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Metabolism of Sulfur-Containing Amino Acids during Sporulation of *Bacillus subtilis*

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Contents

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Introduction

In spore-forming bacteria such as *Bacillus* and *Clostridium* dormant spores are formed in the cells when the environmental and intrinsic conditions are appropriate. Since bacterial spores are extremely resistant to heating, drying, irradiation and chemical agents, they have attracted the attention of microbiologists, in relation to sterilization and preservation of foods.

Not only from the practical point of view but also from basic aspects cencerned with the cell differentiation and morphogenesis the bacterial spore has been studied by many microbiologists and molecular biologists. Several monographs and reviews published recently are dealing with various aspects of bacterial sporulation[1)2)3)4)5)6)7)8)9)10)].

The process involved in the formation of a bacterial spore is diagramammatically illustrated in Fig. 1. As will be seen in this figure the sporulation commences after the cease of logarithmic growth. As the first morphological change during sporogenesis, it can be observed that chromatin bodies extend along the main axis of the cell to form axial threads. Successively, the plasma membrane invaginates from both sides of the cell and surrounds a part of the nuclear materials and the cytoplasm to form the forespore compartment. The inner and outer membranes of the forespore are separated by the accumulation of cortical materials such as mucopeptide, Ca^{2+} and dipicolinic acid (Ca-DPA). After the beginning of the cortical structure formation, a discontinuous deposition of coat materials

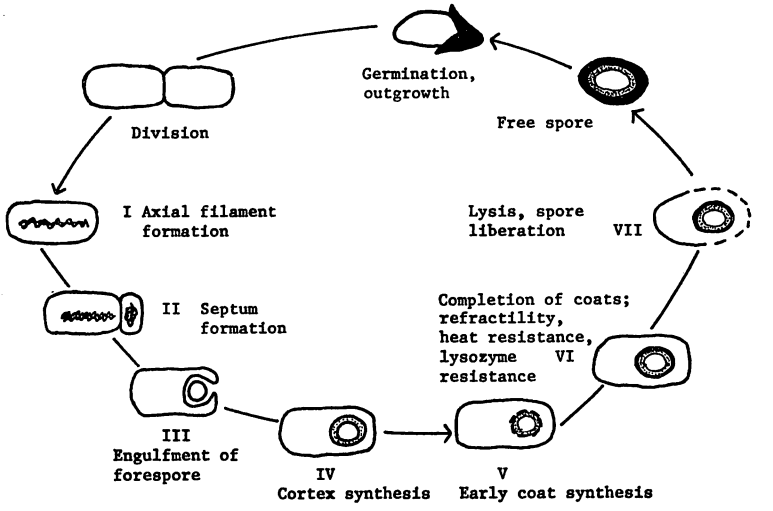

Fig. 1. Life cycle of a sporulating bacillus.

is found around the outer menbrane of the forespore. The mature spore is finally freed from the sporangium by autolysis of the mother cell.

The spore coat among these spore structures occupies about a half of the whole spore in dry weight. The coat fraction is composed of rigid protein with a small quantity of inorganic matter and mucopeptide. The spore coat formation, therefore, is considered to be one of the most important events in the sporulation process.

Many investigators have reported that spore coat is made up mainly of protein which contains all the ordinary amino acids and that the cystine content of spore coat protein is higher than that of vegetative cell protein. For instance, VINTER [11)12)13)14)] reported that spores contained about 26 μg cystine sulfur/mg protein nitrogen and most of cystine was found in the coat fraction while vegetative cells had only about 7 μg cystine sulfur/mg protein nitrogen. KADOTA et al[15)16)] reported that the spore coat of *Bacillus subtilis* after being treated with hydrolytic enzymes contained 292 μg cystine plus cysteine S per mg N, similarly to wool or hair keratin. KONDO and FOSTER[17)] fractionated the spore coat of *Bacillus megaterium* into three fractions, an alkaline soluble fraction, a paracrystal fraction and a resistant residue fraction and found that the paracrystal fraction, the middle particular layer of coats sandwiched with other two fractions, contained a keratin-like basal substance judging from its X-ray diffraction pattern. Recently, ARONSON et al[18)19)20)] have reported that the outer layer of spore coat becomes apparent at the time of the increased cystine incorporation into spore coat and that a large fraction of the cystine incorporation into the outer coat is due to sulfhydryl interchange reactions, which could lead to an altered conformation of the coat polypeptides.

The spore coat is almost insoluble to various chemicals e. g. 8 M urea, N-NaOH, performic acid, phenol and surface-active compounds, but it is solubilized by alkaline thioglycollate or dithiothreitol[18)20)21)]. It has also been reported that the SH reagents stimulated germination of the spore. These facts suggest that the spore coat is composed of chemically stable disulfide-rich proteins.

In the course of studies on the formation of spore coat protein of *B. subtilis*, UCHIDA[22)] revealed that the greater part of sulfur present in vegetative cells was in the form of methionine as a constituent of water soluble proteins and in spores the major part of the sulfur was present in the form of cystine which constituted keratin like protein of the spore coat. The total amount of cystine plus cysteine increased in parallel with the progress of sporulation.

Under such circumstances, the present study was undertaken to elucidate the metabolism of sulfur-containing amino acids and its regulation mechanism during sporulation of *B. subtilis*.

Part I of this study deals with the metabolism of sulfur-containing amino acids and Part II is concerned with the regulation of key enzymes participating in the metabolism of sulfur-containing amino acids.

Part I

Chapter I. Changes in Concentration of Sulfur-Containing Amino Acids during Sporulation

The spore coat is composed of protein in which cystine and cysteine are contained in particularly high concentrations. The cystine plus cysteine content in spores is about five times than that of vegetative cells[12)]. In vegetative cells the greater part of intracellular sulfur is present as methionine in the form of soluble protein,[15)].

In the experiments described in this chapter, changes in the concentrations of methionine and cystine in the cells during sporulation of *Bacillus subtilis* have been examined in an attempt to elucidate the mechanism involved in the formation of the cystine-rich protein of the spore coat. Various sulfur-containg amino acids which are present as the free forms have also been analyzed at different stages of sporulation by use of the paper chromatography and an amino acid autoanalyzer to know the general metabolic pathway of sulfur-containing amino acids in this organism.

Materials and Methods

Organism and cultural conditions. The Marburg strain of *Bacillus subtilis* (ATCC 6051) was employed throughout the work. The chemically defined medium reported by DEMAIN[23)] was employed after being modified slightly. The basal medium was made to have the following composition: L-alanine 890 mg, L-gluta-

mic acid 1470 mg, L-asparagine 1320 mg, Ca-pantothenate 20 mg, K_2HPO_4 8.0 g, KH_2PO_4 1.0 g, NH_4Cl 500 mg, NH_4NO_3 100 mg, Na_2SO_4 50 mg, $MgSO_47H_2O$ 10 mg, $MnSO_44H_2O$ 1 mg, $FeSO_47H_2O$ 1 mg, $CaCl_2$ 0.5 mg, NaCl 40 mg per 1 liter of distilled water. After the pH was adjusted to 7.2 with 1 N NaOH, 500 ml or 1000 ml of the medium was dispensed into 2 liter shaking flask and sterilized. When $^{35}SO_4^{2-}$ was used as the tracer, 0.5 mC of it was added to one liter of medium.

To obtain well-synchronized culture the following procedures were employed. One loopful of cells from the nutrient agar slope was suspended in 10 ml of distilled water and heated at 80 C for 20 min. One ml of this cell suspension was added to 100 ml of the medium and incubated for 12 hr at 37 C. The precultivation was repeated twice and 10 ml of the resulting culture was inoculated to one liter of the medium added with the radioisotopes. The culture was then incubated on a reciprocal shaker at 37 C for the desired periods.

The morphological changes of cells during sporulation were observed microscopically.

Procedures for fractionation of the cells and analysis of intracellular amino acids. The cell grown in the above medium were harvested by centrifugation at different stages of sporulation and washed thoroughly with the mineral salts solution. The washed cells were extracted three times with cold 5% perchloric acid. Perchloric acid in the extracts was neutralized with 5 N KOH and the resulting precipitate was removed by centrifugation. The supernatant solution was adsorbed by Amberlite IR-120 (H type) column (0.9×15.0 cm) and eluted with 2 N NH_4OH solution. The effluent was concentrated by the rotary evaporator. By this treatment NH_3 in the effluent was completely removed.

The free amino acid fraction obtained was subjected was subjected to analysis by use of paper chromatography of an amino acid analyzer (Yanagimoto Model LC-2). The precipitate after being extracted by cold 5% perchloric acid was further treated with 1 N HCl at 90 C for 15 min. The residue fraction obtained with hot acid treatment consisted of proteins and carbohydrates. The residue fraction was hydrolyzed with 6 N HCl at 110 C for 20 hr and concentrated by the rotary evaporator after being filtrated to remove humic substances.

Amino acids in protein hydrolysates were examined by use of paper chromatography and radioautography. Methionine and cysteine in the fraction were determined as follows. Radioactive spots on one-dimensional paper chromatogram were detected by use of a paper chromatogramscanner (Aloka Model PCS-4). Radioactivity of the spot was counted by use of a liquid scintillation counter (Packard Model 314 EX).

The solvent systems used for two-dimensional paper chromatography were[24] ;

(1) isopropanol: formic acid: water (70: 10: 20, v/v)

(2) phenol: water (80: 20, v/v)

Results and Discussion

Changes in concentration of sulfur-containing amino acids in cellular proteins during sporulation of *B. subtilis*

Figs. 2 and 3 show the radioautograms and the scanning patterns of the paper chromatograms of hydrolysates of hot acid insoluble fractions from the cells at different stages of *B. subtilis* grown in the medium containing $^{35}SO_4^{2-}$. The three radioactive spots detected were identified as methionine, methionine sulfoxide and cysteine, respectively. These results indicate that the major part of radioactive sulfur incorporated into methionine in vegetative cells, that the radioactivities in methionine decreased in the course of sporulation and that in mature spores the distribution of radioactive sulfur was restricted to cysteine.

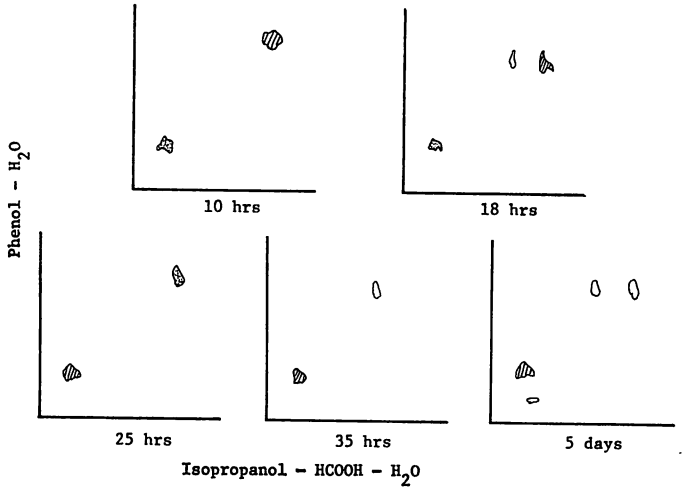

Fig. 2. Radioautograms of hydrolysates of hot acid insoluble fractions from the cells of *B. subtilis* at different stages of sporulation.

Bacterial cells were grown in DEMAIN's medium containing $^{35}SO_4^{2-}$. The fractionation of the cells and the paper chromatography of amino acids were carried out as described previously. The paper chromatograms were exposed to Fuji X-ray films for 3 weeks.

In Table 1, the ratios of concentration of methionine to that of cysteine in cellular proteins are shown. They were calculated from radioactivities of radioactive spots in the paper chromatograms of hot acid insoluble fractions from the cells of *B. subtils* at different stages of sporulation. In vegetative cells, concentration of methionine was found to be about 7 times higher than that of cysteine, and on the other hand in mature spores the ratio of methionine to cysteine was 0.89.

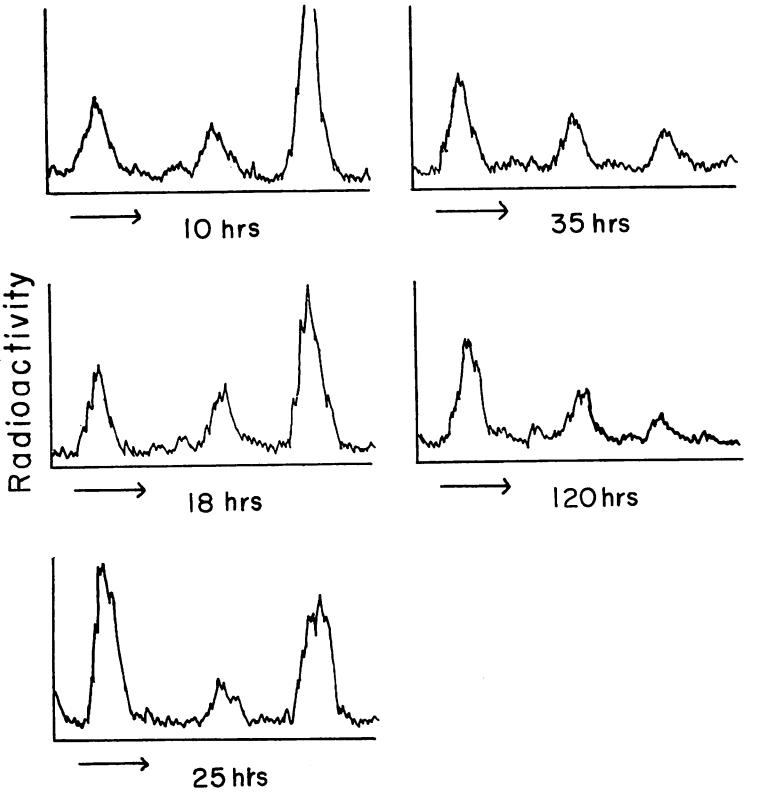

Fig. 3. Paper chromatograms of hydrolysates of hot acid insoluble fractions from the cells of *B. subtilis* at different stages of sporulation.

Radioactivity on one-dimensional paper chromatograms was scanned by use of a paper chromatogramscanner.

Table 1. The ratios of methionine to cysteine in hot acid insoluble fractions from the cells of *B. subtilis* at different stages of sporulation

Incubation time (hr)	10	18	25	35	120
Methionine/cysteine	6.8	3.5	1.4	1.2	0.89
Total count (cpm)	15,122	27,794	24,754	19,254	13,512

Chromatographic separation of methionine and cysteine was carried out on a filter paper (Toyo 51A) in a solvent system consisting of isopropanol-formic acid-water (70: 10: 20). The radioactive spots were cut out and the radioactivities on the paper were measured in a liquid scintillation spectrophotometer.

VINTER[11)13)] reported that the cystine content in proteins of spore of *Bacilli* was about 5 times higher than that in vegetative cells and that the cystine rich proteins were synthesized relatively early during spore formation. KADOTA et al[15)61)] also reported that total amount of cysteine (cystine) increased in paralellel with

the progress of sporulation and that spores contained 108 μg S per mg N. Vegetative cells, on the other hand, contained 26 μg S per mg N. The final preparation of purified spore coats contained 292 μg S per mg N.

In conclusion, most of cystine in the spore is thought to be located in the spore coat as keratine-like protein and its sulfhydryl and disulfide groups may play important roles in the physiological and structural functions of the spore coat.

Intracellular sulfur-containing amino acids in the free form

As shown in the preceding section, the content of methionine in the cells of *B. subtilis* decrease and that of cysteine (cystine) increases in parallel with the progress of sporulation. This fact suggests that methionine in the vegetative cells is converted into cysteine during sporulation. In order to explore the pathway of metabolic conversion from methionine into cysteine and *vice versa* in this organism, sulfur compounds in the cold perchloric acid soluble fractions obtained from the cells were examined at the different sporulation stages.

Fig. 4 shows the paper chromatograms and their scanning patterns of the cold perchloric acid soluble fraction (probably consist of pool amino acids and other low-molecular substances). As seen in the figure, methionine, methionine sulfoxide, cysteine and homocysteine were detected as radioactive spots. In contrast with protein fraction, methionine was found to be higher than cysteine at all the life stages.

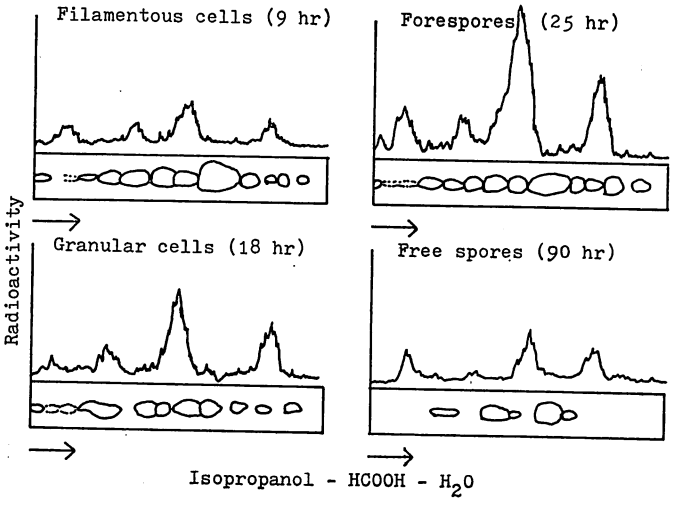

Fig. 4. Paper chromatograms (lower) and their scanning patterns (upper) of cold acid soluble fractions from the cells of *B. subtilis* at different stages of sporulation.

Figs. 5 and 6 show two dimensional radioautograms and column chromatograms of cold acid soluble fractions from cells at different stages of sporulation of *B.*

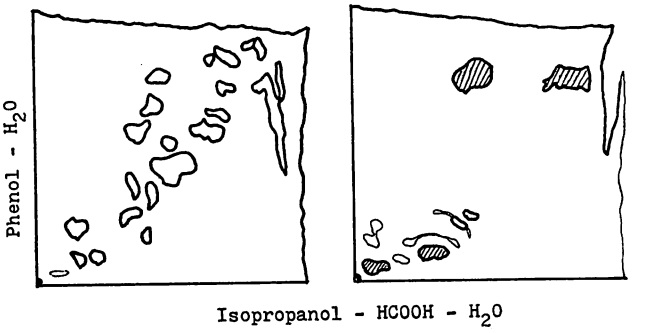

Fig. 5. Paper chromatogram (left) and radioautogram (right) of cold acid soluble fraction from forespore cells of *B. subtilis*.

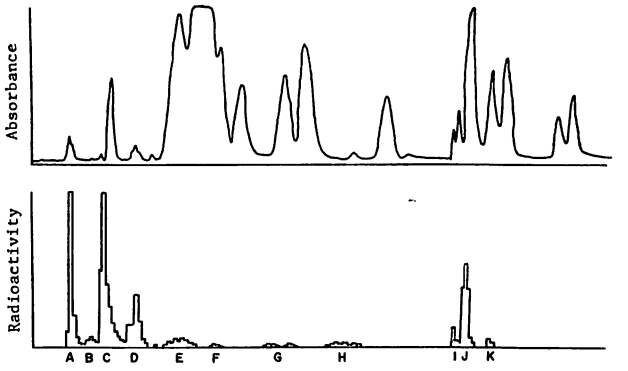

Fig. 6. Column chromatogram by an amino acid analyzer of cold acid soluble fraction from sporulating cells of *B. subtilis* grown in the medium containing $^{35}SO_4^{2-}$.

Peaks: A, cysteic acid plus homocysteic acid; C, methionine sulfoxide; D, methionine sulfone; I, cystathionine; J, methionine.

subtilis. As shown in the figures, 11 peaks of sulfur-containing compounds were detected. These peaks were identified as follows by use of chromatographic techniques;

Peak A, cysteic acid plus homocysteic acid; C, methionine sulfoxide; D, methionine sulfone; I, cystathionine and J, methionine. The other peaks in the pattern have not been identified. The sulfur-containing amino acids detected here are almost same to those known as the intermediates in the biosynthesis of methionine from cysteine in *Salmonella* and *Neurospora*.

Chapter II. Effect of Sulfur-Containing Amino Acids on the Incorporation of $^{35}SO_4^{2-}$ into the Cells during Sporulation

Following pathway for the biosynthesis of sulfur-containg amino acids from sulfate is generally known with some species of *Neurospora*[25)26)27)], *Escherichia*[28)29)30)] and *Salmonella*[31)32)33)].

$SO_4^{2-} \longrightarrow SO_3^{2-} \longrightarrow S^{2-} \longrightarrow$ cysteine $\longrightarrow$ cystathionine $\longrightarrow$ homocysteine $\longrightarrow$ methionine

However, little works have done on the metabolic pathway of sulfur-containing amino acids in *Bacillus* species.

In the preceding chapter, possible intermediates of the methionine biosynthetic pathway in *B. subtilis* have been pointed out. In this chapter it is discussed whether or not these sulfur-containing amino acids act as intermediates in the methionine biosynthesis from cysteine and the cysteine biosynthesis from methionine in the cells of *B subtilis*. For this purpose the effects of addition of cysteine, cystathionine, homocysteine and methionine on the incorporation of sulfate ($^{35}SO_4^{2-}$) into the cell have been examined.

Materials and Methods

Determination of the rate of incorporation of $^{35}SO_4^{2-}$ into the growing cells. *Bacillus subtilis* Marburg strain 6051 was grown at 37 C under shaking in 100 ml of the Demain's medium containing $^{35}SO_4^{2-}$ (0.01 mC) and each of the sulfur-containing amino acids. One ml aliquot of the culture was taken at appropriate intervals and immediately mixed with one ml of 1/15 M phosphate buffer (pH 7.2). After being mixed thoroughly, one ml of the mixture was filtered through Millipore filter (HA, pore size 0.45 μ) and washed with the same buffer. The filters were then placed into vials containing 10 ml of a scintillation solution. The radioactivities of the samples were determined in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 314 EX.

Analysis of sulfur-containing amino acids of the cell proteins The residue fraction obtained by the hot acid extraction was hydrolyzed with 6 N HCl at 110 C for 20 hr in a sealed glass tube and then analyzed. Sulfur-containing amino acids were separated by one-dimensional paper chromatography on Toyo filter paper NO 51 A using the solvent system in which isoprapanol: formic acd: water (70: 10: 20, V/V) were contained. The radioactivities on the chromatogram were measured by use of an Aloka Paper Chromatogramscanner Model PSC-4.

Results and Discussion

Fffect of various sulfur-containing amino acids on the incorporation of $^{35}SO_4^{2-}$.

In Fig. 7 the profiles of incorporation of $^{35}SO_4^{2-}$ by sporulating cells in the

presence of the various sulfur-containing amino acids (5×10^{-4} M) are illustrated. The results show that the uptake of $^{35}SO_4^{2-}$ by the cells was more or less inhibited by these amino acids. Especially L-cysteine inhibited the incorporation of sulfate completely. The inhibition by cystathionine was somewhat weak as compared with the other amino acids. This may be due to difficulty in the permeation of cystathionine through the membrane of *B. subtilis*. Although *B. subtilis* is able to utilize cystathionine as sole source of solfur[34], other utilizable sulfur compounds may be incorporated in preference to cystathioine.

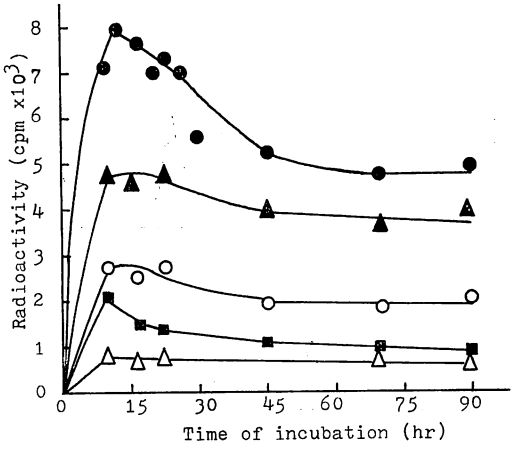

Fig. 7. Effect of addition of sulfur-containing amino acids on the incorporation of $^{35}SO_4^{2-}$ into the cells of *B. subtilis* at different stages of growth and sporulation.
Samples were withdrawn to be filtered through Millipore filters and their radioactivities on the filters were determined.
control; ●, cystathionine; ▲, methionine; ○, homocysteine; ■, cysteine; △.

In order to make clear the pattern of incorporation of sulfate-S into the sulfur-containing amino acids under these conditions, the distribution of ^{35}S in the amino acids of protein fractions was examined. For this purpose the bacterial cells were grown in the medium containing $^{35}SO_4^{2-}$ as sole source of ^{35}S in the presence of cysteine, cystathionine, homocysteine and methionine respectively.

As shown in Fig. 8, in the presence of methionine or homocysteine, radioactivity of $^{35}SO_4^{2-}$ was incorporated into cysteine but not into methionine in both the cases. This finding suggests that L-methionine and L-homocysteine have some regulatory effects on the metabolic pathway from cysteine to methionine in *B. subtilis* and these amino acids are utilized in preference to sulfate. Cysteine and cystathionine, however, did not inhibit the incorporation of SO_4^{2-}-S into methionine. In these cases the total uptake of $^{35}SO_4^{2-}$ was depressed. These facts support the idea that sulfate is converted to cysteine in the amino acid and

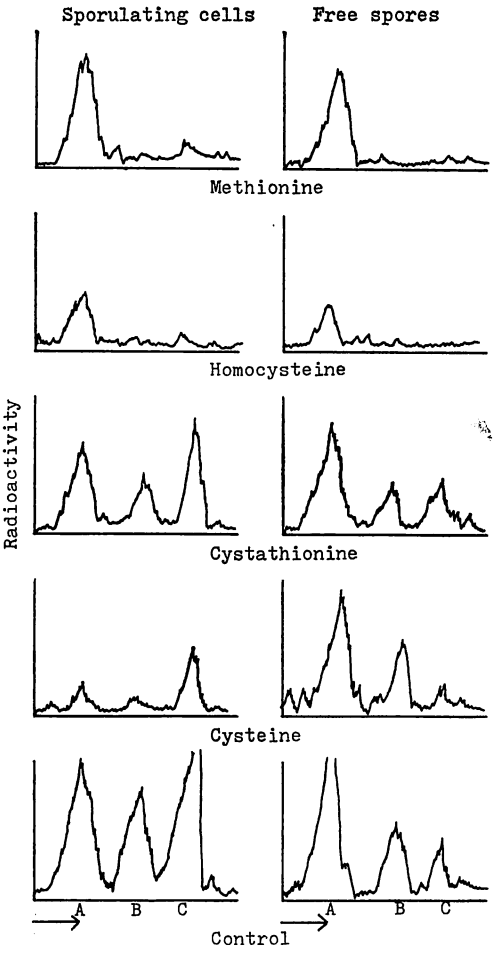

Fig. 8. Radiochromatograms of hydrolysates of protein fractions obtained from the cells of *B. subtilis* grown in the medium containing $^{35}SO_4^{2-}$, in the presence of methionine, homocysteine, cystathionine or cysteine.
Peaks: A, cysteine; B, methionine sulfoxide; C, methionine.

then metabolized to form methionine.

Effect of the addition of cysteine or methionine on the incorporation of $^{35}SO_4^{2-}$ into the cells

Effect of the concentration of L-methionine and L-cysteine on the rate of incorporation of $^{35}SO_4^{2-}$ into the cells of *B. subtilis* is shown in Fig. 9. The incorporation of $^{35}SO_4^{2-}$ into the cells was completely inhibited by L-cysteine at the concentration of 10^{-3}M. L-methionine depressed the uptake of $^{35}SO_4^{2-}$ in parallel with increasing concentration up to 10^{-4} M but it did not show the additional inhibition

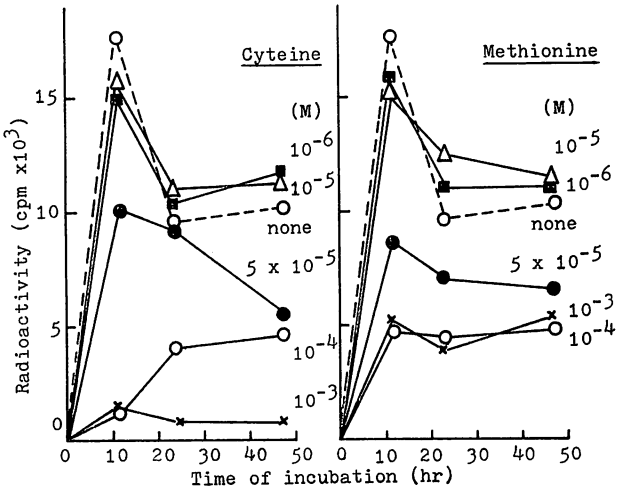

Fig. 9. Effects of L-methionine and L-cysteine on the rate of incorporation of $^{35}SO_4^{2-}$ into the cells of *B. subtilis*.

of the uptake at the concentration of 10^{-3} M or higher.

These results, together with the previously mentioned data, suggest that cysteine inhibits the incorporation of sulfate into the cells and methionine inhibits the biosynthesis of methionine from cysteine.

Based on these results, it is thought that in *B. subtilis* methionine is synthesized from sulfate through cysteine, cystathionine and homocysteine and acts as a regulator in the metabolism of sulfur-containing amino acids as is the case in *Salmonella* or *Neurospora*.

Fig. 10 shows the incorporation of $^{35}SO_4^{2-}$ into the sporulating cells when L-methionine or L-cysteine was added to the culture at various stages of sporulation. As shown in this figure, a large quantity of sulfate was incorporated into the cells when it was added at the logarithmic growth phase. After the growth ceased, the incorporation of sulfate decreased rapidly and the inhibition of the incorporation by L-methionine was not so remarkable as compared with that at the logarithmic growth phase.

These data suggest that at the logarithmic growth phase a large quantity of sulfate was incorporated into the cells and metabolized for the biosynthesis of methionine and that as the incorporation of sulfur compounds decreased during sporulation methionine assimilated in the cells was metabolized to cysteine and utilized as a sulfur source to form spore constituents such as the spore coat protein.

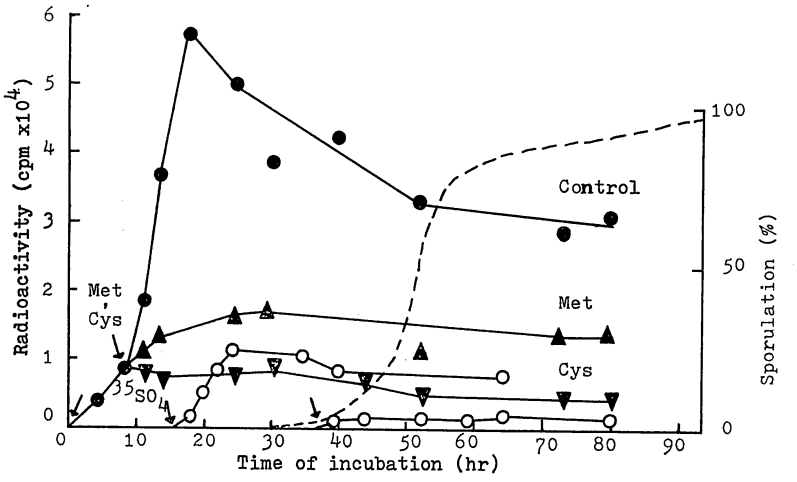

Fig. 10. Incorporation of $^{35}SO_4^{2-}$ into sporulating cells of *B. subtilis* in the presence of L-methionine or L-cysteine.
Cells were grown at 37 C. The additions were made as follows:
●—● $^{35}SO_4^{2-}$ was added at 0 time (control),
○—○ $^{35}SO_4^{2-}$ was added at 18 hr; ⊙—⊙ at 35 hr,
▲—▲ $^{35}SO_4^{2-}$ was added at 0 time and L-methionine was added at 9 hr,
▼—▼ $^{35}SO_4^{2-}$ was added at 0 time and L-cysteine was added at 9 hr.

Chapter III. Incorporation of ^{35}S-Methionine and ^{35}S-Cysteine and their Metabolism during Sporulation

In the previous chapters, the author described that sulfate was incorporated into the cells and metabolized to cysteine and methionine in *B. subtilis* as has been known with microorganisms. L-Methionine and L-cysteine were found to support the growth and sporulation of *B. subtilis* as the sole sulfur source like sodium sulfate. L-Cysteine, however, inhibited the growth and sporulation when it was added at higher concentrations.

In this chapter the incorporation and metabolism of ^{35}S-methionine and ^{35}S-cysteine, and the interconversion between these compounds in the cells are discussed.

Materials and Methods

Uptake of methionine and cysteine. *Bacillus subtilis* (ATCC 6051) was grown in 100 ml of the modified DEMAIN's medium which was added with ^{35}S-methionine (1.08×10^7 cpm, 5×10^{-4} M) or ^{35}S-cysteine (2.55×10^6 cpm, 5×10^{-4} M). Sampling was made by use of Millipore filter as described in the previous chapter.

Experiments on intracellular pool of free amino acids. Growing cells or sporulating cells were harvested from 100 ml of culture by centrifugation at 10,000×g for

20 min. After washing the cells were uniformly suspended in 100 ml of phosphate buffer or DEMAIN's medium containing radioactive sulfur compounds and incubated at 37 C. The uptake of amino acids was examined with the radioactive compounds added to the culture.

Samples were taken at appropriate intervals by removing 1.0 ml of the suspension and immediately added to a definite volume of phosphate buffer at 4 C. The diluted cell suspensions were filtered through a Millipore filter (pore size 0.45 μ) and then the filter was washed with phosphate buffer. Other aliquotes of the samples were added to the same volume of 2 N HCl and placed in a boiling water bath for 15 min. After cooling the suspension was filtered through a Millipore filter.

Results and Discussion

In Fig. 11 the time courses of uptake of $^{35}SO_4^{2-}$, ^{35}S-cysteine and ^{35}S-methionine

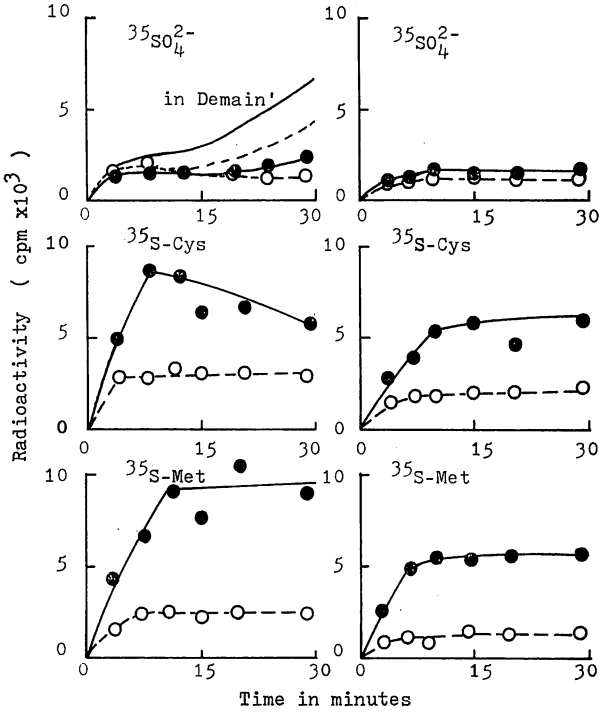

Fig. 11. Uptake of $^{35}SO_4^{2-}$, ^{35}S-cysteine or ^{35}S-methionine by vegetative (left) and sporulating cells (right) of *B. subtilis*.

Cells were incubated with $^{35}SO_4^{2-}$, ^{35}S-cysteine or ^{35}S-methionine in Demain's medium or phosphate buffer. At the time indicated an aliquot of cell suspensions was withdrawn and quickly filtered through a Millipore filter. was washed with buffer solution (●), or with hot acid solution (○).

by vegetative cells and sporulating cells are shown. The difference in height between the two curves (curve of buffer-washed cells and that of hot acid-washed cells) indicates the pool size of sulfur-containing amino acids in the cells. The pool size of cysteine was at approximately the same level in both the growing cells and the sporulating cells. On the other hand, the pool size of methionine in the sporulating cells was small as compared with that in the growing cells. The incorporation of sulfate into the cells was considerably lower than those of sulfur-containing amino acids. However, the incorporation of sulfate into the cells increased rapidly during incubation with Demain's medium.

These data suggest that sulfate was metabolized to cysteine or methionine at a rapid rate and that when the cells were changed from vegetative form to sporulating form the requirement for methionine was diminished but that for cysteine remained constantly.

Since the incorporation of radioactivity into protein fraction was found during the short incubation, the analysis of radioactive sulfur-containing amino acids of protein fraction was made by use of one dimensional chromatography.

The radiochromatograms shown in Fig. 12 indicate that radioactivity was very low or not detected in methionine in the case of hydrolysates of protein fractions from the cells incubated with $^{35}SO_4^{2-}$ or ^{35}S-cysteine for 30 min.

It is speculated that the pool size of methoinine in the cells is large enough to dilute the supply of methionine by *de novo* synthesis from cysteine during 30 min

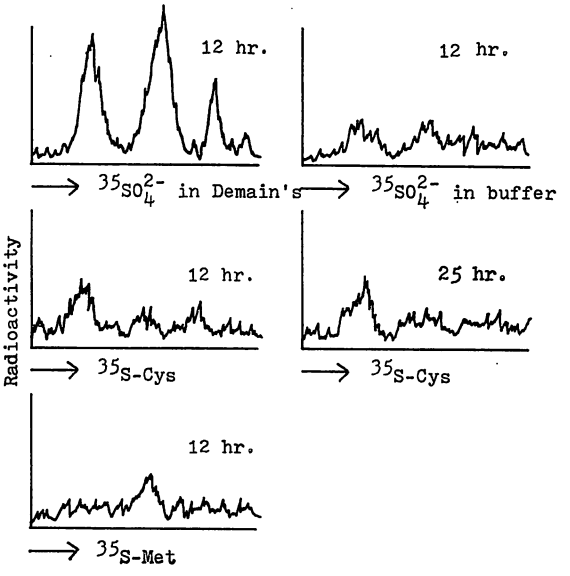

Fig. 12. Radiochromatograms of hydrolysates of hot acid insoluble fractions from the cells of *B. subtilis* incubated for 30 min with the buffered solution containing $^{35}SO_4^{2-}$, ^{35}S-cysteine or ^{35}S-methionine.

Table 2. Incorporation of ^{35}S-methionine and ^{35}S-cysteine into the cells of *B. subtilis*

Incubation time (hr)	Radioactivity (cpm)					
Addition	^{35}S-methionine			^{35}S-cysteine		
	$-SO^{2-}$	$+SO^{2-}$	+cys	$-SO^{2-}$	$+SO^{2-}$	+met
12	2,468	844	917	—	689	—
25	1,798	638	1,971	770	1,354	645
96	1,484	564	602	642	739	—

Cultures were grown in Demain's medium with SO_4^{2-}, cys or met (+) or without SO_4^{2-} (—). ^{35}S-methionine or ^{35}S-cysteine was added as a tracer. One ml aliquot of the cultures was withdrawn and filtered through a Millipore filter. The radioactivity of the filter was determined.

incubation.

Table 2 shows the incorporation of ^{35}S-methionine and ^{35}S-cysteine into the cells in the presence of other sulfur sources. The incorporation of ^{35}S-methionine in the absence of sulfate was larger than that in the presence of sulfate, but the incorporation of ^{35}S-cysteine was not so much affected by the presence of sulfate.

These results are in good accordance with the findings that methionine did not inhibit the biosynthesis of cysteine from sulfate and that sulfate was incorporated into the cells as a sulfur source in the presence of methionine, but cysteine completely inhibited the incorporation of sulfate into the cells.

Then, the author attempted the analysis of sulfur-containing amino acids in hydrolysates of hot acid insoluble fraction of the cells grown under the conditions as mentioned above.

As shown in Fig. 13 the same radioactive patterns of radiochromatograms were obtained whatever ^{35}S-sulfur compounds (such as sulfate, methionine or cysteine) were used as the sole source of sulfur. Radioactivity was not detected in cysteine in the hydrolysates of cellular protein fraction when ^{35}S-methionine and cold cysteine were present at the same time in the culture medium. And also radioactive methionine was not detected when ^{35}S-cysteine and cold methionine were used as sulfur sources.

The results obtained in these experiments indicate that *B. subtilis* is able to utilize sulfate, methionine and cysteine as the sole sulfur source effectively and that the interconversion between methionine and cysteine takes place at all the stages in life cycle. The interconversion between sulfur-containing amino acids is considered to be regulated under certain control mechanism at each stage of growth and sporulation of *B. subtilis*.

In some fungi such as *Neurospora*, and *Saccharomyces* transsulfuration between methionine and cysteine via cystathionine has been reported to be reversible, but in bacteria such as *Salmonella*, and *Escherichia* transsulfuration proceed only from cysteine to methionine[27)35)].

It is thought that *B. subtilis* has an ability to transfer sulfur from methionine

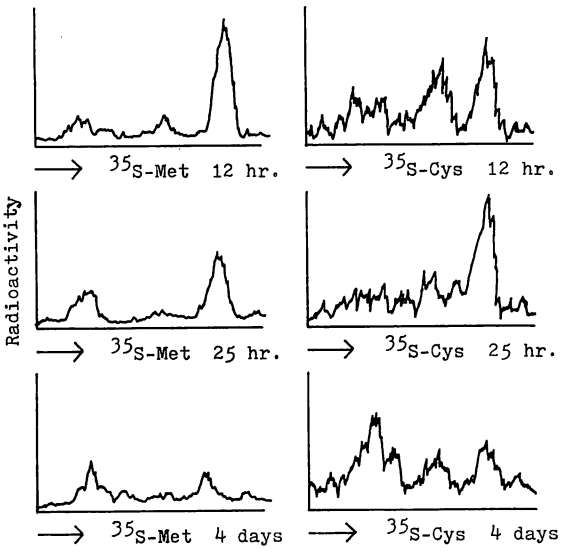

Fig. 13a. Radiochromatograms of hydrolysates of hot acid insoluble fractions from the cells of *B. subtilis* grown in the medium containing ^{35}S-methionine or ^{35}S-cysteine (in the absence of SO_4^{2-}).

to cysteine by way of inorganic compounds such as sulfide or mercaptoethanol, because this organism can grow with methionine as the sole source of sulfur. However, it is still not clear through what pathway sulfur is transfered from methionine to cysteine in *B. subtilis*.

There are a few reports on the process of methionine decomposition by bacteria. SEGAL et al[36)37)] reported that various bacteria and fungi decomposed exogenous methionine to produce methanethiol and dimethyl disulfide but not inorganic sulfur compounds such as sulfide, sulfite and sulfate.

Part II

Chapter IV. Cystathionine Cleavage Enzyme of *Bacillus subtilis*

Cystathionine cleavage enzyme (cystathionase) is one of the key enzymes of transsulfuration pathway in biological system. In bacteria transsulfuration reaction proceeds only in the direction from cysteine to homocysteine. In this pathway cystathionine, an intermediate of the transsulfuration, is cleaved by cystathionine ß-cleavage enzyme through the following reaction.

$$\text{Cystathionine} + H_2O \longrightarrow \text{homocysteine} + \text{pyruvate} + \text{ammonia}$$

Most of the works on cystathionine cleavage enzyme of microorganisms have been done using *Neurospora crassa*[38)39)] *Salmonella typhimurium*[27)33)] or *Escherichia coli*[28)40)]

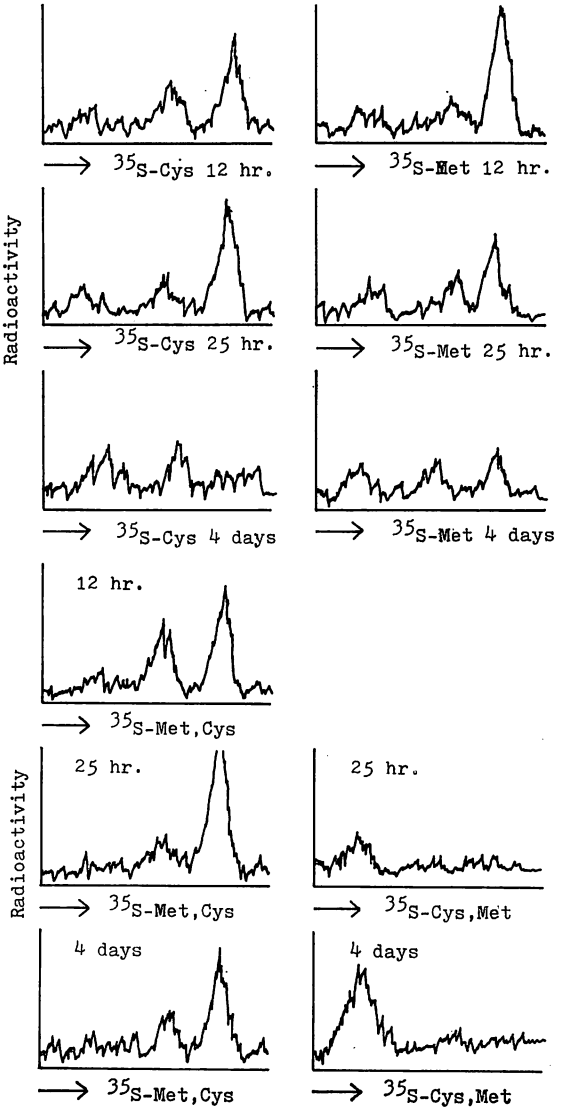

Fig. 13b. Radiochromatograms of hydrolysates of hot acid insoluble fractions from the cells of *B. subtilis* grown in the medium containing ^{35}S-methionine or ^{35}S-cysteine in the presence of SO_4^{2-} (upper), or in the presence of L-cysteine or L-methionine (lower).

as test organisms, and little works have been done with *Bacillus* species.

In the spore-forming bacilli it has been found that vigorous metabolism of sulfur-containing amino acids, necessary to the biosynthesis of cystine-rich protein of spore coat, takes place in the cells during sporulation. It is, therefore, interesting to study with the spore-forming bacilli the metabolism of sulfur-contain-

ing amino acids and the relevant enzymes throughout all the stages of growth and sporulation.

This chapter describes the purification and the characterization of cystathionine cleavage enzyme from the cells of *B. subtilis* which have been carried out as one step toward understanding the regulation mechanism involved in the metabolism of sulfur-containing amino acids during growth and sporulation.

Materials and Methods

Organism and cultural conditions. The Marburg strain of *Bacillus subtilis* (ATCC 6051) was employed throughout the work. The bacteria were cultured in DMAIN'S medium at 37 C as described in the previous chapters. *Bacillus megaterium* QMB strain and *Escherichia coli* B strain were used as reference strains.

Method for obtaining the crude enzyme preparations. The bacterial cells were harvested by centrifugation and washed three times with 0.05 M phosphate buffer (pH 7.5). The cell pellets were suspended in an appropriate volume of the same buffer containing mercaptoethanol and were subjected to sonic disintegration at 20 KC for 7 min. The cell debris and undisintegrated cells were removed by centrifugation at 15,000×g for 15 min.

The cell-free extracts thus obtained was treated with 0.2% protamin sulfate. After standing for 1 hr at 5 C the precipitate was removed by centrifugation. Solid ammonium sulfate was added to the supernatant solution to make 0.55 saturation at 5 C. After standing for 3 hr at 5 C the precipitate formed was removed by centrifugation at 15,000×g for 20 min. The ammonium sulfate concentration of the supernatant was then increased to 0.80 saturation by the addition of solid ammonium sulfate. After standing for 12 hr at 5 C the precipitate was collected by centrifugation at 15,000×g for 20 min and dissolved in 0.05 M phosphate buffer containing mercaptoethanol. The enzyme solution was dialyzed overnight against the same buffer at 5 C.

DEAE-Sephadex A-50 column chromatography. The dialyzed enzyme solution obtained was applied to a column (2.0×25.0 cm) of DEAE-Sephadex A-50 and was eluted with a linear gradient increase inconcentration of KCl. The reservoir containing 250 ml of 0.05 M phosphate buffer containing 0.4 M KCl (pH 7.2) and the mixing chamber consisted of 250 ml of the same buffer without KCl.

Gel filtration with Sephadex G-200. The enzyme solution was passed through a column (1.5×90.0 cm) of Sephadex G-200, equilibrated with 0.05 M phosphate buffer (pH 7.2) and washed with the same buffer.

Hydroxylapatite chromatography. Active fractions from the Sephadex G-200 treatment were applied to a column (1.0×15.0 cm) of hydroxylapatite equilibrated with 0.05 M phosphate buffer (pH 7.2) and then a linear gradient of 50 mM to 500 mM potassium phosphate buffer was used for elution of enzyme proteins.

Polyacrylamide gel electrophoresis. Disc polyacrylamide gel electrophoresis was performed in 5 mm (inner diameter) glass tubes according to the method of Davis

et al[42] with some modifications. A 5 cm running 7.5% acrylamide gel was prepared in a disc gel electrophoresis column and it was covered with 3% spacer gel. Enzyme solution mixed with 0.005% bromophenol blue, was applied to the column. The electrophoresis was done at 2 mA/gel until the dye marker had moved to the end of spacer gel and then at 4 mA/gel in running gel. Protein bands were identified by staining with amido black after electrophoresis.

Assay of the activity of cystathionine cleavage enzyme. Assay of the enzyme activity was made by determing the 2, 4-dinitrophenylhydrazone of pyruvate photometrically according to the method reported by WIJESUNDRA et al[40][41].

Fig. 14 shows the time course of enzyme rection at 37 C.

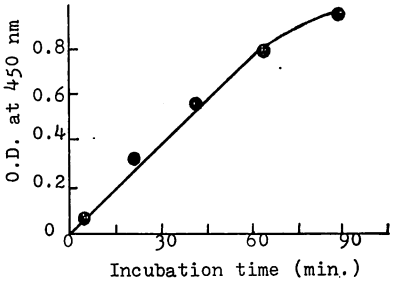

Fig. 14. Time-course of cleavage of cystathionine by the enzyme solution.

Identification of α-ketoacid. Identification of α-keto acid was carried out by using silica gel chromatography[43] and ion-exchange chromatography of Dowex AG (Cl type)[44].

Results and Discussion

Effect of culture conditions on the enzyme biosynthesis.

Table 3 summarizes the effect of various substances added to the culture media on the cleavage activities for various substrates in the crude extracts.

In the case of cells grown under the sulfur-limited condition the specific activity for cleavage of cystathionine was relatively high as compared with the other conditions tested. When glucose was added to the culture the specific activity was found to be very low. It was thought that the limitation of sulfur derepressed biosynthesis of the enzyme proteins, and that some metabolites produced from glucose repressed it or stimulated the production of other extractable proteins.

Separation of isozymes of cystathionine ß-cleavage enzyme by DEAE-Sephadex A-50 column chromatography.

As shown in Fig. 15, chromatogram on DEAE-Sephadex A-50 indicated the presence of two active fractions (Fractions I and II) of cystathionine cleavage

Table 3. Activities of cystathionine cleavage enzyme from the cells grown in various media

Culture medium	Specific activity (mμmoles/min/mg protein)	Relative activity (%)*			
	Substrate				
	(+)-cystathionine	(−)-cysta.	L-cys	Lanth.	SMC
Nutrient broth	6.16 (100)	88	192	62	85
Demain's medium	6.49 (100)	85	140	41	66
+glucose	1.82 (100)	87	178	68	82
+L-methionine	5.93 (100)	107	184	50	86
+L-cysteine	5.96 (100)	83	130	42	62
+DL-djenkolic acid	10.05	—	—	—	—
sulfur limited	9.36 (100)	74	112	83	58

Enzyme assay was carried out under standard conditions as described in the text using the crude extracts from the cells grown in various media for 24 hr. *Relative activity is expressed as percent activity to that for (+)-cystathionine. cysta., cystathionine; cys, cysteine; lanth., lanthionine; SMC, S-methylcysteine.

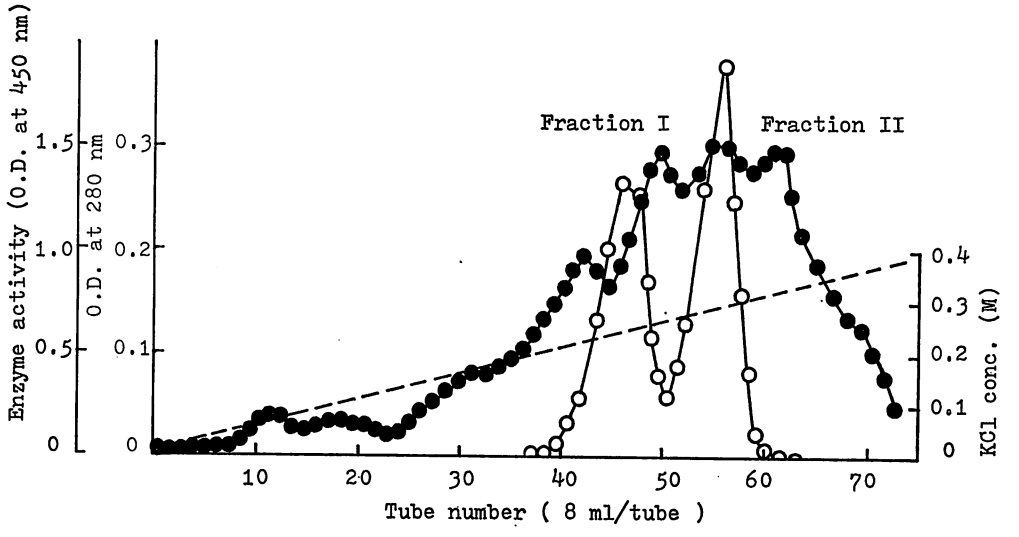

Fig. 15. DEAE-Sephadex column chromatography of cystathionine cleavage enzyme from vegetative cells of *B. subtilis* grown in Demain's medium.
○——○ enzyme activity; ●——● protein conc.; - - - KCl conc.

enzyme. Vegetative cells and forespore cells grown in Demain's medium had both of these two fractions. However, in free spore cells only fractions II was detected.

On the other hand, as shown in Fig. 16, Fraction I was found to be absent in extracts of the methionine-containing medium or nutrient broth medium. In the methionine-containing medium or nutrient broth medium, the relative activities for cystine and S-methylcysteine were high comparing with the case of basal

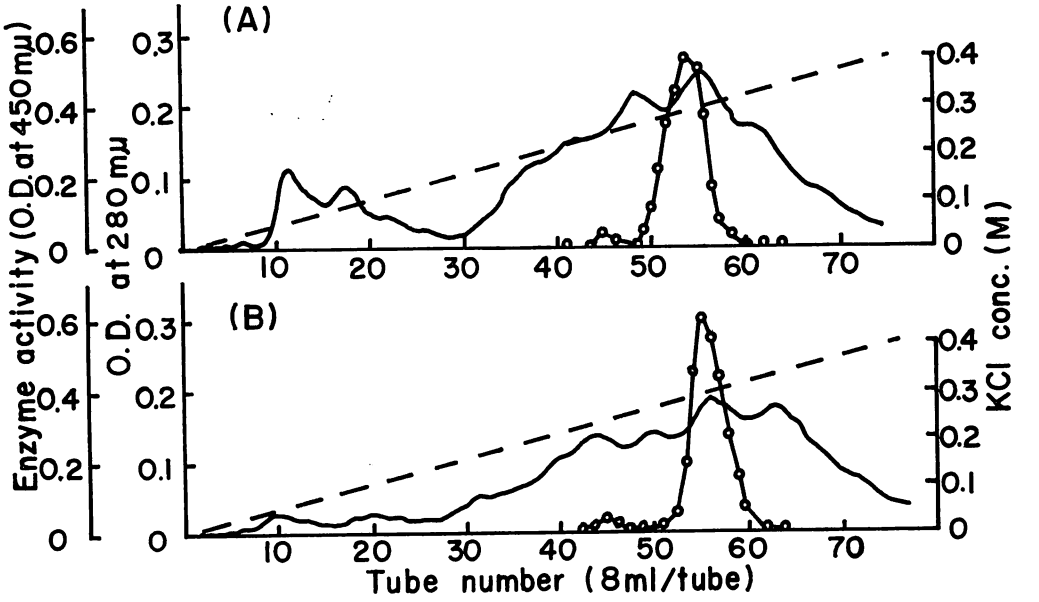

Fig. 16. DEAE-Sephadex column chromatography of cystathionine cleavage enzymes from *B. subtilis* grown in (A) nutrient broth and (B) Demain's medium containing L-methionine.
○——○ cystathionine cleavage activity; —— protein; - - - KCl conc.

medium. These substrates are cleaved by Fraction II of isozymes not by Fraction I. These facts suggest that Fraction I protein is repressed in those case.

Since these isozymes of cystathionine cleavage enzyme were found also in *Bacillus megaterrium* strain as shown in Fig. 17, it is thought to be common in *Bacillus* species. In *Escherichia coli*, only one peak of activity was present, located in a similar position of the elution profile of column chromatography as with Fraction II enzyme of *B. subtilis*.

Identification of the reaction product.

It is known that cystathionine is converted to cysteine and α-ketobutyrate by the γ-cleavage enzyme or to homocysteine and pyruvate by the β-cleavage enzyme. The author attempted to identify α-keto acid (s) produced during the enzymic reaction in order to determine whether the cystathionine cleavage enzyme of *B. subtilis* consists of α-cleavage enzyme and β-cleavage enzyme or β-cleavage enzyme only.

Chromatography using silicic acid gel and Dowex AG (Cl typed) columns of α-keto acid produced by enzymic reaction shows that α-keto acid was pyruvic acid in both the cases of Fraction I and II(Fig. 18). This fact indicates that the cystathionine cleavage enzyme from *B. subtilis* is restricted to the β-cleavage enzyme similarly with *Salmonella typhimurium* and *Escherichia coli*.

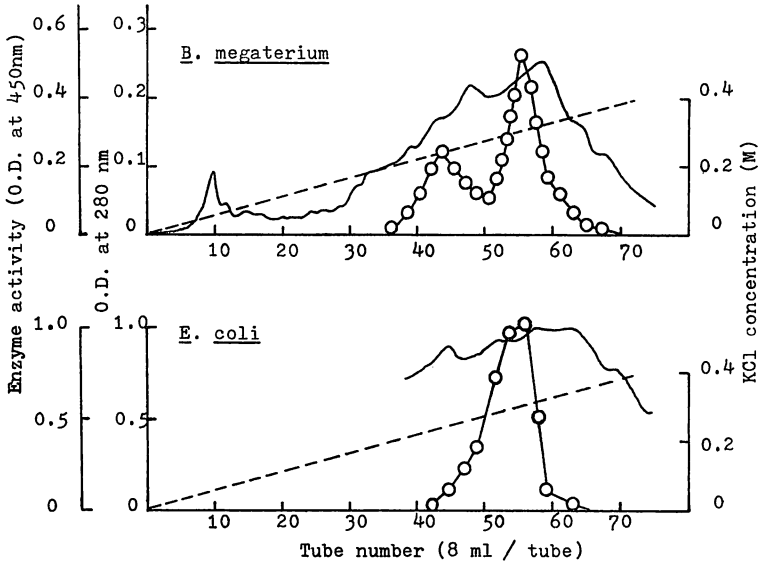

Fig. 17. DEAE-Sephadex column chromatography of cystathionine cleavage enzyme from *B. megaterium* (upper) and *E. coli* (lower).

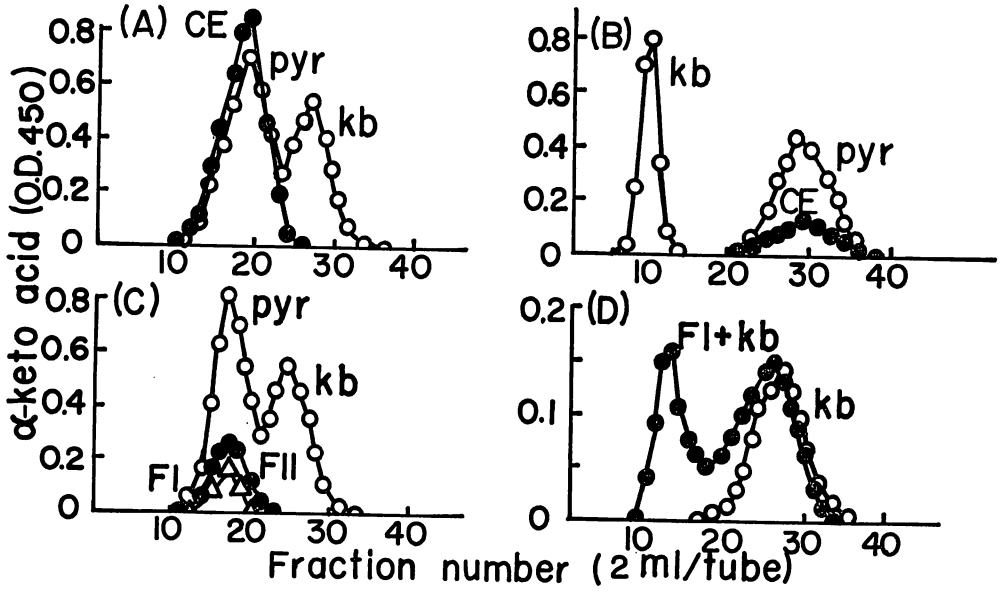

Fig. 18. Identification of the reaction products by column chromatography. A, C, D, Dowex 1×8 column; B, silica gel column. pyr., authentic pyruvate; kb, authentic α-ketobutyrate.

Cystathionine cleavage enzymes of bacteria and fungi have been studied by several workers. However, they regarded the cystathionine cleavage enzyme as a single enzyme and therefore the results obtained by them on the regulation of enzyme biosynthesis and the substrate specificity seemed to require further examination. For example, biosynthesis of cystathionine cleavage enzyme of *S. typhimurium* was reported to be partially repressed by methionine, unlike other enzymes in the methionine pathway[27)33)].

Substrate specificity of isozymes.

To make characterization of two fractions of the cystathionine cleavage enzyme obtained from the cells the substrate specificities were examined. As shown in Table 4, Fraction I had the high substrate specificity to L-cystathionine but Fraction II had a wide substrate spectrum; Fraction II catalyzed also the cleavage of isomers of cystathionine, djenkolic acid, S-methylcysteine and lanthionine to produce pyruvate. The production of pyruvate from cystine or djenkolic acid was two or three times as rapid as that from cystathionine. This fact suggests that in the case of cystine or djenkolic acid two molecules of pyruvate are produced from one molecule of the substrate.

Table 4. Substrate spectra of two types of cystathonine cleavage enzyme (Fractions I and II) from *B. subtilis*

Substrate	Reaction rates (relative to that of L-cystathionine)	
	Fraction I	Fraction II
L(+)-Cystathionine	100	100
(±)-Cystathionine (L-, L-allo)	105	100
(−)-Cystathionine (D-, L-allo)	73	106
L-Cystine	12	236
DL-Djenkolic acid	91	316
L-Cysteine	11	33
L-Serine	4	2
D-Serine	6	2
S-Methylcysteine	6	86
DL-Homocysteine	6	5
DL-Lanthionine	5	80

Enzyme activities were assayed on DEAE-Sephadex fractions for 2 mM substrates and 0.4 μg/ml pyridoxal phosphate in 2.5 ml of 0.2 m Tris-HCl buffer at pH 9.0.

Fraction II protein obtained by DEAE-Sephadex chromatography was subjected to further purification. The purified protein was examined in respect to its catalytic activities for various substrates. The activities of Fraction II enzyme at different purification steps are summarized in Table 5. Fraction II was purified about 1,800 fold over the original extract with a recovery of 3%. As shown in

Table 5. Summary of the purification of cystathionine cleavage enzyme Fraction II from *B. subtilis*

Step	Volume (ml)	Total protein (mg)	Total units (U)×10^5	Specific activity (U/mg)	Recovery (%)
1. Crude extract	1,380	98,500	6.00	6.19	100
2. Ultracentifuged supernatant	1,300	58,900	4.98	8.46	83
3. Ammonium sulfate precipitate (40—80% saturation)	670	26,400	3.76	14.2	63
4. DEAE-Sephadex chromatography (I) and ammonium sulfate precipitate	70	5,040	1.86	36.9	31
5. Sephadex G-200 chromatography (I)	40	2,880	1.07	37.2	18
6. DEAE-Sephadex chromatography (II)	49	1,200	0.768	64.0	13
7. Sephadex G-200 chromatography (II)	10	27.5	0.383	1,390	6
8. Hydroxylapatite chromatography	26.5	1.75	0.178	10,200	3

One unit of activity represents the formation of 1 mμmole of pyruvate per min under standard assay condition.

Table 6. Relative activity of each fraction separated by use of hydroxylapatite column chromatography for various substrates

Fraction no.	Substrate				
	(—)-Cystathionine	SMC	L-Cys	DL-Lanthio.	DL-Djenko.
8	33.7	29.7	31.6	29.3	22.7
10	67.6	67.0	66.1	63.8	61.6
12	94.1	93.2	95.4	94.7	93.3
14	100	100	100	100	100
16	74.2	68.0	72.5	66.8	69.9
18	—	43.7	45.3	44.3	44.5
20	38.2	35.5	37.5	36.6	34.4
22	29.1	27.4	33.8	30.7	39.5
24	25.4	23.0	30.5	26.9	22.8

Relative activity is expressed as percentage to maximum activity for each substrate.

Fig. 19 and Table 6, enzymatic activity peaks for various substrates were in good accordance with one another in the case of both hydroxylapatite chromatography and polyacrylamide gel electrophoresis. These results suggest that the same enzyme protein have wide substrate spectrum for various compounds.

These data on substrate specificity suggest that Fraction I is the proper enzyme participating in the biosynthetic pathway of methionine and Fraction II is an enzyme which decomposes various amino acids containing sulfur-bridge and produces pyruvate.

The substrate specificities of the enzyme preparation obtained from *E. coli* by use of DEAE-Sephadex A-50 column chromatography were also examined. Cystathionine cleavage enzyme from *E. coli* decomposes L-cystine, DL-djenkolic acid

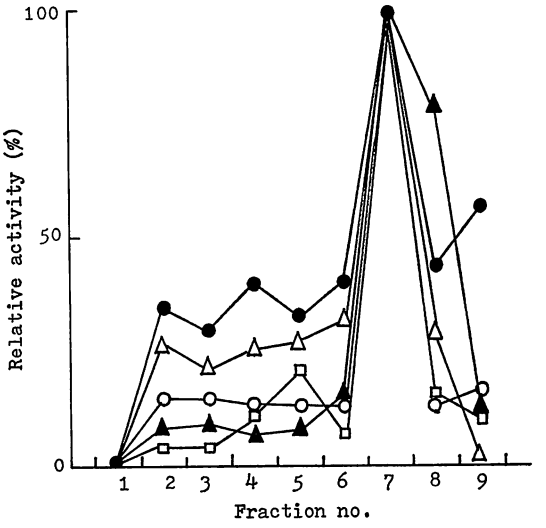

Fig. 19. Cleavage activities for sulfur-containing amino acids of fractions obtained by use of acrylamide gel electrophoresis.
●, (—)-cystathionine; ○, L-cystine;
△, DL-lanthionine; ▲, DL-djenkolic adic;
□, S-methylcysteine.

Table 7. Substrate spectra of cystathionine cleavage enzyme from *E. coli*

Substrate	Reaction rates (relative to that of (—)-cystathionine)
(—)-Cystathionine	100
L-Cystine	80.7
DL-Djenkolic acid	39.3
S-Methylcysteine	0

Enzyme activity was assayed under standard condition using DEAE-Sephadex A-50 fraction.

and DL-lanthionine as cystathionine but not S-methylcysteine as shown in Table 7. The elution profile of *E. coli* enzyme on DEAE-Sephadex column chromatography is similar to that of Fraction II enzyme of *B. subtilis* but *E. coli* enzyme is different from Fraction II enzyme with respect to the substrate specificity. Evidence has accumulated in recent years indicating that cystathionine cleavage enzymes from various microorganisms can decompose a wide variety of amino acids besides cystathionine[45)46)47)].

Gel filtration with Sephadex G-200 of the enzyme protein.

When the crude enzyme solution was applied to a column of Sephadex G-200

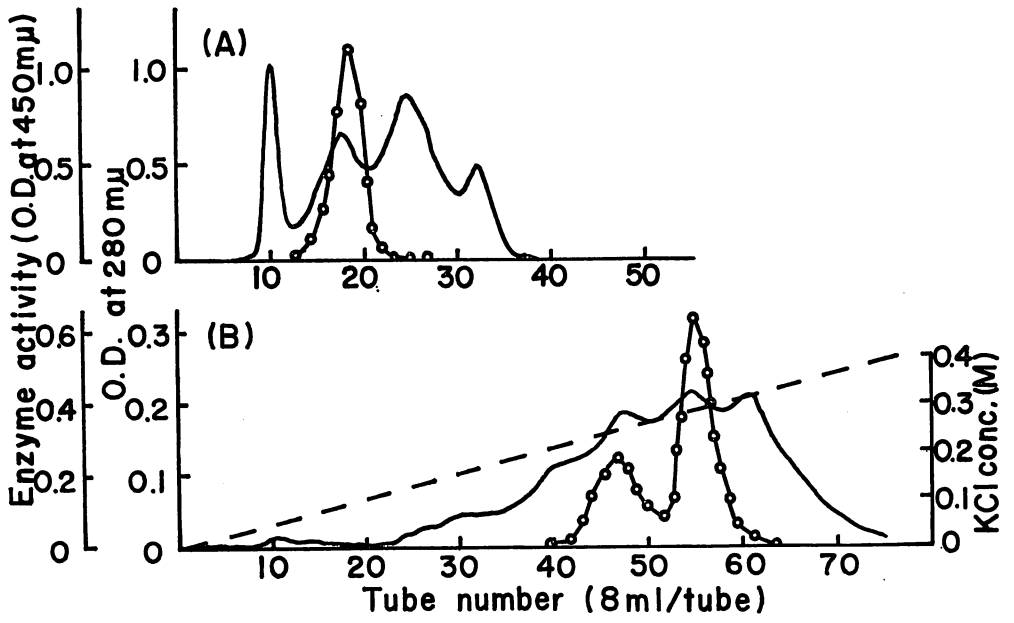

Fig. 20a. Elution patterns of cystathionine cleavage enzymes of *B. subtilis* from a column of Sephadex G-200 (A) and that of DEAE-Sephadex A-50 (B).

and eluted with the phosphate buffer solution the enzyme activity was found in one peak. The active fraction obtained by gel filtration was then charged on the column of DEAE-Sephadex A-50 and developed by a linear gradient Chromatography. As shown in Fig. 20a, two active fractions were found in the pattern.

This indicates that both fractions of cystathionine cleavage enzyme have almost the same molecular weights. The molecular weights were calculated to be about 80,000 from the behavior of the enzyme proteins on molecular sieve chromatography of Sephadex G-200. This value is fairly different from cystathionine cleavage enzyme of rat liver (190,000)[48][49], but similar to cystathionine synthetase of rat liver (78,000)[50].

Effects of pH and temperature on the enzyme activity.

The effect of pH was tested in 0.2 M Tris-HCl buffer. The enzyme reaction required an alkaline pH and the pH optimum was pH was pH 8.8 and pH 9.4 for Fraction I and II, respectively, as shown in Fig. 21.

These pH optima found with *B. subtilis* enzymes are higher than that found with the enzymes of *Neurospora crassa* (pH 7.5) and rat liver (pH8.0). The pH activity curve of cystathionine cleavage enzyme of *E. coli* is fairly consistent with that of cystathionine cleavage enzyme I of *B. subtilis*.

The temperature activity curves of the cystathionine cleavage enzymes are shown in Fig. 22. The enzyme activity was assayed with 2 mM DL-cystathionine

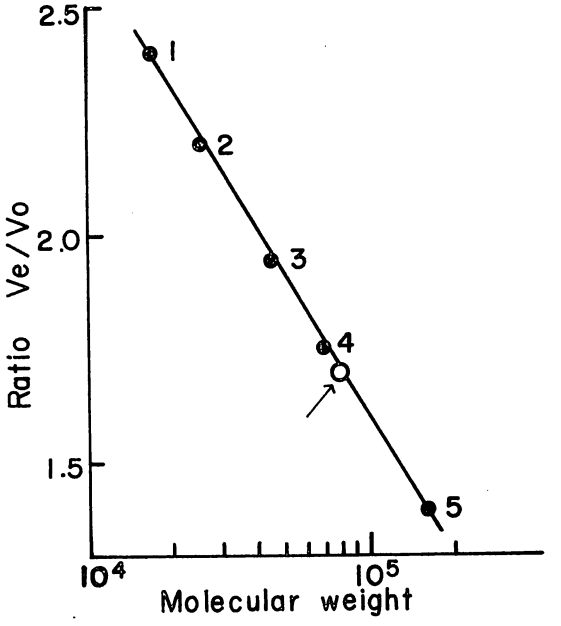

Fig. 20b. Plot of the ratios of V_e to V_o against log molecular weight for proteins on a Sephadex G-200 column (1.5× 90.0c m).
1, myoglobin; 2, chymotrypsinogen;
3, ovalbumin; 4, bovine albumin;
5, γ-globulin.
Arrow indicates the position of cystathionine cleavage enzyme of *B. subtilis*.

at the optimal pH condition. The optimum temperatures in Tris buffer (pH 9.2) were 41.5 C and 38.0 C for Fraction I and II, respectively. The optimal temperature of *E. coli* cystathionine cleavage enzyme was 50 C.

The enzyme solution was incubated in 0.05 M phosphate buffer (pH 7.2) at various temperatures. As shown in Fig.23 cystathionine cleavage enzymes (Fractions I and II) were considerably unstable and a significant decrease in the activity was found for 20 min incubation at above 30 C. A half of the total activity was lost at 43 C for 20 min.

Effect of inhibitors on the enzyme activity.

Effects of various compounds on the enzyme activity were examined using the crude extracts (Table 8).

Sulfhydryl reagents such as p-chloromercuribenzoate (PCMB) and N-ethylmaleimide (NEM) did not inhibit the enzyme activity. On the other hand, NH_2OH and semicarbazide inhibited the enzyme reaction. It is suggested that the cystathionine cleavage enzyme requires pyridoxal phosphate for its activity as has been reported.

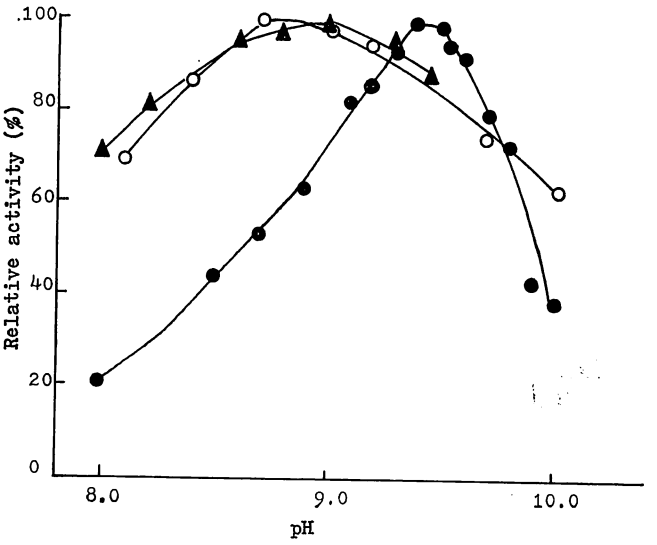

Fig. 21. Effect of pH on the activity of cystathionine cleavage enzymes.
Enzyme was assayed for 2 mM substrate in 2.5 ml of 0.2 M Tris-HCl buffer (pH 8.0—9.5) and 0.2 M borate —NaOH buffer (pH 0.5—10.0).
○——○, cystathionine cleavage enzme I; ●——●, enzyme II in *B. subtilis*; ▲——▲, cystathionine cleavage enzyme in *E. coli*.

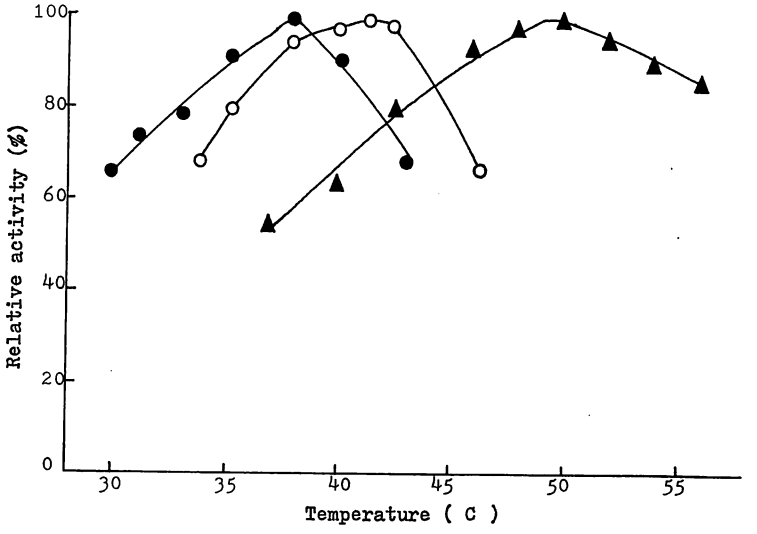

Fig. 22. Activity-temperature curves of cystathionine cleavage enzymes.
Enzyme assay was made with 2 mM substrate, 0.4 or 0.8 μg pyridoxal phosphate in 2.5 ml of 0.2 M Tris-HCl buffer at optimum pH.
○——○, enzyme I; ●——●, enzyme II in *B. subtilis*; ▲——▲, enzyme in *E. coli*.

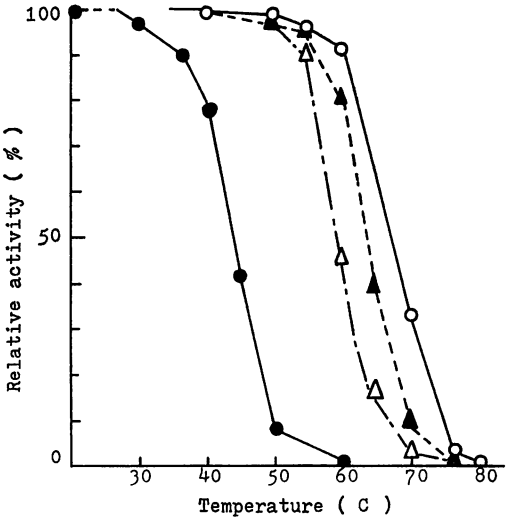

Fig. 23. Stability of enzymes against heating.
The crude enzyme preparations were incubated for 20 min at designated temperature prior to assay.
●——●, cystathionine cleavage enzyme; ○——○, histidine deaminase; ▲——▲, glutamine synthetase; △——△, protease.

Table 8. Effect of inhibitors on the activity of cystathionine cleavage enzymes

Inhibitor	Final conc. (M)	Relative activity	Inhibition (%)
PCMB	1×10^{-3}	108	—
NEM	1×10^{-3}	102	—
Glutathione (SH)	1×10^{-3}	104	—
EDTA	1×10^{-3}	108	—
KCN	1×10^{-3}	11	89
$NaHSO_3$	1×10^{-3}	91	9
NaN_3	1×10^{-3}	102	—
H_2S	1×10^{-3}	97	3
NH_2OH	1×10^{-3}	2	98
Semicarbazide	1×10^{-3}	30	70
Methionine	1×10^{-3}	108	—
Methionine	5×10^{-3}	100	—
Cysteine	1×10^{-3}	76	24
Cysteine	5×10^{-3}	54	46
Homocysteine	1×10^{-3}	71	29
Homocysteine	5×10^{-3}	43	57

Enzyme activity was assayed in a reaction mixture which contained 2 mM substrate, 0.4 μg/ml pyridoxal phosphate and 1 mM or 5 mM inhibitors in 2.5 ml of 0.2 M Tris-HCl buffer at pH 9.4.
PCMB, p-chloromercuribenzoic acid; NEM, N-ethylmaleimide;
EDTA, ethylenediaminetetraacetic acid.

The activity of cystathionine cleavage enzymes increased in proportion to the amount of pyridoxal phosphate in the reaction mixture. The Km value for pyridoxal phosphate was about 1.5 μm (Fig. 24).

Cysteine and homocysteine also inhibited the enzyme activity but methionine did not. As shown in Fig. 25 and Table 9 the activity was inhibited by homo-

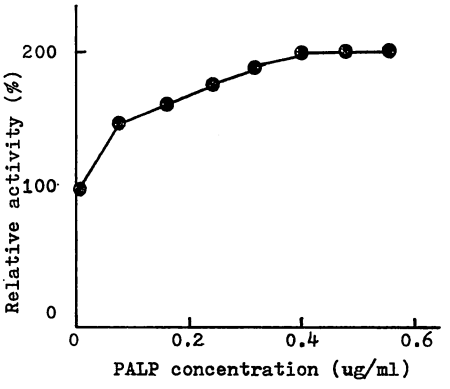

Fig. 24. Effect of pyridoxal phosphate (PALP) concentration on the activity of cystathionine cleavage enzyme Fraction II in *B. subtilis*.

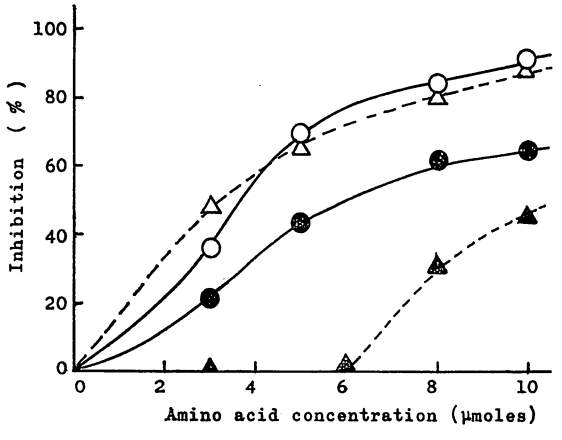

Fig. 25a. Effect of DL-homocysteine and L-cysteine on cystathionine cleavage activities.

Fraction I enzyme, added with DL-homocysteine (—○—) or L-cysteine (—●—); Fraction II, added with DL-homocysteine (--△--) or L-cysteine (--▲--).

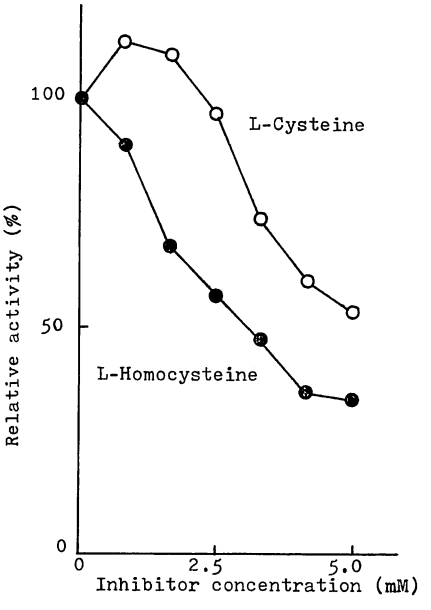

Fig. 25b. Effect of L-homocysteine and L-cysteine on the activity of cystathionine cleavage enzyme Fraction II.

cysteine. Homocysteine in the concentration of 4×10^{-3} M completely inhibited activities of both Fractions. The inhibition percentage by cysteine was not more than 80 %. At lower concentrations cysteine did not inhibit the activity of Fraction II, unlike Fraction I.

Table 9. Effect of inhibitors on the activities of cystathionine cleavage enzymes Fractions I and II

Additions	Relative activity (%)		
	B. subtilis		*E. coli*
	Fraction I	Fraction II	
Control (no addittion)	100	100	100
L-Cysteine	21.8	52.8	43.9
DL-Homocysteine	27.7	34.3	94.4
L-Methionine	99.5	104.9	109.2
L-Serine	94.6	84.3	—
L-Homoserine	100.8	95.3	—
Hydroxylamine	0.07	—	—

The indicated compounds (final concentration 5 mM) were added to the standard reaction mixture.

Table 10. Summary of Km values of three cystathionine cleavage enzymes from *B. subtilis* and *E. coli* for various substrates

Substrate	Km value (mM)		
	B. subtilis		*E. coli*
	Fraction I	Fraction II	
L(+)-Cystathionine	—	8.7	—
(—)-Cystathionine	4.1	10.0	1.25
DL-Djenkolic acid	—	4.4	1.33
L-Cystine	*	5.6	—
DL-Lanthionine	*	2.0	7.7
S-Methylcysteine	*	13.3	*

Km value were determined from double reciprocal plots by the method of Lineweaver and Burk.
—, not tested; *, not cleaved.

Km value of the enzymes.

In order to know affinities of Fraction I and II enzymes to cystathionine, the Michaelis constant (Km) was determined according to the method of Lineweaver and Burk. In the case of Fraction II enzyme, cystathionine, S-methylcysteine, DL-djenkolic acid, L-cystine and DL-lanthionine were used as substrates.

As shown in Fig. 26 and Table 10, the Km values of Fraction I and II enzymes

of *B. subtilis* and cystathionine cleavage enzyme of *E. coli* were 4.1 mM, 10.0 mM and 1.3 mM, respectively.

These results with substrate specificities and Km values suggest that Frac-

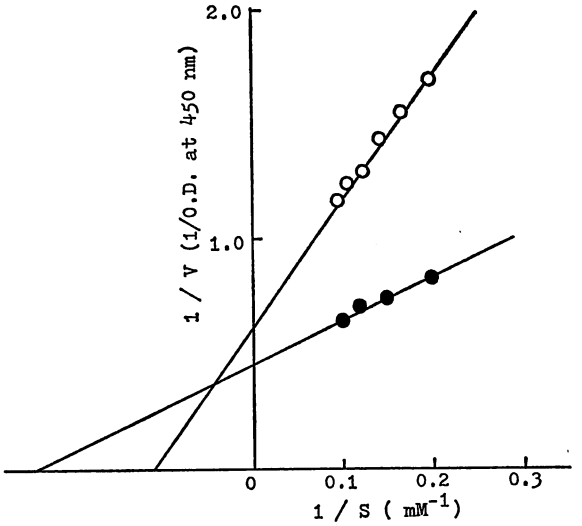

Fig. 26a. Lineweaver-Burk plots cystathionine cleavage enzymes (Fractions I and II) from *B. subtilis* for (—)-cystathionine. Fraction I (—●—), Fraction II (—○—).

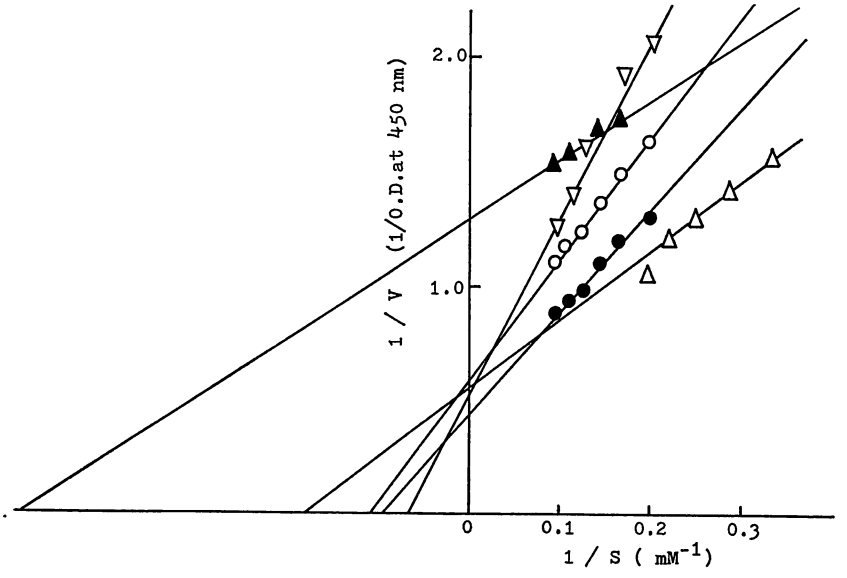

Fig. 26b. Lineweaver-Burk plots of cystathionine cleavage enzyme Fraction II for various sulfur-containing amino acids.
—○—, L-cystathionine; —●—, (—)-cystathionine; —△—, L-cystine; —▲—, DL-lanthionine; —▽—, S-methylcysteine.

tion I is the proper enzyme participating in the biosynthetic pathway of methionine and Fraction II is an enzyme which decomposes various amino acids containing sulfur bridge.

Chapter V. Cysteine Synthetase of *Bacillus subtilis*

Considering the mechanism of sporulation the interconversion between cysteine and methionine during sporulation in the cells is very important. The author, therefore, studied in the preceding chapter the cystathionine cleavage enzyme, one of the key enzymes of transsulfuration in the methionine biosynthetic pathway.

As a results of that study it was suggested that transsulfuration pathway from cysteine to methionine via cystathionine is irreversible and the conversion from methionine to cysteine during sporulation takes place through a pathway similar to that of degradation of methionine to produce mercaptan or sulfide in *B. subtilis*.

In the present chapter the author attempted to make characterization of cysteine synthetase (O-acetylserine sulfhydrylase), the final enzyme in cysteine biosynthetic pathway, in order to make clear the pathway of cysteine biosynthesis from inorganic sulfide in *B. subtilis*.

In bacteria, L-cysteine is synthesized through the pathway shown in Fig. 27. In this pathway inorganic sulfate is reduced to sulfide via two activation steps and two reduction steps and L-serine is acetylated to form O-acetyl-L-serine which then reacts with sulfide to form L-cysteine[51)52)].

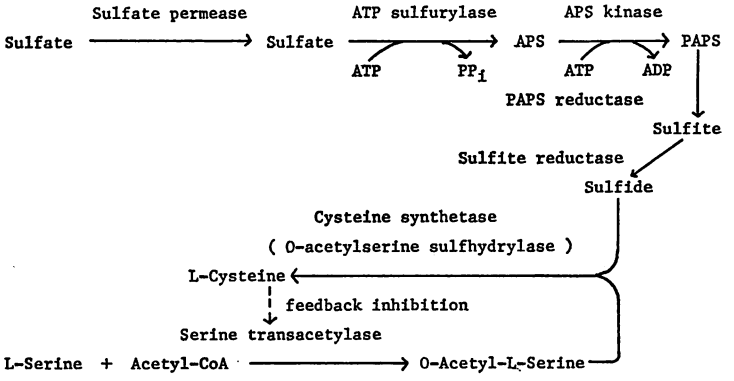

Fig. 27. Pathway of L-cysteine biosynthesis in *S. typhimurium*.
PAPS: 3'-phosphoadenosine 5'-phosphosulfate.

Materials and Methods

Organism and cultural conditions. The Marburg strain of *Bacillus subtilis* (ATCC 6051) was employed. The conditions used for growth and sporulation were the same as previously described.

The basal medium reported by DEMAIN was employed after being modified slightly.

The preparation of crude extracts. The bacterial cells were harvested by centrifugation and washed three times with 0.05 M phosphate buffer (pH 7.2). The cell pellets were suspended in tenfold volume of the same buffer and disrupted by sonication at 20 KC for 7 min. Debris was removed by centrifugation at 15,000×g for 15 min. The cell-free extract thus obtained was immediately assayed for the enzyme activity or committed to further purification.

DEAE-Sephapex A-50 column chromatography. The supernatant solution of the sonicate was treated with 0.2% protamin sulfate and fractionated by adding solid ammonium sulfate. The precipitated protein obtained between 55% and 80% saturation of ammonium sulfate was dissolved again a small amount of 0.05 M phosphate buffer and dialized overnight against the same buffer. The dialyzed enzyme solution was chromatographed on a DEAE-Sephadex A-50 column (2.0× 25.0 cm) with a gradient elution of KCl in the concentration up to 0.4 M

Assay of cysteine synthetase. The activity of cysteine synthetase was determined according to the method of Kredich et al[53] after a slight modification. The reaction system contained 30 μmoles of O-acetyl-DL-serine, 2 μmoles of sodium sulfide, 0.48 μmole of sodium EDTA, 96 μmoles of Tris-HCl buffer (pH 7.2) and an appropriate amount of enzyme in a total volume of 0.6 ml. The mixture was preincubated at 25 C for 5 min. The reaction was started by addition of enzyme solution. After 4 min incubation at 25 C, 3.0 ml freshly prepared 1 mM nitrous acid (1 part 0.1 M $NaNO_2$ to 99 parts 0.4 N H_2SO_4) was added to the reaction mixture, which was then vigorously agitated. The precipitated materials were removed by filtration and 0.2 ml of 2% ammonium sulfate was added to 1.5 ml of the filtrate. After 2 min 2.0 ml of the following mixture were added with mixing; 1 part 2% $HgCl_2$ in 0.4 N HCl, 4 parts 6.88% sulfanilamide in 0.4 N HCl, and 2 parts 0.2% N-1-naphthylene diamine dihydrochloride in 0.4 N HCl. The absorbance of the reaction mixture was read in a spectrophotometer at 540 nm, and an amount of cysteine formed was calculated using a standard curve obtained with authentic L-cysteine.

In Fig. 28, the time courses of enzyme reaction at 30 C and 37 C are shown.

Results and Discussion

Optimal assay conditions.

Unless indicated otherwise, the properties of enzyme were determined with the reaction obtained from the DEAE-Sephadex A-50 column chromatography.

As shown in Fig. 29, the enzyme reaction required an alkaline pH and the pH optimum was between 7.5 and 9.0 in Tris buffer. However, pH 7.5 was used for the standard assay since it was apprehended that O-acetylserine, one of the substrate of enzyme reaction, was labile in the alkaline pH region.

Temperature-activity curve of the enzyme is illustrated in Fig. 30. The opti-

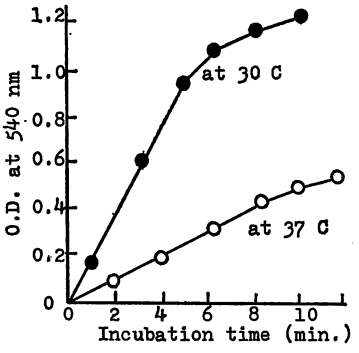

Fig. 28. Time-course of enzyme reaction of cysteine synthetase.

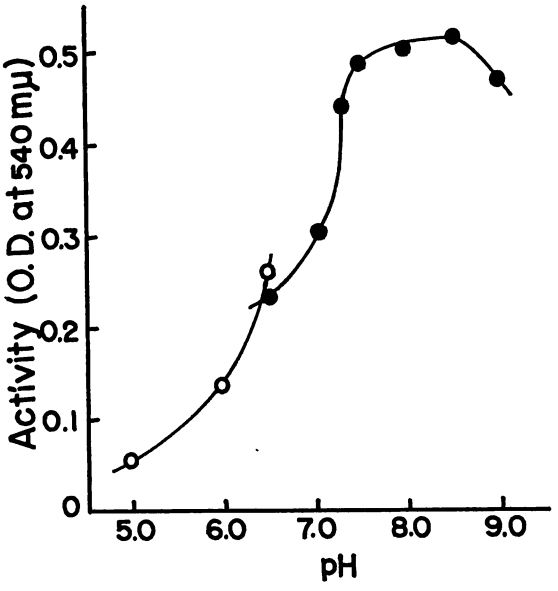

Fig. 29. Activity of cysteine synthetase as a function of pH value.
○——○, 0.15 M phosphate buffer;
●——●, 0.16 M Tris-HCl buffer.

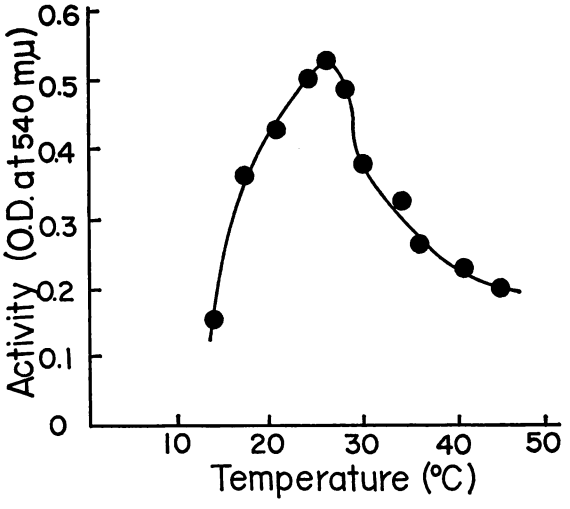

Fig. 30. Effect of reaction temperature on the activity of cysteine synthease.

mum temperature of cysteine synthetase obtained from *B. subtilis* was observed at 25 C. This temperature was lower than that observed for bacterial growth and other enzyme activities.

Effect of the composition of culture medium on the biosynthesis of cysteine synthetase.

In this experiment, the enzyme activity was assayed with crude extracts immediately after the cells were disrupted by sonication and the debris was removed by cetrifugation (Table 11).

Addition of DL-djenkolic acid to the medium or limitation of sulfur in the medium lead to the production of enzyme in higher levels, as has been previously reported with *S. typhlmurium*[52].

Growth on methionine, the final product of the methionine biosynthetic pathway, slightly increased the enzyme level. On the other hand, the enzyme level was found to be low when cells were grown on cysteine as the sulfur source.

Table 11. Effect of composition of culture media on the biosynthesis of cysteine synthetase

Culture medium	Specific activity (mμmoles min^{-1} mg^{-1})	
	12 hr	21 hr
Nutrient broth	227.9	149.6
Demain's medium	197.9	268.1
+L-cysteine (1 mM)	120.6	135.6
+DL-djenkolic acid (1.5 mM)	273.5	437.4
+L-methionine (1.5 mM)	182.9	360.8
sulfur limited	399.4	496.5

Enzyme assay was carried out under standard conditions as described in the text. Crude extracts from the cells grown in various media for 12 hr or 21 hr were used as the enzyme.

These results indicate that cysteine synthetase is derepressed when the bacteria were grown in the sulfur-deficient medium or the medium of which sulfur source is repressed by L-cysteine.

Effect of inhibitors and activators on the enzyme activity.

As shown in Table 12, the enzyme activity was inhibited by methionine and

Table 12. Effect of amino acids and acetate on the activity of cysteine synthetase

Addition	Concentration (M)	% Activity
Control (no addition)		100
+L-Methionine	2×10^{-2}	78.0
+L-Serine	2×10^{-2}	104.0
+L-Homoserine	2×10^{-2}	123.6
+Sodium acetate	2×10^{-2}	175.5

The indicated compounds (final concentration 20 mM) were added to the standard reaction mixture. Activity is expressed as percentage to the value of control.

activated by acetate. The inhibition by methionine at the concentration of 2×10^{-2} M corresponded to 22% of the total activity (Fig. 31). The inhibition by methionine in higher concentration (above at 10 mM) did not exceed 25%. It is not clear whether this phenomenon was caused by the existence of two different enzyme fractions or that of two different states of enzyme protein.

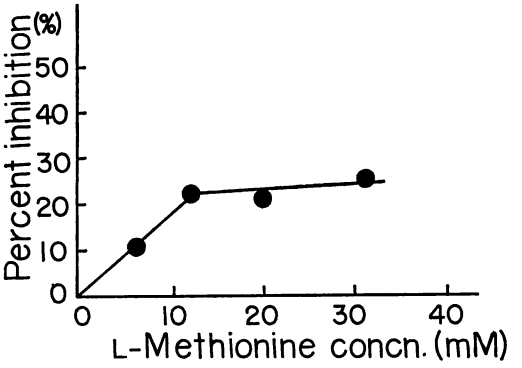

Fig. 31. Effect of L-methionine on cysteine synthetase activity.

Chapter VI. Comparison between Cystathionine Cleavage enzyme and Cysteine Synthetase of *Bacillus subtilis*

In the preceding chapters, two representative enzymes which participate in the metabolism of sulfur-containing amino acids, cysteine synthetase and cystathionine, were examined in many respects.

In the present chapter, some properties and activity changes during sporulation of cysteine synthetase and cystathionine cleavage enzyme from *B. subtilis* were observed and the metabolic changes of sulfur-containing amino acids was discussed on the basis of measurements of the enzyme activities.

Materials and Methods

Enzyme assay. Cystathionine cleavage enzyme and cysteine synthetase were assayed as previously described.

Proteinases and histidine deaminase (histidase) were assayed by the methods of HAGIWARA[54] and TABOR et al[55], respectively.

Determination of protein. Protein was determined by the method of LOWRY et al[56].

Results and Discussion

Precipitation of enzyme proteins with ammonium sulfate.

To the supernatant obtained by the treatment of protamin sulfate, ammonium sulfate was added so as to be invarious saturation percents. The greater part of proteins having the enzyme activities was recovered between 60 and 80 % saturation as shown in Fig. 32.

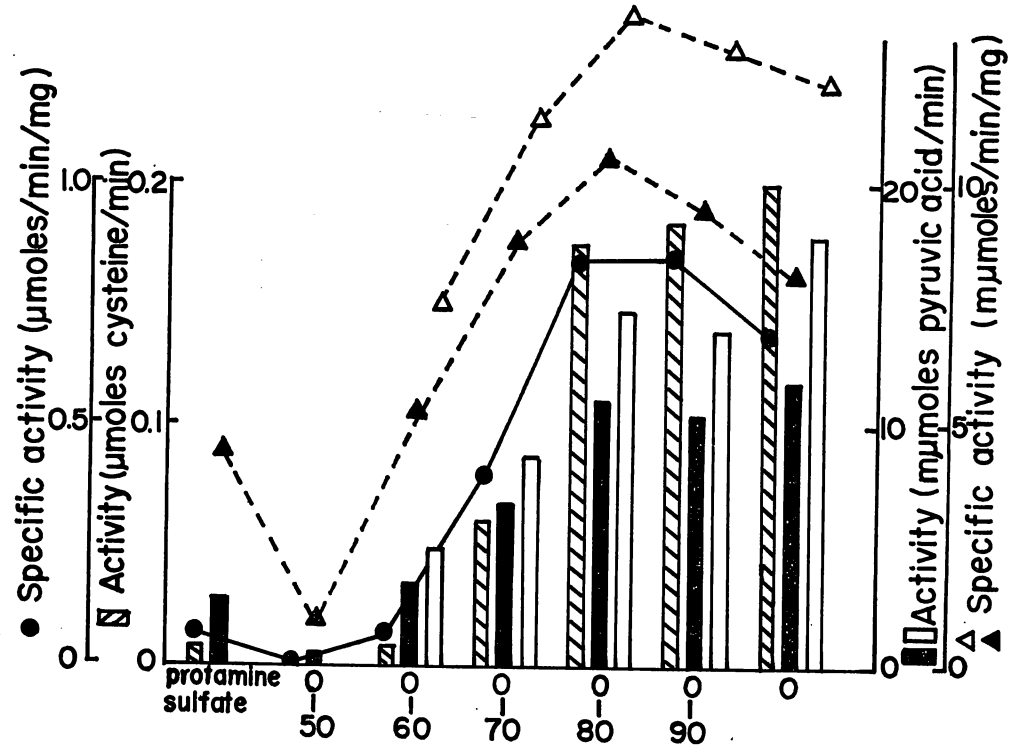

Fig. 32. Fractionation of enzyme proteins with ammonium sulfate.
■ ▲ , cleavage activity for cystathionine;
□ △ , cleavage activity for L-cystine;
▨ ● . cysteine synthetase activity.

After the precipitate was dialyzed overnight against 0.05 M phosphate buffer (pH 7.2), the resulting supernatant solution was employed for various experiments or submitted to the further purification.

Heat stability of the enzymes.

Heat stability of cystathionine cleavage enzyme and cysteine synthetase was examined as follows. The enzyme solution was incubated in 0.05 M phosphate

buffer (pH 7.2) for 20 min at various temperatures. After the enzyme solution was cooled in an ice bath, the activities of cystathionine cleavage enzyme and cysteine synthetase were measured at 37 C and 25 C respectively.

As shown in Fig. 33 cysteine synthetase was relatively stable to heat and inactivated only by the incubation at above 50 C for 20 min. As compared with cysteine synthetase and some other enzyme from *B. subtilis*, cystathionine cleavage enzyme was relatively unstable and a significant decrease in the activity was found by the incubation at higher temperatures than 30 C for 20 min.

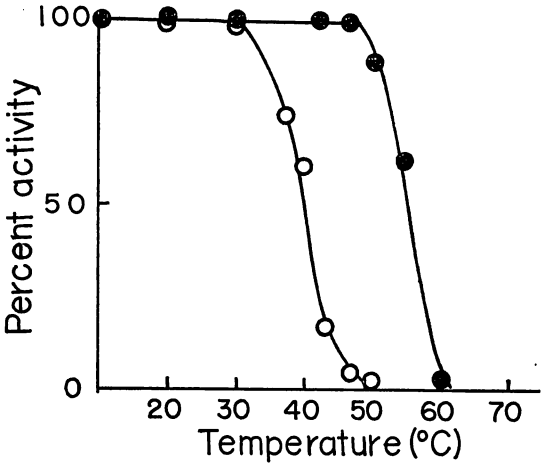

Fig. 33. Heat-stability of enzymes.
○——○, cystathionine cleavage activity;
●——●, cysteine synthetase activity.

Effect of proteases on the enzymes.

It has been reported that after growth was completed the production of proteases became vigorous and that rapid protein turnover and the supply of amino acids took place during sporulation in *Bacillus* species. The latter processes may be closely related to the activities of proteases[57)58)59)60)].

In Fig. 34 the protease production during growth and sporulation of *B. subtilis* is shown with a profile of induction and degradation of an inducible enzyme, histidase.

As an approach to make clear *in vivo* functions of these proteases, an attempt was made to ascertain whether the inactivation of enzymes during sporulation was caused by the proteases. The precipitates obtained by 80 % ammonium sulfate treatment from culture filtrate were used as preparations of extracellular proteases. This protease solution and the DEAE-Sephadex A-50 treated preparations of cystathionine cleavage enzyme and cysteine synthetase, were incubated together at 30 C. In the case of cysteine synthetase, a significant decrease in

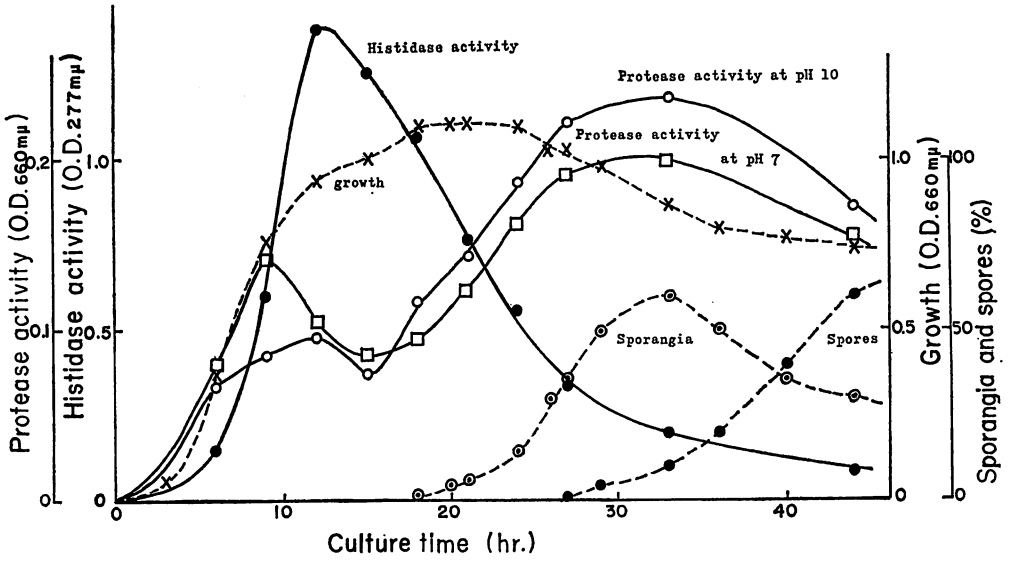

Fig. 34. Changes in protease activity and histidase activity during growth and sporulation of *B. subtilis*.
—□—, protease activity at pH 7.0; —○—, protease activity at pH 10.0; —●—, histidase activity.

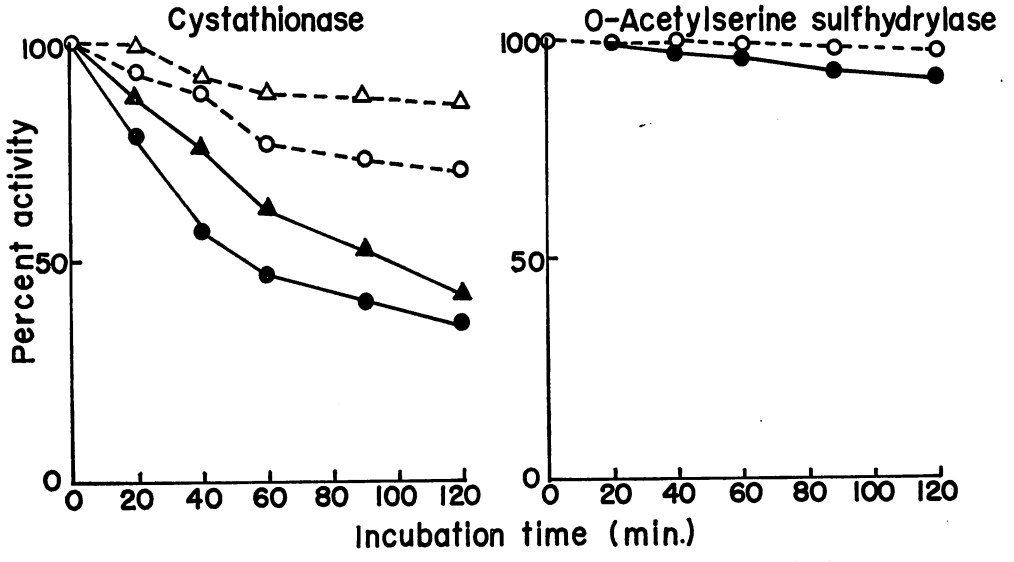

Fig. 35. Effect of extracellular proteases on the activities of cystathionine cleavage enzyme and cysteine synthetase (O-acetylserine sulfhydrylase).
The cell-free extract together with either the preparation or buffer solution were incubated at 30 C. The enzyme activities of the mixture were assayed at the indicated intervals.
—●—, Fraction I with the protease prearation; --○--, Fraction I in Tris-HCl buffer; —▲—, Fraction II with the protease preparation; --△--, Fraction II in Tris-HCl buffer; —●—, cysteine synthetase with the protease preparation; --○--, cysteine synthetase in Tris-HCl buffer.

enzyme activity was not found within 60 min incubation. On the other hand, the activity of cystathionine cleavage enzyme decreased rapidly during incubation and considerable decrease was observed even when incubated with the buffer solution only (Fig. 35)

This result indicates that cystathionine cleavage enzyme is easily attacked by proteases as compared with cysteine synthetase. This experiment, however, disregarded the conditions within the cells such as the presence of the protection mechanism for enzyme protein or the localization of enzymes.

Changes of enzyme activities during sporulation.

The speciffc activities of cystathionine cleavage enzyme and cysteine synthetase were followed during growth and sporulation (Fig. 36). Until early stage of sporulation (20 hr incubation at 37 C) specific activities of both the enzymes remained constant or increased slightly, but in the late stage of sporulation the specific activities of cystathionine cleavage enzyme decreased rapidly, and 3-5 hr later the activity of cysteine synthetase also decreased. The chromatographic patterns of the preparations from vegetative cells, sporulating cells and free spores on the DEAE-Sephadex A-50 indicate that the activity of Fraction I of cystathionine cleavage enzyme was low in sporulating cells and not detected in free spores. On the other hand, Fraction II of cystathionine cleavage enzyme

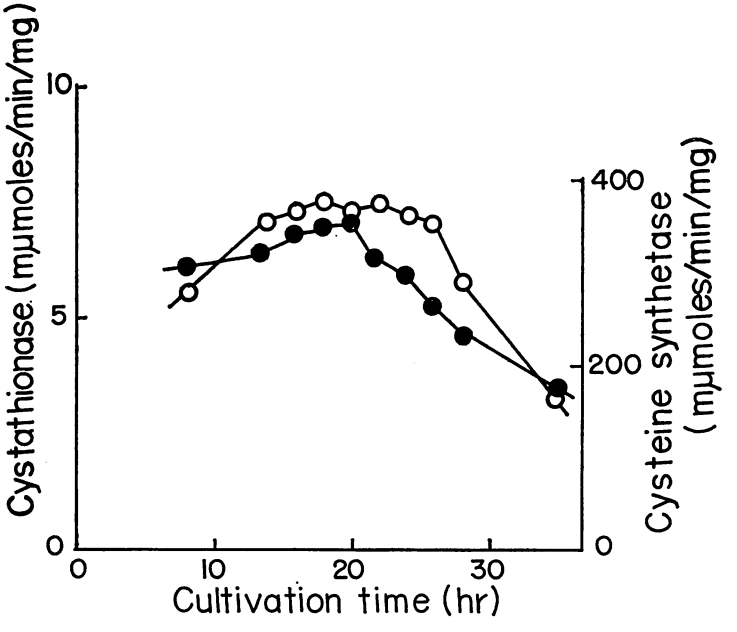

Fig. 36. Changes in specific activities of cystathionine cleavage activity and cysteine synthetase during sporulation.
—●—, cystathionine cleavage activity; —○—, cysteine synthetase.

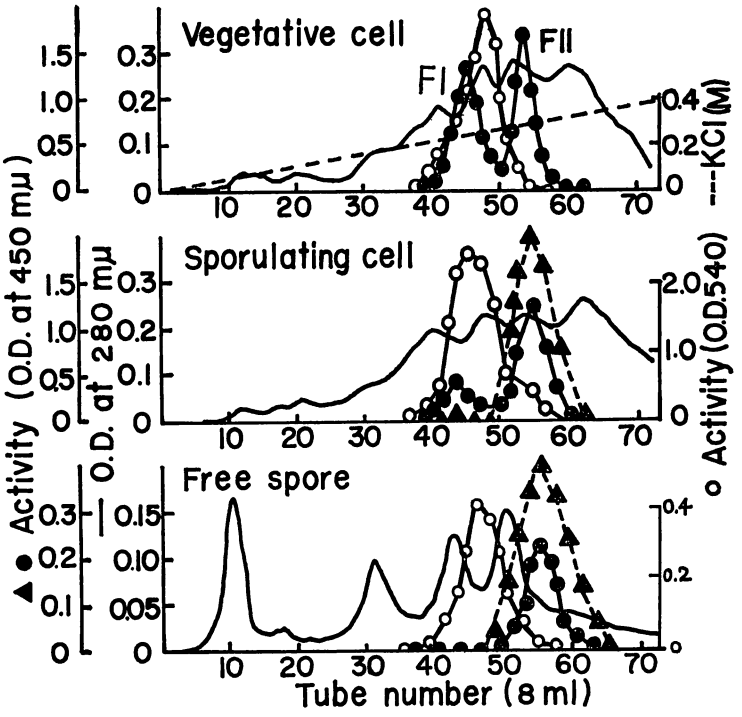

Fig. 37. DEAE-Sephadex A-50 column chromatography of cystathionine cleavage enzymes (Fractions I and II) and cysteine synthetase in cell-free extracts of *B. subtilis* at various stages.
—●—, cleavage activity for cystathionine; --▲--, cleavage activity for L-cystine; —○—, cysteine synthetase activity; ——, protein, expressed as optical density at 280 nm; ---, KCl concentration (M).

and cysteine synthetase were detected even in free spores (Fig. 37).

As shown in Table 13, the presence of activities of cystathionine cleavage enzyme and cysteine synthetase in free spores after heating rules out the contamination of proteins from vegetative cells. The fact that the ratio of cleavage activity for cystine to that for cystathionine is larger in free spores than in vegetative cells also suggests that Fraction II of cystathionine cleavage enzyme mainly accounts for the cystathionine cleavage activity in free spore.

Thus, it is clear that cystathionine cleavage enzyme, specially Fraction I is relatively unstable both *in vitro* and *in vivo* as compared with cysteine synthetase. It could be speculated that the changes in activities of metabolic interconversion between methionine and cysteine during sporulation of *B. subtilis* are explained in terms of stabilities of enzyme proteins. However, this speculation should be further investigated in connection with the other enzymes involved in the metabolic pathway of sulfur-containing amino acids.

Table 13. Effect of heating on *in vivo* activities of cysteine synthetase and cleavage enzyme for cystathionine and cystine

Treatment of cells	Enzyme activity (mμmoles/min/mg protein)		
	Cysteine synthetase	Cleavage activity	
		(+)-cystathionine	L-cystine
Vegetative cells			
No	248.6	4.55	4.71
Heat	0	0	0
Spores			
No	170.0	6.66	9.31
Heat	89.8	3.01	3.89

The bacterial cells and spores were heated at 80 C for 20 min. After quickly cooled in an ice bath, the crude extracts were assayed for the enzyme activies.

Summary and Conclusion

In bacteria belonging to genus *Bacillus* the spore coat contributes to the resistance and dormancy of the spores. It is also known that the spore coat of *Bacillus subtilis* is composed of cystine-rich protein(s).

In the present study the metabolism of sulfur-containing amino acids and changes in the enzymes participating in it during sporulation in *B. subtilis* were studied by use of tracer techniques and enzymological methods. This is an attempt to make clear the mechanism involved in the formation of cystine-rich protein(s) of the spore coat during sporulation.

In Part I, metabolism of sulfur-containing amino acids in *B. subtilis* was investigated by using ^{35}S-labeled compounds as tracers. As to the sulfur-containing amino acids in protein fraction of the cells, it was found that in vegetative cells methionine was the major component and in spores the content of cystine (cysteine) was much larger than that of methionine.

In the intracellular amino acid pool, methionine was always the major amino acid at all the stages of growth and sporulation. Besides methionine and cysteine, homocysteine and cystathionine, the intermediates of methionine biosynthetic pathway, were found in small amount in the amino acid pool.

The incorporation of sulfate-S into sulfur-containing amino acids, in the presence of various sulfur-containing amino acids, was examined by means of tracer techniques. This experiment revealed that the biosynthesis of methionine from sulfate during growth and sporulation was more or less inhibited by the addition of cysteine, cystathionine, homocysteine or methionine to the culture. The incorporation of $^{35}SO_4^{2-}$ into the cells was completely inhibited by L-cysteine at the concentration of 10^{-3} M. In the presence of methionine or homocysteine, radio-

activity of $^{35}SO_4^{2-}$ was incorporated into cysteine but not into methionine.

It is suggested from these results that in *B. subtilis* methionine is synthesized from sulfate through cysteine, cystathionine and homocysteine as the case in *Salmonella* or *Neurospora* and that the metabolism of sulfur-containing amino acids in *B. subtilis* is strongly regulated by methionine.

In the experiments using $^{35}SO_4^{2-}$, ^{35}S-methionine or ^{35}S-cysteine as tracers it was found that *B. subtilis* utilized sulfate, methionine or cysteine as the sole sulfur source and that the interconversion between methionine and cysteine took place in the cells.

These results indicate that the interconversion between methionine and cysteine take place at all the stages in the life cycle and that methionine is metabolized to cysteine directly or indirectly during sporulation.

In order to make clear the metabolic pathway of sulfur-containing amino acids and its regulation mechanism in *B. subtilis*, the key enzymes in methionine biosynthesis were isolated and their properties were studied in Part II.

Cystathionine cleavage enzyme (cystathionase) of *B. subtilis* was fractionated to two fractions (Fractions I and II) by DEAE-Sephadex A-50 chromatography. Fraction I had comparatively high affinity to cystathionine (km; 4.1×10^{-3} M) and was thought to be the proper enzyme in methionine biosynthesis. The pH and temperature optima of Fraction I were at pH 8.8 and 41.5 C, respectively. Fraction II showed relatively wide substrate specificity for various amino acids containing sulfur bridge. The affinity of Fraction II enzyme to cystathionine was relatively low (km; 1.0×10^{-2} M). The pH and temperature optima of Fraction II enzyme were at pH 9.4 and 38 C, respectively.

Both the enzymes (Fractions I and II) were unstable to heating and lost activities on incubation at 30 C for 20 min. Activities of both the enzymes were inhibited by KCN, NH_2OH, semicarbazide, homocysteine and cysteine. Cysteine inhibited fraction I enzyme but not Fraction II enzyme at low concentrations (lower than 2.0×10^{-3} M).

The reaction produced by cystathionine cleavage enzyme were identified, and it was found that the reverse reaction of transsulfuration did not occur in *B. subtilis*: the α-keto acid produced by this reaction was pyruvate only. In the reaction products α-keto-butyrate was not detected.

Cysteine synthetase (O-acetylserine sulfhydrylase) was prepared from cells of *B. subtilis* by ammonium sulfate fractionation method and DEAE-Sephadex A-50 chromatography. This enzyme was compared with cystathionine clevage enzymes in regard to some biochemical properties. Variations in activities of these enzymes were also observed during sporulation.

The optimum pH value and the optimum temperature of cysteine synthetase were pH 7.5 and 25 C, respectively. This enzyme was considerably stable at the temperature below 50 C, and it was not so susceptible to proteases as compared with cystathionine cleavage enzyme.

Production of the enzyme was repressed when the cells were grown in the medium containing cysteine, and it was derepressed by growing the cells in the chemically defined medium deficient in sulfur or the medium containing djenkolic acid as the sole source of sulfur. Activity of this enzyme was inhibited by methionine and increased by acetate.

Cysteine synthetase was relativly stable throughout all the stages of the life cycle. The specific activity of Fraction I enzyme of cystathionine eleavage enzyme, however, decreased rapidly when the sporulation commenced and could not be detected in the free spore. Such difference in stabilities between cystathionine cleavage enzyme and cysteine synthetase is thought to be closely related to the change in metabolic interconversion between methionine and cysteine during sporulation of *B. subtilis*.

At the stages of vegetative growth, the biosynthesis and metabolism of cysteine may be under the metabolic control and cysteine and methionine remain at the constant levels. It is possible that in the developing forespore cysteine accumulates in the cells, due to the unstability of metabolic systems of cysteine. The increased incorporation of cysteine into the spore coat polypeptides may be connected to the spore coat formation. The spore coat will become morphologically apparent at the stage in which the above-mentioned biochemical events take place.

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