Protein Degradation in *Escherichia coli*

I. MEASUREMENT OF RAPIDLY AND SLOWLY DECAYING COMPONENTS*

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SUMMARY

The rate of degradation of intracellular radioactive protein of bacterial cells supported by a membrane filter has been measured directly from the appearance of radioactivity in the carrier-containing perfusate. For this purpose a perfusion apparatus has been constructed from a microsyringe filter holder. The conditions inside the apparatus are physiological and permit exponential growth up to 1 × 10^6 cells. Exchange of extracellular carrier leucine^{14}C with the intracellular leucine-^{14}C pool is so rapid that at least 87% of the amino acid arising from protein breakdown appears in the perfusate and is not recycled into protein even in growing cultures.

Only a limited portion of the cellular protein is subject to rapid degradation. It decays with a half-life of approximately 1 hour and constitutes 2 to 7% of the total cellular protein of cells growing in glucose minimal medium under various nutritional conditions (including growth and starvation). The proportion of the total protein synthesis which is directed to the synthesis of this rapidly degrading protein component increases with decreasing growth rate. At very slow growth rates 10 to 40% of the radioactivity that is incorporated into protein is incorporated into the rapidly degrading protein. Treatment which removes nonproteinaceous material and Pronase digestion of cells before and after the decay of the rapid component substantiates the conclusion that the rapidly decaying component is derived from bacterial protein.

There is another radioactive component in the perfusate which is released at the very slow rate of 0.2 to 0.6% per hour for as long as the experiments are carried out (48 hours). This does not represent degradation of intracellular protein to amino acids since this process is independent of leucine exchange, and 30 to 50% of the radioactivity in the perfusate is acid-insoluble.

In *Escherichia coli*, the rate of degradation of most of the intracellular protein is less than one-thousandth the rate of its synthesis (1–5). However, there are conflicting claims in the literature that the proteins in growing cells do turn over appreciably (6–9). In starvation under various conditions, extensive degradation has been observed (4, 5, 10–12).

Measurements of protein degradation are complicated by recycling of label, changes of growth phase, death, autolysis, and reincorporation by living members of the population. One must also consider the possibility that there is a limited class of intracellular proteins undergoing a continuous process of breakdown and resynthesis in growing bacteria.

We have undertaken a critical re-evaluation of this problem. Here we report the construction of a perfusion system, with cells supported by a membrane filter, to measure directly the rate of release of radioactivity. We find only slight differences between growth and various starvation conditions in the radioactivity release characteristics. There does indeed appear to be a limited class of cellular protein which is subject to rapid degradation ($T_1$ of ~1 hour), and a much slower but continuous process releasing a mixture of acid-insoluble and -soluble radioactivity at a rate of 0.2% to 0.6% per hour.

EXPERIMENTAL PROCEDURE

Materials—Uniformly labeled L-leucine^{14}C (specific activity, >300 mCi per mg) was obtained from Nuclear-Chicago which specified it to be more than 99% pure.

L-Chloramphenicol and Pronase (*Streptomyces griseus* protease, type VI) were purchased from Sigma. Folin and Ciocalteu (13) reagent was purchased from Fisher as 2 x phenol reagent.

Microsyringe filter holders and membrane filters (Millipore HA, 25 mm, 0.45 μ) were purchased from Millipore. The peristaltic pump used to drive fluid through the assembly was purchased from The Holter Company.

**Bacterial Strains and Growth Conditions**—For the chemostat experiments a stable auxotroph of *E. coli* strain B requiring uracil was used. For all other experiments an auxotroph requiring uracil and tryptophan derived from *E. coli* B uracil was used.

All growth media were made from a minimal salt phosphate buffer (Buffer M) which contained per liter: NH₄Cl, 1 g; KH₂PO₄, 3 g; Na₂HPO₄, 6 g; NaN₃, 5 g; and MgSO₄·7H₂O, 100 mg. The pH was between 6.9 and 7.0. Uracil and dl-tryptophan were added as required at a concentration of 20 μg per ml. Routinely, cultures were grown with vigorous aeration at 37°C.

1 *E. coli* B uracil and tryptophan auxotroph was isolated from *E. coli* B uracil auxotroph in this laboratory by Dr. R. N. Peterson.
The perfusate was collected in a fraction collector during long was driven by a pump through the MSFH at a constant rate. Teflon flat gasket (instead of the supplied 0 ring gasket), a aluminium slab which is maintained at 37° with circulating water. membrane filter, a stainless steel support screen, a second Teflon filter is composed from top to bottom is shown in the figure is achieved by using a stirring magnet net within the nonmagnetic stainless steel MSFH. For further details see “Experimental Procedure.”

The glucose medium consisted of Buffer M supplemented with 0.2% glucose as a carbon source and gave rise to a doubling time of 55 to 75 min. The rich medium consisted of the glucose medium supplemented with methionine, alanine, serine, glycine, lysine, proline, aspartic acid, glutamic acid, adenine, guanine, thymine, and vitamin mixtures (doubling time of 35 to 45 min). The poor medium consisted of 0.2% DL-alanine as the carbon source (doubling time of 600 min was observed after 48 hours or a doubling time of 300 min was observed after 120 hours following subinoculation of glucose medium-grown cells).

Chemostats (1-liter or 200-ml) were operated as designed in this laboratory (14). Glucose was the limiting nutrient (0.02% glucose). After attaining constant absorbance the bacterial growth rate was estimated from the rate of outflow.

Bacterial doubling times were estimated turbidimetrically. Standard curves were used to relate the absorbance at 100 nm or 600 nm in a Spectronic 20 colorimeter or at 420 nm in a Cary model 16 spectrophotometer to the cell number or dry weight. For glucose-grown cells, 1.0 mg, dry weight, corresponds to 8.2 × 10^9 cells.

Development of Perfusion Apparatus for Kinetic Study of Release of Protein Radioactivity—A flow apparatus was constructed as shown in Fig. 1. A microsyringe filter holder rests on an aluminium slab which is maintained at 37° with circulating water. The MSFH shown in the figure is composed of a stainless steel cover with a female Luer-Lok connector, a Teflon flat gasket (instead of the supplied O ring gasket), a membrane filter, a stainless steel support screen, a second Teflon flat gasket, and a stainless steel base with an outlet.

Aerated medium, brought to 37° in a coiled glass condenser, was driven by a pump through the MSFH at a constant rate. The perfusate was collected in a fraction collector during long time interval experiments or manually during short time interval experiments. An infrared lamp was positioned at such a height so as to maintain the top of the MSFH at 37°. In later experiments, the need for the aluminum slab, circulating 37° water, the infrared lamp, and coiled glass condenser was avoided by performing the experiments in a constant temperature room. More than one MSFH could easily be operated simultaneously in this case.

The mixing volume above the membrane filter was 0.8 ml and the hold-up volume below the support screen was 0.9 ml. When the apparatus was first assembled and loaded with a solution of ethylene blue or potassium permanganate or leucine-14C and then flushed with solvent, the mixing was found to be inefficient in the fluid space of 0.8 ml above the membrane filter. For this reason a small Teflon-sealed magnetic stirring bar was inserted inside the MSFH above the membrane filter. One end of the stirring bar was held in position by means of a fixed magnet on one side of the MSFH; the other end of the bar was moved back and forth by rotating magnets on the other side of the MSFH as is shown in Fig. 1.

Typical flow rates were about 14 ml per hour for long term and 30 ml per hour for short term experiments. This corresponded to a flow rate of 3.9 min and 1.8 min, at the two flow rates, for the fluid passing through the membrane to appear in the perfusate.

Preparation of Protein—Bacterial cells were processed for protein by a modification of a method by McLean et al. (15). In brief, the bacterial cells were precipitated in 5% trichloracetic acid, washed, and then heated in trichloracetic acid to remove nucleic acid, dissolved in NaOH in the presence of leucine, reprecipitated with trichloracetic acid, and extracted with ethanol and ether.

Determination of Protein—Protein was determined colorimetrically by the method of Lowry et al. (16) with the Folin and Ciocalteu (13) reagent against a standard curve prepared with crystalline bovine serum albumin.

Pronase Digestion—To a 60- to 80-µg sample of protein, 200 µg of Pronase were added in a total volume of 2.2 ml in 0.2 M phosphate buffer, pH 7.78, at 37° in the presence of 0.014 M CaCl₂ (17). Small amounts of toluene were added to prevent bacterial growth. The course of the digestion was followed by measuring trichloracetic acid-insoluble radioactivity.

Measurement of Radioactivity—In all experiments the volume of each of the collected fractions was measured and a known portion was dried and counted on an aluminum planchet either in a gas flow proportional counter or in a gas flow Geiger counter fitted with a Micromil window. Corrections for self-absorption due to the dried solids of the perfusion media were applied. The radioactive bacteria or the trichloracetic acid precipitates on the membrane filters were counted in two ways. For low radioactivities, the wet filter was glued onto an aluminum planchet and counted directly. For higher radioactivities, the filter was dissolved in NaOH and diluted to a known volume (250 ml or 500 ml) with distilled water, and a measured portion was then counted on a stainless steel planchet. Corrections were applied to account for nonspecific adsorption of leucine-14C on the membrane filter, back scatter from the filter or from the stainless steel planchet, self-absorption due to the dried residue of the dissolved filter in NaOH, and coincidence. All radioactivities were finally expressed as counts per min of an infinitely thin sample on an aluminum planchet counted in the gas flow counter.

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2 Buffer M is the minimal salt phosphate buffer.

3 The abbreviation used is: MSFH, microsyringe filter holder.
RESULTS

Bacterial Growth on Membrane Filter—Turbidity measurement of cells recovered from the membrane filter (Fig. 2A) appeared to suggest that bacteria in the MSFH apparatus grew exponentially and at the same rate as in batch culture, up to a total cell density of \(1 \times 10^8\) cells, and this was confirmed by leucine-\(^{14}\)C uptake measurements of previously labeled bacteria growing in radioactive medium (Fig. 2B). Above this concentration, bacterial growth on the membrane filter lags far behind the control batch culture. Failure to grow exponentially above \(1 \times 10^8\) cells may be accounted for by an overcrowding, since a 25-mm membrane filter with an effective diameter of 22 mm (area of \(3.8 \times 10^7 \mu^2\) ) can accommodate a closely packed, continuous monolayer of only \(3.8 \times 10^8\) cells of a rectangular dimension of \(2 \times 0.5 \mu\).

Measurement of Efficiency of Exchange of Internal Leucine-\(^{14}\)C with External Leucine-\(^{14}\)C—Coliform bacteria have an internal pool of free amino acids, constituting 0.25 to 1.5% of the bacterial dry weight (18). Some of the amino acids of this internal pool exchange very rapidly when the same amino acid is present in high concentrations outside the bacteria (19). Leucine has been used in the present studies since it has been reported that leucine exchanges efficiently and that the released radioactivity remains in the form of L-leucine (4). Moreover, leucine does not undergo any catabolic reaction or interconversion but stays free or in peptidic linkage in E. coli (20).

The following experiment was designed to determine the speed of leucine exchange in batch liquid culture in order to estimate to what extent the reincorporation of leucine-\(^{14}\)C originating from protein degradation would be prevented by an external carrier under our experimental conditions. The principle of the experiment is as follows. When a small amount of leucine-\(^{14}\)C is added to a sufficiently concentrated growing bacterial population, there will be a time when a large fraction of the leucine-\(^{14}\)C will be pumped into the cell and yet not all of this internal leucine-\(^{14}\)C will have been incorporated into trichloracetic acid-insoluble material. It at this time nonradioactive leucine is added, at concentrations which are to be used in the studies of protein degradation, then an indication of the relative rapidity of leucine exchange with respect to reincorporation into protein can be obtained by following the increase in protein radioactivity.

Initial experiments showed that, at 37°, within 15 sec of the addition of \(4 \times 10^{-10}\) M L-leucine-\(^{14}\)C to \(4.1 \times 10^8\) bacteria per ml, more than 90% of the isotope was pumped inside the bacterial cell. However, it was all incorporated into trichloracetic acid-insoluble material by this time as measured by the difference between total and trichloracetic acid-precipitable radioactivity of the cells. Since incorporation experiments shorter than 15 sec were deemed unfeasible, the experiment was conducted at 20°, at which all metabolic processes are slowed. It was found that, when \(4.5 \times 10^{-10}\) M L-leucine-\(^{14}\)C was added to \(0.75 \times 10^8\) bacteria per ml, a significant fraction of the free isotope existed inside the cells for up to 40 to 45 sec, whereas 80% or more of the leucine-\(^{14}\)C was pumped into the cell within 20 to 25 sec (Fig. 3A). The total radioactivity inside the cell was measured by filtering a portion of the cell suspension on a chilled membrane filter and then washing repeatedly with ice-cold Buffer M. At the 30th sec L-leucine to a final concentration of \(4.8 \times 10^{-3}\) M was added. This resulted in the rapid decrease of the total radioactivity in the cells but there was no net increase in the level of trichloracetic acid-insoluble radioactivity (Fig. 3B).

As a control, when leucine-\(^{14}\)C was added along with leucine-\(^{12}\)C, only 0.8% of the radioactivity was taken in and 0.5% was incorporated into trichloracetic acid-insoluble material in 32
Fig. 3. Exchange of free pool leucine. Bacteria were grown and experiments were conducted at 20°C. A---A and O---O are the total radioactivities inside and outside the cell and X--X is trichloracetic acid-insoluble radioactivity. A. control. To 40 ml of bacterial suspension (3 x 10⁶ cells), 1 ml of L-leucine-¹⁴C (8.5 x 10⁴ cpm) was added to a final concentration of 4.5 x 10⁻¹⁰ M leucine. At 30 sec 2.8 ml of Buffer M were added as control for B. B. chase. To a 40 ml bacterial suspension of 7 x 10⁹ cells, 1 ml of L-leucine-¹⁴C (8.5 x 10⁴ cpm) was added to a final concentration of 4.5 x 10⁻¹⁰ M leucine. At 30 sec, 2.8 ml of 9 mg per ml of n-leucine-¹²C were added to make a final leucine concentration of 4.8 x 10⁻¹⁰ M. M-9, Buffer M.

Thus, it appears that the carrier does efficiently chase radioactivity out of the pool and prevent the pool radioactive leucine from further incorporation into protein even under those conditions in which protein synthesis is rapid.

To determine this chasing efficiency quantitatively, the experiment was repeated so as to measure accurately total and trichloracetic acid-insoluble radioactivity. At 30 sec, 2.8 ml of Buffer M were added as control for B. A further increase of 3.5% (4.5% in another experiment) in the trichloracetic acid-insoluble radioactivity. This shows that internal leucine is exchanged very efficiently since not more than 10% of the pool radioactivity was incorporated into protein. Thus, internal leucine-¹⁴C originating from protein breakdown should be chased into the medium rapidly by leucine-¹²C, and this exchange should be at least 87 to 90% efficient in growing bacteria.

It was assumed, for these experiments, that transport processes and the rate of protein synthesis have similar temperature coefficients. Hence a similar relationship of efficiency of amino acid exchange should hold at 37°C. It was further assumed that the efficiency of leucine exchange as measured in batch culture also holds for bacteria on membrane filter inside MSFH.

Perfusion of Radioactive Bacteria in Presence of Leucine-¹²C

Bacteria were labeled in batch liquid culture during exponential growth in glucose medium at cell densities of 4.2 x 10⁶ to 7.4 x 10⁷ per ml with 0.05 to 0.2 μCi per ml of high specific activity leucine-¹⁴C, undiluted with carrier leucine-¹²C. Under these conditions 85 to 96% of the radioactivity was taken up within 3 to 10 min. The radioactivity that remained outside (4 to 15%) was not taken up upon further incubation with fresh bacteria. This is probably not due to the presence of contaminants in the leucine-¹⁴C because upon chromatography (butanol-water-acetic acid, 37:25:9), at least 90 to 95% of the tracer corresponded to ninhydrin-positive n-leucine (Rf, 0.63) and the remainder appeared to be trailing material. There is the possibility that leucine-¹⁴C is converted by the bacteria to a peptide which is then excreted into the medium. Observations of this kind have been made with glycine and serine (21). This unincorporated radioactivity was investigated no further.

After the uptake of L-leucine-¹⁴C was complete, the radioactive bacteria were, in some experiments, allowed to grow one or two doublings (doubling time of 73 ± 3 min), after which 0.7 to 7.5 x 10⁶ total cells containing 1.5 to 10 x 10⁴ cpm were radioactivity was in the free pool while there was at most a further increase of 3.5% (4.5% in another experiment) in the trichloracetic acid-insoluble radioactivity. This shows that internal leucine is exchanged very efficiently since not more than 10% of the pool radioactivity was incorporated into protein.

Thus, internal leucine-¹⁴C originating from protein breakdown should be chased into the medium rapidly by leucine-¹²C, and this exchange should be at least 87 to 90% efficient in growing bacteria. It was assumed, for these experiments, that transport processes and the rate of protein synthesis have similar temperature coefficients. Hence a similar relationship of efficiency of amino acid exchange should hold at 37°C. It was further assumed that the efficiency of leucine exchange as measured in batch culture also holds for bacteria on membrane filter inside MSFH.
FIG. 5. Appearance of radioactivity in the perfusate. To a total of 4 x 10^8 cells growing in 9 ml of glucose medium, 1 µCi (1 ml) of leucine-14C was added and after 184 min of incubation 4 ml (0.7 x 10^8 cells, 3 x 10^6 cpm) of bacteria were put into the MSFH, washed twice with 10 ml of glucose media lacking uracil, and supplemented with [u-1-14C]leucine at a final concentration of 300 µg per ml. After rejecting the first 15 min sample, radioactivity in the perfusate was determined and converted to per cent by dividing with the total intracellular bacterial radioactivity at zero hour and expressed as %/HR. This is represented by the height of the histogram for each period of collection. The total intracellular bacterial radioactivity at zero hour was obtained by adding the total released radioactivity from MSFH with the remaining radioactivity on the membrane filter at the end of the collection period. The rapidly decaying component (O) has been corrected for the slow component by subtracting the average hourly radioactivity release after 5 hours. Thus, the total radioactivity (histogram) for the initial 5 hours is composed of rapid and slow components, whereas after this period the total radioactivity represents only the slow component.

In initial experiments (cf. Fig. 5), bacteria were allowed to grow for at least one doubling after the uptake of leucine-14C and before transferring into MSFH in order to eliminate very slow turnover processes. Even then a rapid component was detected for 4 to 6 hours after transferring the bacteria to the membrane (Part A, Table I). After this interval, the rate of release of radioactivity was very small and in many cases essentially constant. It can be seen that the release of this slow component is essentially independent of the perfusion medium used. It is also independent of the amount of cells or radioactivity, both of which span a 10-fold range. It can be concluded that the radioactivity released after 5 hours reflects a slowly decaying process which is substantially invariant with respect to the nutritional status of the organism.

**Estimation of Half-life and Amount of Rapidly Degrading Protein**—By making the assumption that the initial rapid appearance and later slow appearance of radioactivity were separate processes, it was possible to subtract the value of the slow component from the corresponding total radioactivity of the perfusate to obtain the rate of release of the rapid component. In the initial experiments, the rate of release of the slow component in the early phase was assumed equal to the observed rate in the later phase. The rate of appearance of radioactivity after 5 hours was sufficiently constant to serve as an estimate of the rate of release of the slow component. Such an example of the corrected released radioactivity is represented by the open circles in Fig. 5. A straight line fitted through these points was used to estimate the intercept and the first order decay constant and half-life of the rapidly decaying component for this and other experiments listed in Table I. In cases in which the experiments were not as prolonged, a value of 0.5% per hour, the average rate from the 5th to 20th hour of other experiments, was subtracted from the total observed radioactivity in calculating the half-life (T1/2) and the intercept.

The rate of degradation of the rapid component at the time that the cells are put in the apparatus (t = 0) is the intercept of the corrected released radioactivity curve. In the development given in the "Appendix," this intercept is designated as (-dP/dt). It is shown there that, P0, the radioactivity (as per cent of the total radioactivity) present in the rapidly degrading protein at t = 0 is equal to:

\[
P_0 = \frac{\left(-\frac{dP}{dt}\right)_{t=0} \times T_{1/2}}{0.693}
\]

(A)

Estimates of P0 as per cent of total radioactivity at t = 0 are listed in the next to right-hand column of Table I. A further correction for two other factors was necessary to convert this calculated radioactivity to p0, the amount of rapidly degrading protein (as per cent of total cellular protein) actually present in the cells during growth. The first factor is that degradation occurred during the time, Δt, between the period when labeling and when cells were put on the membrane filter. The second factor is analogous to the case of messenger ribonucleic acid turnover; that is, the rapidly degrading protein acquires a higher specific activity after a pulse of short duration t1 than does the stable protein of the cells. To correct for these two factors, values of P0 were multiplied by the following quantity derived in the "Appendix."

\[
P_A = P_0 \times \left[ \frac{e^{\left(\frac{1}{\lambda t_1}\right)}}{1 - e^{-\alpha t_1}} \right]
\]

(B)

In this expression, α is the apparent turnover rate constant of the rapidly degrading protein and λ is the bacterial growth rate constant.

Calculated in this way, the amount of the rapidly degrading protein, P_A, was expressed as per cent of total cellular protein which is presented in the right-hand column of Table I. This table shows that the catalysis of the rapidly degrading protein as calculated is not systematically affected by any physiological condition tested. The half-life varied in this series from 45 to 82 min. The calculated amount of the rapidly degrading protein varied from 7 to 23% of the total protein in experiments in
TABLE I

Effect of various perfusing media on appearance of perfusate radioactivity, half-life, and total quantity of rapidly degrading protein.

| Perfusion medium (glucose medium + DL-leucine) | Observed rate of release of radioactivity | Calculated rapidly degrading protein |
|-----------------------------------------------|------------------------------------------|-------------------------------------|
|                                               | 1st hr | 2nd hr | 3rd hr | 4th hr | 5th hr | 6th hr | 7th hr | 8th hr | 9th hr | Average after 3 hr | Half-life (Tf/2) | Radioactivity (PAo) | Amount (PA) |
| A. Incubation period: Δt = 70-150 min          |        |        |        |        |        |        |        |        |        |                    |                |                    |            |
| Complete                                      | 2.49   | 1.41   | 0.62   | 0.30   |        |        |        |        |        | 0.94               | 70              | 4.44               | 11          |
| Minus uracil                                  | 2.36   | 1.29   | 0.80   | 0.51   |        |        |        |        |        | 45                | 5.95           | 6.25               | 10          |
| Minus uracil & tryptophan                    | 2.45   | 1.65   | 1.19   | 0.70   | 0.54   | 0.65   |        |        |        | 64                | 4.76           | 8.65               | 15          |
| Minus glucose                                 | 2.66   | 1.36   | 0.91   | 0.62   | 0.29   | 0.38   |        |        |        | 63                | 4.85           | 9                  | 11          |
| Minus glucose & tryptophan                    | 3.18   | 2.01   | 0.83   | 0.77   | 0.75   | 0.58   |        |        |        | 63                | 5.30           | 11                  | 13          |
| Minus uracil, tryptophan, and glucose         | 4.24   | 3.86   | 2.68   | 1.88   | 0.72   | 0.72   |        |        |        | 54                | 6.63           | 15                  | 27          |
| Minus uracil & tryptophan & glucose           | 1.44   | 1.19   | 0.73   | 0.47   | 0.42   | 0.42   |        |        |        | 0.50              | 6.52           | 7                  | 8           |
| B. Incubation period: Δt = 3 min               |        |        |        |        |        |        |        |        |        |                    |                |                    |            |
| Complete                                      | 7.02   | 3.08   | 1.91   | 1.56   |        |        |        |        |        | 55                | 12.56          | 7                  | 7            |
| Minus glucose                                 | 8.56   | 3.43   | 2.52   | 1.54   |        |        |        |        |        | 65                | 10.47          | 5                  | 5            |
| Minus glucose & tryptophan                     | 9.00   | 5.00   | 2.95   | 1.49   |        |        |        |        |        | 60                | 16.59          | 7                  | 7            |
| Average                                       |        |        |        |        |        |        |        |        |        | 0.50              | 60.9           | 10.6               |            |
| ±S.E.                                         |        |        |        |        |        |        |        |        |        | ±0.06             | ±3.2           | ±1.6               |            |

* Percentage of total cellular radioactivity present at start of collection.
* Actual amount present in growing cells expressed as percentage of total cellular protein.

Fig. 6. Effect of incubation with leucine-14C prior to transfer to the MSFH apparatus. A, B, and C are three independent experiments. Glucose-grown bacteria were labeled with leucine-14C under conditions in which uptake was completed in a few minutes. Then the cells were permitted to grow for 2.5 to 2.75 hours without carrier leucine. Sample A was then put into the apparatus. Sample B was further incubated with 670 μg per ml of leucine-14C for 2.5 hours when further exponential growth was permitted and then put into the apparatus. Sample C was incubated with 1000 μg per ml of leucine-14C for 4.25 hours under conditions of glucose starvation and then put into the apparatus. A and B were perfused with glucose medium and supplemented with leucine-14C, whereas for C glucose was omitted from this medium.

Additional experiments showed that the amount was independent of the length of the incubation period before the cells were placed on the membrane. Three experiments, essentially identical except for the interval of the incubation period with leucine-14C, are shown in Fig. 6. The decay of radioactivity was the same on the membrane filter inside the MSFH as it was in batch culture. This decay also seems to be independent of growing (B) and nongrowing (C) conditions.

Effect of Alterations in Nutritional Conditions on Rapidly Degrading Protein—In order to assess the effect of inhibition of growth on degrading protein, the release of radioactivity due to rapidly decaying component was compared in a series of paired perfusions. The results are shown in Table II. A total of 4 to 8 x 10^6 bacterial cells which had been grown in glucose medium (average doubling time of 56 min) were labeled with leucine-14C for 10 min (when 70.0 ± 23% of the added radioactivity was taken in) and transferred into two previously equilibrated perfusion apparatus. One MSFH was perfused with complete medium (glucose medium + carrier leucine) and the other lacked either uracil, tryptophan, glucose, or all three; alternatively, chloramphenicol was added at a concentration of 250 μg per ml.

In every set of 10 experiments performed (six of which appear in Table II) the 10-min radioactive eluates, collected for a total of 90 min, were similar in amount in both growth and experimental conditions. In general, the half-lives and the initial rate of release of radioactivities were very nearly equal in every paired experiment. When calculations were performed on the which the extrapolation was large (Part A) and was only 5 to 7% in the experiments in which Δt was 3 min (Part B).

In every set of 10 experiments performed (six of which appear in Table II) the 10-min radioactive eluates, collected for a total of 90 min, were similar in amount in both growth and experimental conditions. In general, the half-lives and the initial rate of release of radioactivities were very nearly equal in every paired experiment. When calculations were performed on the
initial 40-min collected radioactivities a value of 2 to 6% of the total cellular protein was obtained for the total amount of the rapidly degrading protein under all nutritional conditions.

In these experiments the magnetic stirring bar (Fig. 1) was not used in order to prevent possible experimental trauma on cells resting or growing on the membrane filter. The total bacterial concentration after 90 min of perfusion, determined by absorbance measurements after shaking the filter in Buffer M, was $7.8 \pm 0.9 \mu g$, dry weight, for nongrowing and $16.1 \pm 3.3 \mu g$, dry weight, for growing conditions. The total concentrations in batch culture cells were $6.0 \pm 0.4$ and $19.1 \pm 1.4 \mu g$, dry weight, before and after the 90-min interval.

**Effect of Bacterial Growth Rate on Rapidly Degrading Protein**—The doubling time of bacterial culture is obviously related to the rate of protein synthesis. It therefore seemed of interest to measure the amount, $p_A$, the half-life, $T_{1/2}$, and the velocity of synthesis of the rapidly degrading protein. In a series of experiments, cells growing at various growth rates were labeled for 10 to 12 mm and then perused in the MSFH with a medium containing glucose and urea but devoid of uracil (and tryptophan). For batch cultures, two experiments with cells grown at different growth rates were performed simultaneously. For chemostat-grown cultures, during the labeling period the growth rate of an aliquot of culture was maintained by the use of an apparatus that pumped medium at a rate proportional to that of the parent chemostat. The slow component was corrected for directly by subtracting the trichloroacetic acid-insoluble radioactivity (see below) of each 10 min fraction from the total radioactivity of that sample.

It can be seen from Table III that the rapidly degrading protein in bacteria growing with doubling times of 38 to 105 min degraded with half-life ranging from 50 to 78 min and constituted 3 to 4% of the total cellular protein. This amount of the rapidly degrading protein is consistent with the values reported in Table II. On the other hand, for bacteria doubling more slowly, the average half-life of decay was 32 min, while the amount of rapidly degrading protein constituted decreased with decreasing growth rate.

During growth, the rapidly degrading protein is continuously turning over and the amounts calculated in Tables I, II, and III represent the balance between synthesis and degradation. The velocity of synthesis of this component, $V_A$, was calculated from experimental values presented in Table III (see "Appendix" for derived formulas) and is shown in Fig. 7. The velocity of synthesis of the more stable component, $V_s$, is also shown in the figure. In balanced growth, the total velocity of protein synthesis, $V_s + V_A$, is slightly larger than the net rate of protein synthesis. In the units presented here, the net rate of protein synthesis is simply 100 times the bacterial growth rate constant.

It is seen that $V_A/V_s + V_A$, the proportion of the total cellular protein synthesis devoted to the synthesis of the rapidly degrading protein, increases as the bacterial growth rate decreases. The slope of $V_A$ changes at a growth rate constant of approximately 0.1 per hour and goes through the origin (Fig. 7B), whereas at higher growth rates the slope assumes a lower value (Fig. 7A).

**Studies on Protein Nature of Rapidly Degrading Protein**—Although the amount of the rapidly degrading protein that is present in slowly growing cells is very small, the fraction of the isotope in the form of this component immediately after the incorporation of the isotope is large. Thus, $P_A$, the total radioactivity in the rapidly decaying component, was between 4 and 8% in bacteria with a doubling time of less than 105 min, whereas it was between 10 and 40% in the more slowly growing bacteria.
FIG. 7. Velocity of synthesis of proteins. $V_a$ and $V_d$ are the velocities of stable and rapidly degrading protein components both in units of milligrams of protein formed per hour per 100 mg of total cell protein calculated from the values of $\lambda$, $k$ (or 0.693/ $T_{1/2}$), and $p_d$ of Table III according to the equations developed in the "Appendix." The inset in A is expanded in B.

This can also be seen from Fig. 7, since $V_d/V_a$ is the proportion of the radioactivities in the rapidly degrading to the more stable protein components at an infinitely short labeling period.

Thus, preferential labeling of the rapidly decaying component is achieved at slow growths, particularly in chemostats. We used this fact to study some of the properties of this material.

Bacteria growing with 20- and 30-hour doubling times in two

Table IV

| Incubation period with leucine-$^{14}$C | Specific radioactivity in |  
|---------------------------------------|---------------------------|
|                                       | Trichloracetic acid precipitate | Purified protein |
| A. For 20-hr doubling bacteria         | cpm/¡g protein             |                |
| 0                                     | 872                        | 855            |
| 4                                     | 626                        | 631            |
| Loss (%)                               | 28                         | 26             |
| B. For 30-hr doubling bacteria         | 194                        | 163            |
| 4                                     | 124                        | 105            |
| Loss (%)                               | 36                         | 36             |

a Cells were labeled with leucine-$^{14}$C for 20 min in A and for 10 min in B prior to incubation with carrier leucine.

b The loss in specific radioactivity is due to the degradation of the rapidly decaying component during 4 hours of incubation.

200-ml chemostats were incubated with leucine-$^{14}$C for 20 and 10 min, respectively, from which 90 and 30% of the added radioactivity were taken up. The difference in specific radioactivities of protein, before and after incubation with leucine-$^{14}$C for 4 hours in the absence of bacterial growth, is due to the loss of rapidly degrading protein. It is seen in Table IV that the change in specific activities (26 to 28%, Part A, or 36%, Part B) was the same in both the initial acid-insoluble and in the subsequent purified protein fraction. This supports the concept that the rapidly decaying component is indeed protein and not an acid-soluble peptide or such components as cell wall, membrane, or any other nonproteinaceous cellular material.

Additional support for the proteinaceous nature of the rapidly degrading protein comes from the observation that, when the denatured radioactive proteins of Table IV were treated with Pronase, the rate of digestion of the total protein followed the same time course. In every case, at least 95 to 97% of the trichloracetic acid insoluble radioactivity became trichloracetic acid soluble in 72 hours after digestion with excess Pronase. The fraction of the radioactivity in the rapidly decaying component (27 and 36% in the two cases) was thus indistinguishable from the rest of the cellular protein radioactivities (73 and 64%, respectively).

Slowly Decaying Component—As discussed earlier, there appeared a second kinetic component from 5 hours onward (Fig. 5), with an average released rate of 0.5% per hour (Table I). Its half-life of decay was uncertain and hence an estimate of the total extent of this slowly decaying component was not possible. Calculations similar to those used for the rapid component indicated that at least 20% or perhaps all, of the cellular protein may eventually be released as this slowly decaying component if the perfusion were continued for an indefinitely long time.

A portion of this component radioactivity was found to be acid-insoluble. This trichloracetic acid-insoluble radioactivity appeared in the perfusate from the beginning of the collection period. Coprecipitation with varying concentrations of non-radioactive bacterial protein or crystalline bovine serum albumin did not increase this trichloracetic acid-insoluble radioactivity.
tein degradation (to constituent amino acids) and hence its unsoluble radioactivity. insoluble radioactivity correspond very well with leucine-r2C of half trichloracetic acid-soluble and half trichloracetic acid-had a slope almost parallel to that of the total slow component. In the experiment of Fig. 8A the slow component was composed of the total slowly decaying radioactivity, but in slowly growing cells this acid-insoluble value was as low as 1% of the total slow component. In general, the radioactivity due to the total slow component appeared with a rate of 0.2 to 0.6% of the total cellular radioactivity per hour.

**DISCUSSION**

Although measurement of protein degradation by amino acid exchange has been a common method (4, 6, 10, 11), analysis of the efficiency of the exchange process has heretofore never been given. We have demonstrated that under our experimental conditions the replacement of internal leucine-r4C by external leucine-r2C is sufficiently efficient and rapid (Figs. 3 and 4), so that it is possible to measure fully intracellular protein degradation to amino acids even in growing bacteria in which protein synthesis and recycling are large.

The availability of very high specific activity L-leucine-r4C and the development of a perfusion system (Fig. 1), in which bacterial growth was possible (Fig. 2) and in which the appearance of radioactivity could be directly measured in the perfusate (Fig. 8), allowed us to follow unambiguously the kinetics of protein degradation as well as other minor processes of ultrafilterable protein or peptide release.

In addition, our procedure overcomes a number of difficulties inherent in previous attempts in the literature, since (a) the perfusion system leads to a direct measurement of the rate of protein degradation, (b) the released protein or peptide materials are immediately separated from the cells so that reutilization is circumvented, and (c) the cells are never subjected to centrifugation or chilled or otherwise rendered liable to any kind of trauma known to induce bacterial damage or death (22). Consequently, for the first time, an accurate account of several kinds of turnover processes can now be given.

Qualitatively and quantitatively similar amounts of radioactivity appeared with low cell densities (permitting exponential growth), large cell densities, and with cells starving under a variety of conditions (Tables I and II). Two classes of decay processes were detected (Fig. 5); one was limited in amount but decayed rapidly and the other decayed very slowly.

It seemed reasonable that the rapidly decaying component represented a limited class of cellular constituent in all cells. In an earlier work such a component was noted which constituted 2% of the total protein activity of growing cells (1). This was then attributed to a minor turnover of some peptide or other cellular constituent. Since in our experiments it was possible to label this component preferentially at slow growth rates, we are now able to show that this material has the solubility characteristics of cellular proteins (Table IV) and is hydrolyzed upon treatment with Pronase. We do not believe that the rapidly decaying component represents a rapid cell lysis of a limited fraction of the cells because the appearance of this component was dependent on leucine exchange (Fig. 8).

The estimates of the amount and the half-life of the rapidly degrading protein in Part A of Table I are inaccurate for several reasons. First, in order to obtain the radioactivities due to the rapid component, the total collected radioactivity had to be corrected by subtracting an average value of the slow component (values between 5th and 20th hour). There was no way of knowing the actual contribution of the slow component during the initial collection period. Second, since after the uptake of isotope the cells were allowed to grow one or two doubling times...
prior to measurements, a long and possibly inaccurate extrapolation to the time of labeling was necessary. Third, large numbers of cells were used in order to obtain more radioactivity in the perfusate. Probably this effect of crowding is small but yet may be significant.

The first two of these objections were minimized by removing the intervening growth period after the uptake of the isotope (Part B, Table I). In addition, samples were collected during the initial 90 min when the contribution of the slow component radioactivity to the total was quantitatively small. In these experiments the slow component was assessed from the corresponding trichloracetic acid-insoluble radioactivity of each 10-min collection sample (Table III). Experiments of these kinds show that the rapidly degrading protein constitutes 3 to 7% of the total cellular protein in bacteria growing with a doubling time of between 35 and 70 min in batch culture.

The objection to the use of a high cell density (Table I) was removed by the experiments of Table II. Here, few cells were placed on the membrane filter so that normal growth during perfusion could be demonstrated. In these cases, the amount of the rapidly degrading protein was 2 to 6% of the total cellular protein. In addition, these experiments showed that the amount and the half-life of the rapidly degrading protein were independent of the nutritional states during perfusion. This finding is in direct conflict with the reports that under starvation conditions protein degradation takes place at a rate of 2 to 5% per hour for many hours (4, 5, 10, 11). These high reported values may result from the difficulties listed in the earlier sections.

Thus, in all experiments when cells were labeled while growing with doubling times faster than 105 min, the amount of the rapidly degrading protein present in the cells was 2 to 7% of the total protein. Conversely, in extending earlier findings (1, 22) it is shown that, not only during exponential growth but also under starvation conditions, more than 98% of the E. coli protein is not subject to rapid degradation.

The proportion of the cells' capacity for protein synthesis that is used for the synthesis of the rapidly degrading protein varies with growth rate. It is larger under conditions in which growth is slow (Fig. 7). This allows for specific labeling of the rapidly degrading protein and hence provides a means for further study of this material.

The slowly decaying component was best seen when large amounts of radioactive cells were studied under nongrowing conditions (Table I, Figs. 5 and 8). When perfusions were carried out with different nutritional media, the rate of release of radioactivity after 5 hours (when the rapid component had almost decayed to completion) was not substantial and did not vary with the conditions of starvation.

This slowly decaying component does not appear to represent a complete intracellular protein degradation to constituent amino acids since its release is independent of leucine exchange and a considerable proportion of the released radioactivity is trichloracetic acid-insoluble (Fig. 8). Furthermore, the acid-soluble and -insoluble radioactivity release was not altered by the addition of chloramphenicol. This and the persistence of release of the slowly decaying component at a rate of 0.2 to 0.6% per hour for as long as experiments are carried out (48 hours) argue against secretion and degradation of a limited class of protein.

We cannot be certain whether all of the proteins of the cell would eventually decay. The release of this slow component is so slow and fluctuates to such an extent in time that a graphical extrapolation is not possible. We tend to believe that infrequent death followed by autolysis may largely account for this component. Other possible contributions are ribosome breakdown, flagellar loss, or other processes such as the release of protein-lipopolysaccharide-phospholipid complex of membrane fragments (23) as well as the degradation of random cellular protein. Only the latter should be classified as intracellular protein turnover. Including then the total extent of the rapidly degrading protein, intracellular protein turnover must be a minor process indeed.

Although intracellular protein degradation is a quantitatively minor process in E. coli, the proteins involved might have high physiological or evolutionary significance. We do not believe that the production of this degrading protein component has evolved to serve a reserve function, in spite of the fact that this protein breaks down to constituent amino acids and under starvation conditions this can be an important source for the synthesis of inducible enzymes (5, 24). The rapidly degrading protein is eliminated from the cell independently of either the environment of the cells or of the need for the synthesis of new induced proteins. Thus, several hours after cells are starved or stopped from making protein, there would be no reserve left so as to supply the required amino acids and the cells would be almost incapable of responding to any new enzyme synthesis.

The amount of this rapidly degrading protein component is low in slowly growing cells (Table III). Mr. Thomas Alton, in our laboratory, has found that carbon-limited chemostat cells, grown under precisely the conditions used here, have little capability for β-galactosidase synthesis in the absence of a carbon source. Even this small capacity is lost on carbon source starvation with a half-life corresponding to that of the rapidly degrading protein reported here. Detailed analysis shows that the amount of the degrading protein which can give rise to the measured amount of β-galactosidase, under starvation conditions with a gratuitous inducer, based on the assumption that there is no preferential synthesis of β-galactosidase over other cellular components (24), is consistent with the amount reported here.

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APPENDIX

Calculations of Amounts and Velocities of Synthesis of Different Classes of Protein—The proteins of bacterial cells appear to be partitioned into three classes (Fig. 5) with respect to their degradation characteristics:

\[
\text{Total protein isotope} = \text{rapid} + \text{slow} + \text{stable}
\]

\[
P = P_A + P_B + P_C
\]

After the completion of the utilization phase of leucine-\(^{14}\)C, the rapid and slow protein components are observed to degrade according to first order kinetics:

\[
P_A = P_{A0} e^{-k_A t}
\]

\[
P_B = P_{B0} e^{-k_B t}
\]

and

\[
P_C = P_{C0}
\]

where \(P_A, P_B,\) and \(P_C\) are the total radioactivities in rapid, slow,

\(^4\)K. Nath and A. L. Koch, manuscript in preparation.
and stable components, respectively, at time t. The subscript zero denotes the total radioactivities at the time that the cells are placed on the membrane filter, and the k's are the corresponding first order decay constants.

Thus, the sum of all three protein components is:

\[ P = P_{A_0} e^{-k_A t} + P_{B_0} e^{-k_B t} + P_{C_0} \]  \hspace{1cm} (1)

This equation applies equally to growing or nongrowing cells since it deals with total and not specific radioactivities. Since amino acid exchange is very efficient (Figs. 3 and 4), the observed rate of release of total radioactivity will equal \(-dP/dt\). Thus:

\[ \frac{-dP}{dt} = k_A P_{A_0} e^{-k_A t} + k_B P_{B_0} e^{-k_B t} \]  \hspace{1cm} (2)

The rate of release of the slow component does not decrease significantly during the collection period. Therefore, \(k_B\) is so small that \(e^{-k_B t}\) is very nearly equal to 1.

Also,

\[ k_A \cdot P_{A_0} = \left( \frac{-dP_A}{dt} \right)_0 \]  \hspace{1cm} (3)

\[ k_B \cdot P_{B_0} = \left( \frac{-dP_B}{dt} \right)_0 \]  \hspace{1cm} (4)

Making these substitutions in Equation 2, we obtain:

\[ \frac{dP}{dt} = \left( \frac{-dP_A}{dt} \right)_0 e^{-k_A t} + \left( \frac{-dP_B}{dt} \right)_0 \]  \hspace{1cm} (5)

From experimental curves such as Fig. 5, it is possible to obtain \(T_A\), the half-life, and \(\left( \frac{-dP_A}{dt} \right)_0\), the intercept or initial rate of release of the rapidly decaying component and \(\left( \frac{-dP_B}{dt} \right)_0\) for the slowly decaying component. From the first two quantities, \(P_{A_0}\) can be obtained (Equation A of text) by replacing \(k_A\) with 0.693/\(T_A\).

The quantity, \(P_{A_0}\), is the amount of isotope present in the rapidly decaying component at the time the cells are put on the membrane. We have chosen to report it as a percentage of total isotope present at time zero. In some of the experiments, the cells were grown for an interval \(\Delta t\), the period between the end of the isotope incorporation phase and the placement of the cells in the apparatus. During this time, the total isotope in the rapid component will have decreased by a factor of \(e^{-k_A \Delta t}\), if the decay constant is the same within cells in absence of exogenous leucine as it is on the membrane filter in the presence of excess leucine.\(^{23}C\).

The calculation of the amount of protein, \(p_A\), from the total radioactivity present in the rapidly decaying component at the end of the incorporation period involves certain corrections. We remind the reader that the symbol \(P\) has been used for the radioactivity whereas \(p\) has been used for the amount. If a very long labeling period of many generations were used all protein species would have the same specific radioactivities and no correction would be necessary. If a very short labeling period were used, the specific radioactivities would depend on the rates of synthesis and the actual amounts present; the unstable component would always acquire a higher specific radioactivity than the stable component.

We have previously derived the correction factor for this case (Reference 25, Equation 31 or 33). It can be rewritten in the present notation as:

\[ F = \frac{1 - e^{-\alpha t}}{1 - e^{-\lambda t}} \]  \hspace{1cm} (6)

Where \(\lambda\) is the bacterial growth rate constant, \(\alpha\) is the apparent turnover rate constant, and \(t_i\) is the time for the uptake of isotope.

The factor has been written so that it is to be divided into the total radioactivity of the decaying component to yield the amount present in the cell. For very short incorporation periods \(F = \alpha/\lambda\). For very long incorporation periods \(F = 1\). For organisms growing in glucose medium, under our conditions, \(F\) is about 2.

The formula for \(F\) uses two quantities, \(\alpha\) and \(t_i\), on which direct information is not available. \(F\) is relatively insensitive to the uptake time and we have arbitrarily taken \(t_i\) to be the time that it takes for about 50% of the total leucine-\(^1\)\(^{14}\)C to become incorporated into protein. The apparent turnover rate constant, \(\alpha'\), has to do with specific radioactivity of the rapid component \(P_A\) during growth in the absence of exogenous leucine when recycling is occurring. The factor \(F\), therefore, not only corrects for the fact that the turnover over components initially achieve higher specific activities than the stable components, but it also corrects for the effects of recycling through the use of the apparent (\(\alpha'\)) instead of the true (\(\alpha\)) turnover rate constant. The rate constant \(\alpha\) applies in the absence of recycling. The effect of recycling is to slow down the change in specific activities, because breakdown yields precursor molecules that upon resynthesis yield macromolecules of nearly the original specific activity. Thus, \(\alpha'\) is a little smaller than \(\alpha\) and the two are related by (26):

\[ \alpha' = \alpha \frac{V_s}{V_s + V_A} \]  \hspace{1cm} (7)

where \(V_A\) is the velocity of synthesis of the rapid component and \(V_s\) is the velocity of synthesis of the more stable species \((V_s = V_B + V_C)\). Both velocities are expressed as milligrams of protein made per hour per 100 mg of the total cellular protein which also can be written as per cent per hour.

During growth in the presence of carrier leucine, recycling is prevented and the specific activity decreases as \(e^{-\alpha t}\), but at the same time the amount present per unit volume of culture increases as \(e^{\lambda t}\). Because the total radioactivity is the product of the amount and specific radioactivity,

\[ e^{\lambda t} e^{-\alpha t} = e^{-k_A t} \]  \hspace{1cm} (8)

and therefore, \(k_A\) and \(\alpha\) are related as:

\[ \alpha = \lambda + k_A \]  \hspace{1cm} (9)

Applying both corrections\(^{8}\) (dividing by \(F\) for turnover and multiplying by \(e^{\lambda t} e^{-\alpha t}\)), the amount of rapidly degrading protein as per cent of total amount of all cellular proteins is given by Equation B in text.

In addition to the total amounts, we are also interested in the velocity of synthesis of each class of protein. During steady state growth in presence of exogenous amino acid the net rate of synthesis of the rapid component, \(dp_A/dt\), per unit amount of
cellular protein is the difference between synthesis, $V_A$, and degradation, $\alpha p_A$,

$$\frac{dp_A}{dt} = V_A - \alpha p_A$$

In cultures under balanced growth, Component $p_A$ increases as any other cellular component. Therefore, we can write:

$$\frac{dp_A}{dt} = \lambda p_A$$

By equating the right-hand sides of Equations 10 and 11 we obtain:

$$V_A = (\lambda + \alpha)p_A$$

Substituting for $\alpha$ from Equation 9, we obtain:

$$V_A = (k_A + 2\lambda)p_A$$

The velocity of synthesis of the rapidly degrading protein under different growth rates was computed from this formula. The velocity of synthesis of the stable proteins can be calculated in the same way, except that there is no degradation. Thus:

$$\frac{dp_s}{dt} = V_s$$

and

$$\frac{dp_s}{dt} = \lambda p_s$$

Therefore,

$$V_s = \lambda (100 - p_s)$$

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