9	0.7	92.4
115	—	0.7	44.0	49.0	1.5	96.2
115	+	21.7	23.0	50.1	1.3	96.1
135	—	0.7	47.2	38.9	2.0	88.8
135	+	32.2	25.4	44.3	1.4	103.3

* Urea CO_2 + culture CO_2 + NCG + origin of chromatogram.

further evidence for the accumulation of urea in the presence of AHA. The logical conclusion was that urea was an intermediate of NCG degradation by rumen microbes.

Urease inhibition increased with time (Table 3), with a concomitant decrease of ^{14}C in CO_2 when compared to the ^{14}C in CO_2 produced by the control without AHA (Table 3). The disappearance of [*carbamoyl- ^{14}C*]NCG from the medium occurred at approximately the same rate in the presence and absence of AHA. A non-linear increase in the amount of ^{14}C present in urea derived from NCG suggests an increase over time in the effectiveness of AHA as a urease inhibitor. Kobashi *et al.* (1962) reported a time-dependent increase in the effectiveness of AHA as a urease inhibitor. A modified microbial population might, however, be a factor in the present time-dependent effect since the experiment entailed lengthy incubation periods; or the actual rate of AHA degradation may have been less than that measured by Jones (1968) with the result that AHA accumulated from our sequential additions.

Location of NCG degradation activity in rumen contents

Using cell-free rumen filtrate, the recovery of $^{14}\text{CO}_2$ from [*carbamoyl- ^{14}C*]NCG in tubes without Jackbean urease was 0.6%, whereas $^{14}\text{CO}_2$ recovery from [*carbamoyl- ^{14}C*]NCG in tubes containing Jackbean urease was 0.9%. Paper chromatographic analysis of the cell-free rumen contents revealed no qualitative changes in the amount of NCG and there was no indication of the formation of new products. Since considerable care was taken to filter the rumen fluid under anaerobic conditions (Aranki & Freter, 1971) and avoid any potentially detrimental exposure to O_2 , it appears that the activity for NCG degradation may be associated with the particulate, or cell fraction.

Concluding remarks

This study indicated that the degradation of NCG required a viable biological system and the activity was probably associated with the particulate fraction of rumen contents. Very likely an initial cleavage of NCG produces urea which argues well for the use of NCG as a non-protein nitrogen source, since urea is known not to be noxious for ruminants. The chemical bond between the glucose and urea moieties of NCG that would be cleaved is novel in relation to the other types of bonds encountered in biological degradation. Perhaps this uniqueness contributes to the slow degradation by rumen microbes.

The authors wish to thank the National Research Council of Canada for partial funding of this project, Western Co-op Fertilizers Ltd for providing BBGU (a commercial preparation of NCG), the Department of Microbiology, University of Alberta, for use of equipment and J. Wohlebe for guidance during the chemical synthesis of NCG.

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