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Steady-State Measurement of the Turnover of Amino Acid in the Cellular Proteins of Growing *Escherichia coli*: Existence of Two Kinetically Distinct Reactions

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Turnover of cellular protein has been estimated in *Escherichia coli* during continuous exponential growth and in the absence of extensive experimental manipulation. Estimation is based upon the cumulative release into carrier pools of free leucine-*l*-^{14}C over a number of time intervals after its pulsed incorporation into protein. Breakdown rates obtained with other labeled amino acids are similar to those obtained with leucine. Two kinetically separate processes have been shown. First, a very rapid turnover of 5% of the amino acid label occurs within 45 sec after its incorporation, most likely indicating maturative cleavages within the proteins after their assembly. A slower heterogeneous rate of true protein turnover follows, falling by 39% in the remaining proteins for each doubling of turnover time. At 36 C, the total breakdown rate of cellular protein is 2.5 and 3.0% per hr over a threefold range of growth rate in glucose and acetate medium, respectively. This relatively constant breakdown rate is maintained during slower growth by more extensive protein replacement, one fifth of the protein synthesized at any time in the acetate medium being replaced after 4.6 doubling times. Intracellular proteolysis thus appears to be a normal and integral reaction of the growing cell. The total rate equals minimal estimates obtained by others for arrested or decelerated growth but is kinetically more heterogeneous. Quantitatively proteolysis is not directly affected by growth arrestment per se as caused by α-methylhistidine, chloramphenicol, or uncouplers of oxidative phosphorylation, but qualitatively it can gradually become more homogeneous kinetically as a secondary event of starvation. Under more extreme conditions as with extensive washing, prolonged phosphorylative uncoupling, or acidification of the growth medium, the proteolytic rate can increase severalfold.

Although it is generally agreed that much of the intracellular protein of bacteria can be broken down at appreciable rates during starvation, opinion is divided as to the extent of the process when growth is resumed. In studies with different microorganisms, protein label may appear metabolically stable in growing cells some time after the label was originally provided (3). This does not necessarily mean, however, that proteolysis has generally abated, since proteolysis persists in growing *Escherichia coli* in freshly labeled protein in which the preferred, most labile proteolytic substrates are still abundant (11). This finding was met with some agreement by Willetts (19), but breakdown rates, measured this time without cellular washing, appeared lower. A more comprehensive reexamination adapted from the procedure of Willetts (19) has now been made of all sources of turnover of leucine in growing *E. coli*, particularly at extremely early times and under the closest practicable approach to steady-state conditions of growth during measurement of proteolysis. Labeled amino acids other than leucine and different procedures of turnover measurement have been compared. Though intracellular proteolysis can be potentiated to various extents by cellular manipulation, some of it is normal to uninterrupted growth, part indeed occurring so rapidly as to suggest a biosynthetic aspect of peptide removal as distinguished from the slower wear and tear of true turnover.

MATERIALS AND METHODS

E. coli B was grown in a minimal medium of salts (11) and 0.5% glucose or 1% sodium acetate. Cell populations were followed with a Coulter cell counter

or estimated turbidimetrically with a Klett-Summerson colorimeter. In initial experiments, cells were maintained in exponential growth for several days by continuous dilution. When growth became significant during turnover measurement, fresh prewarmed medium was added either in small increments to keep the population approximately constant or in single additions, diluting as much as 12-fold. Proteolysis appeared the same with either procedure. At 9×10^7 to 2×10^8 cells/ml, 51 ng of L-leucine-*1*-^{14}C per ml containing 26 c/mole was added and was consumed within about 1 min. Eventually, 99.5% of the activity became incorporated and resistant to decarboxylation with ninhydrin. At various times thereafter, measurement of proteolysis was initiated with the addition of 500 μg of carrier L-leucine per ml. Immediately and after an additional interval, triplicate or quadruplicate samples, generally of 2 ml, were boiled for 5 min in citric acid at a final concentration of 1 to 2%. Free leucine-*1*-^{14}C that had been produced by proteolysis and trapped by the carrier was decarboxylated by treating the sample with ninhydrin in microdiffusion dishes. The evolved $^{14}CO_2$ was collected over NaOH (11). The activity initially supplied to the cells was determined from a sample to which isotope had been added after the carrier. Protein breakdown was expressed as the percentage value $(r_2 - r_1)/(R - r_1) \times 100$, where R is the total leucine-*1*-^{14}C supplied to the cells and r_1 and r_2 are the amounts of that activity recoverable by decarboxylation at the time of carrier addition and at subsequent sampling, respectively. The leucine-*1*-^{14}C dosage was increased severalfold for the most prolonged measurements or lowered to as little as 3.2 ng/ml for rapid measurement periods of seconds to a few minutes. For the latter, populations were increased to as much as 5×10^8 cells/ml and 2 to 13 ml of culture was decarboxylated. In the most rapid assays, the citric acid was supplemented with concentrated HCl to assure immediate inactivation, the excess then being neutralized before boiling. In supplementary experiments individually cited in the text, cultures were also inactivated with cold 0.5 M $HClO_4$ and the decarboxylation was performed on the soluble portion, neutralizing the $HClO_4$ excess with NaOH after the addition of citric acid. Proteolysis was also measured from the production of trichloroacetic acid-soluble radioactivity of the culture obtained by centrifugation and membrane filtration. $Na_2{}^{14}CO_3$ was counted on planchets in a low background flow counter. Extracts and whole cultures were dried on glass filters in polyethylene vials and counted in liquid scintillant. Samples were counted with an accuracy of at least 5%. Other details of counting and assay have been previously described (11, 12).

RESULTS

Cellular labeling with leucine-1-^{14}C. The ^{14}C-leucine supplied at the routine level virtually replaced the endogenous cellular supply. Thus, a glucose culture at the upper limit of cell density was assayed to have 104 μg of cell protein per ml, of which 8.1% is assumed to be leucine (17). The culture consumed 90% of the leucine-*1*-^{14}C supply of 10 nc/ml or a net 46 ng/ml in a strictly linear fashion with an end point at 33 sec. The growth demand for leucine over a period of 33 sec, as projected linearly from the cell doubling time of that culture (60 min/ln 2), would be 54 ng/ml, not experimentally different from the amount assimilated. Thus, even in this brief interval, the label should be uniformly available within the cell and evenly incorporated in the proteins synthesized. At 3.2 ng/ml, 85% was consumed in 7 sec, this time replacing only one-quarter of the normal endogenous supply of cellular leucine. However, the ^{14}C in the proteins of the soluble, ribosomal and the large and small membrane fragments of pressure-ruptured cells had nearly identical specific activity, when corrected for differences in sensitivity to the protein reagent (10). Before the fractionation, cell growth had been continued with carrier leucine for 15 min to allow for even intracellular distribution of the pulse-labeled proteins after their synthesis (10). The cell proteins would thus appear evenly labeled to some measure at the low isotope level.

Table 1 shows some representative activities, taken from different experiments, of $^{14}CO_2$ obtained from leucine decarboxylation at the earliest and latest intervals of turnover measurement used in calculating the values of Fig. 1 and Table 2. Variations in supplied activity were due to differences in sample size and in the level of isotope provided. In glucose medium, free label found initially (r_1) became large and unwieldy either several generations after labeling or at the earliest labeling intervals (at about 12 sec) unless very dense cultures were used. The r_2 values were greater in acetate medium than in glucose, and measurements became proportionately more accurate.

Short-term proteolysis. The leucine label at the lowest level may be considered to be incorporated essentially in 7 sec in the densest cultures. Within seconds after leucine entered the protein, an extremely rapid breakdown ensued (Fig. 1) over such a limited time as to suggest a biosynthetic function rather than attrition of the protein. This proteolysis, if it followed polypeptide assembly, should be proportional to the amount of substrate still nascent when proteolytic measurement is begun. Free label appearing in cell pools before that time would have been dissipated by largely stable reincorporation. Accordingly, a straight line projected past the earliest breakdown values of Fig. 1 indicated the end of most of this proteolysis at about 45 sec where it meets the projected line of later, more gradual breakdown. The early proteolysis appeared to be almost exhausted during the leucine entrapment

because it increased only slightly when the interval of entrapment was extended from 30 to 60 sec (Fig. 1).

Slowing the cellular growth rate in acetate medium did not seriously alter very early proteolysis, which released 2.6% of incorporated activity into acid-soluble material between 30 and 90 sec after incorporation.

Identification of short-term turnover at the site of cellular protein. How much of the change in decarboxylated leucine activities can be attributed specifically to turnover of protein? Incorporation of the label into transfer ribonucleic acid (tRNA) was judged from the amount of total uptake solubilized in 5% trichloroacetic acid at 90 C for 20 min, which was 10% in mid-uptake and 2.8% when 80% of the label was taken up. About one-fourth of the ester linkage of a preparation of leucine-*1*-^{14}C-charged *E. coli* tRNA was found to be hydrolyzed (susceptible to decarboxylation) by the standard procedure of citric acid boiling and incubation with ninhydrin. Therefore, in the earliest sampling, a minor component, typically less than 1% of the incorporated activity initially estimated as free leucine, might originate from tRNA. This fraction would probably pass into cellular protein by the next sampling, somewhat reducing the estimate of breakdown, or it might exchange its radioactivity with the carrier, increasing the estimate. Accordingly, decarboxylation was performed on only the low-molecular-weight cold $HClO_4$-soluble portion of the culture, and breakdown was at most slightly improved, accounting for 4.4% of the incorporated activity between 10 and 40 sec after labeling. A value of 4.4% was also obtained if trichloroacetic acid was the precipitant, and protein breakdown was measured by direct scintillation counting of the total cold acid-soluble radioactivity. With this method, a maximal breakdown rate of 6.2% of the incorporation was obtained between 6 and 60 sec with a dense culture. Lastly, the samples were collected in NaOH at a net excess of 0.1 N, incubating for 30 min at room temperature before acidification with citric acid. All of the radioactivity in tRNA would then be detected as free leucine. A breakdown of 6.5% was obtained between 10 and 60 sec after labeling in two experiments by decarboxylation. Thus, under suitable conditions, the radioactivity in tRNA had little influence on the turnover rates regardless of whether it was excluded from or introduced into the determinations of nonprotein or free amino acid activity. All determinations gave values that are reasonably similar to a 5% maximum in the initial proteolysis as extrapolated from the intersection of the line of Fig. 1 with the ordinate.

TABLE 1. *Turnover release of radioactivity from protein during steady-state growth*

Growth medium	Interval measured (min)		Leucine-*1*-^{14}C (counts per min per sample)[a]		
	t_1	t_2	Supplied (R)	Decarboxylated r_1	Decarboxylated r_2
Glucose	0.2	1.2	564	27.2	50.8
	0.3	0.8	623	11.0	34.5
	2	4	1,512	17.6	25.7
	5	9	2,865	16.8	28.1
	5	24	962	7.5	19.0
	240	270	7,353	25.4	41.2
Acetate	2	4	1,182	22.6	38.9
	5	9	1,285	11.1	27.0
	621	815	1,070	8.4	26.3

[a] Samples were counted in a flow counter with a low background, <2 counts/min.

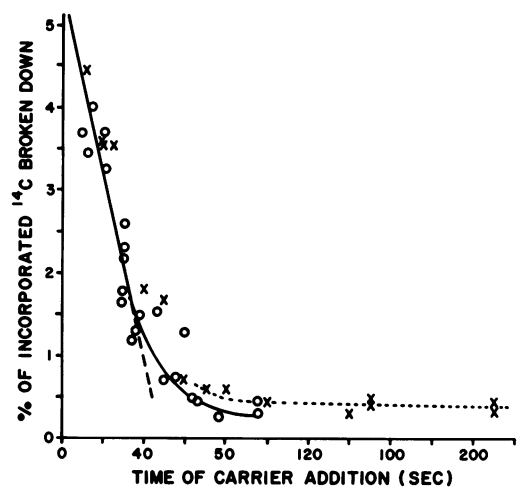

FIG. 1. *Effect of delayed carrier entrapment on the rate of short-term intracellular proteolysis in E. coli. Labeled leucine was added at 0 min and the period of carrier entrapment was 0.5 min* ○ *or 1.0 min* ×. *Experimental points are placed at the time of carrier addition.*

Turnover estimates based on radioactivity of the acid-soluble components agreed with those based on the radioactivity of free amino acid as estimated by decarboxylation. Thus, little significant low-molecular-weight material was produced from protein breakdown other than free leucine, even at the earliest time interval. Moreover, the free leucine, to be on a basis comparable with total acid-soluble material, would have to be generated from acid-insoluble material such as protein and not from a low-molecular-weight

source. Aside from traces in tRNA mentioned, virtually the entire trichloroacetic acid-insoluble incorporation was in protein. All of it was solubilized by digestion with 0.2% Pronase in 0.05 M NH_4HCO_3 (*p*H 7.0 to 7.5), except for 1.9 and 2.8% after 15 sec of incorporation and 5 min of entrapment with carrier, respectively. The residue, probably of undigestible protein, regardless of its identity, was too insignificant and unchanging to have any role in early turnover. Although the high level of leucine used for entrapment of label can be sometimes unphysiological (2), it did not affect cell growth in the least in these experiments. Moreover, supplementing the carrier with a complete amino acid mixture or lowering it to 15 μg/ml in an additional experiment gave the same proteolysis between 30 and 90 sec or at longer time intervals, and most of the breakdown could still be registered with carrier at 5 μg/ml.

Long-term turnover. Intracellular proteolysis during glucose and acetate growth was further determined in a series of nearly contiguous time intervals of from 2 min to over 3 hr each, extending over a total of 4.5 to 4.6 doubling times after labeling (Table 2). Measurements over prolonged intervals agreed experimentally with the sums of several short-term measurements. For each growth rate, breakdown rates varied considerably with the time at which leucine entrapment occurred. The most labile component, in acetate medium, was almost half replaced in 1 hr. There appeared to be a linear negative relationship between the logarithm of both the age of the cell proteins after labeling and their proteolytic rate (Fig. 2). The breakdown rates of cell proteins were severalfold greater in acetate-based growth than in glucose-based growth at comparable ages, but the rates decreased to similar extents in both cases, falling by an average of 39% for every doubling of elapsed time after labeling (Fig. 2). A plot of the logarithm of breakdown rate versus time did not show any early portion of homogeneous first-order decay. A half-life of 13 min reported by Willetts (19) for a fraction designated as unstable protein in *E. coli* probably reflects an apparent homogeneity from the limited and comparatively long time intervals that were used for labeling protein before measurement of breakdown.

With the passage of time, a population of pulse-labeled proteins in a growing cell represents a progressively smaller component of the total cell protein. If labeling, initial addition of carrier, and final harvesting occur at times t_0 (set at 0 min), t_1, and t_2, respectively, the age of the labeled proteins would change from t_1 to t_2 min during entrapment with the carrier, and their breakdown would represent the breakdown of all cell proteins between those age limits. At t_2 min, when the cell mass is maximum, proteins t_1 and t_2 min old would have been synthesized at times $(t_2 - t_1)$ and t_0, respectively. Increases in any of the cell components would be related to the cell growth constant α by $\ln (P_x/P_0) = \alpha(t_x - t_0)$. If P_0, P_{2-1}, and P_2 are the cell masses at times t_0, t_{2-1}, and t_2, respectively, the fraction of the total cell protein generated between times $(t_2 - t_1)$ and t_0 would be $(P_{2-1} - P_0)/P_2 = e^{-\alpha t_1} - e^{-\alpha t_2}$.

In Table 2 are listed the range in protein age encompassed in each schedule of leucine entrapment, the amount of proteolysis within that time range in the relevant proteins, the rate of proteolysis calculated on an hourly basis, the fraction of total cell protein represented by the entrapment schedule, and the absolute proteolytic rate that that fraction would contribute to the total cell protein. Breakdown has been accounted for in 85% of the cell protein. With minor augmentation calculated for intervals not measured and ignoring the special proteolysis (Fig. 1) occurring between 10 and 120 sec, the total turnover breakdown of cell protein amounted to 2.5 and 3.0% per hr, respectively, for glucose- and acetate-based growth.

Effect of cell treatment and growth arrestment on intracellular proteolysis. Slower cellular growth in acetate was accompanied by more far-reaching breakdown in cell protein than found in glucose (Table 2). Breakdown of the proteins, once they were synthesized, was, however, not directly affected by the several changes in growth conditions reported in Table 3. Thus, breakdown of recently synthesized (7 min) or older (60 min) protein was not affected when protein biosynthesis was then blockaded by chloramphenicol or when the cell was depleted of histidyl-tRNA (16) by the addition of α-methyl histidine (Table 3, experiments 1 and 2). At the high levels supplied, α-methyl histidine inhibited growth completely for about 30 min and chloramphenicol would have suppressed even turnover synthesis and minimized adaptive responses. Some adaptation did, however, occur after a 60-min deficiency of nitrogen or carbon, approximately doubling the proteolytic rate of protein 60 to 90 min old (Table 3, experiment 3). The cells had been filter-washed without drainage of medium and the proteolysis shown in fully resupplemented medium was normal.

Breakdown was also increased by other agents, but possibly through physiological dissolution. Complete drainage of medium, even momentarily during filter-washing after labeling, could raise proteolysis from 0.8 to 3.1% per 10 min for 1 hr afterwards, after which it fell to 1.1%. In additional experiments, centrifuging and washing

TABLE 2. *Rate of intracellular proteolysis in growing E. coli as tabulated according to age of cell protein*

Time of carrier entrapment (min) ($t_1 - t_2$)	Growth on glucose					Growth on acetate			
	Protein label released into carrier pool			Breakdown per hr (avg)	Cell fraction $(P_{2-1} - P_0)/P_2$	Total cell protein broken down/hr[a]	Protein label released into carrier pool	Breakdown per hr (avg)	Cell fraction measured $(P_{2-1} - P_0)/P_2$	Total cell protein broken down/hr[a]
min	%			%		%	%	%		%
2–4		0.59	0.63	18.3	0.027	0.49	1.41	42	0.0088	0.37
5–9	0.41	0.31	0.46	5.9	0.051	0.30	1.24	18.6	0.0173	0.32
10–14	0.23		0.26	3.67	0.048	0.18	0.83	12.5	0.0170	0.21
15–24	0.34	0.24	0.55	2.52	0.098	0.25	1.07	7.1	0.037	0.26
25–34	0.38		0.29	2.24	0.084	0.19	0.73	4.9	0.035	0.17
35–49	0.42	0.52	0.44	1.97	0.110	0.22	1.11	4.8	0.052	0.25
50–64	0.35		0.38	1.56	0.088	0.14	0.67	2.87	0.049	0.14
65–84	0.38	0.54	0.40	1.39	0.094	0.13		3.17	0.131	0.41
85–108	0.49		0.41	1.17	0.083	0.10				
109–134	0.46	0.42	0.38	1.01	0.064	0.06		2.16	0.134	0.29
135–164	0.41		0.36	0.80	0.055	0.04				
165–195		0.29	0.24	0.53	0.039	0.02	0.78[d]	1.56	0.060[d]	0.09[d]
191–270							1.42	1.08	0.126	0.14
240–270	0.22			0.44	0.012	0.005				
271–345							0.98	0.79	0.087	0.07
346–420							0.76	0.61	0.065	0.04
421–620							1.98	0.59	0.106	0.06
621–815							1.69	0.52	0.048	0.03
Cell doubling time (min)	47	48	52	(49 avg)			155	180		
Sums measured				(5.30 avg)		0.853	2.13	18.99 (avg)		0.913	2.76
Extrapolated total						1.000	2.49			1.000	3.04

[a] Breakdown per hour (average) × cell fraction $[(P_{2-1} - P_0)/P_2]$.

[b] Experimental values.

[c] Summed and extrapolated from values for shorter intervals.

[d] Not tallied because of the coincidence of this cell fraction with the one below measured at a slower growth rate.

in the phosphate buffer of the medium at 0 C generally doubled or tripled early proteolysis. In Table 3 (experiment 2), acidification can be seen to double proteolysis at *p*H 4.5 and inhibit it completely at *p*H 3.2. 2,4-Dinitrophenol (DNP) had little effect on protein breakdown initially (Table 3, experiment 1) but was stimulatory, as much as fourfold, after more prolonged incubation (Table 3, experiment 2). Levorphanol, which is milder than DNP as an uncoupler of oxidative phosphorylation (4), was also stimulatory. Puromycin at a very high concentration stimulated proteolysis to about the same extent either at very early or at moderate times after labeling (Table 3, experiment 4).

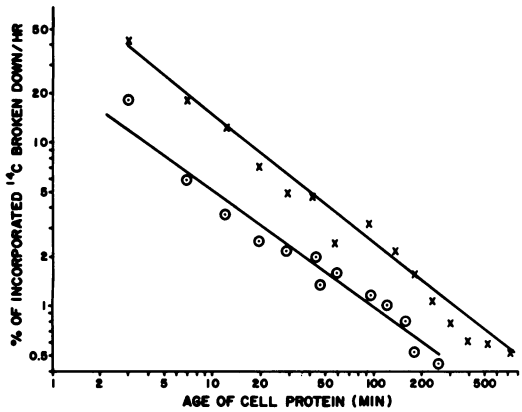

FIG. 2. *Relationship between rate of intracellular proteolysis and cell protein age. Values are obtained from Table 1 and plotted at the median for each interval. Symbols:* (○), *glucose-grown cells;* ×, *acetate-grown cells. The scales are logarithmic.*

Comparison of turnover of amino acid labels. Turnover of ^{14}C-carboxyl-labeled L-isoleucine, L-methionine, L-phenylalanine, L-tyrosine, and L-valine was followed with the standard procedure used for leucine. Residual unincorporated activity was comparatively larger than with leucine and persisted indefinitely or was consumed at reduced rates, sometimes even after carrier addition. Proteolysis with the isoleucine, phenylalanine, and tyrosine labels, which had the lowest backgrounds, amounted to 2.7, 1.7, and 1.1%, respectively, of incorporated activity between 5 and 50 min after incorporation, as compared with 2.1% for leucine. Although more accurate and reliable values remain to be obtained

TABLE 3. *Effect of growth inhibition on intracellular proteolysis of E. coli*

Expt	Treatment[a] (labeling at 0 min)	Proteolysis	
		Time interval	Protein label proteolyzed
		min	%
1	None	7–14	0.51
	10^{-3} M 2,4-DNP, + 6 min		0.60
	3×10^{-4} M CAL, + 6 min		0.52
	1.2×10^{-3} M DL-α-methylhistidine, + 6 min		0.50
2	None	60–90	0.65
	3×10^{-4} M CAL, + 59 min		0.65
	3×10^{-4} M CAL, + 7 min		0.82
	1.2×10^{-3} M DL-α-methylhistidine, + 59 min		0.62
	1.3×10^{-2} M citrate (*p*H 4.5), + 59 min		1.10
	7.2×10^{-2} M citrate (*p*H 3.2), + 59 min		<0.10
	10^{-3} M 2,4-DNP, + 59 min		1.20
	10^{-3} M, 2,4-DNP, + 7 min		2.44
	10^{-4} M, 2,4-DNP, + 7 min		0.75
	10^{-3} M levorphanol, + 59 min		1.00
3	Filter-washing, + 15 min	60–90	
	Growth continued in complete medium		0.65
	Minus NH_3		1.17
	Minus glucose		1.53
4	None	0.5–1.0	1.7
	10^{-4} M puromycin, − 6 min		3.0
	10^{-4} M puromycin, −0.5 min		2.6

[a] CAL, chloramphenicol; DNP, 2,4-dinitrophenol.

with these labels, they indicate that the same magnitude of proteolysis can be demonstrated with a number of amino acids during exponential growth of *E. coli*.

DISCUSSION

Notwithstanding past interpretations to the contrary, intracellular proteolysis in *E. coli* appears to be as inherent to exponential growth as it is to growth arrestment. Except for exposures to label at mid- to late portions of the period of exponential growth for intervals of 1 min or less, growth has been kept at the early exponential stage, and the cellular milieu has been altered in the steady-state measurements only to the extent of providing a labeled amino acid that could be rapidly and uniformly incorporated, followed by addition of carrier at levels that could be as low as 15 μg/ml. Virtually all of the turnover of amino acid label has been shown to take place between the free amino acid and acid-insoluble peptide linkage. Kinetically the turnover appears to be resolved into, first, a return within 1 min of 5% of initially incorporated label into the free leucine pool, and, second, a lower decay rate that continuously decreases but nevertheless remains at a significant activity among the bulk of the cellular proteins. The involvement of the first process in the biosynthetic maturation of proteins is suggested primarily by its speed. Thus, rather than being treated as the cyclic breakdown and resynthesis of a small amount of labile protein, the component has been excluded from the true turnover rate and may represent the excision of a limited number of peptide linkages during the biosynthetic maturation of many proteins. In support of this interpretation, the estimated 45 sec for most of the process to occur is in the same order of magnitude as an estimated 30 sec required for an average alanine amino terminus in the nascent cell proteins of *E. coli* to become exposed to peptidase-like removal of the initiating *N*-formylmethionine residue (13). For β-galactosidase, the one single protein whose translation and *N*-terminal unmasking have both been studied, the time periods of both processes are about the same (7, 8). Amino terminal proteolysis in itself would not appear to account for more than a small part (1) of the 5% peptide removal found in these experiments. Internal peptide cleavage has been implicated in the generation of individual animal and viral proteins from larger precursors (5, 6), and a similar role could be anticipated to occur in bacteria. Such rapid proteolysis may also destroy abnormal or abortive proteins, and drugs such as puromycin or treatments that produce such proteins have been previously shown to increase the rate of long-term proteolysis as much as severalfold (12). Short-term proteolysis is also enhanced but not to any greater extent (Table 3, experiment 4). Thus, although the destruction of incomplete or aborted proteins and polypeptides could be a reasonably attributed role of intracellular proteolysis, it does not appear to be a specialized role of the fast 45-sec turnover. There is no assurance that the added carrier can trap liberated amino acid quickly enough to register the fast turnover completely; hence the estimate of 5% breakdown may be only minimal. The slower, true protein turnover, however, would appear by contrast to be amply registered.

The overall rate of protein breakdown must to a certain extent be a composite of first-order decay rates for a large number of proteins. A decrease of proteolytic rate in older labeled proteins would then indicate the extent to which short-lived molecules are replaced by identical unlabeled ones. However, it is also possible that gradual modifications in some proteins or in the proteolytic apparatus in isolated cellular loci could render some substrates inaccessible to proteolytic attack. No simple interpretation of protein aging or of the kinetics of Table 1 and Fig. 2 would appear warranted, save by determining how strictly breakdown conforms to first-order kinetics in large numbers of individual proteins.

In Table 2 and Fig. 2 there appears to be no defined end point at which proteolysis ends among the cell proteins. There is, however, a fairly constant overall maintenance requirement for protein turnover of 2.5 to 3.0% per hr in steady-state growth which appears uninfluenced by the growth rate (Table 2). Cell proteins are, on the average, older in the more slowly growing cell (Table 2) and are less susceptible to proteolysis than are more recently synthesized proteins. In the slower-growing cell, the total proteolytic rate is kept constant by a fairly proportionate increase in the breakdown of proteins of all ages, somewhat more so in the oldest proteins (Table 2, Fig. 2). The cell proteins thus become more extensively replaced approximately in inverse proportion to the growth rate. The total replacement of the protein originally incorporated in acetate- as opposed to glucose-based growth, equivalent to that decomposed in Table 2, is 2.5-fold greater 195 min after synthesis (12.9 versus 5.1% without extrapolations) and the growth is 3.2-fold slower. After 4.5 to 4.6 doubling times, the comparable cellular replacements become 19.0 and 5.3% (Table 2). To increase the rate of cellular replacement during slower growth, unstable proteins could be synthesized at generally higher cellular levels or the species of pro-

tein that were spared in rapid growth could fall under greater attack. Both alternatives appear to be utilized when growth is abruptly stopped and the most labile substrates are renewed only by turnover synthesis. About one-fifth to one-quarter of the protein synthesized by turnover in rigorously washed amino acid-starved cells is unusually unstable and can probably be replaced as quickly as it is broken down (10). However, the greater remaining proportion of the proteolysis, if the overall rate were maintained, would have to be derived from the proteins that were synthesized during growth. Because the continued replacement of the more labile proteins becomes minimized, the proteolytic rate in stationary cells can now be estimated more simply from long-term proteolysis, as has been conventionally studied in cells labeled over prolonged periods of time. In a detailed study of filter-washed NH_3-starved *E. coli* in glucose, Schlessinger and Ben-Hamida (15) reported an immediate linear breakdown of cell protein at 2 to 3%/hr that was maintained for a number of hours. This would have exhausted the substrates normally attacked in growth within about 50 min (Table 1). In a study by Marr et al. (9) that avoided washing, *E. coli* was induced to grow linearly by progressive glucose starvation in a chemostat and was found to proteolyze 3.1% of its labeled protein per hr. Since a breakdown rate of this magnitude already exists in the growing cell (Table 2), proteolysis in these two studies was not initiated de novo as is generally assumed but shifted to secondary substrates to give the same net rate of hydrolysis. The selectivity of breakdown evident among proteins during growth may still be retained in starvation or the priority may even be altered, but with attack on secondary substrates accelerated as the more labile substrates are exhausted. This stimulated breakdown of the older proteins that would have been spared during growth was indeed evident during glucose and NH_3 starvation (Table 3, experiment 3). The increase does not, however, automatically follow growth stoppage, for example, by α-methyl histidine or chloramphenicol (Table 3, experiments 1 and 2). Presumably the latter agents prevent proteolytic adaptation. Since no net increase in proteolytic activity is needed, adaptation may be required primarily to delocalize protease activity. Alternatively, as has been suggested for bacterial sporulation (14), proteases with new substrate specificities may be formed. The stimulation of proteolysis by general metabolic inhibitors such as DNP and levorphanol (Table 3, experiment 2) or the immediate stimulation by rigorous washing could, in contrast, be attributed to a breakdown in the regulation of the proteolytic apparatus. DNP may therefore be inhibitory in rigorously washed cells (10, 15) or stimulatory (Table 3), or have no effect on proteolysis (18, and Table 2), depending on the degree of dissolution of this regulation. Since DNP can be stimulatory or without effect, no high-energy intermediate or activation need be postulated in intracellular proteolysis, and the event itself could consist of simple catheptic hydrolysis.

The previous high estimates of intracellular proteolysis in experiments that have involved rigorous cell washings are characteristic only of those conditions and must be revised in favor of the present lower values for steady-state growth or for minimal conditions of treatment. However, the qualitative implications of past findings still appear no less valid and verifiable with minimal cellular manipulation, as, for example, the existence of highly unstable peptide linkages (10) and the greater heterogeneity of breakdown rates in growing as compared to resting cells (11).

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