Incorporation of C¹⁴ from Amino Acids and Peptides into Protein by *Clostridium perfringens* Type D

ANDREAS H. W. HAUSCHILD

Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario, Canada

Received for publication 12 July 1965

ABSTRACT

HAUSCHILD, ANDREAS H. W. (University of Toronto, Toronto, Ontario, Canada). Incorporation of C¹⁴ from amino acids and peptides into protein by *Clostridium perfringens* type D. J. Bacteriol. **90**:1569-1574. 1965.—Uptake of C¹⁴ from C¹⁴-labeled amino acids and peptides by *Clostridium perfringens* was measured in culture media containing acid or papain hydrolysates of C¹⁴-labeled *Chlorella* protein. Between 2 and 4 hr of growth, the rate of C¹⁴ uptake from peptides was higher than from free amino acids. Peptides extracted from cells with hot ethyl alcohol contained six to nine times more C¹⁴ after 4 hr of growth with C¹⁴-labeled peptides than with C¹⁴-labeled amino acids. Incorporation of C¹⁴-labeled glycine, serine, threonine, alanine, and proline into both cellular and exocellular protein was two to five times higher when these were supplied as components of dialyzable peptides rather than as free amino acids.

The requirement of peptides for growth (Phillips and Gibbs, 1961) and synthesis of toxins (Tsukamoto, Yamamoto, and Murata, 1963; Mueller and Miller, 1955) and of other proteins (Fox, 1961) has been demonstrated for a number of microorganisms. Recent work (Hauschild, 1965) also demonstrated the importance of peptides for toxinogenesis by *Clostridium perfringens* type D and showed a correlation between the initial molecular size of peptides and toxin synthesis. Such a correlation was shown earlier for bacterial growth by Woolley and Merrifield (1963) and Pittman and Bryant (1964).

The question arose whether peptides in cultures of *C. perfringens* are effective by providing a gradual external supply of amino acids, or whether they may be taken up without being completely hydrolyzed. Snell and co-workers showed that microorganisms of the genera *Lactobacillus* (Leach and Snell, 1960), *Leuconostoc* (Kihara and Snell, 1960), and *Streptococcus* (Kihara, Ikawa, and Snell, 1961) take up C¹⁴ from C¹⁴-labeled dipeptides more rapidly than from the corresponding free amino acids. Levine and Simmonds (1960) obtained similar results with *Escherichia coli*. This study compares the uptake of C¹⁴ from C¹⁴-labeled peptides and amino acids by *C. perfringens*, and the incorporation of C¹⁴ into cellular and exocellular protein. A preliminary report of some of the results has been presented (Hauschild, Bacteriol. Proc., p. 76, 1965).

MATERIALS AND METHODS

Growth and toxinogenesis. The medium contained 2% dextrin, 2% yeast extract, 0.5% Proteose Peptone, 0.2% L-cysteine hydrochloride, 0.2% Na₂HPO₄, and 0.1% K₂HPO₄. The inoculum was grown for 18 hr at 37 C in a medium containing 1% dextrin, 1% yeast extract, 1% Proteose Peptone, 0.1% L-cysteine hydrochloride, 0.2% Na₂HPO₄, and 0.1% K₂HPO₄. It was added to the medium to constitute 5% of the total volume (150 ml). The pH was automatically controlled at 7.0. The methods for measuring growth and toxins were described previously (Pivnick et al., 1964, 1965; Hauschild, 1965).

Hydrolysis of protein-C¹⁴ and C¹⁴ assays. C¹⁴-labeled *Chlorella* protein (New England Nuclear Corp., Boston, Mass.) was suspended in 0.1 M potassium phosphate (pH 7.2), dialyzed against the same buffer at 5 C for 16 hr, and digested with 0.2% crystalline papain (Nutritional Biochemicals Corp., Cleveland, Ohio) at 40 C for 60 min. The mixture was placed in boiling water for 3 min, cooled, and centrifuged. The supernatant fluid was dialyzed through Visking dialysis tubing (average pore size, 24 A) against 25 volumes of distilled water at 5 C for 20 hr, and was thus separated into dialyzable and nondialyzable C¹⁴-labeled peptides.

Complete hydrolysis was effected in vacuo in the presence of 6 N HCl at 110 C for 10 hr. The samples were then dried to remove HCl. The water-soluble material is referred to as C¹⁴-labeled *Chlorella* amino acids.

For experiments on C¹⁴ uptake, Proteose Peptone medium was supplemented with amino acids,

TABLE 1. Distribution of C^{14} among the main amino acids in preparations of free amino acids, dialyzable peptides, and nondialyzable peptides derived from *Chlorella* protein- C^{14}

Amino acid	Per cent radioactivity		
	Free amino acids	Dialyzable peptides	Nondialyzable peptides
Aspartic acid.....	11.9	11.0	11.4
Glutamic acid.....	15.5	16.3	17.2
Glycine.....	4.5	3.7	3.9
Serine.....	5.2	4.4	3.6
Threonine.....	5.2	4.0	3.9
Alanine.....	9.3	10.9	10.6
Proline.....	5.8	5.9	5.8
Valine.....	11.0	11.6	11.4
Phenylalanine.....	8.2	8.2	7.5
Leucine + isoleucine.....	23.5	24.0	24.8
Total.....	100.1	100.0	100.1

dialyzable peptides, and nondialyzable peptides from C^{14} -labeled *Chlorella* protein at 30×10^6 count/min of C^{14} each and <1 mg of total weight per 150 ml of medium. The C^{14} content of the main amino acids, free or in peptide form, was determined after acid hydrolysis of the peptides and paper chromatography by direct counting of the paper spots (Hauschild, Nelson, and Krotkov, 1962). Table 1 shows that the distribution of C^{14} among these amino acids was approximately the same in the three preparations.

Total uptake of C^{14} . Samples of 100 μ liters of culture were filtered through Gelman membrane filters, type GM-7. The cells were washed with 0.9% NaCl, and the filters were glued to planchets, dried, and counted.

Extractions. Cells were centrifuged, washed three times with 0.9% NaCl, and extracted for 3 min in boiling 80% ethyl alcohol. The amino acids of the ethyl alcohol extracts were separated by paper chromatography and assayed for C^{14} , both before and after acid hydrolysis. The difference in the total C^{14} content of the amino acids constitutes the radioactivity of the peptides.

After ethyl alcohol extraction, the sediments were further extracted with 5% trichloroacetic acid at 90 C for 15 min and washed twice with 5% trichloroacetic acid. The residues, which constitute the protein fractions, were assayed for total C^{14} , and were hydrolyzed for C^{14} assays of the major amino acids.

Exocellular protein. Culture supernatant fluids were treated with 350 g of ammonium sulfate per liter at pH 6.5 for 4 hr at 5 C. Under these conditions, the major toxins of *C. perfringens* are precipitated (Hauschild, 1965). After centrifugation, the sediments were washed with ammonium sulfate, suspended in 0.9% NaCl, and dialyzed against 20 liters of distilled water for 16 hr. Exo-

cellular protein was not assayed for C^{14} when nondialyzable peptides were fed, because these peptides were also precipitated with ammonium sulfate. On the other hand, it appears that exocellular protein was not contaminated with dialyzable peptides, since only traces of C^{14} were found in the protein fraction when such peptides were incubated for 60 min with the supernatant fluid of a 6-hr culture of *C. perfringens* and treated as above. No lysis was observed during 6 hr of growth.

Treatment of C^{14} -labeled amino acids with culture supernatant fluid. To determine whether free amino acids are broken down by exocellular enzymes, 2-ml samples of sterile filtrates (passed through Gelman membranes, type GA-7) of supernatant fluids from 2-, 3- and 4-hr cultures were combined with 25 μ liters of a mixture of C^{14} -labeled amino acids (*Chlorella* protein hydrolysate; New England Nuclear Corp.) containing 15×10^8 count/min and were incubated at 37 C for 2 hr. The pH was 7.0 ± 0.1 throughout the incubation period. The amino acids were assayed for C^{14} after 0, 1, and 2 hr.

RESULTS

To determine whether cells of *C. perfringens* are capable of taking up peptides before these are completely hydrolyzed, *C. perfringens* was grown for 6 hr in the presence of C^{14} -labeled amino acids, dialyzable peptides, and nondialyzable peptides. The cultures of a typical experiment described below are designated AA, DP, and NDP, respectively. Growth and toxin

FIG. 1. Growth of *Clostridium perfringens* type D with controlled pH at 7.0.

synthesis were the same in all cultures. A growth curve is shown in Fig. 1. The yields of epsilon, kappa, and theta toxins were 15×10^3 MLD, 60 kappa units, and 32 hemolytic units per ml, respectively.

Figure 2a shows the total radioactivities of the major free amino acids in the culture supernatant fluids. In culture AA, the radioactivity decreased to 73% of the original within 6 hr. In cultures DP and NDP, the radioactivities reflect the balance between formation and utilization of free amino acid- C^{14} . In DP, 42% of the total C^{14} was present in free amino acids after 3 hr of growth. There was little increase during the subsequent 3 hr. In NDP, most of the free amino acid- C^{14} was formed between 1 and 4 hr. It was also found that over the period of 6 hr the radioactivity of each individual amino acid in the supernatant liquid remained higher in AA than in DP or NDP.

FIG. 2. Free amino acid- C^{14} in culture supernatant fluid as percentage of original C^{14} (a) and total radioactivity of cells (b) during growth of *C. perfringens* type D. The media contained C^{14} -labeled amino acids (AA), dialyzable peptides (DP), and nondialyzable peptides (NDP).

TABLE 2. Radioactivity of peptides extracted from cells with hot ethyl alcohol

Culture	C^{14} source	Total ethyl alcohol extract (count/min $\times 10^{-3}$)		Peptides			
				Count/min $\times 10^{-3}$		Ratio*	
		4 hr	6 hr	4 hr	6 hr	4 hr	6 hr
AA	Free amino acid- C^{14}	88	108	13.6	34	—	—
DP	Dialyzable peptide- C^{14}	200	176	83	95	6.1	2.8
NDP	Nondialyzable peptide- C^{14}	242	180	121	124	8.9	3.6

* Ratio of DP-AA (line 2); NDP-AA (line 3).

Figure 2b shows the total C^{14} in *C. perfringens* cells. In cultures DP and NDP, the initial uptake was low, but the average rates of uptake between 2 and 4 hr were approximately 60 and 40% higher than in AA. Since, during this period, the amount of free amino acid- C^{14} in the culture medium was lower in both DP and NDP than in AA, the result indicates that the cells incorporated peptides before these were completely hydrolyzed.

The amount of peptide- C^{14} extracted from the cells with hot 80% ethyl alcohol was determined as the difference in C^{14} of free amino acids after and before acid hydrolysis (Table 2). After 4 hr of growth, the C^{14} content of peptides extracted from cells in cultures DP and NDP was six and nine times higher than that extracted from cells in culture AA. The differences were smaller after 6 hr but were still significant.

The C^{14} contents of cellular and exocellular protein are shown in Table 3. After 6 hr, the C^{14} content of cellular protein was 20% higher in DP than in AA, and the C^{14} content of exocellular protein was 75% higher in DP than in AA. As shown in Materials and Methods, the exocellular protein in NDP could not be assayed for C^{14} . The results in Table 3 show that the incorporation of at least some amino acids into protein is more efficient when these are supplied in peptide form rather than as free amino acids. To determine which amino acids these may be, the proteins were hydrolyzed and their amino acid constituents were assayed for C^{14} .

The C^{14} contents of the protein-bound amino acids are expressed as a percentage of the original C^{14} of the respective amino acids (Table 1) that were supplied either in the free form (AA) or in peptide form (DP and NDP). For cellular pro-

tein, these data are shown in Table 4. A comparison of the data under AA and DP shows that the amount of C^{14} in aspartic acid and in valine relative to the original C^{14} was the same, irrespective of whether free amino acids or dialyzable peptides were supplied. The relative amounts of C^{14} in glycine, serine, threonine, alanine, and proline were higher in DP than in AA, whereas they were lower in glutamic acid, phenylalanine, and the leucines. These differences become more apparent when the data are expressed as ratios of DP-AA (Table 4). The relative amounts of C^{14} in protein-bound amino acids derived from nondialyzable peptides are also shown in Table 4. The result is similar to that obtained with dialyzable peptides. It is concluded that the incorporation of glycine, serine, threonine, alanine, and proline into cellular protein is higher when these amino acids are supplied as peptides rather than as free amino acids.

The C^{14} content of protein-bound amino acids was also assayed after 4 hr of growth. The result was essentially the same.

Table 5 is arranged like Table 4 but shows the

TABLE 3. Radioactivity of protein after 4 and 6 hr of growth

Culture	C^{14} source	Cellular protein (count/min $\times 10^{-4}$)		Exocellular protein (count/min $\times 10^{-3}$) at 6 hr
		4 hr	6 hr	
AA	Free amino acid- C^{14}	212	235	220
DP	Dialyzable peptide- C^{14}	226	282	390
NDP	Nondialyzable peptide- C^{14}	132	182	—

TABLE 4. Radioactivity of amino acids from cellular protein after 6 hr of growth

Amino acid	Radioactivity as percentage of original C^{14}			Ratio	
	AA*	DP*	NDP*	DP-AA	NDP-AA
Aspartic acid.....	4.7	5.2	4.5	1.1	1.0
Glutamic acid.....	8.7	6.0	5.3	0.7	0.6
Glycine.....	5.6	16.3	9.3	2.9	1.7
Serine.....	2.8	16.3	17.8	5.8	6.4
Threonine.....	7.4	15.7	10.0	2.1	1.4
Alanine.....	6.6	12.7	8.7	1.9	1.3
Proline.....	3.7	9.4	6.0	2.5	1.6
Valine.....	7.4	7.6	5.8	1.0	0.8
Phenylalanine.....	12.9	8.3	6.1	0.6	0.5
Leucine + isoleucine..	9.0	6.4	4.1	0.7	0.5

* C^{14} sources as in Table 3.

TABLE 5. Radioactivity of amino acids from exocellular protein after 6 hr of growth

Amino acid	Radioactivity as percentage of original C^{14}		Ratio of DP-AA
	AA*	DP*	
Aspartic acid.....	0.56	1.28	2.3
Glutamic acid.....	1.01	0.88	0.9
Glycine.....	0.53	2.14	4.0
Serine.....	0.64	3.35	5.2
Threonine.....	1.20	2.23	1.8
Alanine.....	0.76	1.44	1.9
Proline.....	0.48	1.70	3.5
Valine.....	1.08	1.43	1.3
Phenylalanine.....	1.12	1.19	1.1
Leucine + isoleucine...	0.84	0.99	1.2

* C^{14} sources as in Table 3.

TABLE 6. Radioactivity of amino acids from cellular and exocellular protein as percentage of free amino acid- C^{14} removed from the fluid of culture AA during 6 hr of growth

Amino acid	Cellular protein	Exocellular protein
Aspartic acid.....	18	2.2
Glutamic acid.....	41	4.8
Glycine.....	6	0.55
Serine.....	10	2.4
Threonine.....	10	1.7
Alanine.....	37	4.2
Proline.....	34	4.4
Valine.....	24	3.5
Phenylalanine.....	81	7.0
Leucine + isoleucine...	43	4.0

relative radioactivities of amino acids from exocellular protein. Incorporation of aspartic acid, glycine, serine, threonine, alanine, and proline was considerably higher when these were supplied in peptide form rather than as free amino acids.

The final radioactivities of the amino acids in the supernatant fluid of culture AA were between 70 and 90% of the original C^{14} , with the exceptions of glycine and threonine which contained <5% and 28%, respectively, after 6 hr of growth. To determine whether this is due to high uptake of these two amino acids by *C. perfringens* cells or to breakdown by exocellular enzymes, C^{14} -labeled free amino acids were incubated with supernatant fluids from 2-, 3-, and 4-hr cultures at 37 C and were assayed for C^{14} . The results showed no significant decrease in the C^{14} content of these two or any other amino acids over a period of 2 hr. Table 6 shows

the radioactivity of the protein-bound amino acids after 6 hr of growth as a percentage of the radioactivity of the respective free amino acids removed from the culture medium during the same period. Beside glycine and threonine, relatively low amounts of C^{14} were also found in serine and aspartic acid.

DISCUSSION

The results indicate marked differences in both uptake and subsequent pathways of carbon derived from either free amino acids or peptides. Figure 2a shows that in cultures DP and NDP the radioactivity of the free amino acids derived from peptides remained well below the radioactivity of the free amino acids in culture AA. However, the rates of C^{14} uptake by cells in DP and NDP between 2 and 4 hr of growth were 60 and 40%, respectively, higher than in AA (Fig. 2b). Such differences were found consistently in four experiments. The results can only be interpreted by assuming that a large proportion of the total C^{14} taken up by cells in DP and NDP was in the form of peptide- C^{14} . Contrary to growth (Fig. 1), uptake of amino acid- C^{14} did not follow a logarithmic curve (Fig. 2b). This may be explained by hydrolysis of unlabeled peptides in the medium and the resulting competition for uptake between C^{14} -labeled and unlabeled amino acids.

The above conclusion regarding uptake of peptide- C^{14} is supported by C^{14} assays of cellular peptides (Table 2). C^{14} -labeled peptides extracted from cells in culture AA may have been formed de novo or by partial hydrolysis of protein. However, such peptides seem to constitute only a small fraction of the total C^{14} -labeled peptides extracted from cells in cultures DP and NDP, because uptake of C^{14} and incorporation of C^{14} into cellular protein were of the same order in the three cultures, whereas the amount of cellular peptide- C^{14} in cultures DP and NDP was several times higher than in culture AA.

Because of interconversion of amino acids, it may be assumed that the radioactivity of the amino acid constituents of protein shown in Tables 4 and 5 are not derived exclusively from the corresponding amino acids in the medium, either free or in peptide form. However, since any C^{14} in these constituents has its origin solely either in free amino acids (culture AA) or in peptide-bound amino acids (DP and NDP), it may be concluded that the incorporation of some amino acids into protein is higher when they are derived from peptides rather than from free amino acids. Among the amino acids assayed, these are glycine, serine, threonine, alanine, and

proline. The ratios of C^{14} in the 10 protein-bound amino acids derived from dialyzable peptides to the C^{14} derived from free amino acids (DP-AA) follow a very similar pattern in their arbitrary order from top to bottom in Tables 4 and 5, which correspond to cellular and exocellular protein, respectively. This indicates that the amino acids incorporated into the two types of protein pass through the same pools.

According to Snell and his coworkers (Leach and Snell, 1960; Guirard and Snell, 1962), the function of peptides in growth is to provide certain amino acids which, in their free form, may either be taken up inadequately or be rapidly broken down in the bacterial cell. Inadequate uptake may be due to low activity of a specific permease or to unbalanced concentrations of amino acids. Table 6 shows that the amount of C^{14} in protein-bound glycine, serine, threonine, and aspartic acid was low compared with the uptake of these amino acids by *C. perfringens* cells. With the exception of aspartic acid in cellular protein, the C^{14} content of these amino acids in both cellular and exocellular protein was consistently lower with free amino acids- C^{14} in the medium than with peptides- C^{14} (Tables 4 and 5). This indicates that these amino acids, in their free form, are rapidly metabolized in *C. perfringens* cells. The reasons for the differences in the C^{14} content of protein-bound alanine and proline after feeding free amino acids or peptides (Tables 4 and 5) are not as yet known.

ACKNOWLEDGMENTS

I wish to thank H. Pivnick for his cooperation and advice, Y. Yano for exceptionally skilled technical assistance, and N. Nolan and C. Smith for the preparation of the graphs.

LITERATURE CITED

- FOX, E. N. 1961. Peptide requirements for the synthesis of streptococcal proteins. *J. Biol. Chem.* **236**:166-171.
- 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. Academic Press, Inc., New York.
- HAUSCHILD, A. H. W. 1965. Separation of *Clostridium perfringens* type D toxins. *J. Immunol.* **94**:551-554.
- HAUSCHILD, A. H. W. 1965. Peptides for toxinogenesis of *Clostridium perfringens* type D. *J. Bacteriol.* **90**:1793-1794.
- HAUSCHILD, A. H. W., C. D. NELSON, AND G. KROTKOV. 1962. The effect of light quality on the products of photosynthesis in *Chlorella vulgaris*. *Can. J. Bot.* **40**:179-189.

- KIHARA, H., M. IKAWA, AND E. E. SNELL. 1961. Peptides and bacterial growth. X. Relation of uptake and hydrolysis to utilization of D-alanine peptides for growth of *Streptococcus faecalis*. J. Biol. Chem. **236**:172-176.
- KIHARA, H., AND E. E. SNELL. 1960. Peptides and bacterial growth. IX. Release of double inhibitions with single peptides. J. Biol. Chem. **235**:1415-1418.
- 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.
- LEVINE, E. M., AND S. SIMMONDS. 1960. Metabolite uptake by serine-glycine auxotrophs of *Escherichia coli*. J. Biol. Chem. **235**:2902-2909.
- MUELLER, J. H., AND P. A. MILLER. 1955. Separation from tryptic digests of casein of some acid-labile components essential in tetanus toxin formation. J. Bacteriol. **69**:634-642.
- PHILLIPS, A. W., AND P. A. GIBBS. 1961. Techniques for the fractionation of microbiologically active peptides derived from casein. Biochem. J. **81**:551-556.
- PITTMAN, K. A., AND M. P. BRYANT. 1964. Peptides and other nitrogen sources for growth of *Bacteroides ruminicola*. J. Bacteriol. **88**:401-410.
- PIVNICK, H., A. F. S. A. HABEED, B. GORENSTEIN, P. F. STUART, AND A. H. W. HAUSCHILD. 1964. Effect of pH on toxinogenesis by *Clostridium perfringens* type C. Can. J. Microbiol. **10**:329-344.
- PIVNICK, H., A. H. W. HAUSCHILD, B. GORENSTEIN, AND A. F. S. A. HABEED. 1965. Effect of controlled pH on toxinogenesis by *Clostridium perfringens* type D. Can. J. Microbiol. **11**:45-55.
- TSUKAMOTO, M., A. YAMAMOTO, AND R. MURATA. 1963. Effect of some synthetic peptides on the alpha-toxin production by *Clostridium perfringens* PB6K. Japan. J. Med. Sci. Biol. **16**:213-216.
- WOOLLEY, D. W., AND R. B. MERRIFIELD. 1963. Anomalies of the structural specificity of peptides. Ann. N. Y. Acad. Sci. **104**:161-171.