3. By elution of the dye from the specific insulin spot and quantitative colorimetric comparison with the similar zone from a standard of known potency, the biological potency of the sample can be estimated.

4. Data are recorded on the comparison between biological potency and the potency given by the chromatographic method for samples of crystalline and crude insulins and for solutions of insulin which had undergone partial decay as a result of prolonged storage under unfavourable conditions. Close agreement has been obtained between the mouse-convulsion method and the chromatographic assay. The potency ratio of a single observation, each on test and standard, was found by statistical analysis to have a standard deviation corresponding to $\pm 4.3\%$.

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Protein Breakdown in *Bacillus cereus*

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In several recent studies, the proteins of *Escherichia coli* have been shown to be stable during growth. Thus Cowie, Bolton & Sands (1950) found that when cells which had been labelled with ^{35}S were grown further in unlabelled medium there was no release of labelled sulphur. Koch & Levy (1955), using a different technique for the detection of protein breakdown, concluded that only a small fraction (about 2%) of the total protein of *E. coli* breaks down during the first 2 hr. of growth, and that after this there is no degradation of protein. Again, it has been shown that during the synthesis of the inducible enzyme β -galactosidase, there is no utilization of material from the preformed proteins of the cells (Rotman & Spiegelman, 1954; Hogness, Cohn & Monod, 1955). These observations led Hogness *et al.* (1955) to suggest that not only bacterial proteins were metabolically stable, but that a similar conclusion might apply to the intracellular proteins of mammalian cells.

However, other investigations have been reported which do not support the general conclusion of Hogness *et al.* (1955). Evidence for intracellular-protein degradation in *E. coli* has been presented by Mandelstam (1957, 1958), who showed that whereas the proteins of rapidly growing cells appeared to be stable, those of non-growing cells were broken down to amino acids at the fairly high rate of 5%/hr. A similar value has been reported by Borek, Ponticorvo & Rittenberg (1958). Also, Halvorson (1958*a, b*), working with yeast, has found that protein breakdown in growing cells is only 4% of that in resting cells. Studies with mammalian systems have been carried out by Steinberg & Vaughan (1956) and Steinberg, Vaughan & Anfinsen (1956), who measured the release of labelled phenylalanine, alanine and leucine from rat-kidney and liver preparations, and its inhibition by dinitrophenol, amino acid analogues and during aerobic metabolism. Recently protein turnover in non-multiplying animal cells has been reported by Harris & Watts (1958), who have measured the incorporation and release of [^{14}C]valine in macrophages.

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Pollock & Kramer (1958), in a search for stable intermediates in the biosynthesis of penicillinase by *Bacillus cereus*, studied the formation of the enzyme in cultures of ^{35}S -labelled cells growing in an unlabelled medium. They found that as much as 10% of the sulphur of the enzyme, formed after transfer to the unlabelled medium, was labelled, and were thus led to consider the possibility of protein breakdown occurring in growing populations of *B. cereus*. It is the purpose of this paper to describe and characterize the protein turnover in *B. cereus*. In the experiments to be reported, turnover has been measured by independent determinations of the rates of synthesis and degradation. The former was measured by the incorporation of labelled amino acids into proteins; the latter by growing the cells in labelled medium and measuring the release of labelled amino acids from the proteins after the cells had been transferred to an unlabelled medium. From the present results, protein turnover in *B. cereus* appears to be essentially similar to that in *E. coli*.

METHODS

Micro-organism. The organism used throughout this work was *B. cereus* strain NRRL 569.

Growth media and buffer solution. (a) Hydrolysed casein medium was prepared as described previously by Pollock (1957). (b) Amino acids–glucose medium (AA/G) contained a mixture of amino acids, glucose and mineral salts (Pollock & Kramer, 1958). (c) Buffer solution contained 0.01M- KH_2PO_4 , 0.0167M- MgSO_4 , 0.01M- Na_2SO_4 and 0.03M-glucose. The pH was adjusted to 7.0 by adding 2N-NaOH and checked with a glass electrode. The glucose was added to provide a source of energy.

Inoculum. Hydrolysed casein medium inoculated with 2 or 3 drops of standard *B. cereus* spore suspension containing 3×10^8 spores/ml. (Pollock, 1950) and left overnight at room temperature.

Labelling of cell proteins with ^{35}S . The inoculum culture was diluted 1/10 in AA/G medium containing 0.2 mM- $\text{Na}_2^{35}\text{SO}_4$, giving a cell density of about 0.03 mg. dry wt. of bacteria/ml. The culture was then allowed to grow in a conical flask to 0.5 mg. dry wt./ml., being shaken at 35° at a rate of 100 full oscillations/min. with an amplitude of 7 cm. The cells were harvested and washed with fresh medium for experiments with growing cells, or with the buffer solution for experiments with non-growing cells. The solutions used for washing contained methionine (600 μg /ml.), cystine (300 μg /ml.) and 0.02M- Na_2SO_4 . Cell densities were determined by opacity measurements with a Hilger Spekker absorptiometer and are expressed as mg. dry wt. of bacteria/ml.

Release of labelled methionine from protein of growing cells. Experiments with growing cells were carried out by resuspending washed cells at a concentration of 0.1, 0.3 or 0.5 mg./ml. (see below) in AA/G medium containing 1.5 mg. of DL-methionine/ml. to 'trap' the amino acid released by protein breakdown. In the earlier experiments, 0.3 mg. of L-cystine/ml. was added to dilute any ^{35}S -

labelled cystine that might have been released, either from protein or glutathione, and possibly converted into ^{35}S -methionine. In later experiments it was found that omission of cystine did not affect the results.

The cell suspension was incubated with shaking at 35° . Duplicate samples (1 ml.) were taken at appropriate intervals and diluted with 10 ml. of water. The bacteria were removed by centrifuging and the radioactivity trapped by the carrier methionine was determined (see below).

Release of labelled methionine from proteins of non-growing cells. Experiments with non-growing cells were carried out by resuspending labelled cells at 0.1, 0.3 and 0.5 mg. dry wt./ml. in buffer solution (c), containing 1.5 mg. of DL-methionine/ml. In some experiments, 0.3 mg. of cystine/ml. was added. As with growing cells addition of cystine did not affect the results. The cell suspension was incubated on a shaker at 35° and samples (1 ml.) were taken as described above.

Methionine released during the incubation is expressed as a percentage of the total methionine present in the bacterial protein at the beginning of the experiment.

Isolation of methionine. Methionine from the culture supernatant was isolated by adsorption on a resin (Zeo-Karb 225 in the hydrogen form) and eluted with ammonia (Mandelstam, 1958). The eluate was concentrated by boiling and transferred to Whatman no. 3 paper. Chromatograms were developed for about 18 hr. with butanol–acetic acid–water (63:27:40). Methionine was eluted from the paper with water and, after evaporation, the radioactivity of the dry residue was measured with an end-window counter giving 10^3 counts/min. with a 10^{-3} μC /mg. of carbon standard of infinite thickness on 1 cm.² The recovery of methionine by this method was 80%.

In order to be certain that the methionine thus obtained was not contaminated with other radioactive substances, it was re-isolated in two experiments by paper chromatography, phenol–ammonia being used as the solvent. Since the specific activity was unaltered by this treatment, one-dimensional separation with the butanol–acetic acid solvent was used as a routine.

Incorporation of amino acids into proteins. Unlabelled cells, grown as described above, were resuspended at about 1 mg./ml. in buffer solution (c) containing either 1.5 mg. of DL- ^{35}S -methionine/ml. (specific activity about 75 counts/min./ μg .) or 0.4 mg. of DL- ^{14}C -leucine/ml. (specific activity about 5 counts/min./ μg .). The suspensions were shaken at 35° for 3 hr. The density of the suspensions was measured during the incubation and samples containing about 20 mg. (dry wt.) of cells were taken every hour. The suspension was centrifuged and the cells were resuspended in 2 ml. of water containing 1.5 mg. of unlabelled DL-methionine/ml. or 0.4 mg. of unlabelled leucine/ml.

Protein was isolated by a modification (Mandelstam, 1958) of the method of Schneider (1945). The radioactivity of the dry proteins was measured at infinite thickness in disks 0.3 cm.² From these measurements the amount of methionine or leucine incorporated has been calculated as a percentage of the total methionine or leucine of the proteins.

Methionine and leucine content of proteins of Bacillus cereus. Protein from *B. cereus* was hydrolysed with a mixture of 6N-HCl and 6N-formic acid at 100° for 24 hr. in a sealed tube. After drying, the hydrolysate was dissolved in water and methionine and leucine were estimated by

microbiological assays (Lewis, 1950) with *E. coli* NCTC 8009 (methionine-less) and ML 328 C (leucine-less). Leucine was also determined by isolation on paper with *tert.* amyl alcohol-water (Work, 1949) and then estimated by the quantitative ninhydrin method of Moore & Stein (1948).

The figures obtained were: methionine 1.4%, leucine 5.6% (microbiological assay) and 5.9% (ninhydrin method), and they were used to calculate the amount of amino acids incorporated or released in the experiments described above. Duplicate estimations did not differ by more than 5%.

Catalase estimations. Catalase was measured manometrically at 30°, with 0.2M-H₂O₂ made up in 0.01M-phosphate buffer, pH 7.0, by the method described by Kaplan (1952, 1955). It was confirmed experimentally that the rate of O₂ evolution from H₂O₂ over a period of 3 min. under these conditions was proportional to the concentration of enzyme over the range 35–800 µl. of O₂/hr.

RESULTS

Breakdown of proteins in growing and non-growing suspensions of Bacillus cereus

The breakdown of protein in labelled cells growing in AA/G medium was studied by following the release of [³⁵S]methionine. Fig. 1 shows the release of [³⁵S]methionine under these conditions (see Methods). Bacterial densities, expressed as mg. dry wt./ml., have been plotted logarithmically in the same figure. It can be seen that the rate of release is about 1.5%/hr. during the first 2 hr. of

Fig. 1. Release of [³⁵S]methionine from protein of growing cells. Cells were grown in AA/G medium containing 0.2 mM-Na₂³⁵SO₄. The cells were washed and resuspended in AA/G medium containing unlabelled 0.01M-Na₂SO₄ and methionine (1.5 mg./ml.). The suspension was incubated at 35°, and the radioactivity of labelled methionine released into the supernatant was determined. The results have been expressed as a percentage of the methionine initially present in the bacterial protein. O, Methionine released; Δ, growth. The growth has been plotted as the logarithms of the bacterial density (mg. dry wt./ml.).

growth and less than 0.5% after this time. The average rate of breakdown obtained from 13 experiments was 1.4%/hr. for the first 2 hr. and 0.5% for the third hour of growth. In all these experiments, the rate of release of methionine in the lag phase (not longer than 20 min.) seemed to be the same as during logarithmic growth.

Non-growing bacteria (cells resuspended in the buffer solution c) presented a quite different picture. The release of [³⁵S]methionine from cells incubated in this condition was about five times as great as in the growing cells and continued for at least 4 hr. (Fig. 2). In some experiments, the process was still continuing at an undiminished rate at the end of 6 hr. Experiments with growing and non-growing bacteria were carried out in which the density of the cell suspensions varied from 0.1 to 0.5 mg. dry wt./ml. The rate of protein breakdown both in growing and non-growing cells was unaffected by the bacterial density.

The effect of chloramphenicol upon the release of methionine from non-growing cells was studied by resuspension of labelled cells in phosphate buffer solution and incubation with 5, 10 or 20 µg. of the drug/ml. At the lower concentrations, the rate of release of methionine (in at least two experiments for each concentration) was constant during incubation for over 3 hr., but was only about 50% of that of the control (without chloramphenicol). Fig. 3 shows the results of one experiment performed with 5 µg. of chloramphenicol/ml. during 2 hr. of incubation. With 20 µg. of chloramphenicol/ml. (Fig. 4) the rate observed was 50% of that of the control for the first hour, but after this initial

Fig. 2. Release of [³⁵S]methionine from proteins of non-growing bacteria. O, Methionine released; Δ, bacterial density (mg. dry wt./ml.). Details were as for Fig. 1 except that cells were suspended in buffer solution instead of growth medium.

period protein breakdown was almost completely inhibited.

The effect of temperature on the rate of amino acid release from proteins in non-growing cells was studied over the range 25–45°. The rate increased linearly from 2.3 %/hr. at 25° to 11 %/hr. at 45°.

Fig. 3. Effect of chloramphenicol (5 µg./ml.) on release of [³⁵S]methionine from non-growing *B. cereus* cells. Bacteria labelled with [³⁵S]sulphate were resuspended in buffer solution (see Methods) containing non-radioactive methionine to trap the labelled amino acid released by protein breakdown. The suspension was incubated by shaking at 35°. Samples were removed at intervals and methionine was isolated as described in Methods. The amino acid released is given as a percentage of that initially present in the proteins. ○, Control; ●, with chloramphenicol.

Fig. 4. Effect of chloramphenicol (20 µg./ml.) on the release of methionine from protein of non-growing bacteria. ○, Control; ●, with chloramphenicol. Details were as described for Fig. 3. Methionine released is given as a percentage of the initial methionine content of the protein.

During the second hour the rate of protein breakdown remained linear at 25° and 35° whereas at 45° there was a marked fall. At higher temperatures (60°) the protein breakdown was less than 0.5 %/hr.

Synthesis of protein in non-growing cell suspensions. Washed cells were incubated in the buffer solution containing [³⁵S]methionine or [¹⁴C]leucine. Bacterial protein was isolated at different periods of incubation and its specific activity estimated. The rate of labelling of the protein was 8 %/hr. for methionine and 7 %/hr. for leucine. The rate of amino acid incorporation remained constant during the first 2 hr. of incubation; after this time there was sometimes a fall in the rate.

The addition of 5 µg. of chloramphenicol/ml. to a cell suspension in the buffer solution containing [¹⁴C]leucine inhibited leucine incorporation into the proteins. During the first hour a proportion (2 %) of the leucine residues of the proteins became labelled and thereafter the rate of incorporation was negligible. In order to determine to what extent amino acid incorporation into the cell wall might be contributing to these results, the radioactivity of the cell wall and of the intracellular protein were estimated independently (Mandelstam & Rogers, 1958). It was then found that more than 95 % of the radioactivity was incorporated in the intracellular protein.

Measurement of cell lysis. An attempt was then made to determine whether the observed protein degradation was intracellular or whether it was preceded by cell lysis, which would be indicated by the appearance of endoenzymes in the incubation medium. Preliminary experiments were necessary to choose an enzyme stable enough for the purpose of this experiment. In order to test whether the enzyme chosen (catalase) lost activity during the incubation or was destroyed by the cells, supernatants of lysed *B. cereus* cells were prepared as follows. Cells suspended in the buffer solution were disrupted in a Mickle disintegrator (20 min.) and centrifuged. The supernatant was then incubated with and without whole cells and its enzymic activity measured at intervals. The results showed that catalase was stable in phosphate buffer, and that it was not appreciably destroyed (< 5 %) by washed suspensions of *B. cereus* cells during incubation at 35° for 2 hr.

Having established that catalase was stable enough for the purposes of our test, the following experiment was designed to determine to what extent the release of methionine or leucine could be explained in terms of lysis. Washed cells were resuspended in the buffer solution and incubated. Samples were taken and catalase in the supernatant was estimated. Table 1 gives the results of two experiments showing an average release of

Table 1. Release of catalase from a suspension of non-growing *Bacillus cereus* cells

Cells were grown in AA/G medium, washed twice and resuspended in buffer solution at a concentration corresponding to 0.6 and 0.45 mg. dry wt./ml. (Expts. 1 and 2 respectively). The suspensions were incubated and the supernatant was assayed for catalase activity by measuring the evolution of O_2 . The total catalase present in the cells initially was 890 and 380 μ l. of O_2 /ml. respectively at the beginning of the two experiments. In the last two columns the catalase released by the cells is compared with the methionine released in similar conditions.

Time (hr.)	Catalase activity of supernatant (μ l. of O_2 /ml./3 min.)		Catalase released/hr. Average of 2 experiments (%)	Methionine released/hr. Average of 12 experiments (%)
	Expt. 1	Expt. 2		
0	6	4	—	—
1	12	4	0.3	7
2	14	4	0.1	6.8
3	15	15	1.4	6.8

catalase from non-growing cells of less than 1%/hr. during the first 2 hr. This compares with an average methionine release of 7%/hr. in a series of twelve experiments done under the same conditions.

These results support the contention that the observed breakdown of protein is not dependent on cell lysis and must therefore be largely intracellular.

DISCUSSION

The results here reported show that protein turnover in *B. cereus* cells occurs at different rates in growing and non-growing bacteria. The average rate of amino acid release in growing cells was about 1.5%/hr. and in washed cell suspensions 7%/hr. Similar differences between rates of protein breakdown in growing and non-growing populations, which suggest that proteins are most stable during growth, have been found by Mandelstam (1957, 1958) for *E. coli* and by Halvorson (1958*a, b*) for yeast. It should be noted that when cells were suspended in a full growth medium, breakdown of protein was not affected by the transition from the lag phase to the growth phase. Furthermore, the rate of breakdown decreased markedly after the first 2 hr. of growth. This would suggest one of the following possibilities: (1) during growth, only a fraction of the bacterial protein can break down; (2) the protein breakdown is inhibited by metabolites accumulating in the cells; (3) cofactors or enzymes, needed for protein degradation, are lost. The possibility that during growth the proteins are stable and that only a fraction of them can break down has been considered by Koch & Levy (1955),

who reported that about 2% of the proteins of growing *E. coli* cells are degraded to amino acids in the first 2 hr. of growth. The second possible explanation has been discussed previously by Mandelstam (1958), and was supported by his finding that ammonium salts and threonine inhibit protein breakdown in the conditions used.

The rate of protein synthesis in non-growing *B. cereus* cells is in quite good agreement with the figures obtained for protein breakdown. Mandelstam (1958) has already found a similar correlation between synthesis and breakdown of bacterial protein for *E. coli*.

With regard to the action of chloramphenicol on protein turnover as a whole, it is interesting to contrast its effect on amino acid incorporation and on amino acid release. Almost complete inhibition of amino acid uptake has been obtained with low concentrations of drug (5 μ g./ml.) similar to those required to inhibit growth and penicillinase formation in *B. cereus* (M. R. Pollock, personal communication). Protein breakdown, on the other hand, is only partially inhibited by this concentration. Higher concentrations (Figs. 3, 4) are required to obtain the same order of inhibition. These results agree with the observations reported by Mandelstam (1958); who has found that in *E. coli* protein degradation is less affected by dinitrophenol, azide and chloramphenicol than is protein synthesis.

The possibility has to be considered that any labelled methionine released during the incubation has been derived from non-protein compounds. A possible source is labelled glutathione, which might be present in the cells after growth in labelled sulphate. Breakdown of glutathione would release cystine and this in turn could lead to the appearance of labelled methionine. However, if this reaction occurred to any significant extent, the addition of cystine carrier to the incubation medium would be expected to reduce the amount of labelled methionine which is trapped. In fact, the addition of unlabelled cystine led to the release of labelled cystine into the medium (showing that it was acting as an effective carrier) but had no effect on the amount of labelled methionine recovered. This suggests that cystine released, either from protein or glutathione, is not converted into methionine under the conditions of these experiments.

The possibility that these amino acids were being released only after cell lysis is unlikely in view of the fact that the extent of lysis, as measured by the release of catalase into the medium, is altogether insufficient to account for the amount of protein breakdown which takes place in non-growing cells. The process is therefore largely, perhaps completely, intracellular.

SUMMARY

1. Protein turnover was studied in growing and non-growing cell suspensions of *Bacillus cereus*.

2. The average rate of protein breakdown in growing cells was about 1.4 %/hr. In non-growing cells the rate of both protein synthesis and protein breakdown was about 7 %/hr.

3. Chloramphenicol inhibited both synthesis and degradation of protein in non-growing *B. cereus* cells.

4. Very little cell lysis occurred and it therefore appears that protein turnover is very largely an intracellular process.

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Mitochondrial Preparations from the Leaves of Tobacco (*Nicotiana tabacum*)

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There is abundant evidence that the tissues of higher plants contain mitochondria and that, when these are extracted by methods similar to those used for animal mitochondria, they are associated with the oxidation and phosphorylation reactions of the tricarboxylic acid cycle (Hackett, 1955; Laties, 1957). Most of the studies on this isolation have, however, been concerned with etiolated or non-pigmented tissues. Very few have been done on chlorophyllous tissue, as the presence of plastids and the vacuolar contents make extraction and purification difficult.

Because of these difficulties some workers have concluded that in green leaves many of the reactions of the tricarboxylic acid cycle are not associated with mitochondria (Brummond & Burris, 1954), or that some of them are located in the plastids (Ohmura, 1955; Sissakian, 1955). But recently mitochondria similar to those from etiolated tissues have been prepared from the leaves of peas (Smillie, 1956a, b; Sissakian, Bekima &

Mosolova, 1957) and other plants (James & Das, 1957). Good evidence is presented, especially in the last of these papers, that the oxidations of the tricarboxylic acid cycle are located exclusively in these mitochondria and not in the chloroplasts.

The present work describes the preparation and some of the properties of an extract of tobacco leaves which is sedimented between 1000 and 10 000 g and which brings about many of the oxidations of the tricarboxylic acid cycle. Although no cytological observations on it are presented, it seems likely that it is a preparation, in a damaged and impure form, of leaf mitochondria. It is therefore referred to as such throughout this paper.

EXPERIMENTAL

Plants. Seeds of *Nicotiana tabacum* (White Burley strain) were germinated in a heated greenhouse in John Innes seed compost, and transplanted as soon as possible into John Innes potting compost. The plants were allowed to grow for at least another 5 weeks before they were used.