Protein Turnover and the Formation of Protein Inclusions during Sporulation of *Bacillus thuringiensis*

By R. E. MONRO*
Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge

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During sporulation of *Bacillus thuringiensis*, a spore and a protein parasporal inclusion are formed within each cell (Hannay & Fitz-James, 1955; Hannay, 1956). The parasporal inclusions are of a regular, dipyramidal form and are referred to as 'crystals'. Similar 'crystals' are formed by a number of related strains of *Bacillus*, all of which are pathogenic to insect larvae. The crystals are composed of protein, and contain no nucleic acid or carbohydrate. The crystals are insoluble in water and do not disperse until the pH is raised to a value above 11·8. After such dispersal, the crystal protein remains in solution over a range of pH values from 6 upwards.

It has been shown (Monro, 1961) that normal sporulation with concomitant formation of protein crystals can take place during incubation under suitable conditions in a nutrient-free medium. It has further been shown that crystal protein antigens are absent from vegetative cells and arise over the same period as the crystals during sporulation. It is thus clear that the crystal protein must be derived from material already present in the cells before sporulation but that such precursor material is antigenically unrelated. In the present paper, chemical analysis and tracer techniques have been employed to investigate whether crystals are formed through modification of antigenically unrelated protein or polypeptide components, or whether proteins break down during sporulation, supplying amino acids for synthesis of the crystal protein. With the aid of a serological method, which has been developed for the rapid, reproducible separation of the crystal protein, it has been found that the crystal protein is synthesized from amino acids during sporulation, and that a process of protein turnover takes place. These results are in agreement with related observations of Young & Fitz-James (1959), who have shown by somewhat similar techniques that the crystal protein of a related strain is synthesized during sporulation.

Further studies show that a general process of protein turnover occurs during sporulation under the conditions employed and that, as well as the crystal protein, a variety of other new proteins are also synthesized. The subject of protein turnover has been reviewed by Mandelstam (1960).

* Present address: Institute for Molecular Biology, Cambridge.

METHODS

The strain of *B. thuringiensis* used throughout the present study, as well as the cultural and sporulation conditions, are described elsewhere (Monro, 1961). Cells were grown in a liquid casein-digest medium to the early stationary phase of growth. The washed cells were then resuspended in a nitrogen-free, buffered salts medium and incubated with aeration. Synchronous sporulation took place in over 75% of the cells within 10 hr.

Light-microscopy, ultrasonic treatment and methods used for the preparation of parasporal inclusions are described elsewhere (Monro, 1961).

Dry weight. Samples of culture were centrifuged in tared tubes. The cells were washed once with water and the sediments dried for 8 hr. at 105°. After cooling over H_2SO_4 the tubes with samples were weighed.

Free amino acid pool. Samples of culture were rapidly filtered on Oxoid membrane filters and washed twice with M-NaCl. The filter was then removed to a centrifuge tube containing cold 0·2N-$HClO_4$ and the cells were dispersed. After 30 min. at 0° the tubes were centrifuged and the ninhydrin-reacting material was estimated by the method of Yemm & Cocking (1955). Results are expressed as μmoles of free amino acids by reference to an alanine standard. Chromatography of extracts showed that over 90% of the ninhydrin-positive material consisted of free amino acids.

Nucleic acid estimation. DNA was estimated by the method of Burton (1956). RNA was estimated from the extinction at 260 mμ of $HClO_4$ extracts of cells (Ogur & Rosan, 1950). Hot and cold extracts were combined since RNA was only partially extracted in the cold. The absorption spectrum of such extracts over the range 215–300 mμ was measured and shown to be typical of nucleic acids. Absorption due to DNA was allowed for in calculating the RNA content. The extinction coefficient of a 0·1% solution of RNA, treated with $HClO_4$ as above, has been assumed to be 28 (Gale & Folkes, 1953).

Alkali fractionation. This has been carried out for spores and crystals by a modification of the method used by Fitz-James, Toumanoff & Young (1958). Sporulated cultures were stored at 5° for 1–2 days, under which conditions vegetative-cell walls and membranes disperse, releasing the spores and crystals. Remaining debris was removed by repeated centrifuging, under conditions adjusted to sediment the spores and crystals but to leave debris in the supernatant. The initial centrifuging and wash were per-

formed at 5° to avoid germination, which is stimulated by the presence of the accumulated breakdown products (Halvorson, 1958). Crystals were removed quantitatively from such spore–crystal mixtures by extraction with alkali. Spores and crystals were suspended in 0·1 N-NaOH, washed once with 0·1 N-NaOH and then twice with water. Viable spores were left behind and there was no trace of the crystals remaining in the sediment. The supernatants were combined and the crystal protein was precipitated by adjustment of the pH to a value of 5·0.

Peptide 'map'. Hydrolysates obtained by tryptic digestion of crystals were examined by high-voltage paper electrophoresis under toluene. Crystals were suspended in citrate–phosphate buffer, pH 3 (McIlvaine, 1921), incubated for 10 min. at 100° and then washed with water. The denatured crystals were resuspended at a concentration of 5 mg. dry wt./ml. in 2% (w/v) NH_4HCO_3 containing trypsin (50 μg./ml.). The suspension was incubated at 38° for 100 min. The freeze-dried digest was subjected to electrophoresis on Whatman no. 1 paper, with a buffer that consisted of pyridine (200 ml.), acetic acid (8 ml.) and water to 2 l. (pH 6·5). It was run for 120 min. at 40 v/cm.

Serological separation of crystal protein. Disrupted cells were extracted with alkali, the insoluble residue (spores and some cell walls) was taken as the spore fraction, and the crystal fraction was obtained by treatment of the supernatant with crystal antiserum. The procedure adopted was as follows: the sample of culture (0·25–0·5 ml.) was exposed to ultrasonic vibration for 1 min. in a volume of about 1 ml. (rods disrupted but crystals and mature spores left intact) and then centrifuged in a 3·5 ml. tube. The supernatant was discarded and the sediment suspended in 0·1 ml. of 0·1 N-NaOH and incubated for 2 hr. at about 20°. The pH was then brought to 7·5 by addition of 0·2 ml. of phosphate buffer, and the suspension was centrifuged. The sediment was taken as the 'spore fraction'. Serum [0·5 ml.; third course anti-crystal serum (Monro, 1961) diluted tenfold with 0·9% NaCl] was added to the supernatant, and the mixture incubated for 1 hr. at 37° with occasional gentle agitation; the suspension was then left overnight at 5°. After centrifuging, the supernatant was discarded, and the sediment taken as the 'crystal fraction'. For estimation of radioactivity spore and crystal fractions were suspended in N-NH_3 solution and dried on planchets.

By use of a purified preparation of ^{14}C-labelled crystals to which unlabelled, sporulated culture was added in variable amount, it was shown that all of the crystal protein was extracted from the spore fraction and over 80% recovered in the crystal fraction. The specificity of the method was also investigated. A culture was labelled with [^{14}C]phenylalanine and samples were taken before and after sporulation. Unlabelled, sporulated culture was added as carrier and the crystal fraction isolated. The amount of radioactivity found in the crystal fraction from the vegetative cells was less than 20% of that from the sporulated cells. It is probable that the small amount of protein recovered in the crystal fraction from vegetative cells was due mainly to the presence of small amounts of vegetative-cell antibodies in the serum (Monro, 1961).

Radioactivity of protein. The protein fraction was isolated by a modification of the method of Schneider (1945). The sample was centrifuged and the sediment suspended in 5% trichloroacetic acid containing about 2 mg. of DL-phenylalanine/ml. After incubation at 90° for 20 min., the suspension was centrifuged, and the sediment washed with acidified ethanol and with ether (radioactivity in ethanol and ether supernatants was negligible). The sediment was allowed to dry and was then dispersed in 250 μl. of N-NH_3 solution and left overnight at 5°. A sample (200 μl.) was dried on a planchet.

RESULTS

Chemical analysis. Estimates of the intracellular content of free amino acids during growth and sporulation are shown in Table 1. The content of free amino acids in sporulating cells was less than that in growing cells. There was a significant increase in intracellular amino acids over the initial stages of sporulation, and during the later stages there was a fall.

An estimate was made of the dry weights of crystals and spores formed during sporulation. A culture was allowed to sporulate. The spores and crystals were freed from vegetative-cell remnants and then separated into spore and crystal fractions by alkali treatment (see Methods). Duplicate dry-weight determinations were made (*a*) on cells immediately after transfer, (*b*) on the spore–crystal fraction, (*c*) on the spore fraction, and (*d*) on the precipitated crystal protein. All fractions were washed once with water before drying. Electron-microsopic examination showed that the fractions were nearly pure.

Table 2 shows the results of the analyses. The crystal fraction, estimated from the precipitated protein, amounted to only about 73% of the estimates obtained by difference. The actual amount of crystal protein presumably lies between the extremes of these two estimates. Table 2 also shows the results expressed as percentage of the initial dry weight of cells and a correction is applied to

Table 1. *Intracellular content of free amino acids*

For analytical procedure, see Methods.

Culture	Time after transfer to sporulation medium (hr.)	Intracellular amino acids* (μmoles/g. dry wt. of cells)
Exponential	—	340
Exponential	—	404
Sporulating	0	144
	0	141
	2	175
	2	174
	4	164
	6	168
	6	167
	8	142
	8	131

* For sporulating cells, amino acid content is referred to as dry wt. of cells in culture at time 0.

Table 2. *Dry weights of cells, spores and crystals*

The procedure followed is described under Methods.

	Dry weight (mg./ml. of culture)	Percentage of initial dry weight	
		Actual	Corrected*
(*a*) Cells at time of transfer	1·73 1·75	100	100
(*b*) Spore–crystal fraction	0·42 0·42	24·1	32·0
(*c*) Spore fraction	0·198	11·4	15·2
(*d*) Crystal fraction by difference (*b* − *c*)	0·22	12·6	16·8
(*e*) Precipitated crystal protein fraction	0·149 0·171	9·2	12·3

* Cell counts on the same culture showed that only about 75 % of the cells gave rise to spores and crystals. Hence a correction factor of 100/75 is employed.

Table 3. *Deoxyribo- and ribo-nucleic acid content*

Estimates are expressed as percentages, referred to the dry wt. of cells at the time of transfer to the sporulation medium.

Time after transfer to sporulation medium (hr.)	DNA (%)	RNA (%)
0	1·18	6·06
	1·14	6·03
2	1·10	4·87
	1·08	4·88
4	1·06	4·25
	1·06	4·33
6	1·04	4·08
	1·04	4·03
8	1·02	3·84
	1·00	3·90

Table 4. *Incorporation of added amino acids during growth*

A sample (0·5 ml.) of culture (early exponential phase) was transferred to a 25 ml. specimen tube containing the labelled amino acid (5–15 c/mole); subsequent growth and sporulation were as normal. Radioactivity in spore and crystal fractions was measured after separation by the serological method. The amount of each amino acid added was 80 000 counts/min.

	Counts/min. recovered in	
Amino acid	Crystal fraction	Spore fraction
Aspartic acid	240	1470
Glutamic acid	370	2950
Proline	140	245
Leucine	110	215
Isoleucine	70	140
Phenylalanine	150	325
Tyrosine	80	80
Serine	160	925
Threonine	870	2085

allow for cells that did not sporulate. Estimates are minimal in view of the unknown loss (less than 25 %) of spores and crystals during differential centrifuging. The spore fraction is subject to a further underestimate as a result of germination and autolysis which occurred in an undetermined percentage of spores during storage at 5° and after ultrasonic treatment. Bearing these errors in mind, it is clear that the dry weight of the average spore and crystal amount to over 15 % and 12·5–17 % respectively of the dry weight of the cell from which they are formed. It can also be calculated that less than 15 % of the crystal protein could arise from free amino acids present in cells at the time of transfer to the sporulation medium.

Table 3 shows that the DNA content of cultures remains nearly constant over the course of sporulation. In contrast, RNA content falls by about 40 %.

Tracer studies

^{14}C-Labelled amino acids, assimilated by cells during vegetative growth, are found in the crystals that are formed during subsequent sporulation (Table 4). Such incorporation is to be expected, since crystals are derived entirely from material present in vegetative cells. However, amino acids added during sporulation are also incorporated into the crystal protein (Table 5). It therefore follows that at least part of the crystal protein is synthesized *de novo* from amino acids during sporulation.

Peptide 'map'. Models can be envisaged in which one part of the protein crystal is synthesized during vegetative growth and a distinct part during sporulation: namely (i) a protein present in vegetative cells is modified by attachment of additional amino acids to it during sporulation, or (ii) the crystals are composed of two proteins, one of which is synthesized during growth and the other during sporulation. Such possibilities have

Table 5. *Incorporation of amino acids added during sporulation*

Sporulating culture (0·25 ml.) was pipetted into a 25 ml. specimen tube containing the labelled amino acid (5–15c/mole). After a period of incubation, excess of non-radioactive amino acid was added (giving a final concentration of about 2 mg./ml.) and incubation continued until sporulation was complete. Spore and crystal fractions were separated by the serological method and counted.

Amino acid	Period of exposure (time after transfer) (hr.)	Counts/min. added	Counts/min. recovered in Crystal fraction	Counts/min. recovered in Spore fraction
Glutamic acid	2–10	33 000	738	1380
Proline	2–4	30 000	834	1184
Phenylalanine	2–2·5	15 000	493	360

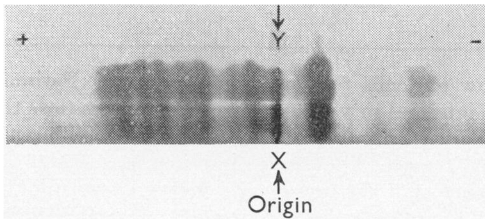

Fig. 1. Peptide 'maps' of labelled crystals. Crystal preparations: X, Cells grown in the presence of [^{14}C]glutamate (10 μc added to growth medium) and then sporulated as usual; Y, unlabelled culture sporulated in the presence of [^{14}C]glutamate (5 μc added 3·5 hr. after transfer). Crystals were separated from both cultures by the method of isopycnic centrifuging. Peptide 'maps' were prepared as described under Methods. Tryptic digests of the crystal preparations were put on the paper in two adjacent 1·2 cm. strips (at points indicated by arrows). After electrophoresis, a radioautograph was taken. In taking the photograph of the radioautograph (shown here), it was necessary to expose the bands from preparation X for a longer period than those from Y. Hence the slight discontinuity in certain regions of the photograph and the differences in background density.

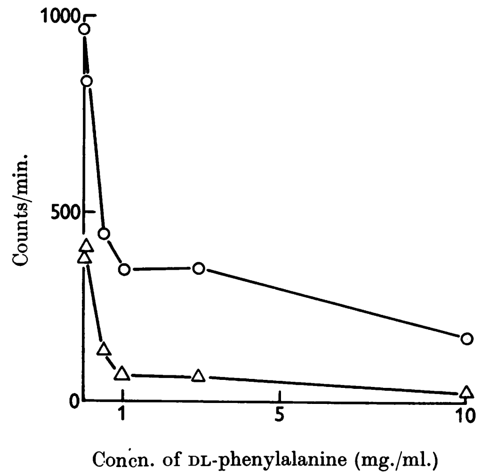

Fig. 2. Radioactivity of spore fractions (○) and crystal fractions (△) after sporulation of cells prelabelled with [^{14}C]phenylalanine: effect of addition of [^{12}C]phenylalanine to the sporulation medium. Cells were prelabelled with [^{14}C]phenylalanine by the procedure described in the text. Samples (0·25 ml.) were then sporulated in the presence of various concentrations of [^{12}C]phenylalanine. After sporulation was complete, excess of [^{12}C]phenylalanine was added and the radioactivity of the spore and crystal fractions estimated by the serological method.

been investigated by studying incorporation into specific regions of the crystal protein molecule(s). Crystals were prepared by the method of isopycnic centrifuging (Monro, 1961) from cells labelled by addition of [^{14}C]glutamate (*a*) during vegetative growth and (*b*) during sporulation. Tryptic digests of the crystals were subjected to electrophoresis on paper and the resulting distribution of tracer was examined by radioautography. Fig. 1 shows that the same peptides are labelled whether [^{14}C]-glutamate is added during growth or sporulation. It can be concluded that incorporation of amino acids added during sporulation results from a general synthesis of crystal protein and not the synthesis of a specific part of the crystal protein. It is not clear from these results what percentage of the crystal protein is synthesized during growth and what percentage during sporulation.

Labelling of vegetative cells. In subsequent experiments cells were labelled with [^{14}C]phenylalanine. Unless otherwise stated, cells from exponential phase culture were resuspended in a synthetic medium containing 17 amino acids (lacking phenylalanine). After incubation for 10 min., [^{14}C]phenylalanine (giving 24 μg./ml. and specific activity as required) was added. After further incubation for 1 hr. cells were resuspended in growth medium. Subsequent growth and sporulation were normal. Incubation after retransfer to the growth medium was for approximately 4 hr. and there was a three- to four-fold increase in dry weight. Chromatography and radioautography of hydrolysates of cells labelled

in this way showed that approximately 80 % of the label was present as phenylalanine, 10 % as tyrosine, and 10 % as glutamic and other amino acids.

Transfer experiment. It is clear from the 'peptide map' experiment described above, that there is at least some *de novo* synthesis of crystal protein during sporulation. The extent of such synthesis has been investigated by studying the incorporation of tracer into crystals during sporulation of prelabelled cells. It should be possible to prevent the transfer of a given labelled amino acid from old to newly synthesized protein by 'swamping' with excess of the corresponding unlabelled amino acid. The percentage fall in radioactivity of the crystal under such conditions would then give a measure of the percentage of the crystal protein that is newly synthesized.

Vegetative cells have been labelled with [^{14}C]-phenylalanine and then sporulated in the presence or in the absence of unlabelled phenylalanine. Fig. 2 shows that the crystals formed in the presence of unlabelled phenylalanine were of greatly reduced radioactivity. Thus the counts/min. were reduced by 85–90 % when DL-phenylalanine was added at concentrations of 1–10 mg./ml. The corresponding counts in the spore fraction were lowered by 70–80 %. Microscopic examination revealed no differences between sporulation in the presence and in the absence of phenylalanine. The possibility is thus eliminated that the fall in radioactivity of the crystal fraction was due to adverse effects of phenylalanine upon sporulation. It can be concluded that at least 80 % of the crystal protein is synthesized *de novo* during sporulation from amino acids which arise through the breakdown of other proteins.

General turnover of protein during sporulation. This was followed by measuring the radioactivity of the total protein and by taking samples at intervals rather than just at the end of sporulation. By this means estimates can be made for the breakdown of protein as well as for the synthesis of new protein (Fig. 3). Cells were labelled with [^{14}C]phenylalanine, and sporulated in the presence or in the absence of unlabelled amino acids (D- and L-phenylalanine, L-tyrosine and L-glutamate at concentrations of 10 mM each). Samples were

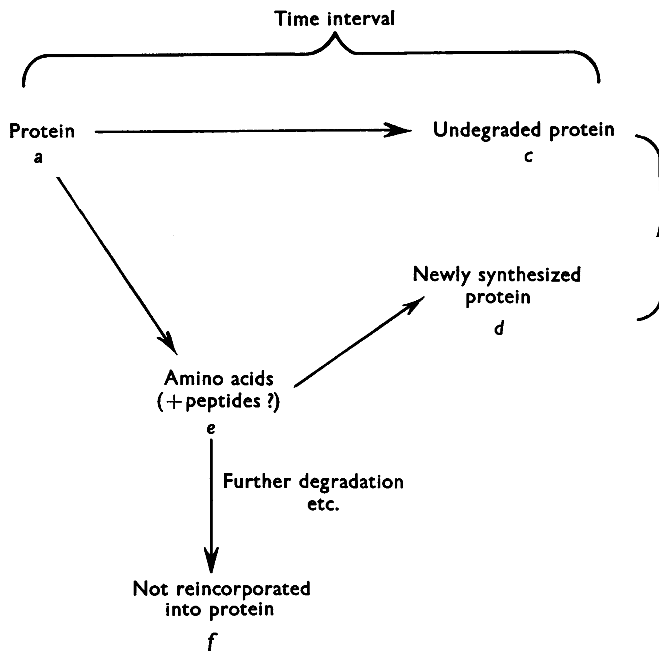

Fig. 3. Scheme to illustrate the method used for estimation of protein turnover. The protein is initially labelled with a [^{14}C]amino acid 'A', and the cells are incubated in the presence or in the absence of excess of unlabelled 'A'. In the former case any labelled 'A' is diluted by the added 'A' and reincorporation of the label into new protein thus prevented. If, then, radioactivity is taken as a measure of the amount of protein containing labelled 'A', direct estimates are made for a (initial protein), b (labelled protein after incubation in the absence of unlabelled 'A'), and c (labelled protein after sporulation in the presence of 'A'). Further: newly synthesized protein $= b-c$; protein broken down $= a-c$; amino acid not reincorporated $= a-b$. Assumptions involved in this scheme are considered in the Discussion.

taken at intervals and the radioactivity of the protein was estimated (see Methods).

Both microscopic examination and analysis for protein (Lowry *et al.* 1951) revealed no differences between cells that sporulated in the absence and in the presence of added amino acids. It is therefore improbable that the presence of the added amino acids had an appreciable effect on the rates of breakdown or resynthesis of proteins in the sporulating cells.

The course of turnover during sporulation is shown in Fig. 4. In the presence of added amino acids there was a continued, rapid fall in the counts/min. in the protein over the first 8 hr. of incubation. There was much less fall with the unsupplemented medium. In Fig. 5 the results are plotted in a different way, showing the course of breakdown and resynthesis. The rates of breakdown and resynthesis were both about 10%/hr. over the first 2 hr. After 2 hr. these processes continued at a diminished rate. The rate of synthesis fell more rapidly than the rate of breakdown, the difference in rates being accounted for by breakdown products' not being reincorporated into the protein fraction. After 8 hr. little further synthesis took place, whereas breakdown continued at a slow rate (about 3·3%/hr.) between 8 and 13 hr. Estimates for breakdown and resynthesis at 13 hr. amount to 67 and 52% respectively. As indicated, estimates for turnover have varied somewhat from one experiment to another. In particular, cultures occasionally lysed to a greater extent than normal and the amount of resynthesis was then correspondingly lower. These results are considered further in the Discussion.

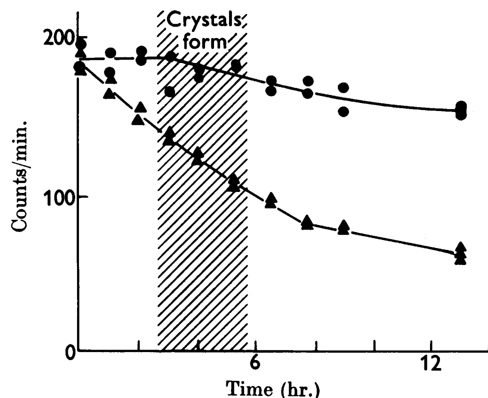

Fig. 4. Turnover of protein during sporulation. Cells were labelled with [14C]phenylalanine and then sporulated in the presence or in the absence of unlabelled amino acids (D- and L-phenylalanine, L-tyrosine and L-glutamate at concentrations of 10 mM each). Samples were taken at intervals for the estimation of radioactivity in the protein fraction. Each point represents a single determination. ●, No amino acids added to sporulation medium; ▲, amino acids added. Cell counts at 13 hr. showed that about 80% of the cells had sporulated, whereas the others appeared to be cell walls without contents.

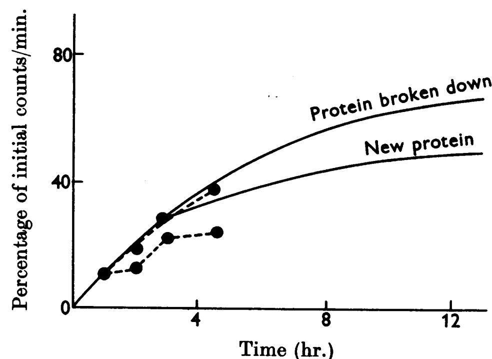

Fig. 5. Protein breakdown and resynthesis during sporulation. Calculated (see Fig. 3) from results shown in Fig. 4. Results are also shown, ●- - -●, of another experiment in which there was more cell lysis than normal.

In order to gain information about the nature of the proteins that break down during sporulation, estimates of breakdown have been made on cells labelled at different phases of growth. [14C]-Phenylalanine was added directly to the growth medium in order to eliminate the possibility of abnormal distribution of protein synthesis during incubation in the synthetic medium (used for incorporation in other experiments). The results (Table 6) show that proteins synthesized during the

Table 6. *Breakdown of protein: effect of labelling at different phases of growth*

Samples (2·5 ml.) of culture (turbidity 0·02 arbitrary unit) were pipetted into each of three flasks and 0·5 μc of [14C]phenylalanine was added to the first flask. When the turbidity reached 0·20, the first and second cultures were centrifuged, the supernatants exchanged and the cells resuspended. The second and third cultures were 'exchanged' in a similar manner when the turbidity reached 0·40. Cultures were transferred at the usual stage of growth to the sporulation medium to which phenylalanine, tyrosine and glutamate were added, as in the previous experiment. Samples (0·5 ml.) were taken at 0 and 5 hr. after transfer for estimation of radioactivity in the protein.

Turbidity at period of exposure to tracer	Counts/min. 0 hr.*	Counts/min. 5 hr.*	Undegraded protein (% of initial counts/min. remaining in protein) at 5 hr.*
0·02–0·20	105	59	55
	117	63	
0·20–0·40	73	52	63
	74·5	41	
0·40–0·65	140	108	78·5
	129	104	

* Time after transfer.

log phase of growth, as well as those synthesized during the transition and early stationary phases, break down during sporulation. There is some indication that proteins synthesized in the early stationary phase break down to a lesser extent than those synthesized in the log phase, but this difference is probably not significant.

DISCUSSION

The chemical analyses show that less than 15% of the synthesis of crystal protein could be accounted for by the free amino acids present in cells before sporulation. Serological work reported elsewhere (Monro, 1961) shows that the crystals are not formed by simple crystallization of protein present in vegetative cells. Further, the 'peptide map' experiment shows that formation of the crystals does not take place through synthesis of one part of the protein crystal during vegetative growth and a distinct part during sporulation. The transfer experiment gave a value of 80% for the amount of crystal protein synthesized *de novo* from amino acids during sporulation. This figure is a minimal estimate in view of the following possible sources of error: (*a*) incomplete equilibration between intracellular and added amino acids; (*b*) approximately 20% of the phenylalanine is converted into other amino acids during incorporation. The transfer of label in the form of these other amino acids to newly forming protein would not necessarily be 'swamped' by the addition of unlabelled phenylalanine since rapid interconversion would be necessary for such 'swamping' to be efficient; (*c*) the precipitation of small amounts of vegetative-cell proteins in the crystal fraction; this contamination may have been as much as 20% of the fraction (see Methods) and could by itself account for the residual counts obtained in the crystal fraction of cells that sporulated in the presence of phenylalanine.

It can be concluded that most, if not all, of the crystal protein is synthesized from amino acids during sporulation. In conjunction with the chemical analyses these results lead to the view that amino acids used for synthesis of the crystal protein are supplied through a breakdown of protein. Crystals, then, can be considered to be formed by a process of 'protein turnover'.

Errors in the time-course experiment could have arisen from the method of labelling used. Thus the label was incorporated only at a particular phase of growth and under abnormal conditions. Such conditions could have led to labelling of only particular protein fractions of the cell. Similar amounts of breakdown to those estimated in the above experiment were, however, estimated for cells labelled at a comparable phase of growth by addition of [^{14}C]phenylalanine to the normal growth medium, and it can therefore be concluded that error did not result from abnormality of labelling conditions. There is a possibility, however, that proteins synthesized during the exponential phase of growth are more labile during sporulation than those synthesized during the transition phase. From the figures in Table 6 it is estimated that this effect could lead to as much as 15% overestimation of turnover.

There is a possibility that proteins synthesized during sporulation are subject to breakdown as are the other proteins in the cell. If this is the case, then the actual amounts of turnover will have been underestimated but the estimates will apply to the net amounts of new and old proteins, referred to the protein content of cells at the time of transfer.

The [^{14}C]phenylalanine content of proteins has been taken as an estimate of the amount of protein containing the label. If there are different percentages of phenylalanine in the initial protein, in the protein that breaks down and in the protein that is resynthesized, then resulting estimates for breakdown and resynthesis would be correspondingly biased.

It is considered by some workers (Young & Fitz-James, 1959) that normal sporulation cannot take place in nutrient-free media. It is held that sporulation in such systems only takes place through the supply of nutrients by lysis of a portion of the cells. However, evidence has been presented elsewhere (Monro, 1961) that normal sporulation took place in the system used in the present studies. All of the cells could be accounted for by microscopic cell counts, and sporulation took place in over 75% of the cells. Negligible cell lysis took place during the early stages of sporulation in many experiments. It might be argued that the conservation of cell numbers could be explained on a basis of turnover on a cellular level, but this possibility is eliminated by the high degree of synchrony observed in sporulating cultures. Further evidence for normal sporulation is provided by the DNA estimations presented in this paper, which are closely similar to those obtained by Young & Fitz-James (1959) for cells sporulating in a nutrient medium.

In the time-course experiment on protein turnover described in Fig. 5, negligible cell lysis was observed during the first 2 hr. It follows that turnover estimations for this period are not confused by lysis. After 2 hr., however, internal lysis took place in a small percentage of the cells, and this lysis amounted to about 20% at 10 hr. Lysis will lead to underestimation of the amount of resynthesis and overestimation of the amount of breakdown that takes place in healthy cells.

Further, the possibility arises that turnover is not entirely an intracellular process, but that synthesis of new protein takes place partly through utilization by sporulating cells of breakdown products from cells which lyse. However, the amount of new protein synthesized could not be accounted for by the amount of cell lysis observed, and it seems probable that turnover taking place during the late as well as early stages of sporulation is an intracellular process. Detailed calculations (Monro, 1959) show that all of the synthesis during sporulation could be accounted for by intracellular turnover, but breakdown products of lysed cells could also have contributed to making up to 50 % of the new protein. It is also estimated that during sporulation of a cell, new protein is synthesized, which amounts to about 50 % of the protein present in the cell at the time of transfer.

The studies on protein turnover show that crystal formation is part of a general process occurring during sporulation, in which there is a radical transformation of the proteins of the cell. Estimates for the initial rate of turnover (about 10 %/hr.) are of the same order as those reported by Urbá (1959) for *Bacillus cereus*, incubated in a nutrient-free medium (about 7 %/hr.). Estimates for the extent of turnover during the whole of sporulation are about 50 % and, if correct, indicate a higher degree of protein turnover than in any system previously reported.

Foster & Perry (1954) have shown that protein degradation and resynthesis occur during endotrophic sporulation of *Bacillus mycoides* but this work is open to some criticism (Young & Fitz-James, 1959). It is probable that bacilli, capable of sporulation, are particularly well equipped for turnover in view of the radical intracellular changes during sporulation, which often takes place under conditions of poor nutrition. An association of well-developed machinery for turnover with the capacity to sporulate would fit in with the higher rates of protein turnover found for *Bacillus cereus* (Urbá, 1959) than for *Escherichia coli* (Mandelstam, 1958). It is possible that, concomitant with the turnover which Urbá observed in resting cell suspensions, the cells underwent the initial stages of sporulation.

The extent of protein breakdown which occurs during sporulation shows that much of the protein of the cell must be susceptible to breakdown rather than just a particular fraction. Further, breakdown is not confined to proteins synthesized during a particular phase of growth. It is probable that the breakdown of RNA during sporulation is even greater than that indicated by the analyses in view of the report by Fitz-James *et al.* (1958) that there is a turnover of RNA during sporulation of *Bacillus cereus* var. *alesti*. The RNA breakdown suggests a general disintegration of ribonucleoprotein particles and there could be a concomitant breakdown of the protein of these particles.

SUMMARY

1. Studies have centred around the formation of protein, crystalline, parasporal inclusions and general protein metabolism in cells of *Bacillus thuringiensis* during sporulation in a nutrient-free medium. Analysis of the metabolic pool shows that insufficient free amino acids to account for synthesis of all the crystal protein are present at the time of transfer to the nutrient-free medium. ^{14}C-Labelled amino acids, added either during growth or sporulation, are incorporated into the crystals. [^{14}C]Glutamic acid, whether it is added during growth or sporulation, is incorporated into the same positions in the polypeptide chains of the crystal protein. When cells are prelabelled with [^{14}C]phenylalanine, the addition of unlabelled phenylalanine to the sporulation medium prevents incorporation of label into the crystals. It is concluded that the crystal protein is synthesized from amino acids during sporulation and that, under the conditions employed, these amino acids are derived largely from the breakdown of vegetative-cell proteins. Further studies show that there is a high percentage of intracellular protein turnover during sporulation, and that other proteins, in addition to the crystal protein, are synthesized.

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