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Turnover of Protein in Growing and Non-Growing Populations of *Escherichia coli*

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The classical theory of the dynamic state of proteins was based upon isotopic experiments with mammals. Recently the concept has been questioned on the basis of experiments with bacterial systems. Three independent groups of workers reported that the proteins of *Escherichia coli* were stable and that the rate of turnover of protein in growing suspensions was negligible (Rotman & Spiegelman, 1954; Hogness, Cohn & Monod, 1955; Koch & Levy, 1955). Hogness *et al.* (1955) went on to suggest that proteins in the mammalian cell also were stable and that the observed turnover was not true intracellular turnover but was due to replacement of material released by secretion or cell lysis.

However, a number of facts pointed to the occurrence of degradation and synthesis of protein in non-growing populations, and suggested that it might not be valid to extrapolate from growing bacteria to mammalian systems where the cell population is, by comparison, virtually static. Thus Podolsky (1953) had reported a slow rate of protein degradation (about 0·25%/hr.) and Melchior, Klioze & Klotz (1951) had found that [^{35}S]-methionine was incorporated into the proteins of washed *E. coli*. Further evidence for the synthesis of protein is to be found in the fact that washed, or even nitrogen-starved, bacteria can synthesize inducible enzymes (for examples see Mandelstam, 1956; Pollock, 1958).

The present paper is a report of experiments designed to measure the extent of protein turnover in non-growing populations of *E. coli* by separate determination of the rates of degradation and of synthesis. Factors affecting both processes have been studied and, in addition, the rates of degradation in growing and non-growing populations have been compared. In this paper the term non-growing will be used to denote suspensions of bacteria in which there is no net synthesis of protein.

The experiments were carried out with mutant strains of *E. coli* with specific amino acid requirements. For the measurement of protein synthesis in non-growing suspensions, leucine- or arginine-requiring strains were used. The bacteria were first starved of the essential amino acid and then incubated with an excess of labelled glycine. The conditions were therefore such that protein synthesis, with concomitant incorporation of glycine, could not take place until leucine or arginine was released by the degradation of existing protein. The validity of this method as a measure of protein synthesis will be considered in the Discussion.

For investigation of protein breakdown a strain of *E. coli* was used which required both leucine and threonine for growth. The proteins were labelled by growing the bacteria in the presence of labelled leucine. The bacteria were then washed and incubated with an excess of unlabelled leucine to trap labelled leucine liberated from the proteins. In the experiments with non-growing suspensions, only a carbon source was added; when a growing population was required the medium was supplemented with ammonium salts and threonine.

METHODS

Organisms. The following strains of *E. coli* were used: 160–37 (requiring arginine for growth); K12 (leu^-thr^-) (requiring leucine and threonine); ML328c (Lac^+) (requiring leucine and able to form induced β-galactosidase). The organisms were grown in synthetic medium (Mandelstam, 1958) supplemented with the required amino acid as follows: L-arginine HCl, 100 μg./ml.; DL-threonine, 600 μg./ml.; DL-leucine, 300 μg./ml. All experiments were done at 35°.

Glycine incorporation. These experiments were done both with strains requiring leucine and strains requiring arginine. The bacteria were harvested during the exponential phase of growth (0·4–0·8 mg. dry wt./ml.), washed and starved of the essential amino acid by incubation at 35° for 30 min. with shaking in the same volume of medium without the amino acid supplement. This procedure sufficed to exhaust the essential amino acid, and caused other amino acids to accumulate, partly intracellularly and partly in the external medium (Mandelstam, 1958). The cells were washed again to remove ammonium salts and extracellular amino acids, and incubated, at a bacterial density of 0·9–1 mg./ml., in 0·05M-phosphate buffer (see preceeding paper) containing Fe^{2+} and Mg^{2+} ions (as in growth medium) and [1-^{14}C]glycine (180 μg./ml.) with a specific activity of about 50 counts/min./μg. Samples of 20 ml. were taken at intervals and centrifuged. The bacteria and the supernatant were then treated separately. For the isolation of protein, the bacteria were suspended in 2·5 ml. of water, 2·5 ml. of 10% (w/v) trichloroacetic acid containing 4 mg. of glycine/ml. was added, and the tubes were heated for 20 min. at 90° to extract nucleic acids (Schneider, 1945). The precipitated protein was washed with 5 ml. of 5% trichloroacetic acid containing 4 mg. of glycine/ml. The protein was then dissolved in 1 ml. of N-NaOH (containing 4 mg. of glycine/ml.) and reprecipitated with 2 ml. of 20% trichloroacetic acid. The precipitate was washed twice with 5% trichloroacetic acid, twice with acetone, twice with ethanol–ether (3:1), heating to boiling each time, and finally twice with ether. The protein was dried at room temperature and the radioactivity measured in 0·3 cm.^{2} polythene disks at infinite thickness (7·5–8 mg. of protein/disk) in a thin end-window Geiger–Müller counter.

Specific activity of glycine. Protein (2–3 mg.) was heated for 48 hr. with 2 ml. of 6N-HCl in a sealed tube at 100°. The hydrolysate was dried in a vacuum desiccator containing conc. H_2SO_4 and solid NaOH. The residue was dissolved in 10 ml. of water, and the amino acids were adsorbed on Zeo-Karb 225 and eluted with aq. NH_3 soln. (see Mandelstam, 1958). The eluate was taken to dryness at 100° and the amino acids were dissolved in 0·2 ml. of water and transferred with two washings of 0·1 ml. to Whatman no. 3 paper. The solution was applied as a line 5 cm. long. Glycine markers were placed on both sides of the sheets and the chromatograms were developed for 4 days with water-saturated *m*-cresol. The position of each glycine spot was determined by treating a 2 mm. strip of the paper with 0·02% ninhydrin. The glycine was then eluted and the eluate dried *in vacuo*. The glycine was dissolved in 0·2 ml. of water and transferred with two washings of 0·1 ml. to 2 cm.^{2} aluminium disks for measurement of radioactivity. Samples were counted at infinite thinness. The glycine on the disk was then dissolved in 1 ml. of water and estimated by the method of Moore & Stein (1948). Duplicate estimations of specific activity agreed to within 10%.

For analysis of the glycine in the culture supernatant, samples (2 ml.) were treated on columns of Zeo-Karb 225 and the same procedure was followed for measurement of specific activity.

Release of labelled leucine from protein. Bacteria requiring both leucine and threonine (K12 leu^-thr^-) were grown in synthetic medium supplemented with DL-threonine and [^{14}C]DL-leucine (approx. 50 counts/min./μg.) from a bacterial density of 0·02–0·80 mg./ml. The leucine of the protein was thus almost all labelled (97·5%). The bacteria were washed twice in the same medium containing 300 μg. of DL-leucine/ml. At each washing the bacteria were shaken for 3 min. at 35° to obtain thorough equilibration between extracellular and intracellular leucine. They were then suspended at a density of 0·15–0·20 mg./ml. in 0·05M-phosphate buffer containing 2% of glucose, Fe^{2+} and Mg^{2+} ions (as in growth medium) and 300 μg. of L-leucine/ml. to trap any labelled leucine released in the subsequent incubation. When a growing population was required, the incubation medium was supplemented with ammonium salts and threonine. The mutant strain with a double amino acid requirement was used to prevent the bacteria from growing by utilizing the leucine carrier as a general source of nitrogen. This precaution was later found to be unnecessary since leucine was not utilized in this way, and almost identical results were obtained with the bacteria which required only leucine. Samples (5 ml.) were taken at intervals and heated at 100° for 20 min. to extract intracellular free amino acids. Water (10 ml.) was added to dilute the solution, and the bacteria were removed by centrifuging. The amino acids in the supernatant were adsorbed on Zeo-Karb 225 as before. The rest of the procedure was as for glycine except that *tert.*-amyl alcohol saturated with water was used for developing the chromatograms. The leucine was eluted from the paper and transferred to 2 cm.^{2} aluminium disks for counting. Radioactivity measurements were corrected for self-absorption. In preliminary experiments the recovery of leucine was 80–85%.

Assay of β-galactosidase. β-Galactosidase activity was determined in toluene-treated cells at 30° by the assay method of Rickenberg & Lester (1955). The substrate, *o*-nitrophenyl-β-D-galactoside, was synthesized by the method of Seidman & Link (1950).

RESULTS

Effect of glucose on the rate of incorporation of glycine into protein of non-growing cells

The mutant requiring arginine, 160–37, was starved of arginine (see Methods) and the incorporation of radioactive glycine followed in the presence and the absence of glucose (2%). Samples were taken at 0, 40, 80 and 120 min.

Table 1 gives the results for the analysis of the radioactive glycine in the incubation medium. The behaviour of the glycine was similar in the presence and the absence of glucose. Its concentration fell fairly sharply in the first 40 min. of the experiment

and then more slowly. The specific activity remained at 56 ± 4 counts/min./μg. for the duration of the experiment. Table 2 shows the values for glycine isolated from the protein. The specific activity increased from 0·09 to 5·0 counts/min./μg. of glycine in the presence of glucose. In the absence of glucose the increase was 20 % less at the end of 2 hr.

From these specific-activity values the percentage of glycine in protein that had been replaced was calculated on the basis that complete replacement would have given a value of 56 counts/min./μg. (i.e. the specific activity of the original glycine). This method of calculation is permissible because the total bacterial glycine is small compared with the labelled glycine added (22 μg. and 180 μg./ml. respectively). In Fig. 1 it will be seen that with glucose present, the incorporation of glycine was almost linear and represented a synthesis of protein equivalent to 9 % of the total bacterial protein in 2 hr. or about 4·5 %/hr. In the absence of glucose, the initial rate was a little lower and it was maintained only for about 80 min. before it began to fall.

The same graph shows the total radioactivity of the protein. It can be seen that, in the presence of glucose, glycine accounted for about 75 % of the total radioactivity. A further 20 % was found in the serine, leaving 5 % unaccounted for. In the absence of glucose there was much more diffuse labelling of the amino acids; glycine accounted for only 50 % of the total radioactivity and the serine for 20 %. The distribution of the remaining 30 % was not further investigated.

Effect of chloramphenicol on the rate of incorporation of glycine into protein. The mutant requiring leucine, ML 328c (Lac$^+$), was starved of leucine and the effect of chloramphenicol (20 μg./ml.) upon the incorporation of glycine was determined. The incubation was carried out in buffer solution containing glucose.

Fig. 2 shows the behaviour of the glycine in the incubation medium. In the control suspension the concentration of extracellular glycine fell rapidly during the first 40 min. and then more slowly. The picture is essentially the same as in the previous experiment. In the presence of chloramphenicol

Table 1. *Concentration and specific activity of glycine in medium during incubation of bacteria in phosphate buffer with and without glucose*

Time (min.)	Concn. of glycine (μg./ml.)	Specific activity (counts/min./μg.)
With glucose:		
0	182	54
40	128	60
80	118	52
120	102	55
Without glucose:		
0	180	56
40	132	56
80	112	54
120	96	58

Table 2. *Specific activity of glycine isolated from protein of* Escherichia coli *incubated with* [1-^{14}C]-*glycine in the presence and the absence of glucose*

Time (min.)	Glycine (μg.)	Specific activity (counts/min./μg.)
With glucose:		
0	51	0·09
40	48	1·9
80	54	3·5
120	53	5·0
Without glucose:		
0	58·5	0·09
40	52	1·65
80	62	3·2
120	66	4·0

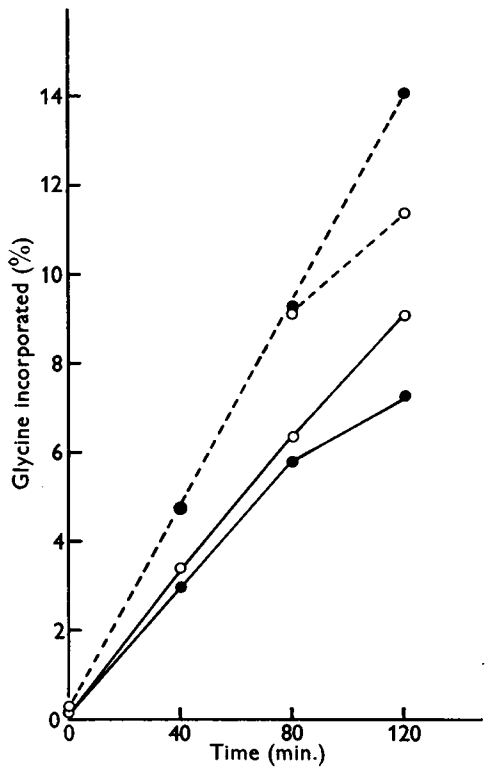

Fig. 1. Incorporation of [^{14}C]glycine into protein by arginine-starved bacteria incubated in the presence and the absence of glucose. Broken lines show the total radioactivity of the protein, i.e. activity due to incorporation of glycine and of other amino acids which have become labelled during the incubation. Continuous lines show incorporation of glycine as such. Values are expressed as percentage of total glycine in protein. ○, With glucose; ●, without glucose.

the concentration of glycine was constant after 40 min. The specific activity of the glycine fell 12–15 % in the course of the incubation.

The extent to which the glycine in the protein became labelled is shown in Fig. 3. In the control suspension, the amount of synthesis was equivalent to 8·5 % of the bacterial protein in 2 hr. The values in the graph have not been corrected for the fall in specific activity of the extracellular glycine, but even so they are in good agreement with those obtained with the strain requiring arginine. In the presence of chloramphenicol the incorporation of glycine gave a value for protein synthesis of 0·65 % in 2 hr., which is less than one-tenth of the control value.

A similar result has been obtained with the arginine-requiring strain.

Effect of 2:4-dinitrophenol on the rate of incorporation of glycine into protein. The effect of 2:4-dinitrophenol (mM) upon the incorporation of glycine into protein was determined with the same procedure as in the previous experiment. The leucine-requiring strain ML 328 c (Lac^+) was used.

Comparison of Figs. 3 and 4 shows that dinitrophenol, like chloramphenicol, depresses incorporation of glycine by over 90 %.

Reversibility of glycine incorporation. Experiments were carried out to determine whether the labelled glycine incorporated into protein could be displaced by unlabelled glycine. The bacteria were starved of the essential amino acid, and then allowed to incorporate labelled glycine in the presence of glucose as in previous experiments. After 2 hr. the bacteria were washed and incubated for 3 hr. in fresh medium containing unlabelled glycine. Samples were taken at hourly intervals.

There was no detectable loss of the incorporated radioactivity.

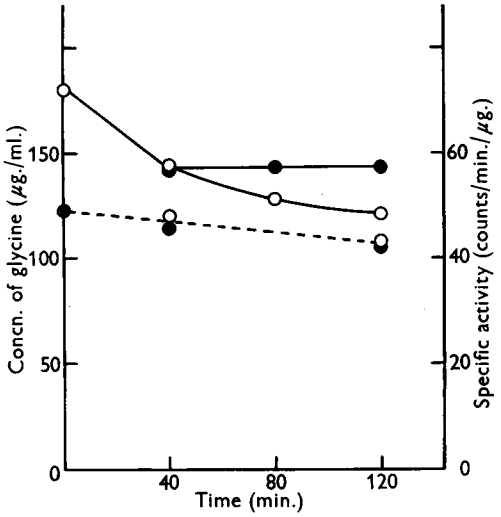

Fig. 2. Concentration (continuous lines) and specific activity (broken lines) of [^{14}C]glycine in medium during incubation of leucine-starved *E. coli* in the presence and the absence of chloramphenicol (see text). ○, Control; ●, with chloramphenicol (20 μg./ml.).

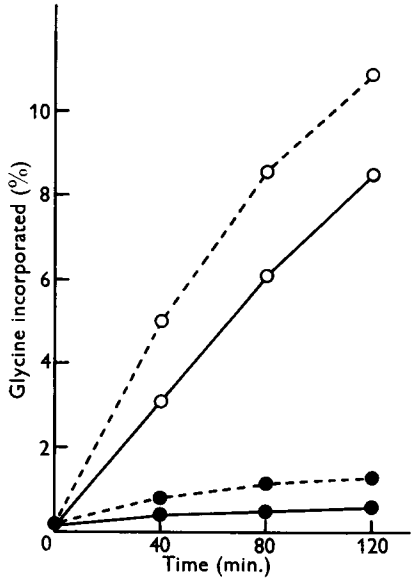

Fig. 3. Effect of chloramphenicol upon the incorporation of glycine into protein of leucine-starved *E. coli*. Continuous lines show glycine incorporation as a percentage of the total glycine of protein; broken lines show the total radioactivity of the protein (see legend to Fig. 1). ○, Control; ●, with chloramphenicol (20 μg./ml.).

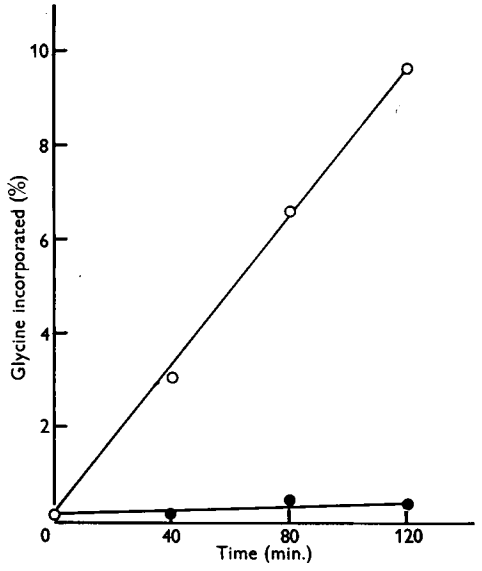

Fig. 4. Effect of 2:4-dinitrophenol upon the incorporation of glycine into protein of leucine-starved *E. coli*. Glycine incorporation is given as a percentage of the total glycine of protein. ○, Control; ●, with 2:4-dinitrophenol (mM).

Degradation of protein in growing and non-growing cells

K12 (leu⁻ thr⁻) was grown in the presence of labelled leucine (49 counts/min./μg.). The bacteria were washed (see Methods) and suspended at a density of 0·15 mg./ml. in complete synthetic medium supplemented with DL-threonine and containing unlabelled L-leucine (500 μg./ml.). A similar flask was set up in which threonine and ammonium salts were omitted so that growth could not occur.

The bacteria which were starved of threonine and ammonium salts increased from 0·15 to 0·20 mg./ml. in 3½ hr. (Table 3). This slow increase in bacterial density, as measured by change in opacity, has been found with most coliform strains during nitrogen starvation and is probably due to formation of polysaccharides. The bacteria which were not starved increased from 0·20 to 0·94 mg./ml. The last column of Table 3 (calculated on the basis that 49 counts/min. = 1 μg. of leucine released) shows that the starved bacteria released more than three times as much labelled leucine as the growing culture.

Estimations of the leucine content of *E. coli* (K12 leu⁻ thr⁻ and ML328c Lac⁺) gave values of 34–38 μg./mg. dry wt. of bacteria. A similar value can be obtained from published data. Thus leucine constitutes 6·1 % of coliform protein (Polson, 1948), and the protein itself 60 % of the dry wt. (Roberts, Abelson, Cowie, Bolton & Britten, 1955). This gives 36·6 μg. of leucine/mg. of bacteria. For purposes of calculation, the value has been taken as 36 μg./mg. From this figure, the radioactive leucine released has been plotted as a percentage of that present at the beginning of the incubation and, in the same figure, the logarithm of the bacterial density has been plotted against time (Fig. 5). It will be seen that the growing bacteria liberated less than 4 % of the leucine of the protein in 3½ hr. and that a substantial proportion of the release occurred during the lag phase. Once growth was well established almost no breakdown of protein was

Table 3. *Release of* [^{14}C]*leucine from the protein of growing and non-growing* Escherichia coli

Time (min.)	Bacterial density (mg./ml.)	Radioactivity in supernatant (counts/min./5 ml.)	Leucine released (μg./5 ml.)
Non-growing bacteria:			
0	0·15	5·5	0·11
30	0·16	28·2	0·57
60	0·16	47·3	0·96
90	0·17	66·4	1·35
120	0·18	89·6	1·82
150	0·19	124	2·53
210	0·20	174	3·56
Growing bacteria:			
0	0·15	5·0	0·10
30	0·16	23·1	0·47
60	0·21	36·5	0·74
90	0·27	43·2	0·88
120	0·37	46	0·94
150	0·50	50·5	1·03
210	0·94	47	0·96

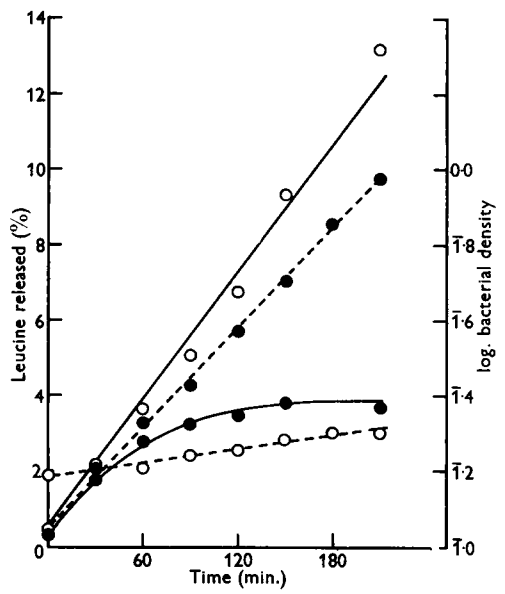

Fig. 5. Release of leucine from protein of nitrogen-starved and of growing *E. coli* expressed as a percentage of the leucine in protein at the beginning of the experiment. ○, Nitrogen-starved bacteria; ●, growing bacteria. The corresponding broken lines are semi-logarithmic plots of the bacterial density (mg. dry wt./ml.).

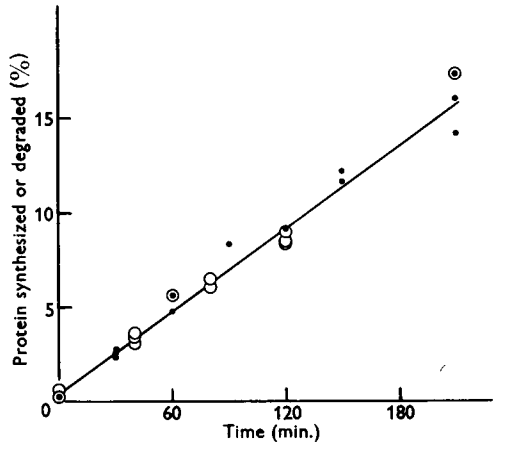

Fig. 6. Comparison of rates of protein synthesis (○) and degradation (●) in non-growing *E. coli*. Synthesis was measured by incorporation of glycine and degradation by release of leucine. The straight line was fitted to all the points by the Method of Least Squares.

detectable. In the non-growing cells the rate of release of leucine was linear for the duration of the experiment. The existence of this inverse relationship between growth and the rate of release of leucine was confirmed with strain ML 328c (Lac^+) (Mandelstam, 1957).

The values of Table 3 and Fig. 5 are 15–20 % too low because the recovery of leucine is not quantitative (see Methods). If the correction is made, the rate of breakdown of protein is about 4·5 %/hr., which is similar to the values obtained for synthesis by measurement of incorporation of glycine. In Fig. 6 the corrected values obtained in three experiments on release of leucine and three experiments on the incorporation of glycine are shown together. It is apparent that the rate of protein synthesis is roughly equal to the rate of breakdown, although values for the latter tend to be slightly higher. All the points are fairly well represented by a single straight line fitted by the Method of Least Squares.

Effect of chloramphenicol on the rate of degradation of protein. The proteins of ML 328c (Lac^+) were labelled as before by growing the bacteria in the presence of labelled leucine. The bacteria were washed and suspended in buffer containing glucose and unlabelled L-leucine. The effect of chloramphenicol (20 μg./ml.) upon the rate of release of labelled leucine was then determined.

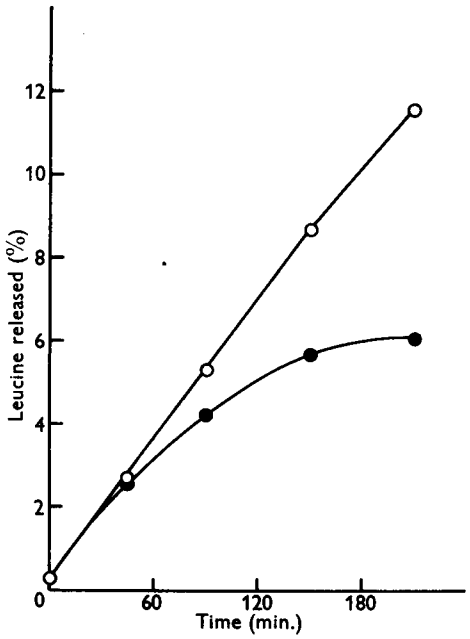

Fig. 7. Effect of chloramphenicol upon the degradation of protein in *E. coli*. Results are given as percentage of leucine released from protein. ○, Control; ●, with chloramphenicol (20 μg./ml.).

It was found that chloramphenicol had a delayed effect (Fig. 7). For the first 45 min. there was virtually no inhibition and even at 90 min. there was only a 20 % difference between the control suspension and that containing chloramphenicol. The inhibitory effect was progressive until, at 2½ hr., the breakdown of protein appeared to be at an end, whereas in the control suspension the process was still continuing at a linear rate. The total amount of protein degraded in 3½ hr. was decreased about 50 % by the inhibitor.

Effect of azide and 2:4-dinitrophenol on the rate of degradation of protein. The effect of sodium azide (5 mM) upon the rate of release of labelled leucine was determined with the same procedure as in the previous experiment; ML 328c (Lac^+) was again used.

The result with azide was very similar to that obtained with chloramphenicol. Again no inhibition was observed during the first 45 min. The release of labelled leucine then fell off rapidly and ceased after 2½ hr. (Fig. 8).

By contrast, dinitrophenol was inhibitory from the start; the rate of protein degradation was about 1 %/hr. as compared with 4 %/hr. in the control.

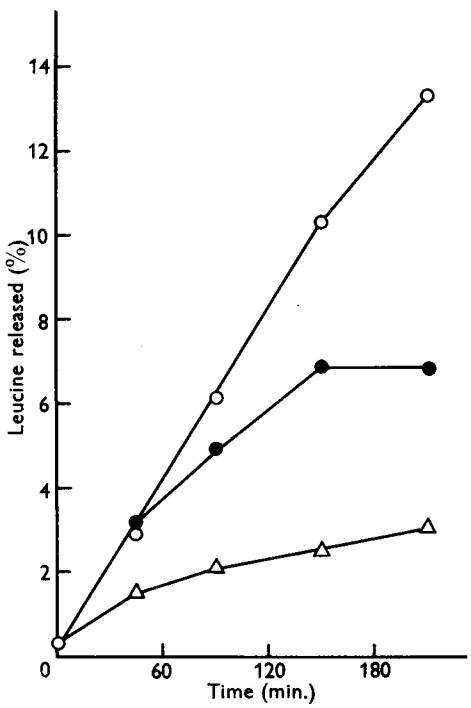

Fig. 8. Effect of azide and 2:4-dinitrophenol upon the degradation of protein in *E. coli* ML328c (Lac^+). Results are given as percentage of leucine released from protein. ○, Control; ●, azide (5 mM); △, 2:4-dinitrophenol (mM).

The effect of dinitrophenol differed according to the strain used. With K 12 (leu$^-$thr$^-$) the result was the same as that obtained with azide or chloramphenicol, that is, delayed inhibition becoming apparent only after 45 min. This difference in response of the two strains to dinitrophenol was consistently reproducible and is the only instance in which a result obtained with one strain could not be repeated with the other. No explanation has been found for this difference in reaction between the two strains.

Effect of ammonium salts on the rate of degradation of protein. The above experiments showed that azide and chloramphenicol might not be acting as primary inhibitors of protein breakdown since their effect only became apparent after about 1 hr. This suggested that their action might be due to the gradual accumulation of some inhibitory metabolite.

The experiments with growing bacteria also pointed to the same conclusion. It therefore seemed possible that, if non-growing bacteria were forced to accumulate metabolites, the breakdown of protein might be inhibited in somewhat the same way as in growing bacteria or as in non-growing bacteria treated with an inhibitor. The experiment was carried out with K 12 (leu$^-$thr$^-$) and the rate of release of labelled leucine from bacteria incubated with glucose and unlabelled leucine was measured under the following conditions: (1) no addition—bacteria were starved of ammonium salts and threonine; (2) with ammonium salts—bacteria were starved of threonine; (3) with ammonium salts and threonine—growing population of bacteria. The ammonium salts and threonine were present in the same concentration as in the growth medium.

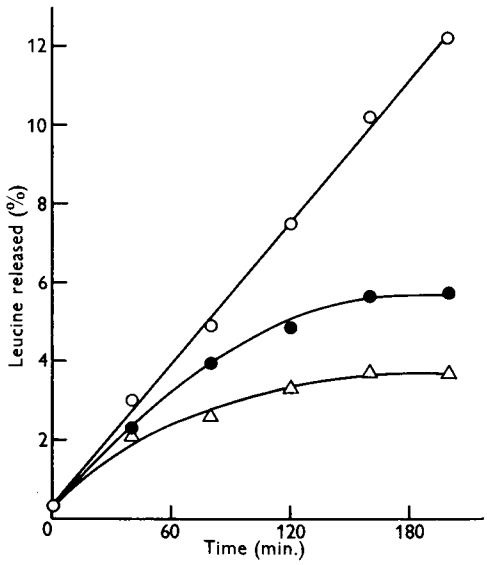

Fig. 9. Effect of ammonium salts, and of ammonium salts with threonine, upon the degradation of protein in threonine-requiring *E. coli*. Results are given as percentage of leucine released from protein. ○, Control, no ammonium salts and no threonine; ●, with ammonium salts, no threonine; △, with ammonium salts, with threonine; ammonium salts and threonine were added to the same concentration as in growth medium.

From Fig. 9 it will be seen that the addition of ammonium salts produced inhibition of degradation of protein whether the bacteria were growing or not; the presence of threonine merely increased the rate of development of the inhibition. In both suspensions the degradation of protein was significantly retarded after 80 min. and appeared to have ceased altogether after 160 min.

Stability of bacterial cells in a non-growing population

An attempt was made to determine whether there was enough lysis in the cells of a non-growing population to account for the observed turnover, or whether the results were due to true intracellular turnover. For this purpose, ML 328 c (Lac$^+$) was grown in synthetic medium with 2 % of lactose as the only source of carbon. The bacteria, when harvested, contained considerable quantities of the inducible enzyme β-galactosidase which had been synthesized in response to the presence of lactose. The bacteria were centrifuged, washed twice to remove lactose and ammonium salts and suspended at a density of 0·2 mg./ml. in buffer containing glucose and leucine, and incubated for 3½ hr. Samples (5 ml.) were taken at the beginning and end of the experiment, and the incubation medium and the bacteria were separately assayed for β-galactosidase activity.

Since there was no lactose present, further synthesis of β-galactosidase was precluded. Consequently, if the turnover of protein measured in the previous experiments had involved lysis of bacteria, β-galactosidase should have been lost from the cells at the rate of 4–5 %/hr. The enzyme thus lost should have been either destroyed, if it were un-

Table 4. *Assay of intracellular and extracellular β-galactosidase activity of a suspension of* Escherichia coli *incubated in the absence of a nitrogen source*

Enzyme activity is expressed as μmoles of *o*-nitrophenyl-β-galactoside hydrolysed/hr./ml. of suspension.

Time (hr.)	Intracellular	Extracellular	Intracellular (%)
0	19·7	<0·05	>99·9
3½	19·3	0·11	>99·9

stable, or recovered in the extracellular solution if it were stable.

Table 4 shows the distribution of enzyme activity. It will be seen that the loss of enzyme was only 2 % in 3½ hr., which is within the error of the method, and that virtually all the activity remained in the cells.

DISCUSSION

It is first necessary to consider the assumptions upon which the experiments were based and the factors that might affect the values for protein turnover obtained by these methods.

The estimate of protein synthesis arrived at by measuring incorporation of glycine could be inaccurate for a number of reasons. First, and most important, an erroneously high figure would be obtained if glycine were incorporated by direct exchange with the glycine residues in the protein (see Gale & Folkes, 1953) and not by protein synthesis. The problem raised by the possible occurrence of exchange has been discussed in an earlier paper (Mandelstam, 1956), where it was pointed out that piecemeal exchange would be difficult to distinguish from protein breakdown and resynthesis. Thus if a pool exists which contains all the amino acids normally present in protein, and if they all undergo independent exchange, the resultant dynamic state will resemble 'true' protein turnover. In principle, however, the two processes are distinguishable, because, whereas exchange could produce labelling of existent molecules, it could not result in the net synthesis of any species of protein molecule. Now, nitrogen- or amino acid-starved bacteria can synthesize inducible β-galactosidase, i.e. there can be net synthesis of a particular type of protein. Furthermore, with incorporation of glycine as a measure of protein synthesis, it can be shown that the ratio of induced β-galactosidase to incorporation of glycine has the same magnitude in starved and in growing bacteria (Mandelstam, 1957). This proportionality between incorporation of glycine and net synthesis of enzyme makes it reasonable to conclude that the incorporation is a measure of synthesis rather than exchange. In addition, Gale & Folkes (1953) showed that exchange is not sensitive to chloramphenicol whereas protein synthesis is. This additional criterion supports the contention that, in the present experiments, little, if any, of the incorporation could have been due to exchange.

An erroneously high result could also arise through contamination of the protein by radioactive glycine. The following facts suggest that this is not the case: treatment of the protein with ninhydrin did not reduce the radioactivity, thus showing that the carboxyl group of the glycine was bound; the zero-time samples had negligible radioactivity; increase in radioactivity was almost wholly prevented by dinitrophenol or chloramphenicol; incorporation of radioactive glycine was not reversed when the bacteria were incubated for 3 hr. in an excess of unlabelled glycine.

An attempt has also been made to control factors that would tend to give too low an estimate of protein synthesis. One such factor is dilution of the labelled glycine by unlabelled glycine produced by metabolism of the cells during incubation. This factor has been controlled by analysis of the glycine in the incubation supernatant. Again, if the labelled glycine did not saturate the protein-synthesizing sites, glycine liberated by breakdown would be reincorporated into protein, and a low estimate would be obtained. This possibility cannot be altogether ruled out, but it was found that doubling the concentration of labelled glycine used in these experiments did not increase incorporation.

Determination of the rate of degradation of protein by measurement of release of leucine could give erroneous results for the same reasons as the experiments with incorporation of glycine. For example, a high result would be obtained if leucine were liberated by exchange as well as by degradation of protein. However, preliminary experiments showed that the incorporation of leucine, like that of glycine, parallels the synthesis of β-galactosidase and is suppressed by chloramphenicol, so that there is no reason to suppose that any significant amount of exchange takes place. It is more difficult to be sure that there is instantaneous equilibration between the extracellular leucine and the labelled intracellular leucine released by protein breakdown, and it is therefore possible that some of the leucine released by breakdown is reincorporated so that the results of these experiments may be lower than the true values.

The necessity for rapid equilibration imposes some limitations upon the method. Thus it cannot be used to determine the effect of carbon starvation upon the rate of degradation, because carbon starvation retards equilibration between extracellular and intracellular leucine (Cohen & Rickenberg, 1956). Chloramphenicol does not have this effect, so that the observed inhibition can be accepted as such. The experiments with azide and dinitrophenol have also been presumed to be valid because, although these compounds retard equilibration, they also block protein synthesis so that leucine that has been released cannot be reincorporated.

On the basis of these assumptions, the experimental results lead to the following conclusions. In non-growing bacteria the rate of protein synthesis is roughly equal to the rate of protein breakdown, and proceeds at 4–5 %/hr.

The processes of degradation and synthesis which together constitute turnover are quite differently affected by metabolic inhibitors. Synthesis is almost completely prevented by such agents as chloramphenicol and 2:4-dinitrophenol and the effect is immediately apparent. Degradation is affected much less and the effect becomes apparent only after an appreciable lag period. Chloramphenicol, azide and dinitrophenol (in K12 leu^- thr^-) produced no effect at all for over 45 min. With ML 328c (Lac^+) dinitrophenol was inhibitory from the beginning, but, even so, breakdown was inhibited less than synthesis. From work with rat-liver slices the conclusion has been reached (Simpson, 1953; Steinberg, Vaughan & Anfinsen, 1956) that reagents which inhibit protein synthesis also inhibit its breakdown. It is apparent that in *E. coli* this statement is true only in a very general way. The inhibition curves suggest that the inhibitors do not affect the catabolic process as directly as they affect synthesis. The slow development of inhibition is more consistent with the assumption that these substances cause the accumulation of inhibitory metabolites within the cell. This interpretation is supported by the fact that the inhibition curve obtained with ammonium salts is almost identical with that produced by azide or chloramphenicol.

When we come to consider the breakdown of protein in growing bacteria the question of equilibration is again of importance, for it is possible that the leucine carrier is utilized for growth as rapidly as it enters the cells, so that it can no longer trap any labelled leucine that may be released. This is probably not the case, because the results for growing bacteria obtained by this method agree qualitatively and quantitatively with those obtained by Koch & Levy (1955). These authors, using a method which does not depend upon equilibration between extracellular and intracellular amino acids, reported that *E. coli* contained a labile component equivalent to 2% of the bacterial protein. This component was degraded in the first 2 hr. of growth, after which no further breakdown of protein could be detected. In the present experiments the labile material was usually somewhat higher (about 3·5%) but again degradation had ended after growth for 2 hr. The degradation observed during this period might be due to a small proportion of non-growing cells in the population. From Fig. 5 it will be seen that breakdown of protein took place most rapidly during the first 30 min. when the bacteria were in the lag phase of growth. In the experiments of Rotman & Spiegelman (1954) and Hogness *et al.* (1955) even this limited turnover was not detected, possibly because of different conditions of growth.

The finding that proteins are more stable in growing than in non-growing cells has been shown to apply to other micro-organisms. Halvorson (1958) has reported very similar results in yeast, and R. Urbá (personal communication) has shown that in growing cultures of *B. cereus* protein breakdown occurs at the rate of 1%/hr. whereas it is about 8%/hr. in non-growing cells.

The difference in the rate of protein breakdown in growing and non-growing cells could be explained in several ways. Thus non-growing bacteria may produce some substance, e.g. enzyme or cofactor, needed for hydrolysis of protein. This is improbable because the mechanism for degrading protein appears to be present and fully active immediately bacterial growth is prevented. The alternative possibility is that the bacteria always possess a mechanism for degrading protein but that during growth some metabolite is formed which inhibits its action. The findings which have already been discussed make this the more likely alternative.

Whatever the explanation may be, it appears that the cell proteins, which are in some way protected during active growth, are rendered labile when the bacteria are starved. The ensuing breakdown and resynthesis ensures a sufficient flow of material through the free amino acid pool to allow the bacteria to synthesize inducible enzymes that may be useful in the changed chemical environment. It has been shown that the turnover process accounts satisfactorily for the adaptive production of β-galactosidase under conditions in which no net synthesis of protein is possible (Mandelstam, 1957).

From the data available it is not possible to tell whether turnover occurs in all cells of a non-growing population or whether all proteins are involved. A number of observations, however, make it likely that bacterial proteins will be found to differ considerably in lability. The present experiments show that β-galactosidase is relatively stable under conditions where other proteins in the same bacteria are being rapidly degraded. Nitratase and tetrathionase are also stable in non-growing cells (Wainwright & Pollock, 1949) and so is lysine apo-decarboxylase (Mandelstam, 1954). It is moreover possible that proteins may be stable under some conditions of starvation but not under others. A case in point is the ethanol-soluble protein of *E. coli* which is destroyed during sulphur starvation (Roberts *et al.* 1955). In our experiments, the ethanol-soluble proteins were examined and found to be stable during nitrogen starvation. The solution of this problem requires the examination of a number of characterized proteins under different conditions of protein turnover.

SUMMARY

1. Methods are described for the estimation of protein turnover in *Escherichia coli* by the use of strains with specific amino acid requirements.

2. In rapidly growing cells there was no detectable turnover of protein; in non-growing cells degradation and resynthesis of protein proceeded at the rate of 4–5 %/hr. for several hours.

3. Chloramphenicol, azide and ammonium salts inhibited protein breakdown after a latent period. The effect of 2:4-dinitrophenol was variable.

4. Protein turnover could not be attributed to lysis of cells.

5. The possible function of protein turnover in bacterial metabolism is discussed.

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Further Studies on the Inhibition of Acetate Metabolism by Propionate

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It has been shown previously (Pennington, 1957, and independently by Masoro, Felts, Panagos & Rapport, 1957) that acetate metabolism in rat-liver slices is strongly inhibited by propionate and, to a lesser extent, by other short-chain fatty acids. These investigations did not reveal the mechanism of the inhibition. The formation of $^{14}CO_2$ from [2-^{14}C]pyruvate was much less affected by propionate than was its formation from [*carboxy*-^{14}C]-acetate (Pennington, 1957), thus indicating that propionate (or a product of propionate metabolism) inhibits acetate metabolism by blocking the formation of acetyl-coenzyme A. It seems likely, however, that other interactions are also involved, since Masoro *et al.* (1957) found that the incorporation of isotope from labelled acetate into higher fatty acids was inhibited by propionate to a lesser extent than the incorporation of isotope into carbon dioxide. This was confirmed by the present authors (unpublished work).

As a step towards elucidating the mechanism of the inhibitory action of propionate, further experiments have been carried out with homogenates and soluble preparations from liver.

EXPERIMENTAL

Preparation and incubation of tissue slices. Tissue slices were cut by hand and incubated as described previously (Pennington, 1957). To facilitate approximate comparison with the activity of the homogenates (which usually corresponded to 100 mg. of wet tissue) the results obtained with slices were corrected to 20 mg. (dry wt.).

Preparation of homogenates. These were prepared by homogenizing the tissue in 9 vol. of 0·25 M-sucrose for 2 min. in a glass homogenizer. The clearance between the walls was about 0·1 mm. and the pestle was driven by a $\frac{1}{50}$ h.p. motor; the specified speed of the motor (without load) was 2000 rev./min. and it was set at about two-thirds of its maximum speed. The tube was immersed in ice–water during the process.