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^{18}Oxygen Probes of Protein Turnover, Amino Acid Transport, and Protein Synthesis in *Bacillus licheniformis**

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SUMMARY

Experiments were performed in which protein turnover was measured by incorporation of ^{18}O from $H_2{}^{18}O$ into protein. Since proteolysis causes the incorporation of ^{18}O into the carboxyl group of amino acids and the incorporation of this amino acid leads to protein with the peptide carboxyl oxygen labeled, turnover can be determined quantitatively. The rate of protein turnover during the first 5 hours after growth was found to be about 22% per hour. Spores contain an amount of ^{18}O that allows the suggestion that the average protein turns over two or more times during sporulation.

To examine the possibility of oxygen exchange during protein synthesis, *Bacillus licheniformis* cells were grown through eight generations, a 200-fold increase in mass, on a medium of glucose plus a mixture of amino acids that were labeled with ^{18}O in the carboxyl groups. The protein of the cells contained oxygen, after appropriate corrections, at an atom per cent excess similar to that of the added amino acids. It is concluded that neither amino acid transport nor protein synthesis involve mechanisms that lead to significant loss or exchange of amino acid carboxyl oxygens.

Values for the rate of protein turnover in microbial cells depend upon experimental and physiological conditions and vary from less than 2 to 18% per hour (1-4). Growing cells generally exhibit a turnover rate of less than 3% per hour while starving or differentiating cells appear to degrade protein at significantly increased rates.

Conventional isotopic measurements of protein turnover are complicated by many factors, the most important being the recycling of labeled amino acid due to inefficient purging with unlabeled exogenous amino acids (1). The inhibition of transport by endogenous amino acids, seen in several systems (5, 6), may lead to a restriction of loss of radioactive amino acids and result in erroneously low values for protein turnover. These factors could be especially important in studies on turnover in differentiating systems in which intracellular pool sizes increase and amino acid transport is restricted (7).

Bacterial sporulation serves as an excellent model for studies of developmental regulation. In the usual laboratory-controlled system, growth is typical of procaryotes and sporulation is prevented by catabolite repression. Upon exhaustion of nutrients, catabolite repression is relieved, and the cell endogenously modifies its metabolism to allow the expression of the sporulation phenome. One striking aspect of sporulation metabolism is the apparent high rate of turnover of RNA and protein (4).

As many *Bacilli* do not contain storage materials, it is assumed that monomers for sporulation energetics and biosynthesis are made available to the cell primarily via protein turnover. *Bacillus licheniformis* endogenously induces several intracellular proteases at the end of the exponential phase of growth, the combined activities of which, *in vitro*, are sufficient to release approximately 30 to 40% of the dry weight per hour as free amino acids (8). Because of this, it was felt that the absolute rate of protein turnover, *in vivo*, should be measured in sporulating cells.

Classical methods for turnover measurement are not satisfactory in this system as it has been shown in *B. licheniformis* that some of the amino acids cannot be purged from the cells because exit transport systems are limiting (7). To circumvent this problem, we have measured protein turnover during sporulation by an alternate method utilizing ^{18}O. The logic of this method is outlined in Fig. 1.

The most significant assets of this method are that turnover values greater than 100% can be determined and that transport of solutes through the cell membrane is not needed. Hydrolysis of peptide bonds followed by the utilization of the resulting amino acids leads to the incorporation of ^{18}O into protein. However, the validity of the method depends on several factors. The first of these is that the equilibration of water between the extracellular medium and the intracellular space of the cell must not be limiting. Also, the enzymes responsible for proteolysis and biosynthesis must not catalyze oxygen exchange reactions between water and amino acid carboxyl groups. Finally, net synthesis of amino acids during sporulation would lead to oxygen incorporation and this should be prevented.

These factors were considered, and one, oxygen exchange dur-

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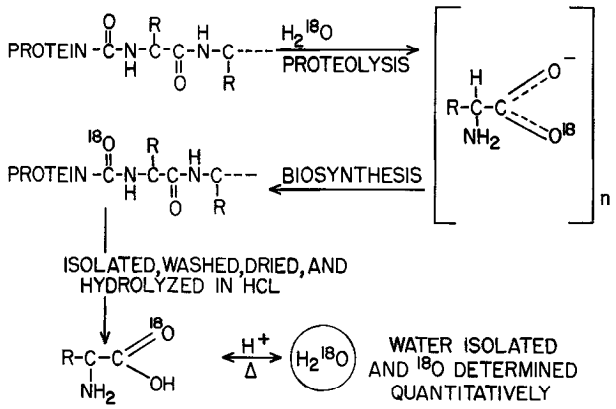

FIG. 1. Scheme depicting the logic behind the use of $H_2{}^{18}O$ in measuring protein turnover.

ing protein synthesis, was tested by ^{18}O-labeled amino acid incorporation studies.

EXPERIMENTAL PROCEDURES

Materials—Water of approximately 1.51 atom % ^{18}O was purchased from Yeda Research and Development Company, Rehovoth, Israel, and from Miles Laboratories, Inc., Elkhart, Indiana. Water of approximately 42.2 atom % ^{18}O was obtained from Miles Laboratories, Inc. Lysozyme was obtained from Worthington Biochemical Corporation, Freehold, New Jersey in the 2 times crystallized, salt-free form. Chromatographically homogeneous L-amino acids were products of Calbiochem, Los Angeles, California, or Mann Research Laboratories, New York, New York. Amersham-Searle Corporation, Arlington Heights, Illinois, was the supplier of all radioactive compounds; the L-amino acids and glucose were of high specific activity and uniformly labeled. All other chemicals were reagent grade.

Growth of Cells—B. licheniformis A-5 was used for all of these studies. Two salt mixtures were used; the first, B-salts, has been described previously (7). The other salt mixture, T-salts, was composed of 100 mg of $MgSO_4 \cdot 7H_2O$, 1.0 mg of $MnCl_2 \cdot 4H_2O$, and 50 mg of $CaCl_2 \cdot 2H_2O$ per liter to which potassium phosphate, pH 7.2, was added to 65 mM. Glucose at 20 mM was used in all media. Nitrogen sources were either 50 mM ammonium lactate, 18 mM urea, or 10 mM NH_4Cl as specified in the text. Growth was measured as absorbance at 540 nm with a Beckman DU spectrophotometer or a PMQII Zeiss spectrophotometer. Visual observations of cells utilized a Leitz microscope fitted with phase contrast optics. Spores were prepared as described previously (7).

Preparation of ^{18}O-Amino Acid Mixture—Three mmoles of each of the L isomers of alanine, arginine, aspartate, cysteine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine were dissolved in 2.0 ml of $H_2{}^{18}O$ at 42.4 atom % ^{18}O to which 2.0 ml of 12 N HCl had been added. After sealing the tube, the mixture was incubated at 110° for 96 hours. The mixture was then precipitated three times from water with methanol-ethyl ether. The final preparation was dissolved in water and analyzed in a Spinco Model 120 B amino acid analyzer by a modification of standard procedures (9). The final composition of this mixture will be shown in the section on "Results."

Analysis of ^{18}O in Amino Acids and Protein—All analyses of ^{18}O were performed with methods similar to those previously described (10–12). Amino acids labeled in the carboxyl groups with ^{18}O were decarboxylated with the guanidine hydrochloride method (12), and the CO_2 was analyzed directly. Control studies with phenol and a secondary aliphatic alcohol showed that the guanidine hydrochloride method does not convert the —OH oxygen of these compounds of CO_2. The ^{18}O-content of water samples was determined by equilibration with CO_2, in some instances with addition of ethanol to increase volume and ease of shaking.[1] The equilibration procedure allowed measurement of the atom per cent excess with a precision of ±0.0005% or better.

TABLE I

Analysis of ^{18}O-amino acid mixture

Sample	Amount analyzed	Diluted with	Atom per cent excess observed	Corrected for dilution (atom per cent excess)
	μmole	*μmoles*		
I	0.354	7.10 alanine	0.756	15.9
II	0.354	7.10 AA mix[a]	0.757	15.9
III	0.354	7.10 AA mix[a]	0.752	15.8
Average				15.9

[a] The composition of the AA mix (amino acid mixture) was identical with that shown in column 2 of Table II.

To facilitate analysis, the amino acid mixture prepared above was diluted with alanine or an amino acid mixture. Table I gives the results of these analyses. The amino acid mixture was prepared from A grade L-amino acids to provide a composition identical with that of the ^{18}O-amino acid mixture. This was confirmed by amino acid analysis on the Spinco model 120 B analyzer. As can be seen in Table I, dilution in either manner provides identical results. The resultant atom per cent excess of 15.9 is approximately 85% of theoretical exchange in the preparation of the ^{18}O-amino acid mixture, but no precautions were taken to completely dry the amino acids. It is concluded that the preparation method produces almost complete random labeling of the carboxyl groups of the amino acids.

In other control experiments, [^{18}O]glutamic acid was prepared by means of the same methods. Analysis showed that both carboxyl groups were labeled and that both exchanged with water in 5.7 N HCl in approximately 96 hours at 110°. In one experiment, about 85% had exchanged in 48 hours at 110°. Thus, 96 hours was used in all preparations and if exchange from protein was being measured, an extra 24 hours was added for hydrolysis.

Analysis of ^{18}O in the peptide carbonyl of protein was as follows. Cells were lysed with crystalline lysozyme (50 μg per 10 mg of cells, dry weight), and the protein was precipitated with trichloroacetic acid (5% final). After standing overnight at 3°, the precipitated protein was sedimented at 2500 × *g* for 10 min and washed three times by boiling for 10 min in 5% trichloroacetic acid, followed each time by centrifugation as above. Subsequently, samples were washed two times by boiling in methanol-chloroform (1:1) followed by centrifugation. After a final wash in anhydrous methanol, the protein was dried overnight in a vacuum desiccator and weighed on a Sartorius microbalance. The dried protein samples were placed in Pyrex tubes (7 × 75 mm), desiccated overnight, and 5.7 N HCl added (0.10 ml per 10 mg of protein). The tubes were sealed and incubated at 110° for 120 hours. To remove the water from the hydrolysates, the tubes were opened and the HCl was neutralized

[1] P. D. Boyer, unpublished procedure.

with dry K_2HPO_4. Anhydrous methanol (1.0 ml per 0.1 ml of hydrolysate) was added and after mixing, the precipitated salt and amino acids were allowed to settle. A sample of the methanol-water solution was equilibrated with CO_2 for ^{18}O-analysis. Control experiments were performed validating the time required for complete CO_2-water equilibration. It should be mentioned that the methanol must be anhydrous and that the washes are necessary as freeze drying will not remove all of the water from precipitated protein.

Measurements of Protein Turnover—For Experiments I and II (Table IV) *B. licheniformis* cells were grown in duplicate in 500-ml flasks containing 60 ml of medium. One hour after the end of the exponential phase of growth, an amount of $H_2{}^{18}O$ was added to each culture equal to the remaining volume. Samples (20 ml) were removed (one from each flask) at the times specified in Table IV and treated as described in the previous section.

Because the cultures in Experiments I and II were diluted extensively, some cell growth was observed after addition of the $H_2{}^{18}O$. This was most pronounced in Experiment I where excess ammonium lactate served as the nitrogen source. Therefore, Experiments III and IV utilized 40-ml cultures in side arm flasks (for optical density measurement) to which approximately 2.0 ml of $H_2{}^{18}O$ (at 42.2 atom %) was added 1 hour after the end of the exponential phase of growth. Turbidity was determined periodically with a Klett-Summerson colorimeter with a 540-nm filter, and values were translated to absorbance by means of a previously established standard curve. Ten-ml samples were removed at the indicated times, centrifuged as before, and the cells were analyzed as before. Samples of the supernatant solutions after centrifugation were sealed and the water analyzed for ^{18}O-content. The total number of samples analyzed in these turnover experiments is specified in Table IV (numbers in parentheses).

Calculations of atom per cent excess of ^{18}O in the peptide carbonyl oxygen are performed as discussed under "Results" and in the footnote to Table III. Amounts of protein turnover were computed by means of the first order relation in which the number of turnovers of an average amino acid residue $= -2 \times 2.3 \log (1/1 - F) = -4.6 \log (1 - F)$ where F equals the fraction of the oxygen that has been replaced. Turnover values are converted to percentage of turnover by multiplying by 100. As is true of first order formulations, high values have greater error and for this reason turnover values of 300 to 500% are approximations.

Analysis of Radioactivity in Individual Amino Acids—Samples containing radioactive amino acids were applied to either column of a Spinco model 120 B amino acid analyzer and eluted as described previously (9). Eluates from the columns were run through a 1.0-ml flow cell (Packard #5724490 packed with anthracene, Packard Instrument Company, Inc., Downers Grove, Illinois) placed in the counting well of a Beckman model LS-100 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, California). A Heath EU-20V recorder (Heath Company Benton Harbor, Michigan) was attached to the ratemeter of the scintillation counter and the recording speed was set to equal that of the recorder on the amino acid analyzer. Eluates were returned to the analyzer from the flow cell for quantitative colorimetric analysis. Total counts under each radioactive peak were located on the recorder chart and determined by summing the relevant counts from a coincident print out system. The entire procedure was standardized with known samples; counting efficiency was about 20%.

For the experiments reported here, ^{14}C- and ^{18}O-amino acid mixtures were analyzed with the above flow system and counts per min per μmole of amino acid calculated. After allowing cells to grow on this mixture, protein samples were isolated as in the ^{18}O-experiments and hydrolyzed in 5.7 N HCl in sealed tubes for 24 hours at 110°. Hydrolysates were dried, *in vacuo*, repeatedly, and the dried material was dissolved in 0.20 M sodium citrate buffer, pH 2.2, and placed on the analyzer column. Specific activities, in counts per min per μmole, were calculated and compared with the original preparations. Data are presented as the percentage of the specific activity of amino acids in hydrolysates compared with the original ^{14}C- and ^{18}O-amino acid preparations. In experiments in which cells were grown on [^{14}C]glucose plus ^{18}O-amino acids, the specific activity of the original glucose was calculated from the specific activity, in counts per min per μmole of carbon, of the muramic acid, glucosamine, and galactosamine in the hydrolysates of cells. The same amino acid analyzer-flow cell system was used. Data are presented as the percentage of the specific activity per μmole of carbon of each amino acid as compared to the average of six values of the hexosamines.

RESULTS

Three types of experiments were performed. In order to make reasonable calculations of protein turnover, the dilution of amino acids labeled with ^{18}O had to be determined. This was done by growing cells on glucose plus an amino acid mixture labeled with ^{14}C and ^{18}O. Assuming that a decrease of ^{14}C-specific activity in amino acids was a measure of the decrease in ^{18}O-specific activity, the efficiency of the incorporation of amino acid carboxyl oxygen could be calculated.

Secondly, a quantitative amino acid analysis of whole *B. licheniformis* protein was needed to allow a calculation of the quantity of peptide carbonyl and total oxygen in the protein. Thus, by combining these analyses, expected ^{18}O in protein from cells grown on a glucose plus ^{18}O-amino acid mixture medium could be calculated and compared with observed values.

Finally, protein turnover studies were performed by adding $H_2{}^{18}O$ to sporulating cultures, isolating the protein after various times, and analyzing for ^{18}O in the protein.

Dilution of Amino Acids during Growth—*B. licheniformis* cells were grown through a 200-fold increase in mass on a medium composed of T-salts, 20 mM glucose, and 8.85 mM ^{14}C- and ^{18}O-amino acid mixture. This medium was chosen for two reasons. First, in order to prevent amino acid catabolism, glucose was added to establish catabolite repression conditions.

Second, it was necessary to perform these dilution experiments under identical conditions to the subsequent ^{18}O-incorporation experiments in which amino acid biosynthesis should be minimal. Thus, an excess of amino acid was added and because of the composition of the ^{18}O-mixture that had been prepared (Table II, column 2) a relatively high total amount was chosen.

In a series of experiments, tracer quantities of different ^{14}C-amino acids were added to samples of this ^{18}O-mixture, and the specific activities of the added amino acids were determined. These separate ^{14}C- and ^{18}O-amino acid mixtures were then used in duplicate sets of growth medium. Fig. 2 shows a typical growth curve. In all experiments, 5.0 μg per ml of lysozyme was added to the growing culture when an optical density of 5.2 to 5.6 was obtained and the culture was allowed to lyse for 30 min. At this time, trichloroacetic acid was added to 5% (w/v), hydrolysates of the purified protein were prepared, and the specific activity of the amino acids was determined and compared with the corresponding values of the original medium.

Column 3 of Table II lists the results of eight duplicate experi-

ments. Arginine, threonine, and isoleucine are incorporated without dilution, but asparate, glutamate, alanine, serine, and cysteine were severely diluted. Since significant equilibration of the carbon skeletons of these amino acids was observed, and because it was impossible to determine if this dilution was brought about by other unlabeled amino acids or by glucose, this dilution-analysis experiment was performed by growing the cells on [^{14}C]glucose plus ^{18}O-amino acids. Column 4 of Table II lists the contribution of glucose carbon in the amino acids of the cell protein. Even though an excess of all of the amino acids was present in the growth medium, a large proportion of some of the amino acids is derived from glucose. For example, over 80% of the aspartic acid and 67% of serine and cysteine originate from glucose.

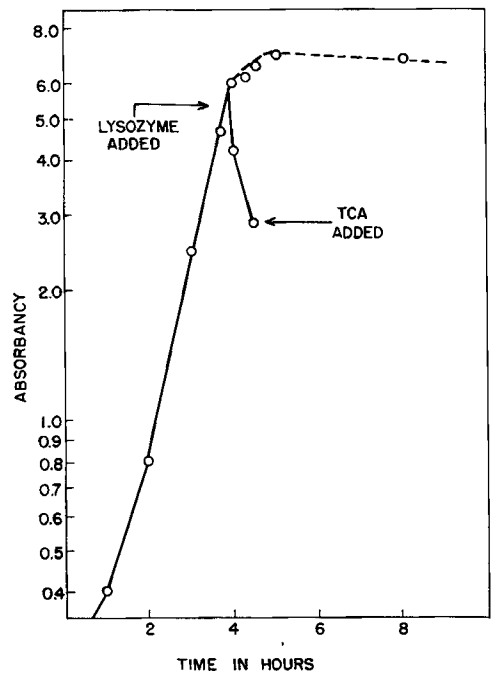

FIG. 2. Growth curve of the cells from Experiment 6, Table III showing the time of addition of lysozyme and trichloroacetic acid (*TCA*). The *dotted line* plots the absorbance of a control culture not receiving lysozyme. See Table III for details.

TABLE II

Dilution of amino acids during growth and composition of whole B. licheniformis protein

Amino acid	Composition of ^{18}O-amino acid mixture[a]	Percentage of amino acid formed from		Composition of whole cell protein[a]
		Amino acid[b]	Glucose[b]	
	mole %			*mole %*
Lys	11.9		2.0 ± 0.1	7.8
His	6.4		3.0 ± 2.0	1.8
Arg	6.8	100.0	0.5 ± 0.5	4.3
Asp	4.5	19.9 ± 1.9	81.6 ± 4.4	9.7
Thr	5.3	100.0	<0.5	4.4
Ser	5.3	36.6 ± 2.6	67.9 ± 4.6	5.4
Glu	5.7	23.2 ± 0.2	53.3 ± 6.3	12.5
Pro	3.7		36.0 ± 2.8	4.4
Gly	7.2		40.6 ± 1.9	7.8
Ala	11.0	25.4 ± 0.1	45.8 ± 2.5	10.7
Cys	1.5	23.5 ± 1.3	67.5 ± 3.2	0.3
Val	6.0		7.3 ± 1.0	7.5
Met	5.1		<0.5	3.9
Ile	8.1	100.0	<0.5	5.5
Leu	6.7		<0.5	8.3
Tyr	1.5		25.2 ± 5.8	2.5
Phe	3.3		<0.5	3.6

[a] Six analyses were performed with an error of 5%.

[b] Duplicate analyses were performed.

All of these experiments were performed with an amino acid analyzer and it was possible, therefore, to repeatedly determine the amino acid composition of whole cell protein. Six of the analyses were averaged and column 5 of Table II presents these results. The average molecular weight of an amino acid in *B. licheniformis* protein was calculated to be 127 by multiplying the mole percentage of each by its molecular weight, followed by the addition of 0.01 of each of these values. From this, the number of peptide bonds per mg of this protein was calculated, producing a value of 7.87 μmoles per mg of protein. Since aspartic and glutamic acids comprise 22.2% of the protein, the total β- and γ-carboxyl groups in the protein is 1.75 μmoles per mg of protein.

The average dilution of amino acids during growth on the salts, 20 mM glucose, 8.85 mM ^{18}O-amino acid medium was calculated by summing, on a mole percentage basis, the average dilution by glucose of each amino acid. Dilution of the β- and γ-carboxyl groups of aspartic and glutamic acids, respectively, was calculated in the same way. Thus, these dilution values are 30% for all α-carboxyl groups and 65% for the β and γ groups. These values, 7.87 and 1.75 μmoles per mg of protein and 30 and 65% dilution, were used in calculating expected ^{18}O in the protein in the following experiments.

Efficiency of ^{18}O-Amino Acid Incorporation into Protein in Vivo—Six *B. licheniformis* cultures (10 ml) were grown on the salts, 20 mM glucose, 8.85 mM ^{18}O-amino acid medium as before (Fig. 2), lysed, and the protein isolated. After drying and weighing, the protein samples were treated in acid for 120 hours at 110°. Table III presents the observed atom per cent excess of ^{18}O in the water of these samples. With the assumption that the oxygen in the carboxyl groups was not exchanged either during transport or during protein synthesis (calculation A), the average expected atom per cent excess of ^{18}O was calculated to be 0.119 and the observed value was 0.109. It is difficult to prevent isotopic dilution during sample preparation, and it is assumed that observed values would be somewhat low. The observed average value is 9% lower than the calculated (A) value but this may not be significant as the experimental error is estimated to be about 10%. Calculation B assumes that the concentration of ^{18}O in the oxygen that is incorporated into protein is halved and produces an average value of 0.066 which is 40% lower than the observed value. Thus, it is suggested that the oxygen in the carboxyl groups was not extensively exchanged during amino acid transport and protein synthesis.

Rate and Quantity of Protein Turnover during Sporulation—Protein turnover studies with $H_2{}^{18}O$ were performed several times on several different media. The results of these studies are shown in Table IV. In Experiment I, the cells were grown on a medium of B-salts, 20 mM glucose and 50 mM ammonium lactate, a medium in which the growth curve is usually "normal." However, in this case, the addition of $H_2{}^{18}O$ at 1 hour after the end of the logarithmic phase of growth resulted in a doubling of the volume and the mass of the cells increased significantly during the next 3 hours. Thus, the calculated turnover rates of 33 to 38% per hour during the first 5 hours probably reflect some net synthesis. Experiment II utilized 15 mM urea in place of the ammonium lactate, and the lack of significant growth during

TABLE III

Atom per cent excess of carbonyl oxygen in protein from cells grown on ^{18}O-amino acids plus glucose[a]

Experiment	Weight of protein	Atom per cent excess		
		Observed	Calculation A[b]	Calculation B[b]
	mg			
1	6.3	0.099	0.108	0.060
2	7.3	0.102	0.122	0.069
3	8.2	0.122	0.134	0.078
4	6.9	0.118	0.118	0.066
5	7.7	0.128	0.130	0.072
6	5.8	0.068	0.099	0.054
	Average	0.109	0.119	0.066

[a] Cells were grown in 10-ml lots on T-salts, 20 mM glucose, 8.85 mM ^{18}O-amino acid mixture, and 10 mM NH_4Cl.

[b] Calculation A is based on no exchange of α-carboxyl oxygen during transport and protein synthesis. Calculation B is based on exchange of 0.5 of α-carboxyl oxygen during transport and protein synthesis. Calculations are based on the equation $Ac_Ad_A + Bc_Bd_B = [A + B + W]c_T$ in which A is the micromoles of oxygen in α-carboxyl groups, B is the micromoles of oxygen in β- plus γ-carboxyl groups, W is the micromoles of oxygen in the water in the 5.7 N HCl, K_2HPO_4, and methanol (5550 μmoles), c_A, c_B, and c_T are the concentration, in atom per cent excess, of the ^{18}O in the α groups, β plus γ groups, and total hydrolysate, respectively, and d_A and d_B are the mole per cent dilutions of the A and B oxygen atom as calculated from Table II. Calculation A for Experiment 1 is as follows:

$$(6.3 \times 7.87 \times 15.9 \times 0.7) + (6.3 \times 1.75 \times 15.9 \times 0.35) = [(6.3 \times 7.87) + (6.3 \times 1.75) + 5550]c_T$$

Solving for c_T provides a value of 0.108 atom % excess.

sporulation was evident. Turnover rates of 21 to 24% per hour were observed. In these two experiments, plus one not reported, the cultures were diluted with an equal volume of $H_2{}^{18}O$ at 1.51 atom %. Since the amount of oxygen in 10 mg of protein is about 1% of the amount in 0.1 ml of 5.7 N HCl, observed values of ^{18}O in the hydrolysates were very low. In fact, 1-hour samples fell within the range of the control values and are not reported.

Because of this, Experiments III and IV were performed in which a small volume of $H_2{}^{18}O$ at 42.4 atom % was added to sporulating cultures. Fig. 3 plots the growth curve of Experiment III. In these experiments a turnover rate of 20 to 22% per hour was found.

In Experiment I, sporulation of the cells in the culture was progressing well as determined by microscopic observation, and the mixture of cells, sporangia, and spores showed about 400% total turnover. Experiment II had sporulated to a lesser extent at 18 hours and 300% turnover was measured. In Experiments III and IV, the cells were allowed to complete sporulation and the spores were well washed before analysis. "Clean" spores exhibited a total of 500% turnover.

Calculations for Experiments I and II were made on the basis of calculations of the known dilution of 1.51 atom % excess $H_2{}^{18}O$ when added to the culture. In Experiments III and IV, the ^{18}O-enrichment of the water in the medium was measured and these data are shown in Table V. Over the range of $H_2{}^{18}O$ used in this experiment about 1% of the excess ^{18}O is lost per hour, presumably by exchange with the atmosphere as the cultures were grown aerobically. These data were used to calculate the ^{18}O atom per cent excess at the time of addition and this zero time value was used in the calculations in Table IV. Corrections were not made for this loss of ^{18}O in the culture medium as the effect is insignificant for the first 5 hours used to calculate turnover rates.

TABLE IV

Protein turnover during sporulation

Time after $H_2{}^{18}O$ addition	Optical density of culture at 540 nm[a]	Observed atom per cent value	Calculated atom per cent excess of peptide carbonyl oxygen[b]	Percentage of turnover	
				Total	Percentage per hour
hours					
Experiment I[c]					
0.0	4.92	0.207 (6)[d]			
3.0	7.98	0.211 (2)	0.276	109	38
5.0	6.07	0.212 (2)	0.370	165	33
18.0	5.65	0.215 (2)	0.570	~400	
Experiment II[e]					
0.0	3.16	0.207 (6)			
3.0	3.61	0.208 (2)	0.173	62	21
5.0	3.72	0.209 (2)	0.298	121	24
18.0	4.80	0.212 (2)	0.488	~300	
Experiment III[f]					
0.0	6.06	0.207 (5)			
3.0	6.10	0.211 (1)	0.64	66	22
48.0	4.54	0.241 (1)	2.04	~500	
Experiment IV[g]					
0.0	5.60	0.207 (5)			
3.0	5.70	0.209 (1)	0.47	60	20
48.0	3.84	0.224 (1)	1.60	~500	

[a] All optical density values have been adjusted to a nondiluted value.

[b] Calculation is the same as in Table III except that the problem is solved for the atom per cent in the peptide carbonyl with the total water value known.

[c] Atom per cent excess of the $H_2{}^{18}O$ was calculated to be 0.657 when added. The 18-hour samples contained approximately 10% spores, 50% sporangia, and 40% vegetative-appearing cells. Cells were grown in B-salts, 20 mM glucose, and 50 mM ammonium lactate.

[d] Numbers in parentheses indicate the number of replicate samples analyzed.

[e] Atom per cent excess of the $H_2{}^{18}O$ was calculated to be 0.657 when added. The 18-hour samples contained approximately 40% sporangia and 60% vegetative-appearing cells. The cells were grown on B-salts, 20 mM glucose, and 18 mM urea.

[f] Atom per cent excess of the $H_2{}^{18}O$ was determined (Table V) and was 2.33 when added. Spore samples (48 hour) were cleaned by centrifugation (5). Cells were grown on T-salts, 20 mM glucose, and 10 mM NH_4Cl.

[g] Atom per cent excess of the $H_2{}^{18}O$ was 1.82 (Table V) after addition. All other information as in Footnote *f* above.

DISCUSSION

It has been shown that exogenous amino acids are transported into *B. licheniformis* cells and subsequently incorporated into protein without measurable exchange of carboxyl oxygen. A similar conclusion resulted from an ^{18}O-study of aminoisobutyrate transport in tumor cells (13). The mechanism of amino acid transport is not known (14, 15), but the carrier hypothesis (see references in Ref. 14) fits well with such an observation in that a covalent bond with the carboxyl group is not proposed. Since

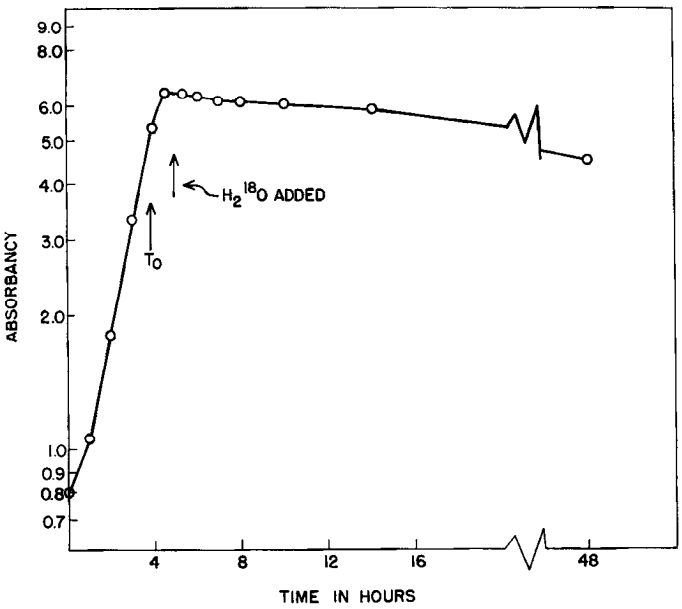

FIG. 3. Growth curve of the culture in Experiment III, Table IV indicating the time of addition of $H_2{}^{18}O$. See Table IV for details.

TABLE V

Analysis of ^{18}O content of water in medium in Experiments III and IV (Table IV)

Experiment	Atom per cent excess in water		48 hours minus 3 hours	Loss per hour	Corrected zero time (atom per cent excess)
	3 hours	48 hours			
III	2.27	1.29	0.98	0.021	2.33
IV	1.78	1.13	0.65	0.014	1.82

amino acid activation (and that of several other acids) involves the synthesis of a covalent bond by a mechanism that leads to the loss of oxygen from the carboxyl group (16), it is considered unlikely that amino acid transport would involve a similar mechanism. Also, it has been shown that the α-hydrogen is not lost during amino acid transport (17), suggesting that a Schiff base between the carboxyl and amino group of the amino acid and a transport protein is not formed since this would be expected to promote α-hydrogen exchange. However, it is not possible to rule out a covalent intermediate during transport if bond cleavage occurs by a novel mechanism and the α-hydrogen is not involved in a resonance form that facilitates exchange.

Of greater significance, however, is the finding that amino acids are incorporated into protein without extensive dilution of carbonyl oxygen. Thus, neither amino acid activation nor the transpeptidation reaction of protein synthesis cause significant dilution of the oxygen that becomes the carbonyl oxygen of peptide bonds in protein. This conclusion was suggested previously from results of a similar but less extensive study (18).

In addition to the absence of exchange reactions during protein synthesis, the validity of the ^{18}O-method for turnover studies depends on the lack of exchange during proteolysis, rapid equilibration of the $H_2{}^{18}O$ with cell water, and the absence of net monomer synthesis during sporulation.

Sporulating *B. licheniformis* cells contain a mixture of protease activities that are quite similar to those of Pronase (8). An extract from sporulating cells catalyzes an autoproteolysis that produces a mixture of free amino acids and oligopeptides that has been refractory to resolution by means that would allow analysis of the ^{18}O-content of the amino acids. Thus, I could not test for exchange during hydrolysis but would suggest that it is probably not significant for two reasons. First, the rate of turnover observed, 20 to 24% per hour during the first few hours of sporulation, is only 20% greater than the value of 18% per hour reported recently (3). Secondly, Kowalsky and Boyer have reported that purified carboxypeptidase does not catalyze an oxygen exchange with carboxyl oxygen during ester hydrolysis (19), Bender and Kemp have shown that chymotrypsin does not catalyze an exchange during ester hydrolysis (20), and the mechanism of five different purified proteolytic enzymes including Pronase and chymotrypsin has been shown to be the same; cleavage at the acyl carbon-oxygen bond of several esters (21). Thus, if exchange does occur it may not be significant, a conclusion reached earlier in similar but less extensive studies on the use of $H_2{}^{18}O$ in measuring protein turnover (22).

The equilibration of water was not tested, but it is known that permeability constants for the transport of water through biological membranes are greater than for known solutes and are in the range of one microbial cell diameter per min (23). Water equilibration would be expected in a few minutes at most; experiments were for periods of 3 hours or more.

Finally, net synthesis of monomers via normal metabolic reactions that lead to oxygen incorporation from water would increase the incorporation of ^{18}O into protein above that due to turnover. That this is indeed possible is demonstrated clearly in Experiment I of Table IV in which a 60% increase in mass occurred due to the dilution of the cells in the presence of nutrient. In this case, turnover values are seen that are approximately 50% greater than those from the remaining experiments. However, when nutrients are limiting (Experiments III and IV) no mass increase is noted and net synthesis is absent. Some equilibration of carbon skeletons of several amino acids would be expected, primarily those that are metabolically "close" to the glycolytic and citric acid cycle pathways but this phenomenon would involve an equilibration between amino acids since no exogenous nutrient is available during sporulation and the cells contain no storage polymers (4). A small amount of ^{18}O from water would enter the amino acid pool as a result of several reactions in the citric acid cycle during the possible conversion of alanine to glutamate carbon or glutamate to aspartate. However, estimates of the magnitude of this type of incorporation are in the order of 5 to 10% of the incorporation due to direct turnover.[2]

Within the limitations discussed above, I tentatively conclude that this ^{18}O-method for measuring protein turnover is valid with an experimental error of not more than $\pm 10\%$. Thus, the rate of protein turnover during the first few hours after the end of exponential growth is 20 to 24% per hour. Spudich and Kornberg measured the decrease in specific activity of [^{3}H]phenylalanine in protein of sporulating *Bacillus subtilis* cells to which excess unlabeled phenylalanine had been added (3). They obtained a turnover rate of 18% per hour. By means of this technique, they also found that a small amount of the label was incorporated into the spores and estimated that 90% of the spore core protein and 95% of the spore coat protein was newly synthesized during sporulation (24). It is possible that isotope recycling occurred in these studies.

Our values for total extent of turnover indicate that the protein in the spores was formed late in sporulation from amino acids

[2] Unpublished data.

that had turned over several times. The values, given in Table IV, of 300 to 500% must be somewhat high. As the spore samples were small and spores are insensitive to lysozyme, isolation of the protein for direct analysis was not attempted and hydrolysates of whole washed spores were analyzed. However, approximately 75% of the dry weight of spores is protein with the remainder being molecules not rich in oxygen except for carbohydrate (25). If all of the carbohydrate in the spores (about 3%) is synthesized from amino acids via gluconeogenesis, it would increase the apparent turnover values about 15% based on relative oxygen content. In no case can a value of total turnover below 200% be envisioned. Thus, the protein in the spore is synthesized from amino acids that have turned over at least twice. If any protein from the vegetative cell is carried into the spore it would have to be very resistant to proteolysis and in small amounts.

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