JOURNAL OF BACTERIOLOGY, May 1967, p. 1499-1508
Vol. 93, No. 5
Copyright © 1967 American Society for Microbiology
Printed in U.S.A.

Oligopeptide Uptake by *Bacteroides ruminicola*[1]

KENNETH A. PITTMAN, SITARAMA LAKSHMANAN, AND MARVIN P. BRYANT

Dairy Cattle Research Branch, Animal Husbandry Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705, Department of Chemistry, University of Maryland, College Park, Maryland 20740, and Dairy Science Department, University of Illinois, Urbana, Illinois 61803

Received for publication 7 January 1967

Bacteroides ruminicola did not take up ^{14}C from exogenous ^{14}C-labeled L-proline or ^{14}C-labeled L-glutamic acid and took up very little ^{14}C from exogenous ^{14}C-labeled L-valine. Growing cultures of *B. ruminicola* rapidly took up ^{14}C from ^{14}C-proline-labeled peptides of molecular weights up to 2,000 and incorporated it into trichloroacetic acid-insoluble cell material. Uptake and incorporation did not occur at 0 C and were reduced or eliminated in glucose-starved cells, depending upon the length of time the cells were starved. The initial rate of uptake of peptides seemed to exhibit saturation kinetics, but it was impossible to establish this conclusively. The initial uptake of ^{14}C from peptides was not affected by chloramphenicol but the incorporation of it into trichloroacetic acid-insoluble cell material was virtually eliminated. Only moderate amounts of trichloroacetic acid-extractable, labeled material were present in cells during peptide uptake, whether or not chloramphenicol was present. ^{14}C-proline was rapidly released from labeled peptides during uptake, whether or not chloramphenicol was present. The amount of ^{14}C fixed into trichloroacetic acid-insoluble cell material was directly related to the size of peptides originally supplied in the medium. It is concluded that *B. ruminicola* possesses a general system for the uptake of peptides, that peptides are rapidly hydrolyzed during or after uptake, and that oligopeptides function only to supply amino acids in a form available to the organism.

The growth of some bacteria has been shown to be dependent upon or to be stimulated by the presence of small peptides. In all such cases, the peptides have been shown to function as carriers of amino acids which are used inefficiently or with difficulty, but not to function as specific growth factors (14). Alterations in the composition of the growth media used sometimes have eliminated peptide requirements (11, 14). Utilization of small peptides occurs by transport of peptides into the cell, hydrolysis of the peptides by internal peptidases, and subsequent metabolism of the free amino acids released (3, 14, 18, 21, 22, 32, 37, 38). Peptides appear to be absorbed by systems distinct from those mediating free amino acid transport (3, 14, 18, 21, 22, 32, 37), and the mere existence of transport systems for peptides different from those for free amino acids often has been sufficient to explain the biological activity of the peptides (14). For example, it has been shown that transport of some dipeptides can occur at greater rates or to a greater extent than transport of the constituent amino acids (11, 21, 37) and that there are bacteria which lack a transport system for a given amino acid but possess transport systems for peptides containing that amino acid (22, 26). In addition, it has been shown that uptake of a required amino acid may be reduced by competition with other amino acids for the same transport system, whereas peptides which contain the same amino acid but which are not in competition with free amino acids in the medium may be taken up rapidly (19, 21, 32).

The use of larger peptides by bacteria has not been understood as clearly as has been the use of smaller peptides. Oligopeptide stimulation of bacterial growth has been demonstrated (12, 27, 36). In general, this stimulation has been shown to be relatively nonspecific in terms of the size and composition of the active peptides (36), and in one case such stimulation was eliminated by alteration of the composition of the growth medium (20). Because of these facts, it has been suggested

[1] Work reported is part of a dissertation submitted by K. A. Pittman to the faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the Ph.D. degree. A preliminary report of this work was presented at the Annual Meeting of the American Society for Microbiology, Atlantic City, April 1965.

that oligopeptide utilization does not differ from the utilization of small peptides (20). On the other hand, bacterial requirements for oligopeptides larger than a certain minimal size have been demonstrated, as has an apparently absolute bacterial requirement for oligopeptides (12, 15, 36). The demonstration of lower size limits for oligopeptide utilization, the fact that such utilization may occur in the absence of the utilization of small peptides or free amino acids, and the fact that the ability of bacteria to utilize small peptides does not ensure their ability to utilize large peptides (13, 23, 31) raises the question of whether or not there is a real difference in the means of bacterial utilization of large peptides as opposed to small peptides and free amino acids.

Bacteroides ruminicola is very efficient in utilizing oligopeptide nitrogen as the sole source of cell nitrogen, but is very inefficient in utilizing small peptide and amino acid nitrogen as a source of cell nitrogen. The oligopeptides are neither required nor stimulatory if large amounts of ammonia are present in the medium (28). Other features of oligopeptide utilization by *B. ruminicola* are similar to those of oligopeptide-stimulated or oligopeptide-requiring bacteria. The research reported here was begun because it was thought that an investigation of oligopeptide utilization by *B. ruminicola* might provide a general basis for understanding oligopeptide function in other bacteria.

MATERIALS AND METHODS

Organisms and culture methods. Escherichia coli B was obtained from the Carnegie Institute of Washington, and *Selenomonas ruminantium* var. *lactilytica* strain HD4 (5) was obtained from Lorraine Gall. *Streptococcus bovis* strain FD10 and *B. ruminicola* subsp. *ruminicola* strain 23 (8) were isolated by this laboratory. All cultures were maintained by the anaerobic culture methods indicated by Bryant and Robinson (6). For uptake experiments, *E. coli* B was grown under nitrogen gas in a salts medium (29) with 0.0001% (w/v) resazurin (34) and 0.5% (w/v) glucose added. The other strains were grown in a defined medium described for the growth of *B. ruminicola* (28), ammonium sulfate being added to a concentration of 7×10^{-3} M. Cultures were prepared for uptake experiments as follows. The afternoon before an experiment was to be run, 5 ml of the appropriate medium was inoculated from a fresh overnight agar slant culture of the organism desired. The next morning, the 5-ml culture was added to 50 ml of the same medium in a 100-ml round-bottom flask. The culture was incubated until the desired absorbancy had been obtained. Dry weights were obtained by determining the absorbance of the cultures in 13-mm rubber-stoppered test tubes in a Bausch & Lomb Spectronic-20 colorimeter at 600 mμ and reading dry weight on calibration curves of dry weight versus absorbancy which had been previously prepared from cultures grown in the same medium.

Materials. Uniformly labeled ^{14}C-L-glutamic acid (75 c/mole) and uniformly labeled ^{14}C-L-valine (99.6 c/mole) were obtained from Volk Radiochemical Co., Burbank, Calif. Uniformly labeled ^{14}C-L-proline (158 c/mole) was obtained from Nuclear-Chicago Corp., Des Plaines, Ill. Sephadex G-25 was obtained from Pharmacia Fine Chemicals, Inc., New Market, N.J. Chloramphenicol (Chloromycetin) was the kind gift of Parke, Davis & Co., Detroit, Mich. Crystalline trypsin was obtained from Sigma Chemical Co., St. Louis, Mo. The other chemicals used in this study were of the best grade commercially available.

Preparation of ^{14}C-peptides. All labeled peptides used in these experiments were from a single preparation of *E. coli* protein, and were obtained as follows. ^{14}C-L-proline (150 μc) was added to 320 ml of a culture of *E. coli* grown in glucose-salts medium and containing about 0.22 mg (dry weight) of cells per ml. The culture was allowed to grow for about 1 hr to 0.29 mg (dry weight) of cells per ml. The cells were then harvested and extracted by the method of Roberts et al. (29), modified to include an extra trichloroacetic acid treatment and extra acid-alcohol and ether extractions, to yield a protein residue containing 53.8 mg. This residue was placed in a small beaker containing a small stirring bar, and 2 ml of 10 M urea was added. The suspension was stirred for 20 min, and enough 10% NaOH was added to clear it (*p*H, approximately 8). The mixture was allowed to stand for about 4 hr with occasional stirring, 6 ml of distilled water was then added with stirring, and the *p*H was adjusted to 8. After standing for 1 hr, the mixture was centrifuged, and the supernatant fluid and a 1-ml distilled water wash of the precipitate were combined. This procedure was necessary to remove a urea-insoluble fraction which was resistant to trypsin digestion. A 1-mg amount of trypsin in 1 ml of distilled water was added to this solution, and the mixture was incubated for 4 hr at room temperature. The entire volume was then placed on a bed (3.65 by 40 cm) of Sephadex G-25, medium grain. The column had been previously equilibrated with 0.050 M NaCl, and the hydrolysate was eluted with the same solution. Fractions of 5 ml each were collected at a rate of 2 ml/min. Samples of 10 μliters were taken from each fraction for liquid scintillation counting, and the fractions were then autoclaved for 5 min at 120 C and stored in a refrigerator. Previous experience had shown that the Sephadex column fractionation was quite reproducible. To avoid wasting the labeled material, analyses were run on the same amount of unlabeled material prepared and fractionated in exactly the same manner. Total nitrogen (35) and amino nitrogen (30) were determined. The ratio of total nitrogen to amino nitrogen was used as an approximate measure of the average size of peptides in each fraction. There was a linear decrease in this ratio, from 16 to 2, from the first emergence of nitrogen on the column to the emergence of urea. Urea did not emerge from the column until almost 95% of the radioactivity had been eluted. Paper chromatography of various fractions in several solvents, followed by radioautography, showed that no free proline was present. Thin-layer chromatography of representative fractions, after hydrolysis with 6 N HCl, showed only

one radioactive spot, the position of which corresponded to the position of free proline.

Uptake studies. The methods used were essentially those used by Britten and McClure (2), both whole-culture samples and 5% trichloroacetic acid-treated samples being membrane-filtered and counted for radioactivity. Filter holders and filters used were from Millipore Corp., Bedford, Mass. Type HA (0.45-μ) filters could not be used because of the slow filtration rate with *B. ruminicola*. Type DA (0.65-μ) filters were acceptable. Filtration time was about 1 sec, and losses through the filter, as estimated by culture and filtrate cell counts, were 0.5 to 1.0%, depending upon the amount of growth in the cultures. Damp filters retained 0.02 to 0.03 ml of solution. To insure anaerobic conditions during an experiment, cultures in 100-ml flasks were gassed continuously with carbon dioxide which was freed from oxygen by passage over hot, reduced, copper filings. Temperature was maintained by suspending the open flasks in a constant-temperature bath. All experiments were run at 37 C, unless stated otherwise. Just before an experiment was begun, 5 ml was removed from the opened culture for absorbance measurement. All radioactive materials were introduced in 0.5-ml volumes from carbon dioxide-equilibrated solutions. Samples of 1.00 ml were removed from experimental cultures with a Cornwall syringe pipette without the filling assembly but with a 4-inch no. 16 needle attached. The Luer Lock was painted over with a neoprene paint to prevent air from leaking into the sample or culture. Cells on filters weighed 0.04 to 0.12 mg/cm^2, and were considered to be infinitely thin. Planchets were counted for 40 or 100 min in an automatic, windowless, gas-flow counter, operating in the Geiger region.

Recovery of ^{14}C-proline. Two methods were used to recover labeled proline from culture supernatant fluids. In one method, the trichloroacetic acid-containing samples from uptake experiments were centrifuged, and the supernatant fluids were extracted with dry ether to remove excess trichloroacetic acid. The solutions were then placed on beds of Dowex 50W-X 8, fine mesh, hydrogen ion-form columns (1 by 10 cm) and washed through with distilled water. The columns were then eluted with 15 ml of 2.5 N ammonium hydroxide (33) and the eluate was dried and chromatographed in two directions on thin-layer plates of Silica Gel H (Brinkmann Instruments, Inc., Westbury, N.Y.), first with an acetic acid-water (10:1, v/v) solvent and second with a liquid phenol-water (85/15, v/v) solvent. Radioautograms were made with Kodak No Screen X-ray film to locate radioactivity. Active spots were scraped off and transferred to vials for direct liquid scintillation counting. Counting was done on two channels, and the counts were corrected for quenching (4). Only one discrete spot, corresponding to free proline, appeared on the chromatograms. The other method used was the preparation and separation of dinitrophenyl derivatives by the method of Matheson (24). Though every effort was made to use this method properly, it was found difficult to control the *R* values of compounds being eluted from the columns. The results were not considered satisfactory. Fractions containing the derivatives were dried and counted on planchets.

RESULTS

Free amino acid uptake. Since *B. ruminicola*, like most of the anaerobic bacteria of the rumen, is quite sensitive to oxygen, or at least to systems of high redox potential, all investigations had to be carried out under as strict conditions of anaerobiosis as possible. To establish positive controls on the experimental technique and degree of anaerobiosis maintained in experiments, preliminary amino acid uptake experiments were run with three organisms known to utilize free amino acids. Growing cultures of *E. coli* and *S. bovis*, facultative anaerobes, and *S. ruminantium*, a strict anaerobe, were supplied with 10^{-5} M ^{14}C-L-proline, anaerobic conditions being maintained throughout the experiments. All three organisms were able to fix ^{14}C into trichloroacetic acid-precipitable cell material, and the amount of ^{14}C fixed was directly related to increase in cell mass (Fig. 1). Under the same conditions, *B. ruminicola* was able neither to concentrate ^{14}C from L-proline or L-glutamic acid nor to fix ^{14}C from these compounds into trichloroacetic acid-precipitable cell material. ^{14}C from L-valine was taken up, but slowly, during the experimental period of 2 hr, the final amount fixed being 8% of that in the medium.

Peptide uptake. The inability of *B. ruminicola* to take up free ^{14}C-proline precluded the possibility of mistaking the uptake of any free ^{14}C-proline which might have been released from labeled peptides outside of the cell for the uptake of the labeled peptides.

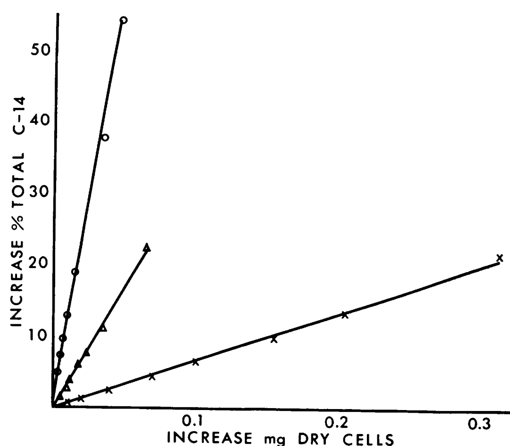

FIG. 1. *Relative uptake of ^{14}C from ^{14}C-L-proline by Escherichia coli (○), Selenomonas ruminantium (△), and Streptococcus bovis (×). All organisms were grown under strictly anaerobic conditions at 37 C. The increase in the percentage of ^{14}C-L-proline per milliliter retained by trichloroacetic acid-insoluble cell material per milliliter was plotted against the corresponding change in dry weight of cells per milliliter.*

Four fractions of ^{14}C-L-proline-labeled peptides of molecular weights of approximately 1,650, 1,200, 700, and 250 g per mole were tested initially. *B. ruminicola* rapidly took up the label from these peptides and incorporated it into trichloroacetic acid-precipitable cell material. Although uptake of the label began immediately, incorporation of label into trichloroacetic acid-precipitable material exhibited a short lag (Fig. 2). The curves representing ^{14}C uptake were of similar shape in all experiments. Experiments with three of the fractions were repeated, and similar results were obtained. In each of the experiments, the concentration of nonprecipitable ^{14}C in the cells rose rapidly to a maximum and as rapidly decreased (Fig. 3). Though this easily extractable material was by definition an intracellular pool (2), the nature of labeled compounds in this pool was not readily apparent. Since intracellular hydrolysis of peptides would be almost certain to precede the incorporation of label into protein, the pool was probably composed of some of the peptides taken up, of partial hydrolysis products of these, and of free amino acids. At the time of maximal pool size, ^{14}C from the larger peptides was about 300 times and from the smaller peptides about 150 times more concentrated in the cells than in the medium.

Because the nature of the pool would be of considerable interest in determining the nature

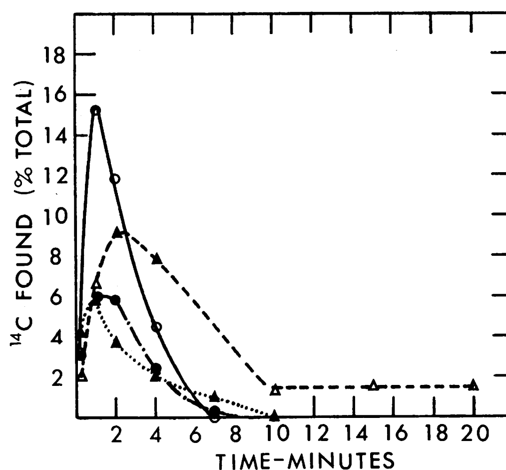

FIG. 3. *Intracellular accumulation of trichloroacetic acid-extractable ^{14}C during uptake of ^{14}C from ^{14}C-proline-labeled peptides by actively growing cultures of Bacteroides ruminicola under oxygen-free carbon dioxide in four experiments at 37 C. Points represent the difference between radioactivity in whole cells and in trichloroacetic acid-insoluble cell material (see Fig. 2). Symbols:* (○) *8.2 × 10^{-7} M peptides; average molecular weight, 1,650; 0.15 mg (dry weight) of cells per ml;* (△) *1.6 × 10^{-6} M peptides; average molecular weight, 1,200; 0.14 mg (dry weight) of cells per ml;* (●) *2.3 × 10^{-6} M peptides; average molecular weight, 700; 0.13 mg (dry weight) of cells per ml;* (▲) *1.8 × 10^{-6} M peptides; average molecular weight, 250; 0.15 mg (dry weight) of cells per ml.*

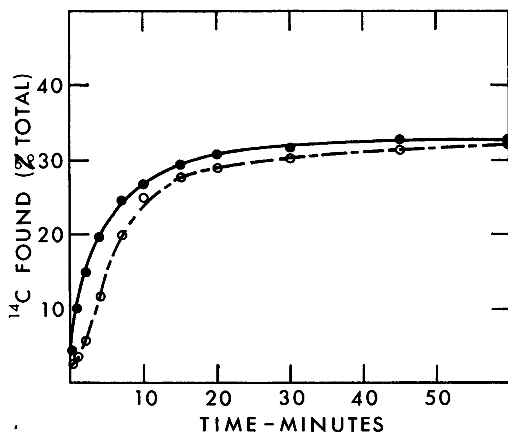

FIG. 2. *Uptake of ^{14}C from ^{14}C-proline-labeled peptides (average molecular weight, 1,200, 1.6 × 10^{-6} M) by actively growing Bacteroides ruminicola (0.14 mg, dry weight, of cells per ml) under oxygen-free carbon dioxide at 37 C. Samples of 1.00 ml of the culture were membrane-filtered to recover whole cells* (●) *or treated with 5% trichloroacetic acid and membrane-filtered to recover insoluble cell material* (○). *The radioactivity on dried filters was compared with the total radioactivity originally present in 1.00 ml of culture.*

of the peptide uptake mechanism, an attempt was made to increase both the size of the pool and its duration by inhibiting protein synthesis. Uptake experiments were designed in which chloramphenicol (10) was added at a final concentration of 20 μg/ml to cultures 10 to 20 min before the labeled peptides were added. It was hoped that large, stable pools of labeled materials would form in the absence of protein synthesis. The results of three such experiments, using a peptide fraction containing peptides of an average molecular weight of 1,200 g per mole, showed that no stable pools were formed even though incorporation of label into trichloroacetic acid-precipitable material was virtually eliminated (Fig. 4). Uptake apparently occurred without inhibition, because transitory pools formed, and because the formation of these pools followed a similar time course and similar concentrations as for the pools formed in experiments with the same peptide fractions without chloramphenicol. A reasonable explanation of the results would be that the peptides were rapidly hydrolyzed to free amino acids after uptake and either that no mechanism was available to retain the amino acids or

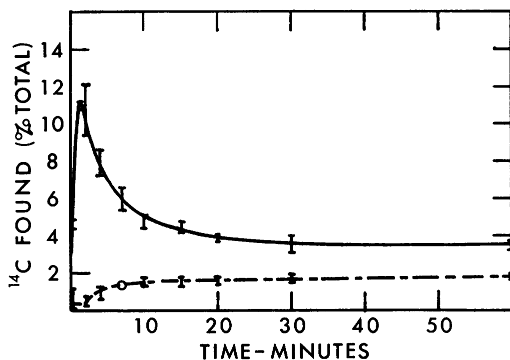

FIG. 4. *Uptake of ^{14}C from ^{14}C-proline-labeled peptides (average molecular weight, 1,200; 1.6 × 10^{-6} M) by Bacteroides ruminicola in the presence of chloramphenicol (20 μg/ml) under oxygen-free carbon dioxide at 37 C. Solid line, radioactivity in whole cells; dashed line, radioactivity in trichloroacetic acid-insoluble cell material as a percentage of total culture radioactivity (see Fig. 2). Vertical bars indicate the range of values observed in three experiments. Dry weights of cells in the three experiments were 0.16, 0.17, and 0.12-mg/ml.*

that specific exit mechanisms for amino acids were present.

Three more chloramphenicol inhibition experiments were run in the same way with a peptide fraction containing peptides of an average molecular weight of 1,250 g per mole. The trichloroacetic acid supernatant fractions from each run were saved and processed to recover ^{14}C-labeled proline. The rapid appearance of free ^{14}C-proline (Fig. 5) indicated that *B. ruminicola* was able to hydrolyze the peptides, and that its inability to form stable pools of labeled material in the absence of protein synthesis was probably due to its inability to retain free, intracellular proline. The amount of ^{14}C-proline recovered was not the same in all experiments. This was probably due to the difficulties experienced in the recovery of the dinitrophenyl derivative of proline.

To determine whether or not cells taking up peptides under normal conditions released free amino acids from the peptides as they did when inhibited with chloramphenicol, cation-exchange and two-dimensional thin-layer chromatography were again used to recover free ^{14}C-proline from culture supernatant fluids. The results again showed that an increase in free proline occurred during ^{14}C-proline-labeled peptide uptake (Fig. 6).

The fact that the uptake of ^{14}C from labeled peptides showed a marked dependence upon temperature (Table 1) suggested the possibility that peptide uptake by *B. ruminicola* was an energy-dependent process. If peptide uptake is an energy-dependent process, then it should be dependent upon the presence of energy sources in the medium. In the anaerobic, defined medium used, glucose was the only source of energy. To test uptake dependence on the presence of glucose, cultures were grown in 5 ml of medium containing growth-limiting amounts of glucose. Growth was followed by absorbance methods until it stopped. Incubation was continued to force the cells to exhaust endogenous energy reserves, and the cultures were then added to fresh media containing no glucose. Regular peptide uptake experiments were then run, using a fraction containing peptides of an average molecular weight of 1,200 g per mole. Three experiments, differing only in the incubation time between growth cessation and the addition of labeled peptides, were run. The times allowed for endogenous metabolism were 2, 4, and 17 hr. Complete inhibition of uptake was obtained only after prolonged starvation of the cultures (Fig. 7). In the third experiment, after 2 hr of incubation with labeled peptides, glucose was added to 0.5% (w/v). The cells began to take up peptides after a lag of 30 min.

An attempt was made to determine what effect, if any, substrate concentration might have on peptide uptake by *B. ruminicola*. A series of ex-

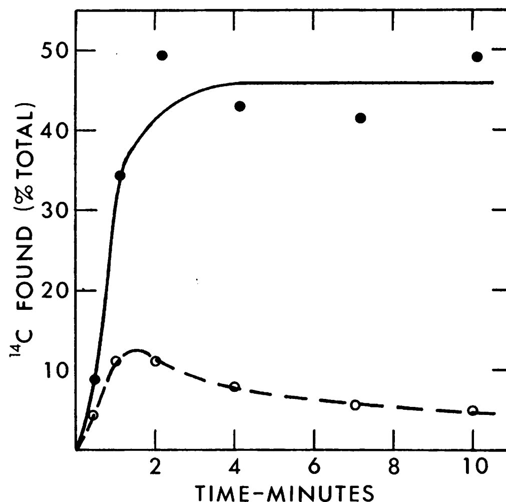

FIG. 5. *Release of free ^{14}C-proline from ^{14}C-proline-labeled peptides (average molecular weight, 1,250, 1.5 × 10^{-6} M) during peptide uptake by Bacteroides ruminicola (0.20 mg, dry weight, of cells per ml) in the presence of chloramphenicol (20 μg/ml) under oxygen-free carbon dioxide at 37 C. Proline was recovered and identified by cation-exchange and thin-layer chromatography. (●) Radioactivity in recovered proline, and (○) radioactivity in whole cells as a percentage of total culture radioactivity (see Fig. 2).*

periments was designed in which unlabeled peptides prepared by the Sephadex fractionation of Casitone (Difco) were used to increase the concentration of peptides in labeled fractions. Since

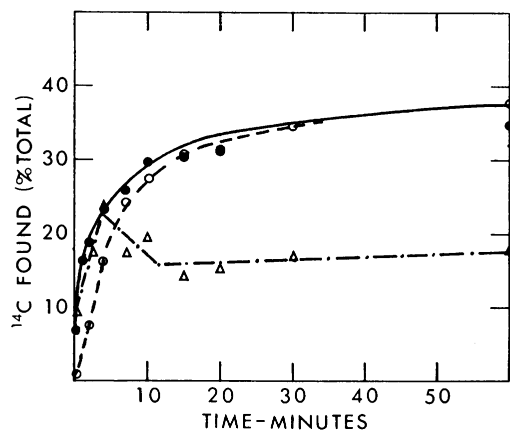

FIG. 6. *Release of free ^{14}C-proline from ^{14}C-proline-labeled peptides (average molecular weight, 800; 3.0 × 10^{-6} M) during peptide uptake by actively growing Bacteroides ruminicola (0.16 mg, dry weight, of cells per ml) under oxygen-free carbon dioxide at 37 C. Proline was recovered and identified by cation-exchange and thin-layer chromatography. (△) Radioactivity in recovered proline, (○) radioactivity in trichloroacetic acid-insoluble cell material, and (●) radioactivity in whole cells, all as a percentage of total culture radioactivity (see Fig. 2).*

TABLE 1. *Effect of tempterature on uptake of ^{14}C from ^{14}C-proline-labeled peptides[a] by Bacteroides ruminicola[b]*

Time[c] (min after ^{14}C-peptide addition)	Temp[d]	^{14}C found[e]	
		Whole cells	Trichloroacetic acid-precipitable cell material
	C		
0.25	0	1.5	—
60	0	2.0	—
120	0	1.7	—
180	0	2.2	—
242	0	1.8	—
248	37	1.4	—
249	37	1.5	—
251	37	2.0	—
255	37	4.1	1.8
265	37	20.4	17.7

[a] Average molecular weight, 1,250; 1.5 × 10^{-6} M.
[b] With 0.04 mg (dry weight) of cells per ml.
[c] Minutes after ^{14}C-peptide addition.
[d] Temperature rapidly changed from 0 to 37 C at 247 min.
[e] Expressed as a percentage of total radioactivity per milliliter of culture.

the labeled peptides being used were undoubtedly heterogenous, we felt that the addition of heterogeneous peptides of the same size class from casein would not bias the results unduly. The results of these experiments were combined with the results of various other uptake experiments, regardless of the size of peptides in the various fractions, and were plotted as the reciprocal of initial uptake velocity versus the reciprocal of initial substrate concentration (Fig. 8). The initial rate of substrate uptake was estimated as accurately as possible by determining the slope of a line drawn through the first two experimental points of a graph of uptake versus time. The slope y intercept of the line was taken to be the value corresponding to 2% of the total concentration of substrate, since this was the average amount retained on unwashed filters. Since cell densities were not the same in all experiments, the individual data on initial uptake velocity were adjusted to the basis of 1 mg (dry weight) of cells. The regression line established by points representing casein peptides plus labeled peptides has a positive slope, but the slope is not significantly different from zero. The regression line established by all the points has a positive slope not much different from that established by the points representing only casein peptides plus labeled peptides, but one which is significantly different from zero at the 1% level of significance.

Although the size of peptides in fractions tested did not seem to have an effect upon the initial

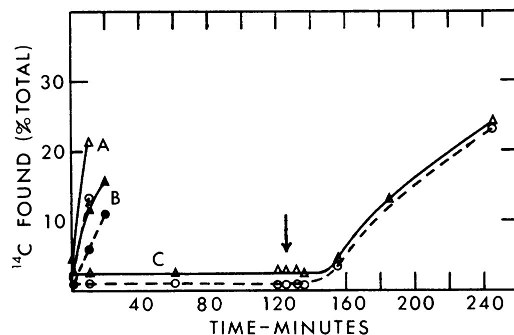

FIG. 7. *Effect of glucose on the uptake of ^{14}C from ^{14}C-proline-labeled peptides (average molecular weight, 1,200; 1.6 × 10^{-6} M) by Bacteroides ruminicola under oxygen-free carbon dioxide at 37 C. Cells starved for 2 (A), 4 (B), and 17 (C) hr before the additional of labeled peptides. Glucose (0.5%, w/v) added at 125 min (arrow) in experiment C. Cultures contained 0.08 (A), 0.05 (B), and 0.03 (C) mg (dry weight) of cells per ml. (△) Radioactivity in whole cells and (○) radioactivity in trichloroacetic acid-insoluble cell material as a percentage of total culture radioactivity (see Fig. 2).*

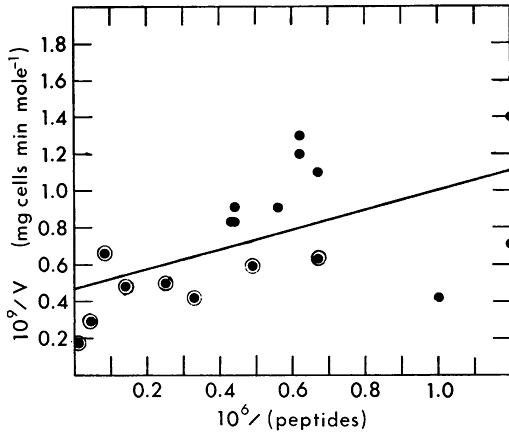

FIG. 8. *Double reciprocal plot of the effect of substrate concentration upon the initial velocity of uptake of ^{14}C from ^{14}C-proline-labeled peptides (molecular weight, 250 to 1,650 in different experiments) by actively growing Bacteroides ruminicola under oxygen-free carbon dioxide at 37 C. Double circles represent experiments in which casein peptides were present in addition to labeled peptides. The regression line,* $1/v$ = *(0.53 mg of cells per min per liter)* $(10^8/S)$ + *0.47* $\times$ 10^9 *mg per min per mole, was calculated from all the points shown.*

rate of ^{14}C uptake, the size of peptides seemed to have an effect upon the amount of ^{14}C finally retained by cells of *B. ruminicola*. Comparison of the results of experiments in which peptides of widely varying sizes were used showed that the amount of ^{14}C fixed per mg of dry cells was directly related to the size of the labeled peptides available (Fig. 9). All data were taken after 30-min incubation of labeled peptides with cultures, at which time no further changes in whole cell ^{14}C or trichloroacetic acid-precipitable ^{14}C were occurring. Initial concentrations of peptides in various fractions differed by a factor of 3, and total carbon in different fractions, by a factor of 2; fractions of intermediate size were generally more concentrated than those at the extremes of size. The possibility that these results are particularly biased by the presence of more than one proline residue per molecule is not great, since only one proline residue would be expected, on the average, in a peptide containing 21 to 22 amino acid residues, the mole per cent of proline in *E. coli* protein being 4.6 (29).

DISCUSSION

The fact that *B. ruminicola* takes up no ^{14}C from proline or from glutamic acid and little ^{14}C from valine is in accord with previous nutritional results (28) showing that the nitrogen of free amino acids cannot be utilized, and with results showing that little ^{14}C from acid-hydrolyzed protein is used during growth in a complex medium (7). Whether or not *B. ruminicola* is permeable to free amino acids is not known with certainty. It is always possible that the acids can diffuse through the membrane but not reach metabolically active areas of the cell, and the fact that proline can leave the cell might support the possibility of its entering by diffusion. The technique used in this study would not detect concentrations of intracellular proline equal to the external concentration. Many bacteria are known to be impermeable to amino acids, and this would support our view that free amino acids cannot readily penetrate by diffusion the cell membrane of *B. ruminicola*.

This study established that oligopeptides are taken up by *B. ruminicola*. The nutritional study (28) showed only that the nitrogen of oligopeptides was utilized for cellular synthesis, but it did virtually eliminate the action of extracellular enzymes as agents responsible for the utilization, since free, extracellular amino acids could not be utilized as nitrogen sources for growth but ammonia could. It was concluded that the nitrogen of peptides somehow got into the cell as a direct consequence of its existence as a part of the peptide molecules. The present experiments with labeled peptides showed that the carbon of peptides also rapidly entered the cell, and that again it was necessary that the carbon be in peptides rather than free amino acids. There is little doubt that some form of transport of even large oligopeptides is carried out by *B. ruminicola*.

What then is the biological function of the peptides taken into the cell? In terms of size and specificity for oligopeptide utilization, the results

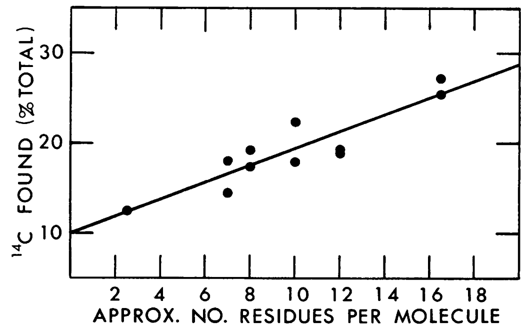

FIG. 9. *Effect of peptide size upon ^{14}C retained in trichloroacetic acid-insoluble cell material after ^{14}C-proline-labeled peptide uptake by actively growing Bacteroides ruminicola. All data were taken 30 min after addition of labeled peptides to cultures. Radioactivity is expressed as a percentage of total culture radioactivity (see Fig. 2) per mg of dry cells.*

obtained with peptides in the previous nutritional study (28) are remarkably similar to the results obtained with the *Lactobacillus bulgaricus* peptide growth factor and to the results obtained in studies on the strepogenin peptide growth factor (15, 36). In the latter studies, it was evidently considered that oligopeptides functioned as growth factors in a vitamin- or hormone-like manner, or at least that the peptide structure was important in itself, and not just as an available form of amino acids. In all of these studies, growth was the only criterion used to determine the response of the organism to oligopeptides added to complex media. *B. ruminicola* would have exhibited much the same need for peptides as did the other organisms studied, if it were grown in a very complex medium, provided that little free ammonia was present. The techniques used in the present study allowed a more critical interpretation of the function of oligopeptides than did growth response. The rapid release of free ^{14}C-proline from a mixture of ^{14}C-proline-labeled peptides of various sizes in which the proline could occupy any position in the peptide in relation to any other amino acid indicates that rapid hydrolysis of all peptides taken up occurs. Proline release appears to begin more rapidly than does the incorporation of ^{14}C into trichloroacetic acid-insoluble cell material, indicating that peptide hydrolysis must precede the utilization of peptide carbon in cell synthesis. If this is true, then the peptides can function only to supply amino acids in an available form, and whatever specificity is shown in utilization is a reflection of the selective nature of the membrane and not of peptide-requiring systems inside the cell. One observation common to all of the studies of oligopeptide utilization in bacteria is that a certain minimal size of peptide must be present before stimulation occurs or before significant amounts of the available nitrogen can be utilized. This study would tend to support those findings, in that less carbon was retained from smaller than from larger peptides. Small peptides appear to be taken up as rapidly as large ones, suggesting that discrimination does not occur in the uptake process. In this particular study, the fact that free amino acid seems to leak out of the cell rapidly might explain the inefficient use of small peptides, especially dipeptides, by the organism, since a greater number of free amino acids would be released per bond hydrolyzed per unit time with small peptides than with large peptides.

Of interest is the mechanism by which *B. ruminicola* is able to utilize oligopeptides. There must be some means to effect passage of the plasma membrane. Free diffusion is unlikely, since bacterial membranes are known to be relatively impermeable to even small hydrophilic molecules, and since it is probable that *B. ruminicola* is impermeable to amino acids. Also, the data collected on initial uptake rates suggest that the rate of entry of peptides into the cells has a maximal value. The rate of free diffusion across a membrane would not saturate, since it is proportional to the concentration gradient. A restricted diffusion through pores cannot be ruled out on the basis of the available evidence, but it seems unlikely that pores large enough to allow peptides to enter could at the same time prevent the passage of amino acids.

It has been found that the rate of uptake of peptides by bacteria may follow saturation kinetics (21, 37). In this regard, the data acquired in this study are far from conclusive, but do suggest that the same relationship may obtain and that the mechanism of oligopeptide uptake by *B. ruminicola* might be similar to that involved in the uptake of small peptides by other bacteria. Considering that the peptide substrates were mixtures, that their sizes varied greatly, and that cell concentrations in different experiments varied somewhat, it is fortunate that any relationship between uptake velocity and substrate concentration was observed. No particular effects of peptide size upon uptake velocity could be observed, and so it is likely that the mechanism of peptide uptake by *B. ruminicola* is quite general with respect to size. Whether or not peptide structure is important in controlling uptake by *B. ruminicola* could not be adequately determined in this study.

The uptake of peptides by other bacteria has been attributed to a carrier-mediated process. Evidence for active transport is often presented. Saturation of uptake velocity is not enough to conclude that the uptake process is consistent with a carrier-mediation hypothesis. It is necessary to demonstrate that substrate appears unchanged in the interior of the cell in a form sufficiently free in solution to be extracted with fairly mild procedures (17, 30). As in many other studies of peptide uptake by bacteria, this study could not show that *B. ruminicola* accumulated peptides, only free amino acids. In a single experiment, an attempt to show intracellular peptide by exchange was unsuccessful, and this also fails to support a carrier-mediated transport mechanism. Though the pronounced temperature dependence shown in the uptake of peptides by *B. ruminicola* may be taken as evidence for active transport, the moderate dependence upon a source of metabolic energy appears inconclusive. Only the most transitory accumulation of labeled material occurred in the intracellular pool, and, since most of this material was free proline and

not peptide, it is impossible to conclude that the reactions involved in uptake required coupling to an energy-yielding reaction. Rapid, intracellular, peptide hydrolysis would in itself ensure low internal peptide concentrations and serve to drive the uptake reactions. Considering the available data, the group-translocation theory of Mitchell and Moyle (9, 25) seems as attractive as any other theory. Such a mechanism should function particularly well for peptide transport because of the essential irreversibility of peptide hydrolysis, and might be useful in explaining the indiscriminate nature of oligopeptide uptake and the fact that fairly large peptides are taken up with as little difficulty as smaller ones. It might be possible to test the pertinence of this theory, if pure membranes similar to those prepared by Kaback and Stadtman (16) could be tested for ability to take up and hydrolyze oligopeptides. The data available at present do not permit any conclusions as to the mechanism of peptide uptake in *B. ruminicola*.

The very rapid loss of an amino acid released intracellularly after intracellular peptide hydrolysis is a somewhat unusual observation. Rapid peptide hydrolysis might cause a rapid increase in the osmotic pressure inside of the cell, so that release of amino acids might be necessary to reduce such pressure. The exit of amino acids independently of uptake and apparently without carrier mediation has been attributed to the fact that internal osmotic pressure causes temporary lesions in the cell envelope (1).

ACKNOWLEDGMENT

The kindness of George Fries of the Animal Husbandry Research Division, U.S. Department of Agriculture, in making available his facilities for measuring radioactivity is gratefully acknowledged.

LITERATURE CITED

1. BRITTEN, R. J. 1965. The concentration of small molecules within the microbial cell. Symp. Soc. Gen. Microbiol. **15**:57–88.
2. BRITTEN, R. J., AND F. T. MCCLURE. 1962. The amino acid pool in *Escherichia coli*. Bacteriol. Rev. **26**:292–335.
3. BROCK, T. D., AND S. O. WOOLEY. 1964. Glycylglycine uptake in streptococci and a possible role of peptides in amino acid transport. Arch. Biochem. Biophys. **105**:51–57.
4. BRUNO, G. A., AND J. E. CHRISTIAN. 1961. Correction for quenching associated with liquid scintillation counting. Anal. Chem. **33**:650–651.
5. BRYANT, M. P. 1956. The characteristics of strains of *Selenomonas* isolated from bovine rumen contents. J. Bacteriol. **72**:162–167.
6. BRYANT, M. P., AND I. M. ROBINSON. 1962. Some nutritional characteristics of predominant culturable ruminal bacteria. J. Bacteriol. **84**:605–614.
7. BRYANT, M. P., AND I. M. ROBINSON. 1963. Apparent incorporation of ammonia and amino acid carbon during growth of selected species of ruminal bacteria. J. Dairy Sci. **46**:150–154.
8. BRYANT, M. P., N. SMALL, C. BOUMA, AND H. CHU. 1958. *Bacteroides ruminicola* n. sp. and *Succinimonas amylolytica* the new genus and species. Species of succinic acid-producing anaerobic bacteria of the bovine rumen. J. Bacteriol. **76**:15–23.
9. CHRISTENSEN, H. N. 1962. Biological transport. W. A. Benjamin, Inc., New York.
10. DAVIS, B. D., AND D. S. FEINGOLD. 1962. Antimicrobial agents: mechanism of action and use in metabolic studies, p. 343–397. *In* I. C. Gunsalus and R. Y. Stanier [ed.], The bacteria, vol. 4, The physiology of growth. Academic Press, Inc., New York.
11. FLORSHEIM, H. A., S. MAKINENI, AND S. SHANKMAN. 1962. The isolation, identification and synthesis of a peptide growth factor for *P. cerevisiae*. Arch. Biochem. Biophys. **97**:243–249.
12. FOX, E. N. 1961. Peptide requirements for the synthesis of streptococcal proteins. J. Biol. Chem. **236**:166–171.
13. GILVARG, C., AND E. KATCHALSKI. 1965. Peptide utilization in *Escherichia coli*. J. Biol. Chem. **240**:3093–3098.
14. GUIRARD, B. M., AND E. E. SNELL. 1962. Nutritional requirements of microorganisms, p. 33–93. *In* I. C. Gunsalus and R. Y. Stanier [ed.], The bacteria, vol. 4, The physiology of growth. Academic Press, Inc., New York.
15. JONES, K. M., AND D. W. WOOLLEY. 1962. Peptides required for the growth of *Lactobacillus bulgaricus*. J. Bacteriol. **83**:797–801.
16. KABACK, H. R., AND E. R. STADTMAN. 1966. Proline uptake by an isolated cytoplasmic membrane preparation of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. **55**:920–927.
17. KEPES, A., AND G. N. COHEN. 1962. Permeation, p. 179–221. *In* I. C. Gunsalus and R. Y. Stanier [ed.], The bacteria, vol. 4, The physiology of growth. Academic Press, Inc., New York.
18. KESSEL, D., AND M. LUBIN. 1963. On the distinction between peptidase activity and peptide transport. Biochim. Biophys. Acta **71**:656–663.
19. KIHARA, H., AND E. E. SNELL. 1955. Peptides and bacterial growth. VII. Relation to inhibitions by thienylalanine, ethionine, and canavanine. J. Biol. Chem. **212**:83–94.
20. KIHARA, H., AND E. E. SNELL. 1960. Peptides and bacterial growth. VIII. The nature of strepogenin. J. Biol. Chem. **235**:1409–1414.
21. LEACH, F. R., AND E. E. SNELL. 1960. The absorption of glycine and alanine and their peptides by *Lactobacillus casei*. J. Biol. Chem. **235**:3523–3531.
22. LEVINE, E. M., AND S. SIMMONDS. 1960. Metabolic uptake by serine-glycine auxotrophs of *Escherichia coli*. J. Biol. Chem. **235**:2902–2909.

23. LOSICK, R., AND C. GILVARG. 1966. Effect of α-acetylation on utilization of lysine oligopeptides in *Escherichia coli*. J. Biol. Chem. **241**:2340–2346.
24. MATHESON, N. A. 1963. An improved method of separating amino acids as N-2,4-dinitrophenyl derivatives. Biochem. J. **88**:146–151.
25. MITCHELL, P., AND J. MOYLE. 1958. Group-translocation: a consequence of enzyme-catalyzed group-transfer. Nature **182**:372–373.
26. PETERS, V. J., J. M. PRESCOTT, AND E. E. SNELL. 1953. Peptides and bacterial growth. IV. Histidine peptides as growth factors for *Lactobacillus delbrueckii* 9649. J. Biol. Chem. **202**: 521–532.
27. PHILLIPS, A. W., AND P. A. GIBBS. 1961. Techniques for the fractionation of microbiologically active peptides derived from casein. Biochem. J. **81**:551–556.
28. PITTMAN, K. A., AND M. P. BRYANT. 1964. Peptides and other nitrogen sources for growth of *Bacteroides ruminicola*. J. Bacteriol. **88**:401–410.
29. ROBERTS, R. B., P. H. ABELSON, D. B. COWIE, E. T. BOLTON, AND R. J. BRITTEN. 1957. Studies of biosynthesis in *Escherichia coli*. Carnegie Inst. Wash. Publ. 607.
30. ROSEN, H. 1957. A modified ninhydrin colorimetric analysis for amino acids. Arch. Biochem. Biophys. **67**:10–15.
31. SHANKMAN, S., S. HIGA, H. A. FLORSHEIM, Y. SCHVO, AND V. GOLD. 1960. Peptide studies. II. Growth promoting activity of peptides of L-leucine and L- and D-valine for lactic acid bacteria. Arch. Biochem. Biophys. **86**:204–209.
32. SHELTON, D. C., AND W. E. NUTTER. 1964. Uptake of valine and glycylvaline by *Leuconostoc mesenteroides*. J. Bacteriol. **88**:1175–1184.
33. SMITH, I. 1960. Desalting and related techniques, p. 40–66. *In* I. Smith [ed.], Chromatographic and electrophoretic techniques, vol. 1, Chromatography. Interscience Publishers, Inc., New York.
34. TWIGG, R. S. 1945. Oxidation-reduction aspects of resazurin. Nature **155**:401–402.
35. UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER. 1957. Manometric techniques, 3rd ed. Burgess Publishing Co., Minneapolis.
36. WOOLLEY, D. W., AND R. B. MERRIFIELD. 1963. Anomalies of the structural specificity of peptides. Ann. N.Y. Acad. Sci. **104**:161–171.
37. YODER, O. C., K. C. BEAMER, P. B. CIPOLLONI, JR., AND D. C. SHELTON. 1965. Kinetic studies of L-valine and glycyl-L-valine uptake by *Leuconostoc mesenteroides*. Arch. Biochem. Biophys. **110**:336–340.
38. YOUNG, E. A., D. O. BOWEN, AND J. F. DIEHL. 1964. Transport studies with peptides containing unnatural amino acids. Biochem. Biophys. Res. Commun. **14**:250–255.