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In Vitro Metabolism of 2,2′-Diaminopimelic Acid from Gram-Positive and Gram-Negative Bacterial Cells by Ruminal Protozoa and Bacteria

ANNA M. DENHOLM AND JOHN R. LING*

Department of Biochemistry, University College of Wales, Aberystwyth SY23 3DD, Wales, United Kingdom

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***Bacillus megaterium* GW1 and *Escherichia coli* W7-M5 were specifically radiolabeled with 2,2′-diamino[G-^{3}H]pimelic acid ([^{3}H]DAP) as models of gram-positive and gram-negative bacteria, respectively. These radiolabeled bacterial mutants were incubated alone (control) and with mixed ruminal bacteria or protozoa, and the metabolic processes, rates, and patterns of radiolabeled products released from them were studied. Control incubations revealed an inherent difference between the two substrates; gram-positive supernatants consistently contained 5% radioactivity, whereas even at 0 h, those from the gram-negative mutant released 22%. Incubations with ruminal microorganisms showed that the two mutants were metabolized differently and that protozoa were the major effectors of their metabolism. Protozoa exhibited differential rates of engulfment (150 *B. megaterium* GW1 and 4,290 *E. coli* W7-M5 organisms per protozoan per h), and they extensively degraded [^{3}H]DAP-labeled *B. megaterium* GW1 at rates up to nine times greater than those of ruminal bacteria. By contrast, [^{3}H]DAP-labeled *E. coli* W7-M5 degradation by either ruminal bacteria or ruminal protozoa was more limited. These fundamental differences in the metabolism of the two mutants, especially by ruminal protozoa, were reflected in the patterns and rates of radiolabeled metabolites produced; many were rapidly released from [^{3}H]DAP-labeled *B. megaterium* GW1, whereas few were slowly released from [^{3}H]DAP-labeled *E. coli* W7-M5. Most radiolabeled products derived from [^{3}H]DAP-labeled *B. megaterium* GW1 were peptides of bacterial peptidoglycan origin. The ruminal metabolism of DAP-containing gram-positive and gram-negative bacteria, even with the same peptidoglycan chemotype, is thus likely to be profoundly different. This may affect the precision of DAP as a marker of bacterial biomass in digesta.**

Bacterial cell walls are structurally diverse, yet those of most gram-positive and gram-negative bacteria contain peptidoglycan. This heteropolymer, which consists of glycan strands cross-linked through short peptides, commonly contains 2,2′-diaminopimelic acid (DAP) in the peptide moiety (18, 31).

This amino acid is thought to be unique to bacteria. This uniqueness has been exploited by using DAP as a marker to measure biomass of bacterial origin, particularly within the digestive tracts of ruminant animals. The main use of DAP has been in the estimation of ruminal outflow of bacterial N contained in duodenal digesta (15, 22, 32, 36). In fact, about 50% of the values for ruminal microbial N yield cited in a comprehensive review (1, 2) were derived from DAP measurements.

For DAP to be an effective marker, it must accurately reflect the content of bacterial biomass in digesta. One potential limitation of DAP concerns the extent of its metabolism within the rumen. The fate of, for example, DAP contained in ruminal bacteria that are lysed or engulfed by ruminal protozoa is uncertain. Preliminary in vitro studies in this area have shown that ruminal microorganisms can metabolize free DAP, principally by decarboxylating it to form lysine (19, 25). Furthermore, bacterially bound DAP can also be substantially metabolized by both bacteria and protozoa from the rumen (23, 26). When bacterially bound DAP in the form of [^{3}H]DAP-labeled *Bacillus megaterium* GW1 was the substrate, several radiolabeled metabolic products were released into the incubation medium (23). The major product of such incubations has been further investigated, and initial results have suggested that it could be a series of DAP-containing peptidoglycan peptides and derivatives (10).

The aim of the present study was to examine in greater scope and detail the in vitro processes, rates, and patterns of DAP metabolism when DAP-containing bacteria were incubated with microorganisms from the rumen. To examine a range of bacterial substrates, these studies used [^{3}H]DAP-labeled mutants, namely, *B. megaterium* GW1 and *Escherichia coli* W7-M5, as models of gram-positive and gram-negative bacteria, respectively.

MATERIALS AND METHODS

When appropriate, all media and solutions were autoclaved at 103 kPa for 15 min. Anaerobic conditions were maintained by gassing (approximately 100 ml/min) with 95% N_2–5% CO_2 that had passed through a column of copper turnings heated at 350°C (20).

Mutant bacteria and growth conditions. *B. megaterium* GW1 (kindly supplied by P. J. White, Department of Microbiology, The University, Sheffield, United Kingdom [U.K.]) and *E. coli* W7-M5 (obtained from the culture collection of the Department of Dairy Science, University of Illinois, Urbana) were used as representatives of gram-positive and gram-negative bacteria. Both mutants are auxotrophic for DAP and lysine (29, 33). Cultures were maintained at 4°C on nutrient agar plates containing (per liter) either 0.02 g of DAP for *B. megaterium* GW1 or 0.02 g of DAP, 0.02 g of glucosamine hydrochloride, and 0.5 g of yeast extract (London Analytical & Bacteriological Media, Salford, U.K.) for *E. coli* W7-M5. Media were adjusted to pH 6.8 for *B. megaterium* GW1 and pH 7.6 for *E. coli* W7-M5. The DAP

* Corresponding author.

and lysine growth requirements were regularly checked by selective plating.

Radioactive labeling of mutant bacteria. Samples of (DL + *meso*)-2,2′-diamino[G-^{3}H]pimelic acid hydrochloride ([^{3}H]DAP; 10.4 kBq/μmol; Amersham International, Amersham, U.K.) were purified by elution from a cation-exchange resin column (23). Under these conditions, [^{3}H]DAP was eluted from 102 to 108 min. These fractions were pooled and filter sterilized (0.22-μm pore size), and their radioactive content was measured in 50-μl subsamples.

Cultures of *B. megaterium* GW1 and *E. coli* W7-M5 were radiolabeled by adding inocula (10% [vol/vol]) to nutrient broth medium (No. 2; London Analytical & Bacteriological Media; 25 g/liter) containing (per liter) various amounts of purified [^{3}H]DAP and 0.02 g of DAP for both mutants together with 0.02 g of glucosamine hydrochloride and 0.5 g of yeast extract for the *E. coli* W7-M5 mutant. These cultures were incubated at 37°C in a rotary shaker (120 rpm). After approximately 20 h of incubation, cells were harvested by centrifugation (16,000 × *g*, 20 min, 5°C). The pellets were washed twice with saline (9 g of NaCl per liter) solution and once with distilled water. The resulting cells were suspended at the appropriate population density in Hungate salt solution (13) (NaCl, 5.0 g/liter; $CH_3COONa \cdot 3H_2O$, 1.5 g/liter; K_2HPO_4, 1.0 g/liter; KH_2PO_4, 0.3 g/liter) and gassed for 10 min before being incubated alone (controls) or with various preparations of ruminal microorganisms.

Rumen microbial preparations. Samples of ruminal digesta were obtained from mature wether sheep each fitted with a permanent ruminal cannula. The animals were given a diet of grain-based concentrate and hay at a rate of approximately 700 and 500 g (fresh weight), respectively, per day in two equal portions at 0800 and 1600 h.

Digesta was removed via the ruminal cannula 3 h after the morning feed and strained through cheesecloth to provide strained ruminal digesta. This was centrifuged (200 × *g*, 30 s), and the supernatant was the preparation designated ruminal bacteria + few protozoa. A portion of this was further centrifuged (2,300 × *g*, 1 min), and the resultant supernatant (ruminal bacteria preparation) was shown by microscopic examination to be free from all protozoa and virtually all plant material. Some of this preparation was then centrifuged (30,000 × *g*, 15 min, 5°C), and the supernatant was called the cell-free bacterial supernatant. When a portion of this was heated (100°C, 30 s) and cooled, it provided the preparation called the heated, cell-free bacterial supernatant.

The pellet obtained from the initial 200 × *g* centrifugation was used to produce the rumen protozoal preparations. The pellet was washed with Hungate salt solution until essentially free from feed material, and then it was suspended in Hungate salt solution at a population density similar to that of the strained ruminal digesta; this preparation was called ruminal protozoa. A portion of this was centrifuged (30,000 × *g*, 15 min, 5°C), and the supernatant was the cell-free protozoal supernatant. When some of this was heated at 100°C for 30 s and cooled, it became the heated, cell-free protozoal supernatant. Samples of the ruminal protozoa preparation were ultrasonically disrupted (Soniprep 150; MSE, Crawley, U.K.; 3 × 15 s at full power, 130 W) to produce the preparation designated sonicated protozoa.

In vitro incubations and sample preparations. Methods for counting bacteria and protozoa, as well as the conditions for incubating them, were similar to those of Masson and Ling (23). Incubations (of 10 or 26 ml) were routinely performed in either Hungate-type anaerobic culture tubes (Bellco Glass, Inc., Vineland, N.J.) or Erlenmeyer flasks fitted with rubber stoppers (Subaseal; Gallenkamp, Loughborough, U.K.). Additional incubations of [^{3}H]DAP-labeled *B. megaterium* GW1 with ruminal protozoa were conducted on a larger scale (65 ml) but with similar procedures.

To every 1 ml of the various control and rumen microbial preparations we added 0.1 ml of cysteine solution (20 g of L-cysteine hydrochloride · H_2O per liter, adjusted to pH 7.2 immediately before use) and 1.5 mg of $NaHCO_3$. After addition of the radiolabeled mutant bacteria, the contents of the vessels were gassed, sealed, and incubated at 39°C for up to 8 h. The contents were then fractionated into pellets (200 × *g*, 30 s) and supernatants. The pellets were stored at −20°C until required for analysis. The supernatants were deproteinized by being mixed with equal volumes of ice-cold picrate (20 g/liter) overnight and then centrifuged (16,000 × *g*, 30 min, 5°C) before removal of the picric acid by anion-exchange column chromatography. The sample eluents were dried under reduced pressure at 37°C, washed with distilled water, dissolved in 0.2 M HCl, and clarified by centrifugation (11,000 × *g*, 5 min).

Isolation and analysis of radiolabeled samples and metabolites. Radiolabeled compounds present in the deproteinized supernatant samples were separated by elution from a cation-exchange resin (Locarte Co., London, U. K.; resin LA/49/36/10) column (215 by 9 mm [inside diameter]). The elution system was a linear gradient (24) with 250 ml of starting buffer composed of 0.2 M pyridine–glacial acetic acid (pH 3.11) and 250 ml of limiting buffer of 2.0 M pyridine–glacial acetic acid (pH 4.22). The buffer flow rate was 0.5 ml/min, and the column temperature was 52°C. With this system, DAP was eluted between 178 and 184 min and lysine was eluted from 458 to 468 min.

Acid hydrolysis was performed by refluxing with 6 M HCl for 22 h. Amino acids and metabolites were separated and quantified with an amino acid analyzer (model 5; Locarte Co.) with ninhydrin detection (22).

The radioactivity contained in microbial samples, column effluent fractions (2 or 4 min), and acid-hydrolyzed samples was detected with scintillant (10 ml of Lumagel; LKB, London, U.K.) and a scintillation counter (SL 30; Intertechnique SA, Plaisir, France). Quenching was corrected by the external-standard channel ratio facility.

Deproteinized supernatants produced from the larger-scale incubations were analyzed by the methods described above. Column effluent fractions that corresponded to a particular peak of radioactivity were pooled and dried under reduced pressure at 37°C to remove chromatographic solvents. Several of these metabolic products were subjected to acid hydrolysis and amino acid analysis, as well as amino acid sequence analysis with 4-N,N-dimethylaminoazobenzene 4′-isothiocyanate and the manual methods of Chang et al. (3).

RESULTS

Metabolism of [^{3}H]DAP-labeled *B. megaterium* GW1 by rumen microbial preparations. Preliminary experiments examined the radioactivity contents of deproteinized supernatants derived from 2-h incubations of [^{3}H]DAP-labeled *B. megaterium* GW1 with various rumen microbial preparations.

The results (expressed as percent total radioactivity added) are shown in Table 1. Those obtained with rumen bacterial preparations were similar to but greater than that of the control. Values increased slightly with the cell-free

TABLE 1. Comparison of radioactivities in deproteinized supernatants after incubation of [^{3}H]DAP-labeled *B. megaterium* GW1 alone (control) or with ruminal microbial preparations

Prepn[a]	% Radioactivity in supernatant
Control[b]	2.7
Ruminal bacteria + few protozoa[c]	4.9
Ruminal bacteria[d]	4.4
Cell-free bacterial supernatant	6.6
Heated, cell-free bacterial supernatant	3.0
Ruminal protozoa[e]	40.2
Cell-free protozoal supernatant	13.2
Heated, cell-free protozoal supernatant	5.1
Sonicated protozoa[f]	58.2

[a] A 22-ml volume of each preparation was incubated for 2 h with 4 ml of [^{3}H]DAP-labeled *B. megaterium* GW1 (2.68 Mdpm/ml; 1.1×10^{10} cells per ml).
[b] [^{3}H]DAP-labeled *B. megaterium* GW1 alone.
[c] Contained 4.0×10^9 mixed ruminal bacteria per ml and $<10^3$ small ruminal protozoa per ml.
[d] Contained 4.0×10^9 mixed ruminal bacteria per ml.
[e] Contained 3.4×10^5 mixed protozoa per ml.
[f] After only 1 h of incubation.

bacterial supernatant but decreased when this preparation was mildly heat treated. Results with ruminal protozoa were higher (40.2%) and highest when sonicated before incubation. The cell-free protozoal supernatant produced a value of only 13.2%, which decreased after heat treatment.

Comparative metabolism of [^{3}H]DAP-labeled *B. megaterium* GW1 and [^{3}H]DAP-labeled *E. coli* W7-M5 by ruminal microorganisms. Both radiolabeled mutant bacteria were incubated alone (control), with ruminal bacteria, or with two population densities of ruminal protozoa.

The distribution of radioactivity (expressed as percent total radioactivity added) found in both acid-hydrolyzed pellets and deproteinized supernatants from these incubations is shown in Table 2. Proportions of radioactivity were present in the control supernatants of both mutants, even at zero incubation times. These control values exhibited little variation with time although the [^{3}H]DAP-labeled *E. coli* W7-M5 values were always some fivefold greater than those for [^{3}H]DAP-labeled *B. megaterium* GW1 (24.6 ± 2.51 versus 5.5 ± 0.24 [mean ± standard error]).

Incubations of the radiolabeled mutants with ruminal bacteria produced supernatants that contained proportions of radioactivity similar to the respective control values.

The proportions of radioactivity in the supernatants from protozoal incubations with [^{3}H]DAP-labeled *B. megaterium* GW1 were responsive to both time of incubation and numbers of protozoa; that is, they increased with both parameters. The comparable [^{3}H]DAP-labeled *E. coli* W7-M5 data were quite different; not only were they very similar to their control values, but they even tended to decline with time and protozoal numbers.

Furthermore, the pellets produced after incubations of ruminal protozoa with [^{3}H]DAP-labeled *B. megaterium* GW1 contained proportions of radioactivities that were approximately constant (10.7 ± 0.43) and apparently unaffected by either incubation time or numbers of protozoa, whereas those from [^{3}H]DAP-labeled *E. coli* W7-M5 incubations were proportional to these experimental parameters.

Products of metabolism of [^{3}H]DAP-labeled *B. megaterium* GW1 and [^{3}H]DAP-labeled *E. coli* W7-M5 by ruminal microorganisms. The metabolites contained in the deproteinized supernatants were separated, and their radioactivities were measured; the elution patterns derived from the incubations of gram-positive and gram-negative bacteria alone (controls) and with ruminal bacteria or ruminal protozoa are contrasted in Fig. 1 and Fig. 2., respectively.

In all of the profiles, the major radiolabeled peak (178 to 184 min) corresponded to [^{3}H]DAP. In the [^{3}H]DAP-labeled *B. megaterium* GW1 incubation series, the control (Fig. 1a) had most of the total profile radioactivity contained in DAP and two earlier eluted (8 to 14 and 28 to 40 min) peaks. Incubation with ruminal bacteria resulted in a limited number of small radioactive peaks (Fig. 1b). The profiles (Fig. 2a to d) resulting from incubation for up to 2 h with ruminal protozoa exhibited not only a steadily increasing number of metabolites but also a general increase of radiolabel contained within these compounds. At 4 h and after (Fig. 2e and f), the radioactive metabolites tended to exhibit a decline in number and content; the notable exceptions were peaks eluted at 44 to 54 and 76 to 86 min and that of lysine (470 to 496 min). The latter steadily increased until at 8 h it contained 6.3% of the total profile radioactivity.

TABLE 2. Comparison of radioactivities in acid-hydrolyzed pellets and deproteinized supernatants after incubations of [^{3}H]DAP-labeled *B. megaterium* GW1 or [^{3}H]DAP-labeled *E. coli* W7-M5 alone (control) and with mixed ruminal bacteria or protozoa

Incubation[a]	Protozoa/ml (10^5)	Incubation time (h)	% Total radioactivity after incubation with: [^{3}H]DAP-labeled *B. megaterium* GW1		[^{3}H]DAP-labeled *E. coli* W7-M5	
			Pellet	Supernatant	Pellet	Supernatant
Control	0	0	ND[b]	5.6	ND	22.5
	0	1	ND	5.8	ND	29.6
	0	2	ND	5.0	ND	21.7
Ruminal bacteria[c]	0	2	ND	6.9	ND	23.9
Ruminal protozoa	3.28	0	ND	6.6	ND	29.1
	3.28	1	9.9	15.5	23.9	27.0
	3.28	2	11.5	22.2	49.3	25.7
	6.57	1	11.4	34.4	30.5	26.8
	6.57	2	10.0	37.2	57.2	18.5

[a] Each incubation contained either [^{3}H]DAP-labeled *B. megaterium* GW1 (130 kdpm/ml; 2.0×10^8 cells per ml) or [^{3}H]DAP-labeled *E. coli* W7-M5 (52 kdpm/ml; 7.2×10^9 cells per ml).
[b] ND, Not determined.
[c] Contained 2.0×10^{10} mixed ruminal bacteria per ml.

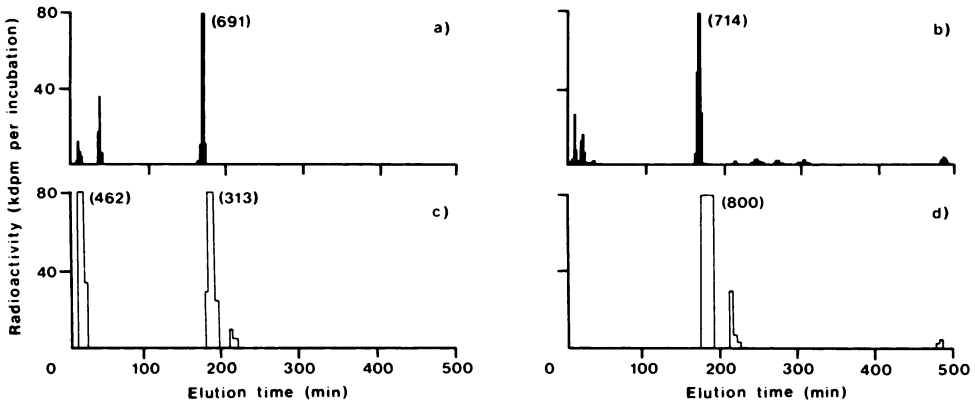

FIG. 1. Elution profiles of radiolabeled metabolites in deproteinized supernatants from 2-h incubations of [^{3}H]DAP-labeled *B. megaterium* GW1 alone (a; control), with mixed ruminal bacteria (b) and of [^{3}H]DAP-labeled *E. coli* W7-M5 alone (c; control) and with mixed ruminal bacteria (d). Values in parentheses are the sums of radioactivity (disintegrations per minute, 10^3) for that particular peak.

The profiles from the [^{3}H]DAP-labeled *E. coli* W7-M5 incubation series were outstandingly different. That of the control (Fig. 1c) contained 53% of its radioactivity in a single early-eluted (4 to 16-min) peak, 36% in DAP, and 12% in a later (212- to 224-min) peak. When ruminal bacteria were incubated with [^{3}H]DAP-labeled *E. coli* W7-M5, no early-eluted peaks were detected (Fig. 1d); only those due to [^{3}H]DAP, a peak eluted between 212 and 224 min, and lysine were evident. Incubation of the radiolabeled mutant with ruminal protozoa from 0 to 8 h produced profiles (Fig. 2g to j) that were similar to that of the control. Most of the radioactivity was contained in just three peaks; the early-eluted peak showed a decline with time, but the other two exhibited no consistent trend. No lysine peak appeared at any time.

Composition of products of metabolism. Several radiolabeled peaks, denoted by letter codes and elution times in Table 3, were collected as column effluent fractions derived from deproteinized supernatant samples after the larger-scale 4-h incubations of [^{3}H]DAP-labeled *B. megaterium* GW1 with ruminal protozoa; the elution profiles were similar to that shown in Fig. 2e. Portions of the pooled effluents from each of the peaks were assessed for purity with a conventional amino acid analyzer elution program; each produced a single, symmetrical ninhydrin-positive peak. The compositions of additional portions were then determined by acid hydrolysis followed by amino acid analysis. All of the radiolabeled peaks contained DAP and at least one of four other amino acids (Table 3). Although the compositions appeared rational (that is, for example, acidic glutamate residues tended to predominate in early-eluted metabolites while the basic lysine residues occurred in late-eluted metabolites), it was not possible to calculate simple amino acid ratios for each incubation metabolite.

Metabolites D, J, M, W, and Y were additionally subjected to amino acid sequence analysis. When these were initially coupled to 4-N,N-dimethylaminoazobenzene 4′-isothiocyanate and run on a thin-layer chromatography system, they all produced single 4-N,N-dimethylaminoazobenzene 4′-isothiocyanate derivatives. However, the interpretation of subsequent analyses was difficult, since often more than one derivatized amino acid was detected at each sequencing cycle. Nevertheless, metabolite J was assigned a tentative sequence of Ala-DAP-Ala.

DISCUSSION

The ideal substrates for studying the ruminal metabolism of bacterially bound DAP would be ruminal bacteria specifically radiolabeled with the amino acid in question. Several attempts to produce such substrates were made at the outset of this study. However, when mixed bacteria from the rumen were incubated with [^{3}H]DAP for up to 8 h, 85% of the incorporated radiolabel was in the form of tritiated lysine. In addition, several individual strains of ruminal bacteria (kindly supplied from the culture collection of the Department of Dairy Science, University of Illinois, Urbana) were screened for their ability to incorporate [^{3}H]DAP. The maximum cell-associated radioactivities (expressed as percent [^{3}H]DAP added to the incubation media) were found to be low for *Streptococcus bovis* JB1 (3.7%), *Ruminococcus flavefaciens* FD1 (0.7%), *Lachnospira multiparus* D32 (0.8%), and *R. albus* 7 (0.4%). Because of such limited uptake, the search for appropriate bacteria species of ruminal origin was abandoned. Instead, *B. megaterium* GW1 and *E. coli* W7-M5 were used as representatives of gram-positive and gram-negative types of bacteria.

Incubation of these mutants alone (Table 2, control values) indicated some intrinsic difference between them. Such incubations of [^{3}H]DAP-labeled *E. coli* W7-M5 resulted in unexpectedly large and rapid releases of radioactivity into the supernatants (22.5% to 0 h) compared with only small releases (5.6% at 0 h) from [^{3}H]DAP-labeled *B. megaterium* GW1. When samples of [^{3}H]DAP-labeled *E. coli* W7-M5 were disrupted in a French pressure cell, 22% of their total radioactivity was solubilized. This readily released radioactivity may have been due to [^{3}H]DAP in the cytoplasmic and periplasmic pools rather than the cell wall pool. By contrast, at least 98% of the radioactivity in [^{3}H]DAP-labeled *B. megaterium* GW1 is known to be bound in cell wall material (23). This feature of [^{3}H]DAP-labeled *E. coli* W7-M5 may have contributed to the high and essentially constant results from controls and from most of the rumen microbial incubations. In addition, the method of radiolabeling both mutants may also have contributed some residual [^{3}H]DAP; certainly, the radioactivities of controls were due largely to [^{3}H]DAP (Fig. 1a and c).

Neither of these mutant bacterial species is numerically important in the rumen (14), yet their efficient incorporation

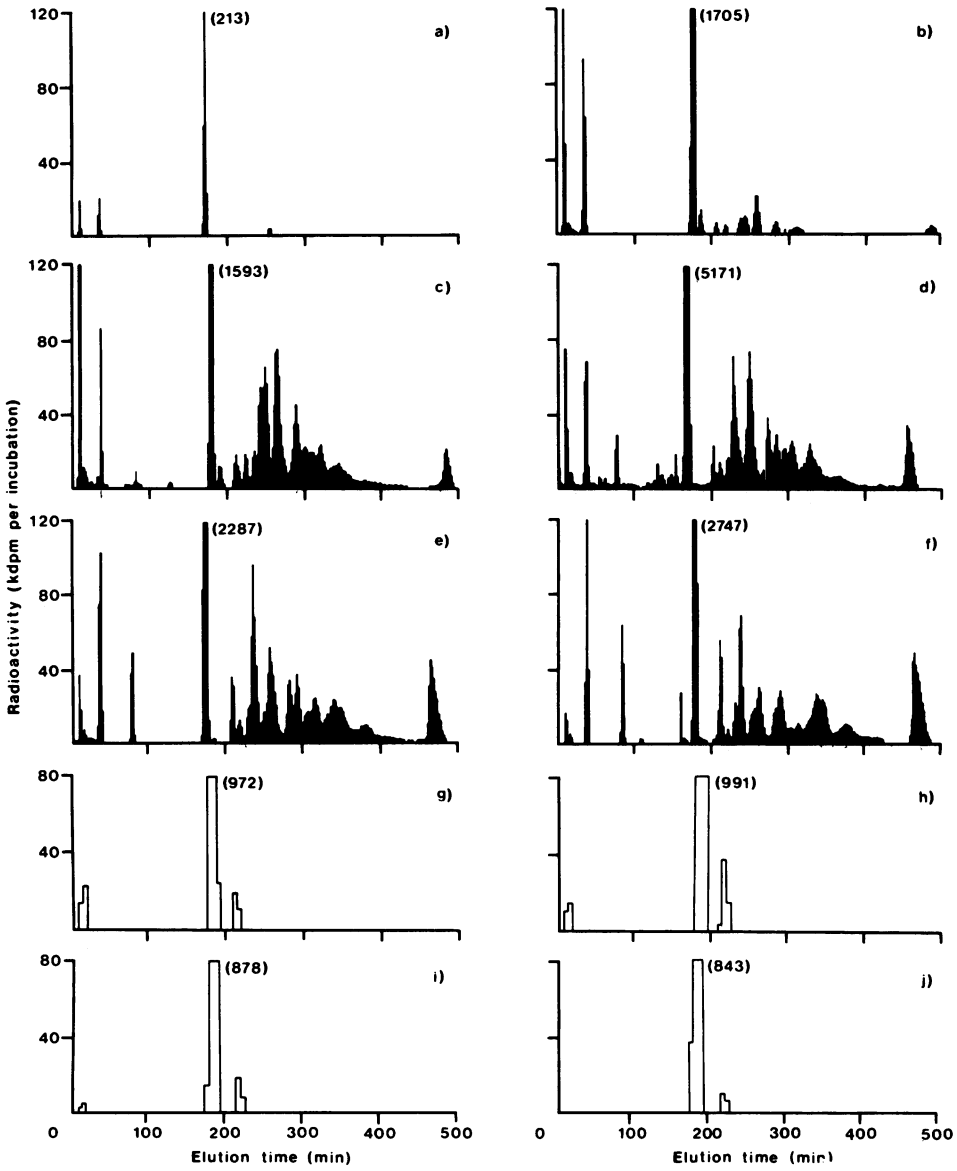

FIG. 2. Elution profiles of radiolabeled metabolites in deproteinized supernatants from incubations of [^{3}H]DAP-labeled *B. megaterium* GW1 with mixed ruminal protozoa for 0 h (a), 0.5 h (b), 1 h (c), 2 h (d), 4 h (e), and 8 h (f) and of [^{3}H]DAP-labeled *E. coli* W7-M5 with mixed ruminal protozoa for 0 h (g), 0.5 h (h), 4 h (i), and 8 h (j). Values in parentheses are the sums of radioactivity (disintegrations per minute, 10^3) for that particular peak.

of [^{3}H]DAP, their lack of endogenous [^{3}H]DAP metabolism, and their chemotypically similar peptidoglycan peptide moiety (type A1γ, which consists of Ala-Glu-DAP-Ala [30]), together with their fundamental difference of cell wall morphology do make them suitable, though perhaps not ideal, models for the study of the metabolism of gram-positive and gram-negative types of bacteria that inhabit the rumen.

If the appearance of radioactivity in the deproteinized supernatants of the incubations is assumed to be a measure of the degradation of radiolabeled bacterial cell walls, then Table 1 shows that ruminal protozoa are the major effectors of such a process. This relative importance of ruminal protozoa in the degradation of bacteria has been previously reported (6, 10, 11, 17, 34). Jarvis (17) demonstrated that most bacteriolytic activity in ruminal digesta was indeed protozoal in origin but that "soluble lytic activity" also existed in protozoan-free and bacterium-free suspensions. Such activity may in fact be attributable to bacteriophages and mycoplasmas (27, 28), but the present study suggests an additional source, namely, that of procedural artifact. When ruminal bacteria and protozoa were subjected to high-speed centrifugation (30,000 × *g*) at low temperature (5°C) and after some inevitable exposure to oxygen, the resulting cell-free supernatant preparations produced relatively high (Table 1, 6.6 and 13.2%, respectively) degradative activities. Exposure to low temperature (4°C) alone appears to be a cause of protozoal lysis (12). Thus, the centrifugation procedure at low temperature (27,000 × *g* at 1°C) used by Jarvis (17) may have produced the so-called soluble lytic activity. Nevertheless, this lysis and subsequent release of enzymes from ruminal microorganisms, especially protozoa, may not be a feature solely of traumatic in vitro experimental proce-

TABLE 3. Elution times and amino acid compositions of some radiolabeled metabolites isolated from the deproteinized supernatant after larger-scale 4-h incubations of [^{3}H]DAP-labeled *B. megaterium* GW1 with mixed ruminal protozoa

Metabolite	Elution time (min)	μmol of amino acid/incubation				
		DAP	Glu	Ala	Gly	Lys
A	8–20	0.60	15.12	11.76	—[a]	—
B	24–32	1.07	0.67	0.58	0.63	0.46
D	76–84	1.28	8.08	1.92	0.96	—
J	256–264	1.36	—	2.98	—	—
K	268–276	0.68	0.34	0.89	0.22	0.23
M	280–300	0.62	0.23	0.67	0.59	—
P	308–316	0.65	—	1.11	0.74	0.33
Q	320–336	0.91	—	1.41	—	0.46
W	348–360	0.60	—	1.11	—	0.23
Y	376–388	0.55	0.24	0.75	0.21	0.42

[a] —, Less than 0.03 μmol of amino acid.

dures; such occurrences seem to be part of normal ruminal function (7, 21).

The interpretation of experiments using protozoa derived from ruminal digesta is complicated by the fact that the protozoal preparations, however well washed with buffer solution, always contain some intracellular and adherent bacteria (5). The metabolic activities of these associated bacteria may be estimated; average counts of 250 bacteria attached to the surface of each protozoan and 30 viable bacteria inside each ruminal protozoan have been reported (16, 35). Assuming a total of 280 bacteria per protozoan, the associated bacteria in the ruminal protozoa preparation of Table 1 would amount to 9.5×10^7/ml of preparation. Since the ruminal bacteria preparation contained 4×10^9 bacteria per ml and resulted in 4.4% degradation of the radiolabeled *B. megaterium* mutant, the ruminal bacteria associated with the protozoa would (on the basis of equivalent metabolic activities of the two bacterial pools) account for only 0.1% degradation. Thus, the conclusion still stands; ruminal protozoa are the major effectors of bacterial cell wall degradation.

The metabolism of DAP-radiolabeled bacteria was further studied by monitoring the changes in the radioactive contents of various incubation fractions (Table 2). Radioactivities unaccounted for after the addition of the values of these fractions would be expected to appear in the picrate precipitate pellets largely as unmetabolized, radiolabeled mutant cell material. Analysis of a series of these acid-hydrolyzed picrate pellets confirmed this—summations in excess of 95% of the total radioactivities incubated were achieved.

The distributions of radioactivites between the incubation pellets and supernatants when [^{3}H]DAP-labeled *B. megaterium* GW1 was the substrate were proportionally similar but numerically lower than those reported by Masson and Ling (23). These lower values may be attributed to differences in the ratios of mutant bacteria to protozoa of the two studies. For example, Masson and Ling (23) commonly incubated 3,200 *B. megaterium* GW1 cells per protozoan, whereas results in Table 2 were derived from ratios of only 600:1 or 300:1.

On the assumption that bacterial engulfment by protozoa precedes bacterial degradation, the radioactivity from the protozoal pellet plus the supernatant represents the radiolabeled bacteria engulfed. Such measurements from incubations with [^{3}H]DAP-labeled *E. coli* W7-M5 were confounded by high control values, so engulfment values for this mutant may be estimated from protozoal pellet radioactivites alone. Engulfment rates for mixed ruminal protozoa derived from the 2-h values of Table 2 were 150 *B. megaterium* GW1 and 4,290 *E. coli* W7-M5 organisms per protozoan per h, i.e., within the ranges reported by Coleman and Laurie for these two bacterial species (4, 9). The radioactivity value of the supernatant may be used to estimate the radiolabeled bacteria degraded. By using these values from the 2-h incubations of the present study and those reported by Masson and Ling (23), it is possible to further define the relationship between the numerical ratio of *B. megaterium* GW1 organisms to the mixed ruminal protozoa incubated and the rate of bacterial degradation. These data show that degradation is a saturable process with a maximum rate, determined by linearization, of 4,830 *B. megaterium* GW1 organisms per protozoan per h.

It is beyond doubt that differences exist in the rates at which cell wall polymers of various bacterial species are metabolized by ruminal microorganisms, particularly protozoa. These differences do not appear to be related to bacterial size, shape, or simply the Gram reaction (8). Nevertheless, some biochemical or morphological or both types of differences of cell wall structure must distinguish bacteria that are rapidly digestible from those that are recalcitrant. The comparisons from the present study are especially germane, since the molecular composition of the peptidoglycans from both bacterial mutants was common. And although the cell walls of gram-positive bacteria typically contain 10 times more peptidoglycan than those of gram-negative bacteria, such a feature cannot account for the different rates of engulfment and degradation or degradation products from these two bacterial types (Fig. 2). The present study thus confirmed the findings of Masson and Ling (23) that bacterial cell walls, at least from gram-positive *B. megaterium* GW1, can be extensively metabolized by ruminal microorganisms, and it extended them by identifying the major metabolic product as a series of metabolites mainly peptidyl in composition.

Investigations of the compositions and stoichiometries of several of these metabolites from [^{3}H]DAP-labeled *B. megaterium* GW1 incubations with ruminal protozoa were not conclusive (Table 3). The sequencing of some products was largely ambiguous; one reason for this is that the presence of DAP as a diamino acid can lead to branched structures of peptides containing more than one N-terminal amino group. Nevertheless, the fact that most products contained DAP, glutamic acid, and alanine is consistent with the peptidoglycan structure of *B. megaterium*. The detection of glycine and lysine was more surprising; perhaps glycine is added by protozoa as a transport strategy; the presence of lysine suggests the possibility of DAP decarboxylation while it is

still bound in peptide form. Certainly, the range of metabolism products shows that ruminal protozoa must contain an array of enzymes that includes at least peptidase, glycosidase, amidase, and decarboxylase activities.

The relevance of this in vitro study to bacterial cell wall metabolism in vivo will depend on the extent to which the processes and rates of engulfment, degradation, and release of products from mixed ruminal bacteria are like those of the gram-positive and gram-negative mutants reported here. The present study does endorse previous observations from this laboratory (11, 23), namely, that a significant proportion of the DAP in the ruminant digestive tract may be either free or bound in low-molecular-weight compounds and therefore not associated with bacterial cell wall material, let alone intact bacterial cells. The implications of this for the use of DAP as a marker of bacterial biomass in vivo are currently being assessed.

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