Intracellular Protein Breakdown in Non-Growing Cells of *Escherichia coli*

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1. When *Escherichia coli* leu$^-$ was incubated at 35° in a medium based on minimal medium, but with the omission of phosphate ions, or glucose, or NH_4^+ ions and leucine, intracellular protein was degraded at a rate of about 5%/hr. in each case. If Mg^{2+} ions were omitted, however, the rate of degradation was 2·9%/hr. 2. Under certain conditions of incubation, protein degradation was inhibited. The inhibitor was neither NH_4^+ ions nor amino acids, and its properties were not those of a protein, but it might be an unstable species of RNA. 3. Although a large part of the cell protein was degraded at about 5%/hr. during starvation of NH_4^+ ions and leucine, some proteins were lost at more rapid rates, whereas others were lost at lower rates or not at all. 4. In particular, β-galactosidase activity was lost at about 8%/hr. during starvation of NH_4^+ ions and leucine, whereas D-serine-deaminase and alkaline-phosphatase activities were stable. During starvation of Mg^{2+} ions, all three enzyme activities were stable.

When *Escherichia coli* is incubated in a medium lacking a required amino acid, a source of nitrogen, or sources of carbon and nitrogen, intracellular protein is broken down at 2–5%/hr. (Mandelstam, 1957, 1958*b*; Chaloupka, 1960; Proctor, 1962). During exponential growth, however, no protein is degraded (see Mandelstam, 1958*b*, 1963). The proteolytic system seems to be present, but in an inactive state, in growing cells, and to be activated by certain conditions including starvation (Mandelstam, 1958*b*; Chaloupka & Liebster, 1959; Chaloupka, 1960; Pine, 1965; Willetts, 1965, 1967).

Protein degradation is probably essential both for adaptation of micro-organisms to different environments and for their survival during any intervening period of starvation. It provides a mechanism for the supply of amino acids in non-growing cells: these may be further degraded to provide energy required for cell maintenance (see Dawes & Ribbons, 1964), or used for the synthesis of proteins, possibly including new enzymes that will allow growth in the new environment.

In this paper the first series of experiments described was designed to investigate the mechanism of protein breakdown, and its activation and inactivation. For these the average rate of protein breakdown under various conditions was obtained by measuring the rate of release of [^{14}C]valine from prelabelled cells. The second series was designed to show whether or not all proteins are equally subject to degradation. This was done in two ways: by examining the stability of proteins formed under different physiological conditions, and by examining the stability of several individual enzymes.

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MATERIALS AND METHODS

Organisms and medium. E. coli ML328c *lac*$^+$*leu*$^-$ was grown in a defined medium based on that described by Mandelstam (1958*a*). The medium consisted of 0·1M-KH_2PO_4 adjusted to pH7·0 with NaOH, and contained the following additions per ml.: glucose (1mg.); NH_4Cl (1mg.); $(NH_4)_2SO_4$ (1mg.); $MgSO_4$ (20μg.); $Fe(NH_4)_2SO_4,6H_2O$ (1·56μg.); citric acid (1·68μg.). It was supplemented with DL-leucine (300μg./ml.) and valine, as described below, all incubations were with shaking at 35°, and media were warmed to this temperature before use.

Radioactivity. DL-[1-^{14}C]Valine was obtained from The Radiochemical Centre (Amersham, Bucks.).

The radioactivity of samples plated at infinite thinness on aluminium planchets was determined with an Automatic Sample-Changer Counter (Harwell type 1112A, with a thin end-window Geiger tube detector). At least 1000 counts were collected, and day-to-day variations in the efficiency of counting were corrected by counting a standard at the same time.

Induction and assay of enzymes. β-Galactosidase was induced with isopropyl thio-β-D-galactoside (50μg./ml.) during cell growth. It was assayed in toluene-treated cells by the method of Rickenberg & Lester (1955).

To obtain cells containing alkaline phosphatase, a culture was incubated for 3hr. in a growth medium buffered with 0·1M-tris and containing no phosphate, to de-repress the enzyme. The cells were then grown for two generations in

normal defined medium before use. The enzyme was assayed in washed toluene-treated cells by the method of Torriani (1960).

D-Serine deaminase was induced with D-serine (200 μg./ml.) during cell growth. An equimolar concentration of glycine was included in the medium to prevent growth inhibition by D-serine. The enzyme was assayed in toluene-treated cells by the method of Pardee & Prestidge (1955). Preliminary experiments showed that it was not necessary to include pyridoxal phosphate, or AMP, or glutathione, or $MgSO_4$ in the assay mixture.

Determination of the concentration of intracellular amino acids. Culture samples containing about 2·5 mg. of cells were filtered through Oxoid membrane filters (4 cm. diam.). The central tube of the membrane filtration apparatus was surrounded by a tube of greater diameter, and the annular space was filled with ice during the filtration and extraction.

The cells were washed five times with 3 ml. of KCl (0·2 M) at 0°. This lowered the concentration of extracellular NH_4^+ ions to a minimum without extracting the amino acid pool (Britten & McClure, 1962). The suction was then stopped, and the cells were resuspended in 4 ml. of trichloroacetic acid (5%, w/v) at 0° in the filtration apparatus. After 10 min. the extract was collected in a tube placed below the outlet from the filter, which was washed through three times with 0·5 ml. volumes of trichloroacetic acid (5%, w/v).

The extract was evaporated to dryness in a boiling-water bath. Under these conditions, the trichloroacetic acid was hydrolysed to chloroform and CO_2. Complete removal of the trichloroacetic acid was ensured by twice redissolving the residue in water (0·5 ml.), and drying down again.

The amino N content of the residue was estimated by the method of Jacobs (1960). A standard curve was constructed by using a mixture containing equal weights of the 20 common amino acids.

Preliminary experiments showed that amino N was not lost during removal of trichloroacetic acid from amino acids by this method.

Measurement of protein degradation. Protein degradation was followed by measuring the liberation of radioactive valine from labelled cell protein.

Cells were grown from a small inoculum (taken from a slope culture) in defined medium containing radioactive DL-valine (0·05 μc/ml.; 30 μg./ml.). Preliminary experiments showed that more than 95% of the radioactivity in the labelled cells was in the protein fraction, and that more than 98% of the radioactivity of this fraction was present as valine. The cells were harvested during exponential growth and washed twice in the starvation medium to be used but containing unlabelled DL-valine (300 μg./ml.). They were resuspended at a culture density of about 0·5 mg./ml. in the starvation medium. This contained L-valine (200 μg./ml.) to prevent reincorporation of radioactive L-valine liberated by intracellular protein breakdown. It was unlikely that equilibration of intracellular and extracellular valine was a limiting factor, since amino acid equilibration is very rapid in *E. coli* under the conditions used (Britten & McClure, 1962). This assumption was strengthened by the fact that the rate of protein degradation found by the method used here was similar to the rate determined by a method not dependent on equilibration (Borek, Ponticorvo & Rittenberg, 1958). Also, increasing the concentration of L-valine to 400 μg./ml. did not increase the recovery of radioactive L-valine.

At the beginning of the experiment, culture samples (1 ml.) were added to an equal volume of trichloroacetic acid (10%, w/v). These were heated at 90° for 30 min. to extract nucleic acids, and the protein precipitates filtered off on membrane filters. Each filter was washed successively with three 1 ml. volumes of trichloroacetic acid (5%, w/v) containing DL-valine (150 μg./ml.), trichloroacetic acid (5%, w/v) alone and acetic acid (1%). This treatment removed free radioactive DL-valine and then the trichloroacetic acid. The filters were glued while damp to aluminium planchets, dried, and their radioactivity was determined. This gave a measure of the radioactivity originally present in the cell protein.

The radioactive valine released from the cells was determined by taking culture samples (3 ml.) at intervals during the experiment. These were cooled to 0° to prevent further metabolism, and the cells removed by centrifugation and discarded. The supernatants were diluted to 10 ml. and passed through columns of Zeo-Karb 225 (H^+ form) (20 ml.). The columns were washed with water to remove inorganic salts, and the adsorbed amino acids eluted with an excess of 2 N-NH_3. The solutions were evaporated to dryness, and the residues dissolved in water and transferred to aluminium planchets. These were dried and their radioactivities measured. Corrections were made for loss of radioactive valine during preparation on the samples (about 20%).

The amount of radioactive valine in the culture medium, expressed as a proportion of that originally present in the protein of the cells, was taken as a measure of protein degradation.

Measurement of cell lysis. It was necessary to determine whether the labelled valine was released by intracellular breakdown or by lysis of cells followed by extracellular proteolysis. For this purpose, liberation of β-galactosidase, an intracellular enzyme, into the culture medium during experiments was used as an index of cell lysis (Pollock, 1961). Control experiments showed that various concentrations of a crude cell-free extract of β-galactosidase were stable when incubated under the conditions used, in the presence or absence of cells.

The rate of liberation was always less than 0·3%/hr., and was usually less than 0·1%/hr. Therefore it could be assumed that cell lysis followed by extracellular proteolysis did not contribute significantly to the results obtained.

Measurement of protein degradation in cell-free extracts. Exponentially growing labelled cells were washed and resuspended in the starvation medium at 0° at a cell density of about 50 mg./ml. They were disrupted by ultrasonic treatment for 5 min. in a 250 w oscillator (constructed at the National Institute for Medical Research) at 25 kcyc./sec., the temperature being kept between 0° and 5°. Unbroken cells were removed by centrifuging three times at 1300*g* for 10 min. at 0°, the pellets being discarded each time. The extract was then diluted tenfold with the starvation medium.

During the measurement of protein degradation, the procedure given above was slightly modified. Since the concentration of the extract was equivalent to about 5 mg. of cells/ml., smaller samples (0·2 ml.) were taken for estimation of the original radioactivity of the protein. This prevented both overloading of the membrane filter and increase of the self-absorption during counting. The samples (3 ml.) for the determination of the supernatant radioactivity were mixed with an equal volume of trichloro-

acetic acid (10%, w/v) at 0°, and the precipitate was removed by centrifugation. The supernatant was extracted five times with 4ml. volumes of ether to remove the trichloroacetic acid, and the ether remaining in aqueous solution then removed by heating at 100° in a water bath. The samples were then passed through Zeo-Karb columns and treated as described above.

RESULTS

Protein degradation during nutrient deprivation. Protein degradation was measured during incubation of cells in a medium lacking glucose, or phosphate ions, or NH_4^+ ions and leucine, or Mg^{2+} ions.

Protein was broken down under all of these conditions, at the rates shown in Table 1. In each case, degradation began immediately and continued at the given rate for at least 4hr.

Effect of inhibitors on protein degradation in whole cells and in extracts. Protein degradation was measured during incubation of cells for either 3 or 4hr. in a medium lacking NH_4^+ ions and leucine and containing various inhibitors (Table 1). Similar experiments were done with cell-free extracts (Table 2).

Di-isopropyl phosphorofluoridate and *p*-chloromercuribenzoate were strong inhibitors. $\alpha\alpha'$-Bipyridyl and EDTA inhibited by about 50%, but 2,4-dinitrophenol had no effect. The percentage inhibition was similar in intact cells and in cell-free extracts (cf. Tables 1 and 2).

Table 1. *Protein degradation during incubation of cells in different media*

Cells, prelabelled during exponential growth with [^{14}C]valine, were incubated in the medium given in the first column for 4hr. Samples were taken every hour for the determination of protein breakdown. The inhibition is expressed as a percentage of the rate measured during starvation in a medium lacking NH_4^+ ions and leucine. The following abbreviations are used in the Tables: PCMB, *p*-chloromercuribenzoate; DFP, di-isopropyl phosphorofluoridate.

Additions to and omissions from growth medium	Protein degradation (%/hr.)	Inhibition of protein degradation (%)
None	0·6	—
−Glucose	5·3	—
−Phosphate	4·8	—
$-NH_4^+$, −leucine	4·8	0
$-Mg^{2+}$	2·9	—
+Chloramphenicol (50 μg./ml.)	1·0	—
$-NH_4^+$, −leucine, +chloramphenicol (200 μg./ml.)	0·7	85
$-NH_4^+$, −leucine, +2,4-dinitrophenol (1 mM)	4·8	0
$-NH_4^+$, −leucine, +$\alpha\alpha'$-bipyridyl (5 mM)	2·8	41
$-NH_4^+$, −leucine, +EDTA (10 mM)	1·9	61
$-NH_4^+$, −leucine, +PCMB (10 mM)	1·1	76
$-NH_4^+$, −leucine, +DFP (100 mM)	0·7	86

Table 2. *Protein degradation during incubation of cell extracts in different media*

Extracts of prelabelled cells prepared by ultrasonic treatment were incubated for 3 or 4hr. in the medium given in the first column. Samples were taken every hour for the determination of protein breakdown. The inhibition is expressed as a percentage of the rate measured during incubation in a medium lacking NH_4^+ ions and leucine. As stated in the text the values of 6% and 10% are not considered to be significant.

Additions to and omissions from growth medium	Protein degradation (%/hr.)	Inhibition of protein degradation (%)
None	1·6	—
$-NH_4^+$, −leucine	1·7	0
−Leucine	1·6	—
$-NH_4^+$, −leucine, +20 amino acids (100 μg./ml. each)	1·6	—
$-NH_4^+$, −leucine, +chloramphenicol (200 μg./ml.)	1·6	6
$-NH_4^+$, −leucine, +2,4-dinitrophenol (1 mM)	1·5	10
$-NH_4^+$, −leucine, +$\alpha\alpha'$-bipyridyl (5 mM)	1·0	40
$-NH_4^+$, −leucine, +EDTA (10 mM)	0·9	49
$-NH_4^+$, −leucine, +PCMB (10 mM)	0·4	78
$-NH_4^+$, −leucine, +DFP (100 mM)	0·3	84

Effect of ammonium ions and of amino acids on intracellular breakdown of protein. When cells were incubated in a medium lacking both NH_4^+ ions and leucine, protein degradation continued for at least 4hr. at a rate of 4·8%/hr. If cells were incubated in a medium lacking only leucine, degradation began at about this rate, but gradually decreased until after 4hr. the rate had fallen to 0·6%/hr. (Fig. 1).

The long period necessary for the rate of degradation to become minimal during leucine starvation suggested that NH_4^+ ions did not inhibit protein degradation directly, an idea supported by the fact that NH_4^+ ions had no effect on the rate of degradation in a cell-free extract (Table 2).

When the cells were starved of leucine alone, other amino acids would have been synthesized from the NH_4^+ ions and glucose present and, since net protein synthesis was impossible, these amino acids would have accumulated in the cell (Mandelstam, 1958*a*). Proctor (1961) suggested that, when these had reached some critical concentration, they might have inhibited protein degradation.

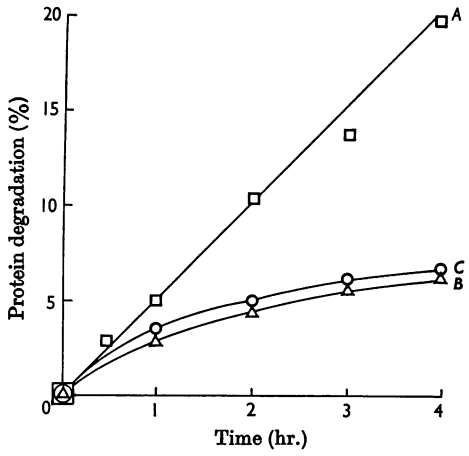

Fig. 1. Protein breakdown during incubation of cells in a medium lacking NH_4^+ ions and leucine (□, curve *A*), in a medium lacking leucine (△, curve *B*), and in a medium lacking NH_4^+ ions and leucine but containing the other 19 amino acids found in protein (100 μg./ml. each) (○, curve *C*).

To test this idea, protein degradation was followed in cells incubated in a medium lacking NH_4^+ ions and leucine but containing all of the other common amino acids. The amino acids did not inhibit degradation any sooner than NH_4^+ ions (Fig. 1). Also, the rate of protein breakdown in a cell-free extract was unaffected by the 20 common amino acids (Table 2). Therefore direct inhibition of protein degradation by amino acids seemed unlikely.

Further, if amino acids inhibited proteolysis, one would expect to find that the rate of protein degradation was inversely related to the amount of material in the free amino acid pool. Accordingly, the intracellular amino acid concentration was measured during incubation in different media (Table 3). A correlation of this type was not observed.

Effect of chloramphenicol and of ribonucleosides on protein breakdown. It was shown above that the decreased rate of protein degradation observed during leucine starvation in the presence of either NH_4^+ ions or amino acids was not due to direct inhibition by these substances. It seemed possible, however, that they were used to synthesize a protein that then inhibited or inactivated the proteolytic enzymes. The leucine necessary for synthesis of this postulated protein could have been provided by protein breakdown.

Experiments were accordingly carried out with chloramphenicol to see if protein synthesis was necessary for inhibition of protein degradation. An essential preliminary was to show with cell-free extracts that chloramphenicol did not affect

Table 3. *Amino acid pool of starved cells*

Cells were incubated for 4hr. in the medium given in the first column, and samples taken every 30min. for the determination of the concentration of free intracellular amino acids. The averaged results are given. The values for the rate of protein degradation are taken from previous experiments.

Omission from growth medium	Amino acid pool (μg./mg. of cells)	Protein degradation (%/hr.)
None	18·8	0·6
$-NH_4^+$, −leucine	11·8	4·8
$-NH_4^+$, −leucine, +chloramphenicol (200 μg./ml.)	13·1	0·7
$-Mg^{2+}$	41·7	2·9
−Glucose	21·8	5·5
−Phosphate	15·3	5·8
−Leucine (first 2hr.)	35·9	2·1
−Leucine (second 2hr.)	18·4	1·2

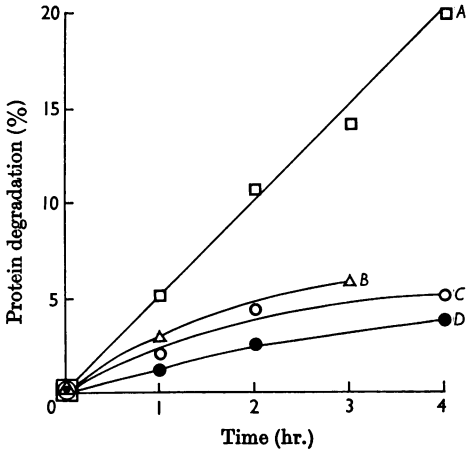

Fig. 2. Protein breakdown during incubation of cells in a control medium lacking NH_4^+ ions and leucine without addition (□, curve *A*), in a medium lacking NH_4^+ ions and leucine but containing adenosine, guanosine, cytidine and uridine (100 μg./ml. each) (△, curve *B*), in a medium lacking leucine but containing NH_4^+ ions and chloramphenicol (200 μg./ml.) (○, curve *C*), and in a medium lacking NH_4^+ ions and leucine but containing chloramphenicol (200 μg./ml.) (●, curve *D*).

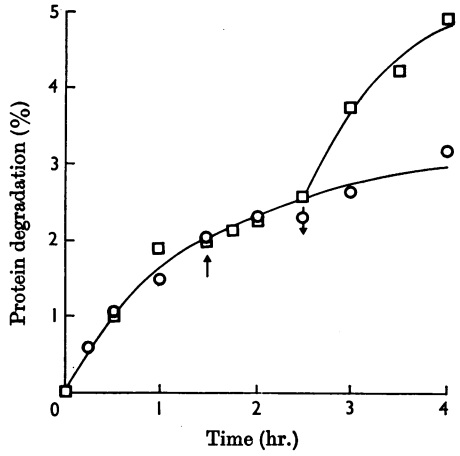

Fig. 3. Effect of addition and removal of amino acids on protein degradation. Prelabelled cells were incubated in a minimal growth medium containing L-valine (200 μg./ml.). After 1·5 hr. (first arrow) the medium was enriched by adding the 20 L-amino acids found in protein (50 μg./ml. each). After a further 1 hr. (second arrow) the cells were harvested, washed and returned to minimal growth medium containing L-valine (200 μg./ml.). Protein degradation was followed (□) and compared with that of cells incubated throughout in minimal growth medium containing only L-valine (200 μg./ml.) (○).

degradation (Table 2). Experiments were then done with cells starved of leucine in the presence of chloramphenicol, and these showed that the rate of protein degradation declined in much the same way as it had done in the absence of chloramphenicol (cf. curve *B* in Fig. 1 and curve *C* in Fig. 2).

In addition, when cells were incubated in a medium lacking NH_4^+ ions and leucine but containing chloramphenicol, the antibiotic decreased the rate of protein degradation markedly almost from the start (cf. curves *A* and *D* in Fig. 2). Since the concentration of chloramphenicol used was sufficient to produce virtually complete inhibition of protein synthesis in this strain under these conditions (Mandelstam, 1958*b*), the results suggested that the observed inhibition of degradation was not due to the accumulation of a newly synthesized protein inhibitor. Also, the effect of chloramphenicol could not be attributed to an effect on the degradative enzyme itself (Table 2), so other possibilities were considered. It is well known that chloramphenicol, though stopping protein synthesis, allows the synthesis of ribonucleic acid, and curve *B* in Fig. 2 shows that addition of the four ribonucleosides produced an inhibition of protein degradation in much the same way as adding chloramphenicol or omitting only leucine from the medium. Cells could not grow when incubated in defined medium lacking NH_4^+ ions but containing DL-leucine (300 μg./ml.) and the four ribonucleosides (100 μg./ml. each). The results were consistent with the idea that a species of RNA molecule could be the inhibitor (see the Discussion section).

Effect on the rate of protein breakdown of transfer from a rich to a poor medium. These experiments were performed to see whether protein degradation was initiated after transfer of cells from a richer to a poorer growth medium, rather than to a medium allowing no growth at all.

Cells prelabelled with [^{14}C]valine were grown in a minimal growth medium that was enriched after 90 min. by adding th 20 common L-amino acids (50 μg./ml. each). After growth for 60 min. in the supplemented medium, the cells were harvested, washed and incubated in fresh minimal growth medium containing only L-valine. Growth continued throughout the experiment, though at a faster rate in the supplemented medium. Protein degradation was followed by taking samples at intervals and measuring the valine released in the usual way. The addition of amino acids had no effect on the rate of protein degradation, but the transfer of cells back to minimal medium led to an immediate increase that lasted for about 1 hr. (Fig. 3). Control experiments showed that the actual process of washing and transfer did not lead to an increased rate of protein breakdown.

Degradation of proteins synthesized under different physiological conditions. The following experiment was done to determine whether proteins synthesized under different physiological conditions were degraded at the same rate. Cells were labelled with radioactive valine (I) during exponential growth, or (II) during starvation of NH_4^+ ions and leucine, when a limited amount of protein synthesis takes place due to turnover, or (III) during regrowth after this period of starvation. The cells were then starved of NH_4^+ ions and leucine for a second time, and the degradation of the proteins synthesized under each of the above conditions was determined by measuring release of labelled valine. The details were as follows. (I) Cells were grown from a small inoculum to a culture density of 0·5mg./ml. (II) They were harvested, washed and incubated for 6hr. at the same culture density in a medium lacking NH_4^+ ions and leucine, and containing L-valine (100μg./ml.). (III) They were then harvested, washed and incubated in fresh growth medium for 1hr. The cells were growing exponentially at the end of this period, during which the culture density increased by about 20%.

The proteins synthesized during any one of these three consecutive stages were labelled by including radioactive DL-valine (0·06μc/ml.; 250μg./ml.) in the medium during that stage.

At the end of stage III the cells were harvested, washed and incubated at a culture density of 0·5mg./ml. in a medium lacking NH_4^+ ions and leucine and containing L-valine (200μg./ml.). Protein degradation was followed for 4hr. (Fig. 4).

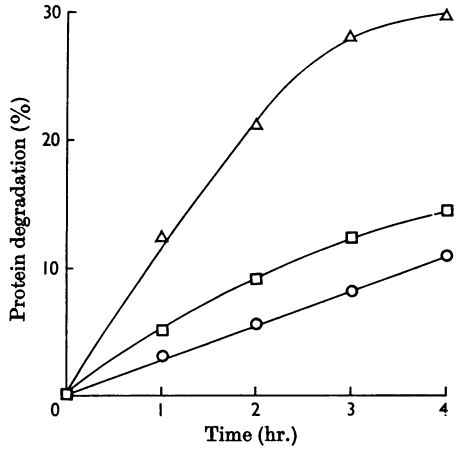

Fig. 4. Degradation of proteins synthesized under different physiological conditions. Cells were labelled with radioactive DL-valine (0·06μc/ml.; 250μg./ml.) during exponential growth (○, stage I), in a medium lacking NH_4^+ ions and leucine (□, stage II), or during regrowth of the starved cells in mimimal growth medium (△, stage III). Degradation of the protein synthesized in each of these three physiological states was then determined by starving the cells for a second time in a medium lacking NH_4^+ ions and leucine and measuring the release of labelled valine.

The rates of degradation of the proteins labelled under these conditions were very different. The protein synthesized in the first growth phase and remaining after starvation of NH_4^+ ions and leucine for 6hr. (I) was degraded throughout at 2·8%/hr., whereas that synthesized during starvation (II) was degraded at a rate that fell from 5·1%/hr. during the first hour to 2·2%/hr. during the fourth hour. The protein synthesized during regrowth of the starved cells for 1hr. (III) was degraded at 12%/hr. during the first 3hr. and 1·7%/hr. during the fourth hour.

These differences must reflect a variation in the susceptibility of these proteins to proteolysis, rather than variations in the proteolytic system, since the cells had been subjected to identical conditions of growth and starvation in each case.

Degradation of individual enzymes. The three enzymes studied were chosen because, first, they were either inducible or repressible. Thus, during incubation in starvation media, conditions could be chosen to prevent their synthesis that would otherwise obscure any loss of activity. Secondly, their enzymic activity was accurately and easily measurable in cell-free extracts under standard conditions. Therefore small differences in activity could be measured. However, it must be noted that loss of enzyme activity by irreversible denaturation, rather than proteolysis, could not be excluded.

The enzymic activities of β-galactosidase, alkaline phosphatase and D-serine deaminase were followed during incubation of cells in a medium lacking NH_4^+ ions and leucine, or Mg^{2+} ions.

During starvation with respect to Mg^{2+} ions all three enzymes were completely stable, and during starvation with respect to NH_4^+ ions and leucine only β-galactosidase activity was lost (Fig. 5). It had previously been observed that *E. coli* became more resistant to toluene treatment after nitrogen starvation (Koch, 1963), and it seemed possible that this might explain the decreased β-galactosidase activity. However, the following experiments indicated that this was not the case.

First, efforts to destroy any residual barrier by the use of longer periods of toluene treatment (2 or 4hr. instead of 1hr.) or of a fivefold higher concentration of *o*-nitrophenyl galactoside during the assay were unsuccessful. Secondly, use of a different method to disrupt the cells did not affect the result. Thus a time-course of the liberation of β-galactosidase activity by ultrasonic treatment showed that after 10min. there was no further liberation of enzyme activity and that the maximum enzymic activities of cells starved for 0, 2 or 4hr.

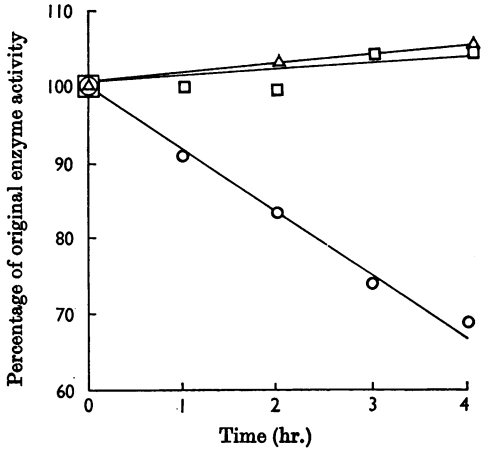

Fig. 5. Stability of enzymic activity during starvation of preinduced cells in a medium lacking NH_4^+ ions and leucine: β-galactosidase (○); alkaline phosphatase (□); D-serine deaminase (△).

were similar to those found after toluene treatment. After centrifugation of the ultrasonic extracts to separate the remaining whole cells and large cell debris, all of the β-galactosidase activity measurable without toluene treatment was in the supernatant and corrections for the decreased breakage obtained with starved cells (by measurement of the liberation of DNA into the supernatant of the ultrasonic extract) were much too small to account for the observed loss of enzymic activity.

Finally, cells were regrown in the absence of inducer after different periods of starvation: after 2hr. they were growing exponentially, and were therefore in similar metabolic states. However, there was no increase in the measured β-galactosidase activity of the starved cells, though further loss of activity was prevented.

These experiments all suggested that there had been an irreversible loss of β-galactosidase activity during nitrogen starvation.

DISCUSSION

The similarity of the rates of protein degradation during various types of starvation suggests that a single mechanism is involved, and that this is 'switched on' by any conditions of starvation. The rate during nitrogen starvation was similar to that previously found by Mandelstam (1958*b*), measuring release of radioactive leucine rather than valine. It continued unchanged for at least 8hr. (Willetts, 1965).

The rate during starvation with respect to Mg^{2+} ions may have been lower because Mg^{2+} ions are essential for rapid amino acid equilibration or because they are required as a cofactor for the enzyme system. Britten & McClure (1962) found that Mg^{2+} ion deficiency decreased the ability of *E. coli* to form amino acid pools, suggesting that the rate of amino acid equilibration might be decreased. However, it was shown in the present study that the bivalent-metal ion chelating agents, αα′-bipyridyl and EDTA (Dixon & Webb, 1964, p. 343), inhibited protein degradation to a similar extent in cells and cell-free extracts. This indicates that Mg^{2+} ions may be necessary as a cofactor, rather than for rapid equilibration.

Proteolysis, both in cells and cell-free extracts, was strongly inhibited by both *p*-chloromercuribenzoate and di-isopropyl phosphorofluoridate. Since these substances are known to be inhibitors of many proteolytic enzymes (Cecil, 1963; Dixon & Webb, 1964, p. 343), this provides added evidence that the mechanism of intracellular protein breakdown is direct enzymic hydrolysis. 2,4-Dinitrophenol, known to be an inhibitor of ATP production (Borst & Slater, 1961; Dixon & Webb, 1964, p. 305), had no effect, suggesting that ATP was not required for protein breakdown (see Halvorson, 1964). This is what would be expected if degradation occurred by simple enzymic hydrolysis.

The percentage inhibition of protein degradation by the last five inhibitors listed in Tables 1 and 2 was the same in both cells and extracts. This suggested that the proteolytic system studied in extracts was the same as that studied in whole cells.

Mandelstam (1960) and later Halvorson (1964) thought that the proteolytic enzymes might be carried in a latent form by the ribosomes; when cells were starved the ribosomes disintegrated, liberating the enzymes. This hypothesis was based on an analogy with latent ribosomal nucleases (Elson, 1959; Bolton *et al.* 1959), the known instability of ribosomes during starvation (Dagley & Sykes, 1958; Mendelsohn & Tissières, 1959) and known ribosomal peptidases (Bolton *et al.* 1959; Matheson, 1963; McCorquodale, 1963). However, the ribosomal peptidases are probably too restricted in their specificity to give complete hydrolysis of proteins, and in any case their activity is not latent in some cases and only partly so in others. The association of proteolytic enzymes with the soluble, membrane and cell-wall components of the cell, as well as with the ribosomal fraction (Chaloupka, 1961*a*,*b*; Willetts, 1965), also argues against this hypothesis, though cell disruption always completely activates the enzymes and may cause their redistribution among the cell components. Halvorson (1964) suggested that energy was necessary for activation of the proteolytic system, since 2,4-dinitrophenol inhibited protein breakdown in yeast. Mandelstam

(1958*b*) and Pine (1965) also found that 2,4-dinitrophenol inhibited protein breakdown in *E. coli*. However, in the present studies 2,4-dinitrophenol had no effect on protein breakdown in whole cells or cell-free extracts. The reason for the discrepancy between these results is unknown.

Another hypothesis suggests that protein breakdown is controlled by an inhibitor of the proteolytic enzymes (Halvorson, 1964). The inhibitor might inhibit as such, or it might serve to bind the proteolytic enzymes to a cell component such as the cell membrane, thus separating them from their substrates. Whatever the mechanism, it seems likely that the proteolytic enzymes are present in an inactive state both in growing cells (Mandelstam, 1958*b*) and in leucine-starved cells. When cells incubated under either of these conditions were transferred to a medium lacking both NH_4^+ ions and leucine, the proteolytic system was immediately fully activated even though very little protein synthesis was possible (Willetts, 1965).

Pine (1967) suggested the hypothesis of a 'preferred substrate', i.e. a type of protein forming a small proportion of the total and particularly susceptible to the action of degradative enzymes. This might explain the very low rates of degradation that may be observed in growing cells, but it would not explain results of the type reported here.

The results presented in this paper suggest that the postulated inhibitor of protein breakdown is neither NH_4^+ ions nor amino acids. Although these inhibited intracellular degradation, their action was slow, and, further, they did not inhibit degradation in cell-free extracts. It seems reasonable to conclude that they are only precursors of the real inhibitor. The amino acids may have been simply deaminated to give NH_4^+ ions, which then served as a precursor of the inhibitor. The next possibility considered was that the inhibitor was a protein. However, high concentrations of chloramphenicol did not prevent synthesis of the inhibitor in leucine-starved cells, so that this also seems unlikely.

If the inhibitor is a macromolecule rather than a small molecule, the most likely possibility remaining is that it is RNA in nature, and there are three small pieces of indirect evidence to support this. The first is that chloramphenicol inhibits protein breakdown in nitrogen-starved cells, and it is known that under these conditions 'stringent' control of RNA synthesis would be released (Ezekiel, 1964). The second is that the four ribonucleosides inhibited breakdown. It was shown that they could not act as a nitrogen source for cell growth, so it is unlikely that they were first deaminated to give NH_4^+ ions that were then used for synthesis of the inhibitor. The third is that during transfer of cells from a supplemented to a minimal medium, or from a higher to a lower growth rate during continuous culture (Willetts, 1967), protein degradation took place only during the periods when it would be expected that RNA synthesis, and hence synthesis of the postulated inhibitor, did not occur.

The suggestion of Mandelstam (1958*b*) that individual proteins might differ considerably in their rates of degradation was supported by experiments that measured the rates of degradation of proteins synthesized under different conditions. In particular, the proteins synthesized during regrowth of cells after a long period of starvation of NH_4^+ ions and leucine were very susceptible to degradation during further starvation (though not during further growth; Willetts, 1965).

Two of the enzymes studied here, alkaline phosphatase and D-serine deaminase, were stable during starvation of either NH_4^+ ions and leucine, or of Mg^{2+} ions. This agrees with the results of other authors, who found that nitratase and tetrathionase (Wainwright & Pollock, 1949) and lysine apodecarboxylase (Mandelstam, 1954) were stable in non-growing cells. The activity of the other enzyme studied here, β-galactosidase, was stable during starvation of Mg^{2+} ions, but was lost during starvation of NH_4^+ ions and leucine. The loss was irreversible, though it could not be proved that actual proteolysis of the enzyme protein took place. Proctor (1962) also found a decrease in the β-galactosidase activity of cells starved under similar conditions, though Mandelstam (1958*b*) did not. The reason for this difference is not known.

It is clear from this discussion and from the results illustrated in Figs. 4 and 5 that different proteins vary in their susceptibility to degradation, and that the same protein may differ in its susceptibility to degradation under different conditions of starvation. The underlying reasons remain unknown.

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