Protein Degradation in Escherichia coli

II. STRAIN DIFFERENCES IN THE DEGRADATION OF PROTEIN AND NUCLEIC ACID RESULTING FROM STARVATION*

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SUMMARY

Based on leucine exchange measurements of cells labeled with [14C]leucine, we had previously reported (J. Biol. Chem., 245, 2889 (1970)) that only a limited class of protein of Escherichia coli B is subject to a first order process of rapid degradation. The rate of degradation (half-life of 60 min or faster) and the total amount of protein undergoing degradation (2 to 7%) was the same during growth and during various kinds of starvation. We extend this finding and report here that the release of this "rapidly degrading protein" is unaltered not only during growth and starvation but also during growth in an enriched medium (step-up), in a poor medium (step-down), and during the diauxic phase of induced β-galactosidase synthesis.

The extent of this observed degradation (less than 10%) is lower than reported by other workers (20 to 35%). This discrepancy is not a consequence of experimental manipulation or cellular damage but reflects differences in bacterial strains. Although strains ML and K-12 under conditions of growth release radioactivity similar in amount and half-life to the rapid protein degradation process observed in strain R, during several conditions of starvation, degradation of an additional class of cellular protein can be measured by leucine exchange in these two strains but not in B. The starvation-induced protein degradation occurs at a rate of 2.5 to 6% per hour, and the amount of the cellular protein that degrades as a result of starvation amounts to 20 to 40% of the total bacterial protein. Simultaneous with this starvation-induced protein degradation is the excretion of nucleic acid degradation products into the medium amounting to 40 to 60% of the ordinarily stable nucleic acid of growing cells. Starvation-induced nucleic acid degradation occurs in all strains equally. With the findings of these strain differences, much of the conflict in the literature can be explained satisfactorily.

We conclude that a continuous process of protein degradation occurs in bacterial cells at all times, and an additional process of protein degradation concomitant with nucleic acid degradation is initiated following starvation of nutrients. Some of the properties of the degradation processes under starvation conditions are as follows. While almost all of the protein degradation products released in the presence of carrier leucine are acid soluble, a large portion of nucleic acid degradation products released from the cells are precipitable by cold trichloroacetic acid. Both protein and nucleic acid degradation occur under starvation of either glucose, nitrogen, or phosphate. Inhibition of protein synthesis by chloramphenicol at 100 μg per ml inhibits the starvation-induced protein degradation, but does not affect the degradation of the rapidly degrading protein.

Protein turnover in Escherichia coli involves degradation and subsequent resynthesis. The degradation phase can be measured by the exchange of degradation products with added exogenous carrier amino acids (1, 2). Although there have been many conflicting claims in the literature concerning protein turnover in bacteria during growth and starvation, it is becoming apparent that there are a number of different processes leading to protein breakdown and release. These processes vary in extent, in rate, in the degree of degradation, and in the substrate protein.

The fastest process reported to date is one involving the clevage of 5% of the peptide bonds formed de novo within 40 sec after formation (3). It has been suggested that this represents the removal from newly synthesized peptide chains of terminal amino acids which are coded by the message, but which are absent in functional proteins.

We have investigated another process that leads to the ultimate degradation of less than 10% of the total proteins of exponentially growing bacteria (1). This limited class of protein was previously designated as "rapidly degrading protein." The degradation results in a final radioactive product that efficiently exchanges with exogenous carrier amino acid. This class of proteins is degraded in a first order process with a half-life of about 1 hour during growth or when growth is inhibited by various nutritional starvation or by the addition of chloramphenicol. Although we do not yet know the chemical nature of the proteins subject to this process of degradation they may well be comprised of malformed, nonfunctional, or incomplete polypeptide chains.

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The subject of this paper is to document an additional intracellular degradation process which involves 20 to 40% of the protein of cells grown in glucose minimal medium, and is concomitant with extensive degradation of nucleic acids that are usually stable in growing cells. Both these processes are induced by a variety of starvation conditions.

There are yet slower processes of radioactive release from [14C]-leucine-labeled cells (1, 4). There is a slow and continuous release of radioactive compounds (0.2 to 0.6% per hour) which appear to be partly acid insoluble as well as partly reutilizable by growing bacteria. This persistent process is independent of added carrier amino acid and hence this process occurs without amino acid exchange. We feel, therefore, that it represents excretion of mostly incompletely degraded protein products and does not represent intracellular protein turnover to the level of amino acids. Additional slow release of radioactive protein will occur after cell death and subsequent lysis.

The experimental measurement of any of those processes requires correction for, or the elaboration of methods to eliminate, the others. In our previous work, we found less intracellular degradation than had been reported by many other workers (7-13). Moreover, we found that the process we were studying was not altered by various states of growth or starvation as had been so often described (7-13). In trying to resolve these discrepancies, we found that E. coli strain B affords an experimental system for the study of the second process alone, i.e. the breakdown of the rapidly degrading protein. In this strain, the third process or starvation-induced degradation does not lead to products which would exchange with exogenous amino acids. By studying the difference in the properties of strain B with respect to strains ML and K-12, the properties of the starvation-induced process could be elucidated.

**EXPERIMENTAL PROCEDURE**

Most of the materials and methods used here have been outlined previously (1).

**Materials**—Uniformly labeled L-[14C]leucine (specific activity 312 mCi per mmole) was purchased from Amersham-Searle, Des Plains, Illinois, [8-14C]guanine (37.5 mCi per mmole) was a product of Schwarz Bioresearch. Rifampicin was obtained from Mann.

**Cells and Growth Conditions**—Besides E. coli strain B and B u-try-, other strains used were ML 30 (i+z+y+a+) and ML 308 (i+z+y+a+) originally obtained from Dr. J. Monod; K-12 CP 78 (RC-stringent and leu-, arg-, thr-, his-, and thiamine-) obtained from Dr. J. Friesen; K-12 W0 (RC-relaxed, met-) obtained from Dr. E. Borek; and K-12 AB259 (HfrH, thiamine-) from Dr. A. Markowitz.

Cells were grown in a minimal salt phosphate buffer, Buffer M, in 0.2% glucose and supplemented with the required nutrients.

**RESULTS**

Further Studies on Effect of Starvation on Degradation of Rapidly Degrading Protein in E. coli Strain B u-try—Although the use of the perfusion apparatus previously described (1) leads to accurate and unambiguous determinations of protein turnover from direct measurements of the released radioactivity in the perfusate, it was desirable to find a less cumbersome method in order to perform many experiments simultaneously. For this purpose several different approaches were developed.

In one kind of experiment in which experimental trauma to the cells (14) was avoided, the amount of radioactivity external to the [14C]leucine-labeled cells was measured in the presence of an excess of nonradioactive leucine after starvation was self-initiated by cellular consumption of a nutrient, added in limited amount. In such experiments three components contributed to the radioactivity in the medium (Fig. 1). The first is the 5 to 6% radioactivity external to the cells remaining from the isotope incorporation phase prior to the addition of carrier leucine. The second is the release of nearly 6% of the radioactivity in the initial 4-hour period because of the turnover of the rapidly degrading protein. The third is a continuing slower release of radioactivity at a rate of nearly 0.5% per hour because of the slow processes. These values are consistent with the earlier values obtained from the perfusion apparatus (1). Furthermore, as seen in Fig. 1, neither the rapid release of radioactivity nor the subsequent continuing...
slow process was found to depend on the physiological conditions of the cell.

In a second kind of experiment, exponentially growing cells were labeled with high specific activity leucine for 10 to 15 min and then filtered on a membrane filter, washed, and resuspended in appropriate media containing carrier [14C]leucine. As seen in Table I, irrespective of the growth or starvation status of the cells, an average of 9 to 10% of the total radioactivity appeared during a 31/2 to 4-hour period. This value includes the rapidly degrading protein as well as a small contribution from the slower processes. The acid-insoluble radioactivity which generally represents about one-third of the total radioactive release caused by slower processes (1, 4) appeared at a rate of 0.1 to 0.2%/hr. Therefore, the amount of radioactivity associated with the rapidly degrading protein was obtained by subtracting a value of 1.2 to 2.4% for the slower processes from the value of 9 to 10%. To convert this amount of radioactivity to the amount of protein that has undergone degradation, these results must be divided by a factor of about 2 (see Reference 1) to correct for the fact that during the short incorporation phase the labile proteins have a higher specific activity than do the more stable ones.

These experiments confirm that at least 90% of the radioactive cellular protein in E. coli strain B does not become labile after starvation for nitrogen, carbon, magnesium, required uracil, required tryptophan, or after inhibition of protein synthesis with chloramphenicol.

The latter method of bacterial filtration and resuspension in the desired medium was generally used in subsequent experiments since results obtained by this method were similar to the nutrient medium was generally used in subsequent experiments. In order to study the effect of nutritional alterations under conditions where the bacteria can still grow, cells of E. coli B w-try* or B growing as indicated in Table II were isotopically labeled for 10 min, filtered, washed on the filter and then either resuspended

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**Table I**

**Amount of cellular protein subject to degradation in E. coli B w-try* under various nutritional conditions**

<table>
<thead>
<tr>
<th>Nutritional condition (glucose minimal medium + nL-leucine)</th>
<th>Acid-soluble radioactivity in the filtrate at 40 min</th>
<th>4 hrs</th>
<th>48 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>- Uracil</td>
<td>3.34</td>
<td>9.48</td>
<td>9.91</td>
</tr>
<tr>
<td>- Tryptophan</td>
<td>2.96</td>
<td>7.67</td>
<td>7.98</td>
</tr>
<tr>
<td>- Glucose</td>
<td>3.64</td>
<td>8.72</td>
<td>9.55</td>
</tr>
<tr>
<td>- Nitrogen</td>
<td>3.46</td>
<td>11.51</td>
<td>10.82</td>
</tr>
<tr>
<td>- Mg++</td>
<td>3.58</td>
<td>9.78</td>
<td>11.24</td>
</tr>
<tr>
<td>Complete</td>
<td>2.74</td>
<td>8.65</td>
<td>9.64</td>
</tr>
<tr>
<td>Complete</td>
<td>3.58</td>
<td>8.97</td>
<td>10.30</td>
</tr>
</tbody>
</table>

Average ± standard error: 3.33 ± 0.13; 9.17 ± 0.47; 9.92 ± 0.40

Acid-insoluble radioactivity: 0.20 ± 0.02; 0.43 ± 0.05; 0.46 ± 0.05

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**Table II**

**Effect of step-up and step-down on protein degradation of E. coli strain B**

<table>
<thead>
<tr>
<th>Medium for protein degradation measurement (glucose minimal medium + nL-leucine)</th>
<th>Amount of rapidly degrading protein ( P_{AS} ) ( b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli B w-try*</td>
<td>T1 = 900 min (Experiment 5)</td>
</tr>
<tr>
<td>Complete</td>
<td>5.67 ± 0.21</td>
</tr>
<tr>
<td>B. Minus glucose</td>
<td>4.34</td>
</tr>
<tr>
<td>C. Minus glucose, plus DL-alanine</td>
<td>4.78</td>
</tr>
<tr>
<td>D. Minus glucose, uracil and tryptophan</td>
<td>4.92 ± 0.82</td>
</tr>
<tr>
<td>E. Plus adenine, thymine, guanine, and vitamin mixtures</td>
<td>4.46</td>
</tr>
<tr>
<td>F. As in E plus 13 amino acids</td>
<td>5.75 ± 0.15</td>
</tr>
</tbody>
</table>

Previous growth and isotope incorporation medium: F E glucose-limited chemostat

\( P_{AS} = \frac{P - k_BT}{1 - e^{-k_BT}} \)

where \( P \) is the percentage of total radioactivity released at time \( t \), \( k_BT \) is the degradation rate constant \((0.693/T1)\) of the rapidly degrading protein, and \( k_B \) is the rate of release of the slow component expressed as percentage of the original protein label per hour.

For the cells growing in batch culture (Experiments 1 to 4), the half-life of degradation of the rapidly degrading protein was taken as 60 min and \( k_B \) as 0.46%/hr (1) and for the slowly growing chemostat culture (Experiment 5), they were taken as 30 min and 0.2%/hr, respectively (4).

The formula is not sensitive to the values assumed for \( k_B \) and \( k_T \).

The percentage of total cellular protein constituting the rapidly degrading protein, \( p_A \), was obtained by dividing \( P_{AS} \) by a factor of about 2, calculated as described previously (1), to correct for the difference in specific activity between labile and stable proteins attained during a short period of labeling.

Each value of \( p_A \) reported in Table II is the mean average of

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four values calculated from the four measured values of \( R \) at different times. They were averaged since they showed no trend with time. This fact that there was no trend with time also implies that the half-lives and rates assumed for the calculation were approximately correct.

The steady state amount of rapidly degrading protein, \( p_a \), depends on the bacterial doubling time during the period of labeling. In bacteria with a doubling time of 36, 62, and 900 min, \( p_a \) ranges between 5 to 8, 4 to 6, and 0.5 to 0.6%, respectively. No consistent trend could be observed from conditions of either step-up, step-down, starvation, or unaltered growth in the postlabeling medium. There is, however, one exception: \( p_a \) is consistently higher for the step-down to alanine as carbon source than in any other physiological manipulation of Experiments 1 to 3.

Since an accelerated rate of protein degradation has been reported by Willetts (15) during diauxic lag, protein degradation was measured during induced synthesis of \( \beta \)-galactosidase in E. coli B u\(^{-}\)try\(^{-}\). The release of acid-soluble radioactivity from induced, uninduced or diauxic phase cells was found not to vary significantly. In all cultures, by 5 hours, a total of 6 to 8% of acid soluble radioactivity was released.

Mandelstam’s Method—Since the results reported previously (1) and above were in marked discrepancy with those reported by several other laboratories, the widely used Mandelstam procedure for measuring protein turnover (13) was repeated, and the results compared with our previous values. The Mandelstam procedure involves measurement of the release of radioactive amino acid, previously incorporated by growing cells for several generations, after several centrifugations in the cold so as to wash away unincorporated extracellular radioactive amino acids. Since previous work from this laboratory had indicated that cold centrifugation could result in cellular damage (14), the effect of such treatment on the extent of protein radioactivity release was next examined.

Cells were incubated with \([\text{14C}]\)leucine for 10 min and a portion was transferred into the perfusion apparatus described previously (1). Another portion was centrifuged for 10 min at 15,000 \( \times \) g at 0\(^{\circ}\), either once or three times; after each centrifugation the suspended cells were equilibrated at 37\(^{\circ}\) for 2 min. After the final resuspension, the cells were transferred to another perfusion apparatus. The rate of radioactivity release in the presence of \([\text{14C}]\)leucine of the centrifuged cells was not significantly different from the untreated control cells for at least 1\(\frac{1}{2}\) to 2 hours. (The radioactive profiles were almost identical with the results shown in Fig. 5 of Reference 1.)

Fig. 2 shows the results of an experiment comparing Mandelstam’s method with our own filtration procedure in E. coli B u\(^{-}\)try\(^{-}\) after cold centrifugation treatments. Bacteria growing with a doubling time of 68 min in glucose minimal medium were grown for at least 12 generations in the presence of 100 \( \mu \)M \([\text{14C}]\)leucine (5.7 \( \times \) 10\(^{-3}\) Ci per ml). At a bacterial concentration of 8.7 \( \times \) 10\(^{-8}\) g dry weight, per ml, three 10-ml portions were centrifuged at 15,000 rpm (SS-2 rotor) for 10 min at 0\(^{\circ}\). The pelleted bacteria were resuspended in 10 ml of minimal medium devoid of glucose, supplemented with 360 \( \mu \)g per ml of \([\text{14C}]\)leucine and equilibrated at 37\(^{\circ}\) for 3 min. The operation of centrifugation at 0\(^{\circ}\), equilibration at 37\(^{\circ}\), and recentrifugation was repeated prior to the commencement of degradation measurements in the presence or absence of glucose. As control, two portions from the original radioactive bacteria were filtered at room temperature (24\(^{\circ}\)) and washed with 3 volumes of minimal medium (devoid of glucose) in the presence of leucine, and finally resuspended in the same medium with or without supplement of glucose. The measurement of released radioactivity was carried out as outlined in the legend to Fig. 1. The cells that were not centrifuged are represented by: \( \bigcirc \), for growing and \( \triangle \), for uracil- and tryptophan-starved cells; centrifuged cells are represented by: \( \times \), for growing and \( \bullet \), \( \square \), for two batches of uracil- and tryptophan-starved cells.

Thus, in addition to the two types of protein degradation processes in strain B already described, there is a third process elicited as a result of glucose starvation. It will be shown below that other types of starvation also elicit this response in various mutants of E. coli strains K-12 and ML.

Effect of Glucose Starvation on Protein Degradation in Various Strains of E. coli—Further use of strain CP 78 was abandoned in favor of other K-12 strains that are auxotrophs for only one nutrient, such as W6 and AB 259. Growing cells were inoculated for 10 min with \([\text{14C}]\)leucine and protein degradation was measured in the presence of \([\text{14C}]\)leucine. As seen in Table III, glucose starvation resulted in an additional release of 9 to 14% of the original protein radioactivity in 5 hours in strains ML and K-12, but not in strain B. In all strains, under growing conditions, the protein degradation was the same.

To minimize artifacts of experimental manipulation, starvation was next achieved by growing the cells in medium containing a limiting amount of glucose, 200 \( \mu \)g per ml. \([\text{14C}]\)Leucine was added at least 45 to 60 min prior to complete utilization of glucose. In such experiments bacteria were subjected to only one pipetting operation, while centrifugation, filtration, or temperature fluctuation is avoided.

It can be seen in Fig. 4 that the released radioactivity is significantly greater under conditions of glucose starvation than under
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Fig. 3. Measurement of protein degradation in E. coli strain K-12 CP 78 after cold centrifugation treatments. CP 78 was grown for at least 12 generations (doubling time of 58 min) in glucose minimal medium supplemented with arginine, histidine, and threonine (50 µg per ml each), thiamine (40 µg per ml), and 100 µM [14C]leucine (7 × 10^-8 Ci per ml), then three portions were centrifuged at 0°C as described in the legend to Fig. 2. They were finally suspended in complete medium, X; in medium lacking only glucose, ■; and in medium lacking required arginine, histidine, threonine, and thiamine, ◇. Two additional portions from the original radioactive bacterial culture were filtered, washed with an equal volume of Buffer M containing [14C]leucine, and subsequently resuspended in the same medium but containing glucose and required nutrients in one case, ◇, and only glucose and leucine in the other, △.

TABLE III
Release of acid-soluble radioactivity from various strains of E. coli after incorporation of [14C]leucine for 10 min

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Doubling time in glucose minimal medium</th>
<th>Released radioactivitya after</th>
<th>Filtration</th>
<th>Centri-</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Growth</td>
<td>Starvation</td>
<td>Excess release</td>
</tr>
<tr>
<td>ML 30</td>
<td>60</td>
<td>%c</td>
<td>7.59%</td>
<td>21.71%</td>
<td>13.62%</td>
</tr>
<tr>
<td>ML 308</td>
<td>60</td>
<td>%c</td>
<td>8.36%</td>
<td>20.35%</td>
<td>11.99%</td>
</tr>
<tr>
<td>K-12 W6 (met-)</td>
<td>70</td>
<td>%c</td>
<td>10.14%</td>
<td>19.40%</td>
<td>9.26%</td>
</tr>
<tr>
<td>K-12 AB 259</td>
<td>98</td>
<td>%c</td>
<td>20.08%</td>
<td>29.05%</td>
<td>8.97%</td>
</tr>
<tr>
<td>B</td>
<td>48</td>
<td>%c</td>
<td>7.72%</td>
<td>9.13%</td>
<td>1.41%</td>
</tr>
<tr>
<td>B u-try-</td>
<td>57</td>
<td>%c</td>
<td>9.00%</td>
<td>13.13%</td>
<td>4.13%</td>
</tr>
</tbody>
</table>

a In 5 hours.
b Excess released radioactivity caused by glucose starvation.
c Percentage of total cellular radioactivity present at start of collection.
d Released radioactivity in 4 hours.

conditions of exponential growth in strains ML 30 (and 308), K-12 AB 259, and W6, but not in B (and B u-try-). These results confirm those shown in Table III, indicating that the effect of glucose starvation described above for strains ML and K-12 is not an effect of experimental manipulation.

The enhanced degradation during energy starvation in strains ML and K-12 represents a specific starvation-induced intracellular protein turnover and not cytoplasmic leakage or cell lysis, since there was no increase in the amount of acid-insoluble or acid-soluble radioactivity released in the absence of exogenous carrier leucine. It can be concluded that the starvation results in the activation of an intracellular process leading to free amino acids or to entities transported in and out of the cell by a carrier common for leucine transport.

Fig. 4. Measurement of protein degradation in E. coli strains B, ML, and K-12 during starvation after glucose exhaustion. To 10 ml of exponentially growing bacteria (4.4 to 5.3 × 10^8 g per ml) in medium containing 0.02% glucose, 0.1 ml of L-[14C]leucine (1 Ci) was added. After 22 to 41 min (bacterial doubling time ranged from 48 to 62 min) at a bacterial concentration of 6.8 to 9.8 × 10^8 g per ml each culture was divided into three equal portions. One portion was supplemented with glucose and carrier leucine, O——O; another was supplemented with leucine alone but not glucose ■——■; whereas the third portion was supplemented neither with glucose nor with leucine, △——△. The first portion gave a measure of protein degradation during 5 hours of bacterial growth and subsequent stationary phase, the second portion represented protein degradation during glucose starvation, and the third portion represented excretion processes during glucose starvation that occurred in the absence of amino acid exchange. In the last two portions, there was no increase in turbidity after 1 hour of commencement of measurements. Results with ML 308 and B u-try- (not shown above) were similar to those of ML 30 and B, respectively.

Release of Radioactivity from Stable Nucleic Acid In order to look for the release of other cytoplasmic macromolecules or their degradation products under similar starvation conditions, exponentially growing cells were labeled for 15 min with 6 µM [8,14C]guanine (0.225 µCi per ml), centrifuged, and washed once in the cold, and then grown further for 60 min at 37°C in fresh medium containing 0.4 µM [14C]guanine. This culture was again centrifuged, washed, and resuspended for measurement of radioactivity release at various intervals after filtration on a membrane filter.
TABLE IV
Release of radioactivity from breakdown of "stable nucleic acid"

Exponentially growing E. coli cells were first grown in the presence of \[^{14}C\]guanine for 15 min and then in the presence of \[^{14}C\]guanine for 1 hour prior to the measurements of release of extracellular radioactivity.

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Doubling time in glucose minimal medium (min)</th>
<th>Radioactivity released (^b) (in 3 hours)</th>
<th>Excess release caused by starvation (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML 30</td>
<td>48</td>
<td>2.79</td>
<td>11.88</td>
</tr>
<tr>
<td>ML 308</td>
<td>48</td>
<td>4.78</td>
<td>10.02</td>
</tr>
<tr>
<td>W6 met-</td>
<td>43</td>
<td>3.73</td>
<td>12.57</td>
</tr>
<tr>
<td>AB 259 thiamine</td>
<td>63</td>
<td>3.17</td>
<td>10.99</td>
</tr>
<tr>
<td>B</td>
<td>58</td>
<td>1.99</td>
<td>15.41</td>
</tr>
<tr>
<td>B ur·try</td>
<td>66</td>
<td>1.69</td>
<td>14.31</td>
</tr>
</tbody>
</table>

\(^a\) In the Millipore filtrate.

\(^b\) Percentage of total cellular radioactivity present at start of collection.

at an accelerated rate of 2 to 3% per hour during conditions of glucose starvation.

Addition of sonicated ML 30S filtrate at a final concentration of 0.02 mg, dry weight, per ml and 0.14 \(\muCi\) \[^{14}C\]guanine to prevent reincorporation of released extracellular radioactivity, did not alter the levels released during growth or starvation.

**Effects of Glucose Starvation on E. coli Strain ML 30 in Perfusion Apparatus**—For better resolution, further measurements of protein and nucleic acid degradations were performed in the perfusion apparatus. Cultures of ML 30 growing with a doubling time of 45 min were labeled with \[^{14}C\]leucine for 10 min and then two equal portions were transferred onto the membrane filters, one perfused with and the other perfused without \[^{14}C\]leucine. In a parallel experiment, cells were labeled with \[^{14}C\]guanine for 15 min, filtered, washed, and resuspended for further growth in \[^{14}C\]guanine. After 1 hour, the culture labeled with guanine was transferred into a third perfusion apparatus.

As seen in Fig. 5, in the presence of glucose, cells labeled with leucine released radioactivity in a first order process with a degradation half-life of nearly 45 min similar to the rapidly degrading protein component described earlier. Cells labeled with guanine released radioactivity at a rate of 0.95 to 1.55% per hour. This is the highest rate of release of guanine-labeled radioactivity that has been observed in growing bacteria; in most experiments it ranged between 0.5 to 1.0% per hour.

At the end of 60 min, the perfusion medium was changed to a medium lacking glucose. Maximum stimulation of the degradation process from either the leucine exchange-dependent process or the nucleic acid component was detected only after 20 min of glucose starvation. (The wash-out time of the perfusion apparatus was about 2 min.) In both cases, the rate of release was 5 to 6% per hour. Again these are somewhat higher values than have been observed in other perfusion experiments.

The leucine exchange-independent fraction of radioactivity appeared at a rate of 0.35 to 0.65% per hour in the presence of glucose, and remained unaltered after starvation. About 20% of this radioactivity was reincorporated into growing bacteria.

With guanine as the label, a significant proportion of the total radioactivity released could be precipitated by trichloroacetic acid in the cold throughout the experiment (see below).

**Effect of Readdition of Glucose to E. coli Strain ML 30**—In reciprocal experiments to those presented in the previous section, when the cells were first perfused for 60 min with media lacking glucose and then glucose was added back, radioactivity appeared in the perfusate as shown in Fig. 6. In the absence of glucose, radioactivity from protein degradation is released at the high linear rate of 5 to 6% per hour when measured by leucine exchange. In the absence of exogenous leucine, radioactivity is released at a rate of 1 to 2% per hour. This value is higher than that obtained for the slower degradation process during growth and is perhaps caused by partial excretion of acid-soluble radioactivity consequent to excessive protein degradation resulting from starvation. During the same period of glucose starvation, the radioactivity originating from degradation of nucleic acid is released at an average rate of 1.5 to 3% per hour.

Upon addition of glucose, the rate of radioactivity release in the presence of leucine decreases immediately and drops exponentially with a half-life of about 30 min. Similarly within 60
Fig. 6. Appearance of radioactivity in the absence and subsequent presence of glucose from cultures of *E. coli* strain ML 30 labeled with leucine and guanine. Labeling with [14C]guanine was carried out as described in the legend to Fig. 5. Two additional portions of growing bacteria were labeled with [14C]leucine for 10 min and then transferred into two apparatuses, one perfused with and one without carrier leucine. All three apparatuses were first perfused for 60 min with medium lacking glucose and then with medium containing glucose.

min of addition of glucose, the rate of radioactivity release from cells labeled with guanine drops to the low value of 0.5 to 0.8% per hour observed in growing cells. In the absence of carrier leucine, products from protein radioactivity are released at the rate of 0.2 to 0.5% per hour.

The experiments in Figs. 5 and 6 indicate that the mechanism or mechanisms for degradation of protein and nucleic acid, induced by starvation, can be initiated after a short lag following the onset of glucose starvation, and can be inhibited as well with a similar lag upon removal of the starvation condition. Based on the observations in the presence and in the absence of carrier leucine it can further be suggested that most of the amino acids produced as a result of protein degradation during starvation can be reutilized inside the cells, indicating an efficient process of protein turnover under such a starvation condition.

Effect of Addition or Removal of Glucose during Perfusion of *E. coli* Strain B—When experiments similar to Figs. 5 and 6 were repeated with strain B, the appearance of radioactivity from cells labeled with guanine followed a pattern similar to that described for strain ML 30. The radioactivity in the perfusion medium appeared at 0.4 to 0.8% per hour in the presence and at 1.5 to 2.7% per hour in the absence of glucose. However, the profile of leucine release from protein degradation was different. The rate of exchange of carrier leucine with intracellular [14C]leucine did not increase upon the onset of starvation. In both these experiments the slower degradation process as evidenced by the rate of release in the absence of carrier leucine was 0.2 to 0.7% per hour.

Kinetics of Starvation-induced Degradation in *E. coli* Strain ML 30—In order to assess the kinetics of the cellular release of radioactivity resulting from starvation-induced degradation processes, experiments similar to those just described were performed but with two modifications. Perfusion in the presence of glucose was carried out for 2 hours, then all the apparatuses were flushed twice with 8 to 12 ml of medium lacking glucose and further perfused for 7 hours in the same medium.

As seen in Fig. 7, the release of starvation-induced protein degradation products, instead of being linear as suggested by the shorter duration measurements, follows the kinetics of a first order process with a half-life (0.693/t<sub>1/2</sub>) of nearly 43 hours and an intercept (−dP<sub>s</sub>/dt<sub>0</sub>) of 6.6% per hour when extrapolated to the time of onset of starvation. If it is assumed that the protein degradation induced by starvation does not take place during bacterial growth, and that degradation of the rapidly degrading protein in the presence of glucose is virtually complete prior to the commencement of starvation, then the total amount of bacterial protein actually subject to starvation-induced degradation is (6.6% per hour) × (43 hours)/(0.693) or nearly 40% of the total bacterial protein. If correction is made for the contribution of the slower processes and extrapolation is restricted to the time of commencement of starvation-induced degradation rather than to the time of initiation of starvation proper, then this value of 40% changes to about 30%.

In the presence of glucose, the degradation half-life of rapidly degrading protein in strain ML 30 is nearly 45 min (See Figs. 5 and 7), similar to that obtained with strain B.

Effect of Chloramphenicol and Rifampicin on Starvation-induced Protein and Nucleic Acid Degradation—Since the release of radioactivity from cells labeled with guanine represents the excretion of degraded nucleic acid from living and actively metabolizing
cells, an estimate of the true magnitude of the intracellular nucleic acid degradation can be obtained only when intracellular resynthesis from the degradation products can be prevented. It is not known whether the exchange of the intracellular nucleic acid precursors with those added extracellularly is fast or slow as compared with intracellular resynthesis. For this reason, excretion of nucleic acid degradation products was measured in the presence of 250 μg per ml of rifampicin and chloramphenicol were used at final concentrations of 250 μg per ml and 100 μg per ml, respectively. △-△, complete medium containing rifampicin; ○-○, lacking glucose; △-△, lacking glucose, containing rifampicin; ■-■, complete containing chloramphenicol; ×-×, lacking glucose, containing rifampicin and chloramphenicol; ●-●, complete.

For measurement of the starvation-induced degradation processes in the presence of these inhibitors, cellular protein and nucleic acid were labeled for 10 min in parallel cultures. After 1 hour of further growth, the cells were centrifuged, washed, and divided into seven portions, specified in Figs. 8 and 9. The patterns of release of radioactive activity from cells labeled with guanine, shown in Fig. 8a, can be divided into several categories. The first category includes those cases where the starvation-induced protein degradation occurred at a continuous rate of nearly 3% per hour. This was observed either with or without rifampicin in the absence of glucose, or with rifampicin in the presence of glucose (A to C). In the second category are those cases where there is no additional release of radioactivity as the result of starvation (D to F). These occur in the presence of chloramphenicol where the release of the rapidly degrading protein is unmasked. Also in this category is the release of radioactivity under conditions of bacterial growth (G). When the same experiment was performed with E. coli B u-try- only one pattern of radioactive release was obtained under all conditions (Fig. 8b), involving about 4 to 9.5% of the total radioactivity (2 to 5% of the total protein). Neither chloramphenicol nor rifampicin had any significant effect on protein degradation in this strain (see also Fig. 1).

Thus, inhibition of protein synthesis by chloramphenicol prevents the appearance of starvation-induced protein degradation, whereas inhibition of nucleic acid synthesis has no inhibitory effect.

The patterns of release of radioactivity from stable nucleic acid during 4 hours of measurements can be grouped in three classes (Fig. 9a). In the first class, radioactive release of 40 to 56% of total nucleic acid radioactivity is observed upon inhibition of nucleic acid synthesis (A to C). In the second class, radioactive release of 16 to 20% is observed during growth inhibition by means other than nucleic acid synthesis inhibition (D to F). In the third class, 2.4% of total cellular nucleic acid radioactivity is observed under conditions of normal growth (G).

Similar results are obtained with E. coli strain B u-try- (Fig. 9b). The total radioactivity released during 4 hours was: 32 to 60% in cells treated with rifampicin, 12 to 16% in presence of a growth inhibitor which does not primarily affect nucleic acid synthesis, and 3.6% in growing cells.

Thus, under conditions of starvation which do not specifically prevent nucleic acid synthesis, it is probable that only one-half to one-third of the nucleic acid degradation products are excreted from the cell. The remainder is probably reincorporated into the nucleic acid that may as well undergo turnover.

The high value of radioactive release shown in Fig. 9 by cells treated with rifampicin in the presence of glucose is reproducible but cannot be explained at this time. The large release of 30 to 60% nucleic acid radioactivity is not caused by bacterial lysis brought about by rifampicin, since only 4 to 12% of the protein radioactivity (see Fig. 8) is released in filterable form. Furthermore, in the absence of the drug but under prolonged glucose star-
The points not joined by any line indicate periods of increment in bacterial turbidity. The joined points indicate periods of no appreciable change of turbidity. The thick dashed lines indicate the period of exponential growth.

**Effects of Other Kinds of Starvation on Protein and Nucleic Acid Degradation**—Starvation-induced protein degradation occurred in strain K-12 CP 78 only under the condition of glucose starvation and not in the absence of required amino acids and thiamine (Fig. 3). But as this degradation process also took place in the presence of rifampicin (Fig. 8) the protein degradation induced by starvation is not a consequence of glucose starvation alone but may occur under a variety of conditions of bacterial growth inhibition.

Fig. 10 shows the results of protein and nucleic acid degradation during starvation of either glucose, nitrogen, phosphate, magnesium, or sulfate. Nitrogen-, magnesium-, and sulfate-deficient media were prepared by appropriate substitutions in Buffer M, whereas Kennell’s medium (16) was used for phosphate deprivation.

Although the starvation-induced degradation processes in Fig. 10 appear to the same extent under conditions of glucose, nitrogen, and phosphate starvation, in the absence of magnesium or sulfate, no starvation-induced degradation is observed. There was significant increase in bacterial turbidity in the two latter cases, and the cultures may not have been fully depleted of their required nutrients.

**Characterization of Released Guanine-labeled Radioactivity**—The addition of cold trichloroacetic acid to the excreted radioactivity of cells labeled with guanine resulted in a substantial recovery of the released radioactivity in an acid-insoluble form. The proportion of the acid-insoluble radioactivity was larger in the presence of glucose. On an average, 10 to 25% of the total radioactivity released by growing (and later stationary state) cells was acid insoluble as compared with 5 to 15% for starving cells.

When a fresh inoculum of the same strain was grown in a portion of the collected radiouctive medium, an average of 40% of the excreted radioactivity from growing cells and 60% from starving cells could be reincorporated after extensive growth. Thus, although a large portion of the excreted products from nucleic acid degradation is acid insoluble, most of the remaining portion exists in a readily utilizable form, presumably free bases or nucleosides. Moreover, the action of the intracellular nucleases is more complete under conditions of starvation as compared with conditions of growth (and stationary phase).

**Uptake of [14C]Leucine by Cells Grown in Presence of Leucine**—The finding of a small but limited class of protein that undergoes degradation (rapidly degrading protein) even in growing cells of *E. coli* is contrary to most of the literature. Since in the commonly used method bacteria are grown for several generations in the presence of glucose. On an average, 10 to 25% of the total radioactivity released by growing (and later stationary state) cells was acid insoluble as compared with 5 to 15% for starving cells.

When a fresh inoculum of the same strain was grown in a portion of the collected radiouctive medium, an average of 40% of the excreted radioactivity from growing cells and 60% from starving cells could be reincorporated after extensive growth. Thus, although a large portion of the excreted products from nucleic acid degradation is acid insoluble, most of the remaining portion exists in a readily utilizable form, presumably free bases or nucleosides. Moreover, the action of the intracellular nucleases is more complete under conditions of starvation as compared with conditions of growth (and stationary phase).

**DISCUSSION**

In keeping with our earlier observations (1) it is seen that in *E. coli* strain B under various physiological conditions there is a

**Table V**

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>mL[14C] Leucine in growth medium</th>
<th>Uptake of L-[14C] leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>39 sec</td>
</tr>
<tr>
<td>I. B u' try'</td>
<td></td>
<td>9,796</td>
</tr>
<tr>
<td>II. K-12 W6</td>
<td></td>
<td>15,035</td>
</tr>
<tr>
<td>III. ML 30</td>
<td></td>
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</tr>
</tbody>
</table>

**Uptake of radioactive leucine by bacteria grown in presence of [14C]leucine**

Bacterial cells grown in absence and presence of 630 µg per ml of L leucine were centrifuged and washed twice with medium devoid of leucine. Uptake measurements were performed in the presence of 1 µM L-[14C]leucine (8000 cpm). Bacterial concentration in experiments I, II, and III were 96, 140, and 280 µg, dry weight, respectively. Addition of leucine prior to centrifugation to cells grown in its absence had no effect upon [14C]leucine in incorporation.
limited fraction of bacterial protein that is rapidly degraded. This amounts to 2 to 8% of the total cellular protein (Figs. 1 and 2 and Tables I and II) in close agreement with our previous value of 2 to 7% (1). In the following discussion a value of 2 to 8% will be assumed for this rapidly degrading protein. The degradation of this protein is unaffected by nutritional deficiencies or by inhibition of protein or nucleic acid synthesis (Fig. 8). Furthermore, this rapidly degrading protein occurs almost to the same extent in all strains of *E. coli*, but in most strains it is obscured except under conditions of growth (Table III), or after the addition of chloramphenicol (Fig. 8), when other intracellular degradation processes are absent.

Although unrecognized as such, evidence in the literature for the existence of a limited labile class of protein similar to rapidly degrading protein that represents 2 to 8% of the total cellular protein in various strains of *E. coli* is overwhelming (3, 13, 17–21). Thus, after a 10-min incorporation of [14C]leucine, 7% of the total radioactivity was released by growing cells of strain 113-3, with nearly half of this radioactivity appearing by 60 min (17). In other words, a class of protein was degraded with a half-life of 1 hour, which constituted nearly 3 to 4% of the total strain 113-3 protein synthesized. A low value of 2% was reported in growing strain B/15 by the use of an internal trap. In this experiment, the increase in radioactivity in purines as a result of incorporation of [14C]glycine originating from protein breakdown was measured (18). The highest reported value of 8 ± 2% in strain B, D-84 (requiring arginine) was obtained by measuring the loss of acid-insoluble arginine radioactivity during a period of 30 hours of exponential and later stationary phase of growth (19).

Further support for our kinetics of degradation of the rapidly degrading protein comes from Pine's report on protein turnover in strain B (3). Upon calculating his data, we find that in addition to the reported extremely fast component and the slow component, there is a component with a half-life of 55 min representing 1.6% of the protein in glucose-growing cells, and with a half-life of 95 min representing 4.7% of the protein in acetate-growing cells.

Failure to recognize this limited class of protein degradation during growth of all commonly studied strains of *E. coli* most likely stems from the fact that it is a minor process compared with the starvation-induced protein degradation. Also, in the commonly used method for protein degradation (13) cells are grown for several generations in the presence of a low specific radioactive amino acid prior to the addition of carrier amino acid for degradation measurements. Repression of the amino acid transport system may result in an inhibition of amino acid exchange as seen with strains ML and K-12 when grown in the presence of leucine (Table V), and would cause an underestimate of the actual extent of the degradation of the rapidly degrading protein. Thus, when strains K-12 and ML were grown in radioactive leucine or valine (amino acids of the same family) and then degradation was measured in the presence of carrier amino acid it was observed that about 3 to 4% of the cellular protein radioactivity was released within 2 to 4 hours and no more thereafter (13, 21). This value should correspond to a value of 8% if correction for inhibition of amino acid exchange is applied. We were fortunate in working with strain B since the leucine exchange efficiency in this organism was not affected by previous growth in leucine. In addition, this strain partially or completely lacks the capability of any additional protein degradation under various conditions of physiological starvation and thus, enabled us to clearly establish the amount of the rapidly degrading protein component. This strain also acted as a control in the subsequent findings of a different class of degrading proteins which is induced upon starvation in strains ML and K-12.

The appearance of radioactivity from cellular protein at a rate of 2 to 6% per hour (Table III, Figs. 4 and 5) as a consequence of starvation is a well accepted fact in the literature (2, 7). Thus, under conditions of nitrogen, glucose, or other starvation, degradation rates of 6.5% per hour in an undefined strain (11), 4 to 5% per hour in strains ML and K-12 (10, 13), 3 to 6% per hour in strain K-12 W6 (22), 3.2% per hour in strain PS (23), 3% per hour in strain 113-3 (17), and 2 to 5% per hour in strain K-12 (9) have been reported. Furthermore, in strain ML, a protein degradation rate of 6% per hour during the diauxic lag of β-galactosidase synthesis (15) and a rate of 3.2% per hour in strain PS during restricted growth on glucose (23) have been reported.

Our measured value for protein degradation under starvation conditions varied within the range of 2 to 6.5% per hour, despite careful attempts to reproduce experimental conditions. These values include all the reported values in the literature. Because of this wide range of measured degradation rates, interpretations of experiments based on values of protein degradation should be made with caution. The finding that spermidine treatment of *E. coli* strain TAU produced a 25% increase in protein degradation has been interpreted as a specific stimulation of protein degradation by spermidine (24); yet for a protein degradation rate of 4% per hour, for example, a 25% increase would merely be a value of 5% per hour, values within our normal limits of estimation.

The initial rate of release of radioactivity from starvation-induced protein degradation is comparable with that from rapidly degrading protein (Figs. 3 and 8 and Reference 17), and hence, if measurements are not carried out for a long period, their separate identity may not be recognized. Even a total radioactivity release of 5 to 8% caused by the degradation of rapidly degrading protein may be interpreted as a release of 1 to 2% per hour caused by starvation-induced protein degradation if measurements are made for periods of 5 hours or less and under starvation conditions alone. Thus, with [35S]methionine in strain B, it has been reported that the rate of protein turnover is 1 to 2% per hour under conditions of nitrogen starvation (25), which may be solely caused by the rapidly degrading protein. In exponential and stationary phase growth a very low value of 0.2% per hour has been reported (26). In addition to the probable poor exchange of extracellular methionine in these experiments, a lowering in specific activity of this amino acid inside the cell caused by interconversion to other amino acids (27) may account for this low protein degradation value.

The slowly decaying component observed in [14C]leucine-labeled cells and characterized by radioactive release in the absence of amino acid exchange (<0.5% per hour) can also be related to various reported observations. Since a substantial amount of this component is acid insoluble, the component may be related, in part to bacterial death and lysis, or to the specific release of cytoplasmic materials.

In one report, during deprivation of lysine in an auxotroph of *E. coli*, lipopolysaccharide-lipid-protein complexes that originated from the cell's outer membrane were liberated into the medium (28, 29). With [3H]leucine it was later shown that the protein component of this excreted complex corresponds to 0.3% of the total cellular protein per hour (30). In another report, when a
The degradation of stable nucleic acid has also been reported in the literature. When deprived of ammonium salts the greatest decrease in the cellular contents of strain K-12 occurred in the ribonucleic acid fraction (32). The ribose appeared to be metabolized, while uracil radioactivity accumulated in the suspension medium to an extent of 27% in 24 hours. Ribose of adenosine has also been shown to be utilized in a similar way (33). RNA degradation, measured by previously labeled ribose utilization, commences immediately when E. coli NCTC 5928 was starved in water or phosphate buffer (34), and these cells showed no loss of viability during 12 hours of starvation. A ribonucleic acid degradation rate of 1% per hour for at least 20 hours has been reported in strains B and K-12, previously labeled with [14C]uracil (35). These values of uracil release correspond with our finding of a radioactive release of 40 to 60% in 24 hours from cells labeled with guanine under conditions of glucose starvation.

The release of radioactivity from stable nucleic acid is not in contradiction with the stability of ribosomal and transfer RNA in growing E. coli (18, 36) established by internal traps and density labeling. Under conditions of exponential growth (and stationary phase), less than 5% of the stable nucleic acid is broken down in every generation. During this period less than 1% of the cell’s nucleic acid is released to the environment. Most of what is released appear to be free bases or nucleosides since almost half of the excreted radioactivity can be readily incorporated by unlabeled growing cells.

Since we have no effective way to exchange the intracellular nucleotides with an exogenous pool, during starvation the released cellular [14C]guanine radioactivity would represent the excess nucleic acid degradation over and beyond that which is reutilized in intracellular nucleic acid synthesis. Such an excess degradation corresponds with a radioactive release of 1.5 to 6% per hour (Table IV, Figs. 5, 6, and 9) under conditions of glucose starvation or inhibition of protein synthesis. The actual extent of nucleic acid degradation can only be obtained when reutilization of the degradation products during turnover is completely inhibited, but then it is necessary to assume that the resynthesis is not linked with degradation.

It has been reported that the apparent degradation of RNA increases 3- to 5-fold when RNA synthesis is inhibited with proflavin sulfate or 2,4-dinitrophenol (35). We find a similar 3-fold increase in the rate of release when synthesis is inhibited by rifampicin (Fig. 9). It was further observed that more than 80% of the total stable nucleic acid was released within 24 hours as a consequence of degradation. This proportion of stable nucleic acid must correspond to a large fraction of the total ribosomal nucleic acid (37).

Degradation of ribosomal RNA in nongrowing E. coli strain K-12 cells at a rate of 5% per hour has been indicated by Mandelstam and Halvorson (38). Based on our findings, it is postulated that intracellular ribosomal RNA degrades at an initial rate of 10% per hour for at least 6 hours and that all ribosomes are unstable under conditions of starvation. Similar findings have also been reported with [32P]-labeled RNA in E. coli B (39). After exhaustion of inorganic phosphate, 32P in RNA decreased by 30% for 3 to 4 hours. The ultracentrifugation profile after this period indicated a loss of ribosomal peaks. The large proportion of protein degradation occurring under conditions of starvation has been suggested to be the result of the inherent instability of ribosomes under conditions of growth inhibition (35, 38, 40). Thus, the net effect of turnover in E. coli under conditions of starvation is the transfer of nucleic acid and protein from ribosomes to soluble components and to other cell organelles.

In conclusion, a very large amount of conflicting data in the literature on protein turnover in bacteria can now be resolved. There is a rapid degradation process involving a small amount of protein which is independent of growth or starvation and is exhibited by all strains. In a variety of starvation conditions, ribosomes, and perhaps certain species of cellular protein are degraded. Only in certain strains does this degradation continue to the level of amino acids where they can be exchanged with carrier amino acid added to the culture medium. This turnover to the level of amino acid cannot be observed in E. coli strain B with our method of leucine exchange measurements.

However, based on the finding that all strains respond identically with the release of nucleic acid radioactivity it is suggested that a mechanism exists by which ribosomes are degraded as a result of various kinds of starvation so as to permit the organism to respond and adapt to its new environment.

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Protein Degradation in *Escherichia coli*: II. STRAIN DIFFERENCES IN THE DEGRADATION OF PROTEIN AND NUCLEIC ACID RESULTING FROM STARVATION

Kamalendu Nath and Arthur L. Koch


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