Intracellular Protein Breakdown in Growing Cells of *Escherichia coli*

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1. When *Escherichia coli* was grown exponentially in a defined medium at 35°, the rate of protein breakdown was initially rapid, but decreased to 0.6%/hr. after about 30 min. The latter rate was maintained for at least 3.5 hr. 2. The initial rapid rate may have been due to the presence of a small protein fraction (about 1%) that was degraded with a half-life of 13 min. 3. The rate of protein degradation was the same during balanced growth at low rates imposed in a bacterium. However, it increased during the period immediately after a decrease of the growth rate.

Although intracellular protein is broken down at about 5%/hr. in non-growing cells of *Escherichia coli*, previous reports have suggested that there is little or no breakdown in exponentially growing cells (see Mandelstam, 1958, 1963).

The present paper describes experiments that demonstrate that protein is broken down slowly during exponential growth. A continuous culture apparatus was used to show that the rate of degradation is not a function of the growth rate, but is accelerated during the transition period after a fall in the growth rate.

MATERIALS AND METHODS

Organisms and media. *E. coli* ML328c *lac⁺leu⁻* and a prototrophic revertant of this were grown in the synthetic medium described in the preceding paper (Willettts, 1967). Neither organism could use leucine or valine as a source of nitrogen.

Radioactivity. L-[¹⁴C]Valine and DL-[¹⁴C]valine were obtained from The Radiochemical Centre (Amersham, Bucks.). The measurement of radioactive amino acids released during protein degradation has been described in the preceding paper (Willettts, 1967).

Measurement of protein degradation. The method has been described in the preceding paper (Willettts, 1967).

Bacterium. This apparatus was that used by Rogers (1957), except that the Marriott bottle and capillaries were replaced by a variable-flow-rate pump.

It proved impossible to use the leucine auxotroph for these experiments, since the DL-leucine necessary for growth strongly inhibited incorporation of the small amount of L-[¹⁴C]valine added to label the cells, probably because both are transported into the cell by a single permease (Cohen & Rickenberg, 1956). In previous experiments (Willettts,

1967), a high concentration of DL-[¹⁴C]valine (30 µg./ml.) was provided during the whole growth period to label the cells, and any residual radioactive valine was removed by washing the cells. In these experiments, however, incorporation of the L-[¹⁴C]valine had to be both rapid, to avoid excessive dilution out of the radioactive cells before measurement of protein breakdown, and complete, to lower the initial radioactivity of the supernatant as much as possible since the cells could not be washed. Therefore a prototrophic revertant was used in all bacterium experiments. This revertant strain degraded protein at a lower rate during incubation in a medium lacking NH₄⁺ ions, but was qualitatively similar to the parental strain.

A synthetic medium based on that described above, but without L-leucine, and containing a growth-limiting concentration of ammonium salts (40 µg. each of the chloride and sulphate/ml.) was used. This allowed the cells to grow to a culture density of about 0.2 mg./ml.

After continuous culture of the cells (100 ml.) overnight, L-[¹⁴C]valine (0.01 µC/ml.; 0.2–0.5 µg./ml.) was added directly to the culture vessel. This amount of valine was incorporated in 2–6 min. Preliminary experiments showed that protein labelled under these conditions was degraded at about the same rate as protein labelled for much longer periods.

L-Valine and L-leucine (150 µg./ml. each) were added 15–30 min. after the [¹⁴C]valine had been added, both to the culture and to the reservoir of medium, being fed into the culture to prevent reincorporation of radioactive amino acids liberated by protein breakdown. L-Leucine was included since the prototroph may have been able to convert part of the labelled L-valine into labelled L-leucine (Roberts, Cowie, Abelson, Bolton & Britten, 1955). Simultaneously, if required, the rate of supply of fresh medium, and thus the growth rate, was changed.

When the flow rate was decreased, the concentration of organisms increased slowly and continuously during the next 4 hr., i.e. before a new equilibrium value had been reached. For example, a maximum increase of 50% in cell density was found after 4 hr. at a mean generation time of 4 hr. Apart from affecting the values for the mean genera-

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tion time slightly, the increase in cell mass does not affect the overall experimental results of the interpretation. However, in these experiments the cells should be regarded as being in a state of transition over the whole period during which samples were being taken.

Culture samples (2×3 ml.) were collected at intervals of 15–30 min. during the next 4 hr., and the radioactivities of the cells and medium in each sample determined for the measurement of protein breakdown.

If we write c for the total radioactivity per unit volume of culture, we have in the circumstances of the experiments, and assuming perfect mixing:

$$\frac{dc}{dt} = -Dc$$

where D is the dilution rate (the ratio of flow rate to culture volume). Hence

$$c \propto e^{-Dt}$$

and if we measure c at times t_1 and t_2 we have:

$$c_2/c_1 = e^{-D(t_2-t_1)}$$

and

$$D = \frac{1}{t_2 - t_1} \cdot \log \left(\frac{c_1}{c_2} \right) \quad (1)$$

In fact, a plot of $\log c$ against t showed that the residence-time distribution was sensibly exponential over the periods of interest.

Also, if b and s are the amounts of radioactivity per unit volume of culture associated with the cells and with the medium respectively:

$$b + s = c \quad (2)$$

at all times. The activity associated with cells diminishes for two reasons: (i) wash-out of cells at a rate D ; (ii) protein degradation at a rate k . Thus:

$$\frac{db}{dt} = -(k + D)b$$

$$b \propto e^{-(k+D)t}$$

and as before:

$$b_2/b_1 = e^{-(k+D)(t_2-t_1)};$$

$$k = \frac{1}{t_2 - t_1} \cdot \log \left(\frac{b_1}{b_2} \right) - D \quad (3)$$

Thus k can be determined from eqns. (1) and (3), or various combinations of eqns. (1), (2) and (3).

RESULTS

Protein breakdown during exponential growth. Cells, labelled as described by Willetts (1967), were washed and resuspended at a culture density of about 0.05 mg./ml. in an unlabelled growth medium containing L-valine (200 μ g./ml.). Protein degradation was followed for 4 hr. during exponential growth of cells (Fig. 1).

After an initial degradation of 1.3% in 30 min. the rate of protein breakdown decreased to 0.6%/hr., and continued at this rate for the next 210 min.

Fig. 1. Protein breakdown during exponential growth. Prelabelled cells were resuspended in growth medium containing L-valine (200 μ g./ml.), and growth (○) and protein degradation (□) were followed.

Similar results were obtained in 16 different experiments. This showed that protein degradation, although slow, does in fact occur during exponential growth.

Mandelstam (1958) suggested that the initial rapid rate of protein breakdown in exponentially growing cells was associated with a lag phase of growth. Such a lag phase was also observed in the present experiments; it may have been caused by the centrifugation and washing processes (Koch, 1959).

However, another possibility was that a small fraction of the proteins synthesized during exponential growth (about 1%, from Fig. 1) was comparatively rapidly degraded during continued growth. The following experiment indicated that this was the case.

Three cultures were grown in parallel, in media containing 5, 10 or 20 μ g. of L-leucine/ml. At cell densities of 0.1, 0.2 and 0.4 mg./ml. respectively, radioactive DL-valine (0.04 μ C/ml.; 1.0 μ g./ml.) was added to the cultures. At these cell densities, the L-leucine was almost exhausted, and the low concentration remaining did not inhibit the uptake and incorporation of the radioactive DL-valine. After 10 min. an excess of unlabelled DL-valine (300 μ g./ml.) and more L-leucine (80 μ g./ml.) were added, the first to prevent further labelling and the second to allow continued growth. The three cultures were allowed to grow to 0.5 mg./ml. and were then harvested. It was assumed that they were identical at this point, except that incorporation of the pulse of [14 C]valine had occurred 2 hr., or 1 hr., or just before harvesting. The cells were

Fig. 2. Demonstration of a protein fraction rapidly degraded during exponential growth. Three cultures were grown in parallel, and the cells labelled with a pulse of DL-valine ($0.04 \mu\text{C}/\text{ml.}$; $1.0 \mu\text{g.}/\text{ml.}$) 2 hr., 1 hr. or just before harvesting (see the text). The cells were washed and resuspended in fresh unlabelled growth medium containing L-valine ($200 \mu\text{g.}/\text{ml.}$), and the rate of release of labelled valine was measured: $\circ$, labelled 2 hr. previously; $\triangle$, labelled 1 hr. previously; $\square$, labelled just before harvesting.

washed and resuspended at a culture density of about $0.05 \text{ mg.}/\text{ml.}$ in fresh growth medium containing L-valine ($200 \mu\text{g.}/\text{ml.}$), and protein degradation was measured for 2 hr. during subsequent growth of these cultures.

The results (Fig. 2) show that the initial rapid rate of degradation was not found if the cells were grown in unlabelled medium for 1 or 2 hr. before measurement of protein degradation. This suggested that the initial rapid rate of protein degradation when the measurement was done immediately after labelling was due to the presence of a radioactive protein fraction that was rapidly degraded. In the cultures labelled for 1 or 2 hr. earlier, the labile fraction would have been completely degraded before measurement of degradation began. The different initial rates could not have been due to the growth lag, since this was the same in all experiments.

Effect of variation of the growth rate. Protein was degraded in the prototrophic revertant at $2.5\%/ \text{hr.}$ during starvation with respect to NH_4^+ ions, and at $0.6\%/ \text{hr.}$ during exponential growth in synthetic medium. Protein degradation was measured in cells growing exponentially at different rates in a bactogen, to see if the rate of breakdown was higher in cells growing at lower rates, and if it changed immediately after the growth rate was decreased.

The experimental method is described above: mean generation times of 1, 2 and 4 hr. were investigated; longer mean generation times with a corresponding slower efflux of culture prevented a sufficiently rapid collection of samples. The results are described in Table 1.

Table 1 *Protein breakdown in cells growing at different rates in a bactogen*

The prototrophic strain was grown overnight in the bactogen at the growth rate given in the first column. Labelled L-valine ($0.01 \mu\text{C}/\text{ml.}$; $0.2\text{--}0.5 \mu\text{g.}/\text{ml.}$) was added to the culture. After 15–30 min. L-valine ($200 \mu\text{g.}/\text{ml.}$) and L-leucine ($200 \mu\text{g.}/\text{ml.}$) were added both to the culture vessel and to the medium in the reservoir, and the growth rate was either left unchanged or changed to the value given in the second column. Samples of the effluent culture were collected every 15–30 min. for 4 hr. for the determination of protein breakdown. Values were calculated from the equation given in the Materials and Methods section.

Mean generation time (hr.)		Protein degradation (%/hr.)
During labelling	During measurement	
1	1	0.6
2	2	0.5
4	4	0.6
1	2	0.7
1	4	1.8
1	∞	2.5

Variation of the mean generation time from 1 to 4 hr. had no effect on the rate of protein degradation, as long as it was left unaltered throughout the experiment. However, when the rate of growth was decreased immediately before measurement, the rate of protein degradation increased to an extent dependent on the difference in growth rate.

In all of these experiments, degradation began immediately, and there was an initial rapid rate of degradation (about 4% during the first hr.). This was probably due to the labile protein fraction described above.

DISCUSSION

The validity of the method used for the determination of the rate of protein breakdown in growing cells has been discussed previously (Mandelstam, 1958). The results obtained are similar to those found by a method not involving equilibration between internal and external amino acids (Koch & Levy, 1955), suggesting that equilibration is not a limiting factor. It can be calculated that the concentration of unlabelled L-valine present in the medium during measurement of protein degradation was enough to prevent reincorporation of significant amounts of the liberated radioactive L-valine.

The results obtained confirm previous reports (see Mandelstam, 1958, 1963) that the rate of protein breakdown in exponentially growing cells is low, but establish that it has a value of $0.6\%/ \text{hr.}$ for at

least 3 hr. Previous work has suggested that the proteolytic system responsible for degradation in starved cells is already present in growing cells, but in an inactive state (see Mandelstam, 1963; Willetts, 1965). Possibly an imperfect mechanism of inactivation allows this low rate of degradation. It must be noted that these experiments do not give any information about the stability of the remaining 96% of the cell protein not degraded during the first 4 hr. of growth.

The initial rapid rate of protein degradation in growing cells has been described previously (Cowie, Bolton & Sands, 1950; Koch & Levy, 1955; Mandelstam, 1958). Mandelstam (1958) suggested that it was due to a proportion of non-growing cells in the population, because it was associated with the lag phase of growth. However, the results obtained in the present study show that the growth lag is not the relevant factor, and that the observed facts can be more easily explained by the presence of a small, rapidly degraded, protein fraction. Further support for this idea can be deduced from the experiments with the bactogen, in which the cells were neither centrifuged nor washed, but were grown continuously throughout both labelling and measurement of protein breakdown. Despite the absence of a growth lag, the initial rapid rate was still observed. In fact it was even greater here, possibly because the period between labelling and measurement of protein degradation was shorter, so that a larger proportion of the rapidly degraded fraction was radioactive at the beginning of the measurement. It was calculated that the half-life of the rapidly degraded protein fraction in both types of experiment was about 13 min., and that about 5% of the protein synthesized during growth must be of this type. In the bactogen experiments, the sole difference between the conditions of culture during labelling and measurement was the addition of excess of L-leucine and L-valine to the medium; it seems unlikely that this would have any effect, since the addition of an excess of each of 20 amino acids to a culture growing in a synthetic medium did not affect the rate of protein breakdown (Willetts, 1967). However, one cannot exclude the possibility that the rapidly liberated radioactive L-valine was not in peptide linkage, although in all experiments except the bactogen experiments every effort was made to eliminate free radioactive L-valine by washing the cells thoroughly in a medium containing an excess of unlabelled valine.

Pine (1967) found that, over short time-periods, pulse-labelled protein was degraded at the same rate under all conditions of growth or starvation. He therefore proposed that protein breakdown is not inhibited during cell growth, but rather that a smaller proportion of the cell proteins is susceptible to the proteolytic enzymes in growing cells. The

present results also suggest that the rate of protein degradation is about 5%/hr. in growing cells as well as in starved cells (Willetts, 1967), and that in growing cells the substrate used is continually synthesized as 5% of the cell protein, whereas the rest of the cell proteins are degraded at the much lower rate of 0.6%/hr. During starvation, however, at least 35% of the cell proteins can be degraded, and there is no preference for the fraction identified operationally as newly synthesized protein (Willetts, 1965; Pine, 1967).

The mechanism that controls the preferential degradation of a particular protein population during cell growth remains unknown. One possibility is that during growth there is some sort of compartmentalization of the proteolytic enzymes that is destroyed on starvation or cell disruption, so that the enzymes are found in all subcellular fractions (Chaloupka, 1961; Willetts, 1965). Although, during starvation, protein of all subcellular fractions is degraded (Pine, 1965; Willetts, 1965), it is not known if the fraction preferentially degraded during cell growth is associated with a particular subcellular component. The idea of substrate restriction during cell growth may not be basically at variance with an inhibition of protein degradation, possibly resulting from an inactivation of the proteolytic enzymes caused by their binding to some cell component, as proposed in the preceding paper (Willetts, 1967).

The bactogen experiments suggest that the rate of protein degradation is the same during balanced growth of cells at any growth rate. This result agrees with that of Pine (1967), who found that the rate of protein breakdown was the same in cells growing at different rates in different media. If, however, the rate of growth was decreased immediately before measurement of the rate of protein breakdown, by decreasing the rate of supply of medium, the rate of protein breakdown increased during the resulting transitional state. This result is similar to that obtained in a different experiment (Willetts, 1967) in which the rate of protein degradation did not change when a culture of cells growing in synthetic medium (with a mean generation time of 60 min.) was enriched with an excess of each of 20 amino acids (to give a mean generation time of 40 min.), but increased sharply when cells that had grown in the rich medium were transferred to minimal medium. There was no growth lag after either of these transfers.

It seems, then, that absence of growth is not a necessary condition for a rapid rate of protein breakdown and that the precipitating factor is a fall in the growth rate.

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