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Effect of Methionine on Sporulation of *Aspergillus niger*

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Introduction

Numerous investigations of fungi have been reported, while there are little biochemical studies on the process of reproduction of fungi. As to the sporulation, there are only a few reports, and most of them are connected with indirect observations such as the effect of environmental factors or nutritional conditions on sporulation but not with direct analysis of metabolic changes during sporulation. HAWKER (1) surveyed the physiology of fungal reproduction and defined many of its out-standing problems, however, direct investigations on metabolism were not carried out at all. As an obvious physiological necessity, sporulation requires the transport of materials needed for spore formation from the mycelium to the developing spores (2, 3). As to the role of nitrogen in sporulation, MORTON *et al.* (4) stated that 'absence of nitrogen is an important factor in inducing, or at least permitting, sporulation to occur' and MORTON has assumed that the primary aerial stimulus to sporulation is associated with some physical change at the cell surface (5). Some metabolic changes are often coincident with the onset of sporulation, e.g., changes in respiratory rate (6) and nitrate level (7), appearance of tyrosinases (8) or other enzymes (9), formation of carotenoids (10), accumulation of colinesulfate (11), and changes in amino acid composition (12). On the other hand, TANAKA *et al.* have shown the fine structure of developmental hyphal cell and conidio-bearing apparatus of *Aspergillus niger* by electron microscopy (13, 14). Recently BEHAL *et al.* (15, 16, 17) and HEPDEN *et al.* (18) suggested a possible relationship between nucleic acid metabolism and fungal sporulation. Furthermore, KUMAGAI *et al.* reported that the mycochrome plays an important role with photocontrol of conidial development in fungi, *Alternaria tomato* (19).

It must be emphasized that the sporulation is not a simple, or even a single process, but a metabolic sequence conditioned by a different set of

biochemical and other factors. As mentioned in the earlier report (20) suitable methods of growth control to separate effectively the process of sporulation from the mycelial growth have been required for further studies. The results of such a study are presented here. This paper deals with methionine metabolism in relation to effects of amino acid analogs and base analogs on sporulation.

Materials and Methods

Cultures of organisms

Aspergillus niger A 1015 was grown at 30°C and harvested on suitable stages. Details of the culture was described in the earlier paper (20).

Methionine requiring mutant of *Aspergillus niger* was obtained by ultraviolet irradiation in our labolatory. The mutant was able to grow without the onset of sporulation in the basal medium but did not grow in Czapeck medium. Addition of L-methionine of suitable concentration to culture medium caused the sporulation of the mutant. The details are described in 'Results'.

Assay for methionine adenosyl-transpherase

The enzyme was prepared from the mycelium (about 20 g of wet weight). Substantially the procedure of enzyme preparation was made by the method of CANTONI (21, 22). The mycelium were homogenized with 2.5 volumes of ice cold 0.01 N acetic acid by glass homogenizer. The homogenate was centrifuged at 4,000 r.p.m. for 30 min. and the precipitate was removed. The supernatant was again centrifuged at 15,000 r.p.m. for 20 min., and the precipitate was removed. The supernatant fluid retained fat particle (acetic acid extract) was fractionated by means of ammonium sulfate. The fraction ($AmSO_4$ppt 1), which precipitated between 35 and 52 per cent saturation were collected and used directly. Before use, the ammonium sulfate paste was dissolved in a small volume of cold 0.02 M phosphate buffer at pH 6.4, and dialyzed two times for 5 hours against 100 volumes of 0.02 M phosphate buffer at the same pH. At the end of dialysis, a small precipitate was removed by centrifugation, and the pH was adjusted to 7.6 by careful addition of 1.0 N KOH. The protein concentration of the supernatant fluid was adjusted to 2 to 8 mg per ml with water. Protein was determined by

Abreviations used: $AmSO_4$, ammonium sulfate; S-AM, S-adenosylmethionine; ATP, adenosine triphosphate; GSH, reduced glutathione; 8-AG, 8-azaguanine; p-FP, DL-p-fluorophenylalanine; 5-BU, 5-bromouracil; 5-MT, DL-5-methyltryptophan; DL-ET, DL-ethionine; DYDA, Diketohydrindylidene-diketohydrindamine.

the spectrophotometric method of WARBURG and CHRISTIAN (23).

The enzyme activity was measured in the reaction mixture of the following composition: 0.02 M ATP; 0.04 M L-methionine; 0.008 M reduced glutathione (GSH); 0.3 M $MgCl_2$; 0.13 M tris-HCl buffer, pH 7.6; and enzyme protein, about 1 to 3 mg; final volume 1.4 ml. The reaction for 15 min. at 37°C was stopped by the addition of two fold volumes of 6 per cent perchloric acid, and aliquots of the protein-free filtrate were used for the determination of S-adenosylmethionine (S–AM). S-Adenosylmethionine in protein-free fitrate treated by resin (Dowex 1 chloride form X–2 or X 8) was measured in a spectrophotometer. At 260 nm, a molar extinction coefficient of 16,000 was used to calculate the concentration of S-adenosylmethionine.

Extraction and estimation of S-adenosylmethionine from mycelium

S-Adenosylmethionine from mycelium was extracted with 2 to 4 volumes of 1.5 N perchlororic acid at room temperature. The chromatographic separation of S–AM from the acid extract and the estimation were carried out in the manner of SCHLENK (24, 25) except that the sulfuric acid (1 N–5 N) gradient method was used for Step II fraction of ionexchange chromatography, and that the effluent (3.2 N–4.5 N) was usually determined as S–AM fraction in the present paper.

Analytical procedures

For the identification of the S–AM, one-way ascending paper chromatography were accomplished with ethanol-water-acetic acid (65: 34: 1, v/v) (24) and isoamyl alchol-5 per cent KH_2PO_4 (26) as solvents. Paper chromatography of S–AM hydrolyzate (pH 4–6, 100°C, 30 min) was carried out with ethanol-water-acetic acid (65: 34: 1, v/v). Thiomethyladenosine must be hydrolyzed by 1 N hydrochloric acid for 60 minutes at 100°C prior to the test. For detection of adenine in thiomethyladenosine hydrolyzate one-way ascending paper chromatography was carried out with n-buthanol-0.6 N NH_3 (6: 1, v/v) (27) and isopropanol (680 ml)–11.6 N HCl (176 ml)–water to 1000 ml (28) as solvents. Adenine like compounds located were eluted with 0.1 N HCl and identified with ultraviolet spectrometry. In these paper chromatography, Toyo Roshi filter paper 51–A was used.

The purine linked or free ribose in the acid hydrolyzate were estimated with the orcinol test of BROWN (29).

Amino acid was measured by the ninhydrin reaction of YEMM and COCKING (30).

Manometric procedures

Disks of mycelium, 5 mm in diameter, were cut and washed in deionized

water at room temperature.

Rates of mycelium respiration were determined by measuring the oxygen uptake of about twenty mycelium disks (dry weight approximately 5–10 mg) suspended in 4×10^{-2}M potassium phosphate buffer (2.0 ml., pH 7.0) at 30°C. The oxygen uptake was measured by WARBURG manometer for 2 hours.

Chemicals

Amino acids (L– or DL–) were purchased from Nippon Rikagaku K. 8-Azaguanine (8–AG), DL-p-fluorophenylalanine (p-FP), and glutathione (GSH) were obtained from Nutritional Biochemical Corp., 5-bromouracil (5-BU) and DL-ethionine (DL–ET) from Tokyo Kasei K.K., DL-5-methyltryptophan (5–MT) and adenosine triphosphate (ATP) from Sigma Chemicals Co.

TABLE 1 Effects of amino acid- and base-analogs in different concentrations on sporulation of the mold

Condition of culture	Compound					
	Concentration	Sporulation at 24 hr after addition of analogs Visual units[a]				
	M	DL-ET	p-FP	5-MT	8-AG	5-BU
Replacing medium[b]	None	+++++	+++++	+++++	+++++	+++++
	1×10^{-4}	++	+++++	+++++	+++++	+++++
	2×10^{-4}	0	+++++	+++++	+++++	+++++
	5×10^{-4}	0	+++++	+++++	+++++	+++++
	1×10^{-3}	0	+++	+++	++++	+++++
	2×10^{-3}	0	+++	+++	++++	+++++
	5×10^{-3}	0	++	++	+++	++++
	1×10^{-3}	0	++	++	+++	++++
Growing medium[c]	None	+++++	+++++	+++++	+++++	+++++
	1×10^{-4}	+++++	+++++	+++++	+++++	+++++
	2×10^{-4}	+++++	+++++	+++++	+++++	+++++
	5×10^{-4}	++++	++	+++	+++	+++++
	1×10^{-3}	++	+	++	++	++++
	2×10^{-3}	+	0	0	±	+++
	5×10^{-3}	0	0	0	0	±
	1×10^{-2}	0	0	0	0	±

a +++++ : Optimum sporulation.
b Curtures were grown in 20 ml of basal medium at 30°C. At 20 hr of growth culture medium was replaced by 20 ml of each compound solution.
c Each compound was supplied in 20 ml of culture medium at 20 hr of growth.

Results

Effects of the base analogs and amino acid analogs on the mold development

DL-Ethionine (DL–ET), p-fluorophenylalanine (p–FP), 5-methyltryptophan (5–MT), 8-azaguanine (8–AG), and 5-bromouracil (5–BU) have been tested for the ability to alter mold development. At the inductive period of conidiophore formation, each analog was supplied to the basal medium at the various concentrations. In replacing culture, the medium was replaced to the solution of analog at various concentrations. The effects of concentrations are shown in Table 1. In replacing medium, 8–AG, 5–BU, 5–MT, and p-FP (5×10^{-3}M to 1×10^{-2}M) showed the inhibition of sporulation with higher concentrations that in growing medium, while DL–ET showed complete inhibition of sporulation with the concentration of 2×10^{-4}M.

In Table 2, the effects of analogs used are shown as the types of physiological response. Briefly, two general types of response were elicited by these compounds at the suitable concentration.

TABLE 2 Effects of the analogs on growth

Compound	Concentration	Growth[a] (at 24 hr after addition of analog)	Sporulation (at 24 hr after addition of analog)
	M	mg dry wt.	visual units[b]
None	——	614	+++++
DL-Ethionine	5×10^{-3}	592	0
p-Fluorophenylalanine	2×10^{-3}	283	0
5-Methyltryptophan	2×10^{-3}	390	0
8-Azaguanine	2×10^{-3}	558	±
5-Bromouracil	4×10^{-3}	577	±

a Cultures were grown in 20 ml of basal medium at 30 °C. Each compound was supplied in culture medium at 20 hr of growth, when the dry weight of mycelium was 76 mg.

b +++++ : Optimum sporulation.

Type I: General retardation. Compounds of this type (p-fluorphenylalanine, 5-methyltryptophane) exerted a general inhibitory effect on the rate of growth and sporulation.

Type II: Inhibition of sporulation. Compounds of this type (DL-ethionine, 8-azaguanine, 5-bromouracil) inhibited sporulation, but had little effect on growth. Especially, ethionine inhibited the sporulation without any influences on mycelial growth and differed from other amino acid ana-

logs. From the available evidence it is suggested that the sporulation may be induced by affecting the nucleic acid and protein metabolism, and that special attention should be given to methionine.

Effect of L-methionine supplied to medium on sporulation

An attempt had been made to determine methionine requirement on growth and sporulation with methionine requiring mutant of *Asp. niger*. The results of two sets of experiment, in growing medium and in replacing medium, are shown in Table 3. In replacing medium, about 3 μg of L-

TABLE 3 Effects of L-methionine on sporulation of methionine requiring mutant[a]

Condition	Methionine concentration	Sporulation at 2 days after addition of methionine
	M	visual units[b]
Replacing medium	None	0
	5×10^{-5}	±
	1×10^{-4}	++++
	1.66×10^{-4}	+++++
	3.33×10^{-4}	+++++
	5×10^{-4}	+++++
	1×10^{-3}	+++++
	2×10^{-3}	+++++
	5×10^{-3}	+++
	1×10^{-2}	++
Growing medium	None	0
	5×10^{-5}	0
	1×10^{-4}	±
	2×10^{-4}	+
	5×10^{-4}	++
	1×10^{-3}	+++++
	1.66×10^{-3}	+++++
	2.5×10^{-3}	+++++
	5×10^{-3}	++++
	1×10^{-2}	++

a Cultures were grown in 20 ml of the basal medium. The maximum growth was observed after 5 days culture without sporulation. In the experiment L-methionine was supplied in each culture medium after 2 days of growth.

b +++++: Optimum sporulation.

methionine per mg dry weight mycelium stimulated the initiation of sporulation, while, in growing medium, about a 2-fold methionine (6 μg) was necessary. Furthermore, L-methionine, 10 μg per mg dry weight, caused the maximum sporulation in replacing medium, but in growing medium, approximately a 10-fold methionine was required.

It seems that the degree of sporulation was proportional to concentration of methionine supplied to the circumambient medium. However, excess methionine (5×10^{-3}M–1×10^{-2}M) inhibited the sporulation in either growing

TABLE 4 Effects of excess amino acids on sporulation in normal strain of the mold

Compound (1×10^{-2}M)	Inhibition on sporulation at 24 hr after addition of amino acid	
	In replacing medium[a]	In growing medium[b]
L-Leucine	No.	No.
DL-Alanine	No.	No.
L-Glycine	No.	No.
DL-Serine	No.	No.
L-Threonine	No.	No.
L-Tryptophan	No.	No.
L-Histidine	No.	No.
L-Arginine	No.	No.
L-Asparatic acid	Yes. } a little; reversible;	No.
L-Asparagine	Yes. } a little; reversible;	No.
L-Glutamic acid	Yes. } a little; reversible;	No.
L-Cysteine	Yes. } remarkable; reversible;	Yes. } remarkable; reversible;
L-Lysine	Yes. } remarkable; irreversible;	No.
L-Methionine	Yes. } remarkable; irreversible;	Yes. } remarkable; irreversible;
Amino acids mixture[c] (except methionine)	No.	No.
Amino acids mixture) (with methionine)	Yes. } remarkable; irreversible;	Yes. } remarkable; irreversible;

Cultures were grown in 20 ml of the basal medium at 30°C.

a At 20 hr of growth, culture medium was replaced by 20 ml of each amino acid solution or amino acids mixture.

b Each amino acid was supplied in 20 ml of culture medium at 20 hr of growth.

c Mixture of amino acids as follow: L-leucine; L-histidine; DL-alanine; L-asparatic acid; L-asparagine; L-glycine; DL-serine; L-threoine.

or replacing medium.

Further experiments on methionine effect were carried out using normal strain. The results were compared with effect of other amino acids on sporulation. Each amino acid in various concentrations (1×10^{-4}M–1×10^{-2}M) was added to medium at the stage of conidiophore induction. These are brought together for ready comparison in Table 4. Other amino acids except L-lysine were not so effective as methionine on sporulation. Lysine inhibited the sporulation in replacing medium only. In general, amino acids including

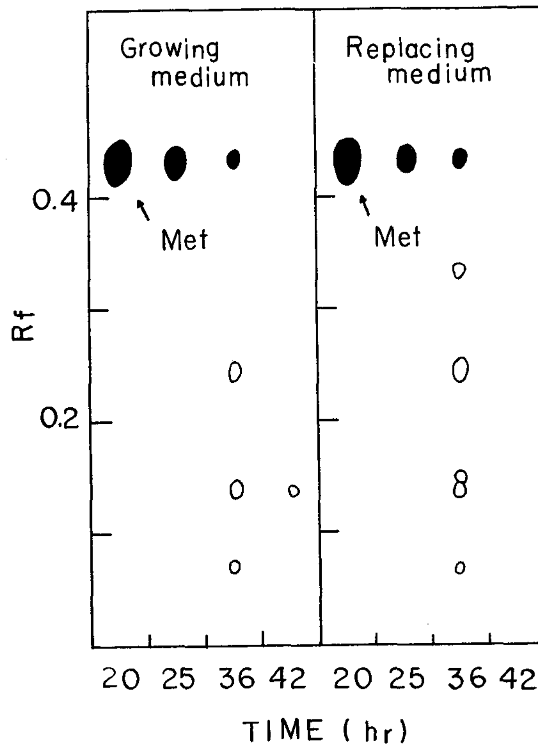

Fig. 1. Chromatographic detection of L-methionine in medium during the incubation. The 20 ml culture were used through the experiment. In growing medium L-methionine (final concentration 3.33×10^{-3}M) was added in the culture medium at 20 hr of growth. In replacing medium, culture medium was replaced by 20 ml of 3.33×10^{-3}M L-methionine solution at 20 hr of growth. Each 0.05 ml of medium was sampled in appropriate intervals during incubation and developed in n-butanol—acetic acid—water (4: 1: 5, v/v) by one-way ascending paper chromatography used Toyo Roshi filter paper No. 50. Solid symbol: L-methionine.

L-methionine had little effect on sporulation at low concentrations (1×10^{-4}M–5×10^{-3}M). However, the results that high concentrated methionine inhibited the sporulation agree very closely in two strain.

It should be noticed that only the amino acid mixture with methionine affected the sporulation, and that the other amino acids except methionine did not dilute the methionine effect.

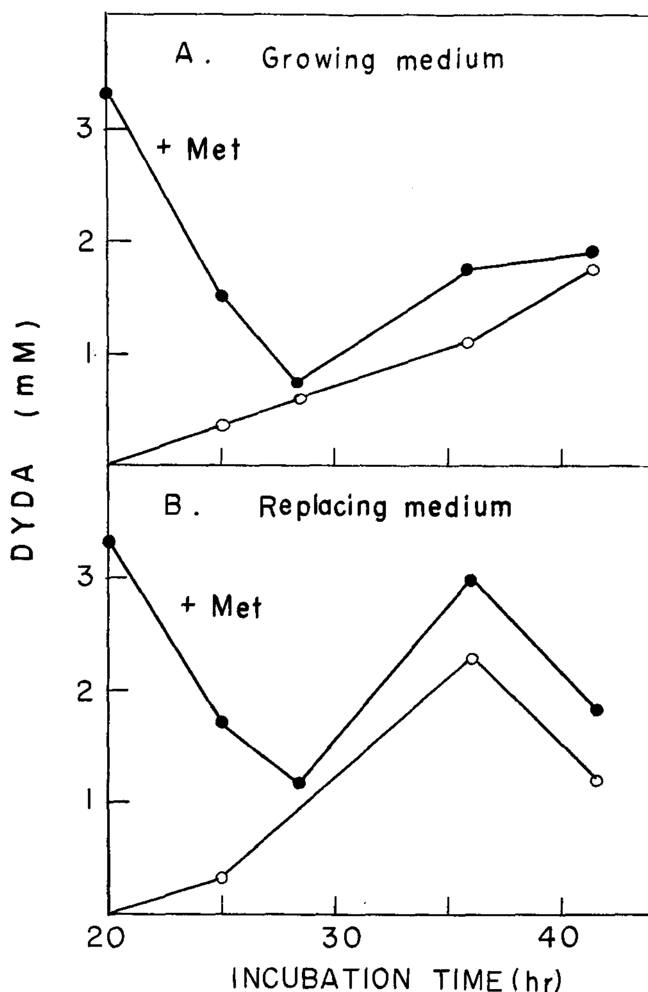

Fig. 2. Changes in the amount of ninhydrin reacting substances in medium. Cultures were grown as described in fig. 1. Values of DYDA concentration of culture medium at 20 hr are taken as 0. ●——●, with L-methionine; ○——○, without L-methionine.

Uptake of L-methionine by mycelium of normal strain

The uptake of methionine by mycelium was studied for methionine metabolism. The results of two sets of experiments, in growing medium and in replacing medium, are shown Fig. 1 and Fig. 2, which bring out the fact that the methionine in medium was rapidly incorporated into mycelium under two conditions. Fig. 1 represents the decrease of methionine in medium chromatographically and Fig. 2 shows the changes of ninhydrin reaction substances (DYDA, Diketohydrindylindene-diketohydrindamine) in medium contained L-methionine. It appears that the rapid decrease of initial DYDA concentration in medium dependent upon the cellular accumulation of methionine. In addition, the increase of the ninhydrin reacting substance was observed in control medium. The observation suggests the output of some ninhydrin reacting substances from mycelium.

Moreover, amino acid like unknown compounds was detected with paper chromatography (Fig. 1).

Effect of L-methionine on the respiration of mycelium

Above observations led to a study of the effect of methionine on the metabolism of mycelium. As an example the effect of methionine on respiration of mycelium was tested manometrically.

In normal strain, the optimum pH value was about 7.0 for endogenous respiration of mycelium disk. The activity of endogenous respiration was high ($Q_{O_2}=15.0$) at earliar stage of conidiophore, whereas the activity decreased ($Q_{O_2}=7.0$) after sporulation. Before and after sporulation, L-methionine (5×10^{-3}M–2×10^{-2}M) or 2–4 dinitrophenol (5×10^{-5}M–2×10^{-4}M) had little effects on the rate of oxygen uptake of mycelium disks, and also L-methionine had no influences on the respiration rate of mycelium which starved between 24 hr and 48 hr in deionized water at 30°C. Furthermore, the effects of phosphate buffer concentrations on the rate of respiration was not found in these experiments.

In methionine requiring mutant, the value of Q_{O_2} of mycelium cultured two days without methionine was 7.2. After addition of L-methionine (1×10^{-3}M) to 2 days culture, mycelium disks led to an increased rate of endogenous respiration. The value of Q_{O_2} became about 12.4 after 15 hr of L-methionine addition (conidiophore inductive stage). But the activity of endogenous respiration falled in 3 of Q_{O_2} value after 3 days culture.

On the other hand, the effect of L-methionine (1×10^{-3}M–2×10^{-2}M) on the rate of oxygen uptake of mycelium disks (2 days culture) was observed. A miner component of endogenous respiration (about 15–30 per

cent) was stimulated by methionine (1×10^{-3}M–2×10^{-2}M). The specificity of L-methionine stimulation was studied by testing the effect of other compounds on the respiration of mycelium disks. The following compounds were tested at various concentrations (1×10^{-3}M–2×10^{-2}M): L-leucine; D-glucose; succinic acid; fumuric acid; citric acid; hydroquinone; pyrocatechol. L-Leucine and D-glucose had the effect of respiration stimulation as shown in methionine, but other compounds had little effect on respiration. Consequently, L-methionine alone was not the compound which excerted stimulation.

However, the respiration of the mycelium disks, which had been incubated for 15 hr with methionine after 2 days of culture, was slightly (about 20%) inhibited by methionine (5×10^{-3}M). The respiration was significantly about 60 per cent inhibited by glucose (2×10^{-2}M). The effects of other compounds were not investigated.

The results of experiments showed that L-methionine had only a little effect on the rate of endogenous respiration of the methionine requiring mutant grown without methionine.

S-Adenosylmethionine formation in normal strain in vivo

(1) *Chromatographic separation*

L-Methionine (final concentration 3.33×10^{-3}M) was added to medium at the stage of conidiophore induction (about 18–21 hours). *Asp. niger* was continuously grown in medium added methionine and harvested at various intervals. Chromatographically a S–AM like compound was separated from the acid extract of mycelium. Fig. 3 shows the result of chromatographic separation using sulfuric acid gradient method for S–AM Fraction. The result suggests that the S–AM like compounds have been separated effectively from thiamine. In further investigation, it could not be found the S–AM like fraction in methionine requiring mutant, which starved methionine.

(2) *Identification of S-adenosylmethionine*

S-Adenosylmethionine like compound was identified by following method. The compound in chromatographic effluent was used for identification. In paper chromatography, u. v. absorbing compound in effluent (3.2 N–4.5 N in H_2SO_4) migrated as a single ninhydrin reacting spot with an R_f value of 0.30 in ethanol-water-acetic acid=65: 34: 1 (ascending technique). Similarly the compounds showed an R_f value of 0.80 in isoamylalcohol-5 per cent KH_2PO_4 as solvent. The R_f values in these solvent systems agreed closely with those obtained in analysis of CANTONI (26) and SCHLENK (24). Further-

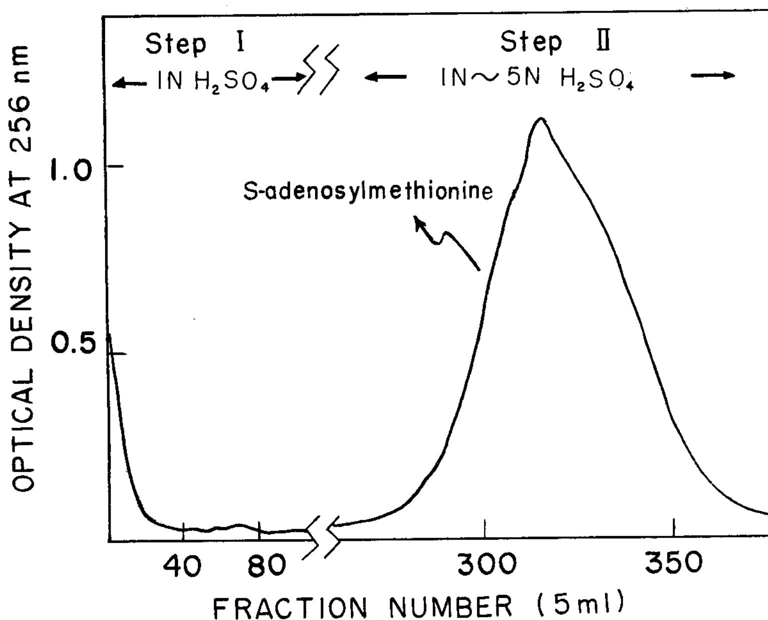

Fig. 3. Chromatographic separation of S-adenosylmethionine like compound in mycelium extract. The 200 ml culture was used in the procedure. L-Methionine (final concentration 3.33×10^{-3}M) was added in medium at 24 hr of growth. S-Adenosylmethionine like compound was extracted from mycelium at 15 hr after of L-methionine addition. The compound was separated by the procedures as described in Materials and Methods.

more, the paper chromatographic analysis of the hydrolized compound (pH 4–6, 100°C, 30 min.) in ethanol-water-acetic acid (ascending technique) revealed the u. v. absorbing spot (R_f 0.75) and ninhydrine reacting spot (R_f 0.65). Each of the two products has been identified as thiomethyladenosine and homoserine by CANTONI (26). In the present investigation, the thiomethyladenosine like compound on paper was cut out and eluted in 0.1 N hydrochloric acid. The eluted compound was hydrolized with 1 N hydrochloric acid at 100°C for 60 min. The hydrolized thiomethyladenosine like compound was identified as adenine with paper chromatography and spectrometry. The molar ratio of adenine, ribose, and amino acid in the compound of effluent was shown in Table 5.

The same results were obtained in the identification of the S-adenosylmethionine like compound from other incubation stage (30 hr. with methionine).

TABLE 5 Analysis of S-adenosylmethionine-like compound present in chromatographic effluent[a]

	Adenine	Ribose	Amino acid
μmoles / ml	0.31	0.34	0.28

a Mixture of No. 330–334 fraction shown in Fig. 3 was used. Analytical procedures are described in Materials and Methods.

These results suggest that the compound obtained by chromatographical separation is S-adenosylmethionine.

(3) *Accumulation of S-adenosylmethionine in mycelium*

Endogenous S–AM contents are shown in Table 6, which have a high value at the early stage of sporulation (31 h).

TABLE 6 Level of endogenous S-adenosylmethionine in mycelium at different growth stages

Growth		Amount of S-adenosylmethionine per g wet weight
hr	g wet weight	μmoles
20	7.2	0.062
27	10.1	0.082
31	12.8	0.092
40	22.4	0.069
44	24.7	0.054

Mycelium from 200 ml culture were used throughout this experiments. S-Adenosylmethionine was extracted and estimated as described in Materials and Methods.

After addition of L-methionine (500 μg per ml medium) S–AM was extracted from mycelium and estimated at various incubation time. The results of two sets of experiments, in growing medium and in replacing medium, are shown in Fig. 4. It seems that the amount of S–AM in mycelium increases initially and then decreases in either case, although S–AM level is higher under the growing condition than in replacing condition.

Furthermore, the effects of 8-azaguanine for S–AM accumulation in mycelium are shown in Fig. 5.

When 8-azaguanine was added to medium at the stage of conidiophore induction, the compound caused the accumulation of endogenous S–AM of mycelium during incubation. However, when 8-azaguanine was added with

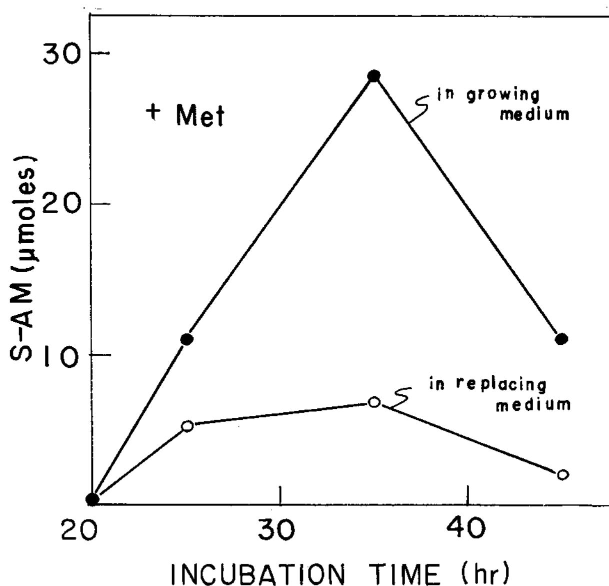

Fig. 4. S-Adenosylmethionine formation in the presence of excess L-methionine. The 200 ml cultures were used in the experiments. In growing medium L-methionine (final concentration 3.33×10^{-3}M) was added at 20 hr of growth. In replacing medium, culture medium was replaced by L-methionine solution (3.33×10^{-3}M) at 20 hr of growth. S-Adenosylmethionine from mycelium was assayed as described in Materials and Methods.

L-methionine S-AM accumulated at a higher level over additive effect of each compound. It may be suggested that 8-azaguanine inhibits further metabolic changes of S-AM without any influences on S-AM formation. Throughout above mentioned experiments, most of S-AM formed were found in mycelium.

Activities of methionine adenosyl-transpherase of normal strain in vitro

Although the endogenous S-AM contents show a very low value (Table 6), the data related to S-AM accumulation (Fig. 4, 5) suggest indirectly that metabolic system of S-AM may be active on sporulation.

Table 7 shows the possible existence of S-AM synthesizing system for transmethylation in acetic acid extract of the mycelium. The results suggest that the rate of S-AM formation in spore-forming mycelium may be increased. However, if acetic acid extract contained a methyltransfer system, S-AM could not accumulate in reaction mixture. Specific inhibitor of

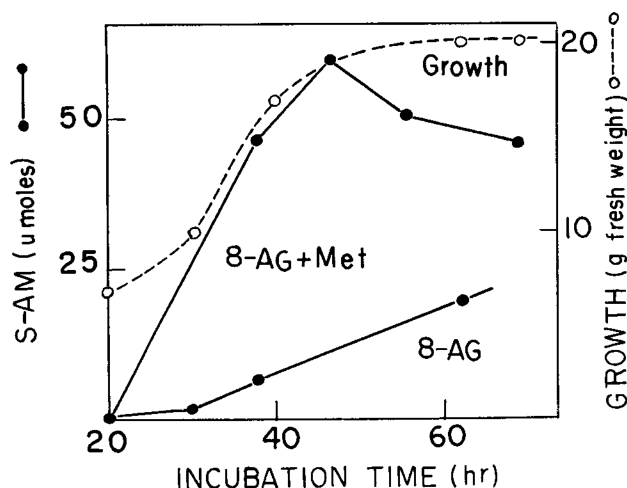

Fig. 5. Effect of 8-azaguanine on accumulation of S-adenosylmethionine in mycelium. At 20 hr of growth 8-azaguanine (final concentration 2×10^{-3}M) or 8-azaguanine with L-methionine (3.33×10^{-3}M) was added in 200 ml culture medium. S-Adenosylmethionine from mycelium was assayed as described in Materials and Methods.

TABLE 7 Activity of methionine adenosyl-transpherase in acetic acid extract

Origin of extract	Activity, μmoles S-AM per mg protein per 60 min.
Mycelium in 19 hr culture	0.028
Mycelium in 40 hr culture	0.206

Assay procedures are described in Materials and Methods.

methyltranspherase was not yet found. In the experiments described below, the ammonium sulfate fractions ($AmSO_4$ ppt 1) were used as a source of the methionine adenosyl-transferase. The fraction did not contain soluble methyl accepter. Table 8 shows the change of the enzyme activity during the sporulation. The fraction from young mycelium or older spore-forming mycelium had a lower activity of S-AM formation. The activity was not detected in the absence of ATP, methionine, GSH, Mg^{2+} or enzyme.

In this experiment, influences of ATPase in $AmSO_4$ ppt 1 were tested indirectly. For example, the 2 fold amounts of ATP did not change the

TABLE 8 Activities of methionine adenosyl-transpherase at different stages of the mold development

Expt. No.	Incubation time of mycelium as enzyme sourse	Specific activity	$100\% \times \frac{\text{Proteins in AmSO}_4\text{ ppt fraction}}{\text{Proteins in acetic acid extract fraction}}$
	hr	μmoles S-AM/mg protein/15 min.	
I	20	0.013	6.0
	24	0.016	—
	27[a]	0.119	6.8
	34[b]	0.109	6.5
	37	0.873	10.7
	50[c]	0.000	16.2
II	24	0.017	—
	40	0.080	—

Assay procedures are described in Materials and Methods.
a Early stage of conidiophore formation.
b Early stage of sporulation.
c Stage after optimum sporulation.

low enzyme activity of the fraction from young mycelium (culture for 20 hrs.). This was an indirect evidence that the disappearance of ATP by ATPase did not suppressed the S–AM formation.

CANTONI suggested that, in the course of the activation reaction, pyrophosphate was formed, and furthermore that it accumulated, in the absence of sufficient pyrophosphatase, in amounts large enough to inhibit the synthesis of S–AM (22). However, it was noteworthy that there was a marked effect of pH on this inhibition; at low concentration of pyrophosphate the inhibition was evident only at pH level below 7.0 (22).

In this experiment, it was observed that the final pH of reaction falled in 7.2 or 7.0 from 7.6. Table 8 showed the proportion of $AmSO_4$ ppt 1 protein fraction in total protein of acetic acid extract. The activities of the enzyme could not be found in other fraction.

Discussion

As shown in Tables 1, 2, the sporulation of *Aspergillus niger* was by affecting the nucleic acid and protein metabolism. However it must be emphasized, that there exists as yet no direct evidence for the involvement of the specific nucleic acid and protein metabolism in the induction of the

sporulation. In recent years, it has been found by assay for DNA–RNA hybridization in our laboratory that a portion of the messenger-like RNA (s) synthesized during the conidiophore formation of the fungi differs from that during conidia formation. (YOSHIOKA *et al.*, unpublished).

On the other hand, ethionine inhibited the sporulation without any influences on mycelial growth. Ethionine differs from other amino acid analogs in tendency to the response type II. It has been reported by many workers that ethionine in intact animals, higher plants, and other organisms cause a considerable morphological or histological changes (31, 32, 33, 34). It is suggested from our experiments that in the metabolic processes of sporulation a specific methionine metabolism may exist other than its incorporation into proteins.

The earlier paper by PARKS has shown that yeast grown on a defined medium supplemented with ethionine produces S-adenosylethionine (35). One of explanations of sporulation inhibition shown in this work may be a competition probably exists between the ethyl and the normal methyl donors for appropriate acceptors.

It has been shown by BEHAL *et al.* that 6-ethylthiopurine and ethionine specifically inhibited the sporulation of *Aspergilla niger* and methionine alone was the most effective counteractant of inhibition. (15, 16, 17). In their experiment, although BEHAL *et al.* have proposed the hypothesis that the inhibitors interfere with a specific aspect of methionine metabolism which limits the production or activity of condensing enzyme, it seemed reasonable to assume that the inhibitors mainly act as ethyl analogs in metabolic system of S-adenosylmethionine.

On the other hand, the fact that excess methionine in culture medium inhibits the sporulation (Table 3, 4) has an interesting bearing on the nature of the methionine metabolism on sporulation of this fungi.

The data of S-adenosylmethionine formation *in vivo* and *in vitro* suggest that if relatively large amounts of methionine in mycelium act as a trap of ATP under the presence of methionine adenosyl-transpherase, the resulting decrease in ATP may contribute for elucidating the problem of inhibition of sporulation. When the value was calculated from Figs. 4 and 6, at least about 6 per cent of methionine incorporated into mycelium was metabolized to S–AM under the growing condition.

DAVIES has reported that increased respiration by washing disks of plant storage tissue has been found to be inhibited by L-methionine (36). However, our results investigated on respiration of the methionine requiring mutant do not indicate a possibility that methionine is a coupling agent

between oxidation and phosphorylation in mycelium as DAVIES assumed.

It is clear that there exist systems of formation and further metabolic changes of S-AM in mycelium (Fig. 6). Changes in the amount of S-AM will depend on various biochemical conditions in mycelium as follow: ATP, methionine and Mg^{2+} level in mycelium; methionine adenosyl-transpherase activity, S-AM methyltranspherase (s) or catabolic enzyme (s) activities; S-AM incorporation to spore. The fact that 8-azaguanine arrests the fall down of S-AM level suggests that the compound inhibits the activity of a system of further metabolic changes of S-AM rather than that of S-AM synthesis (Fig. 7). The relation between S-AM accumulation and sporulation inhibition by 8-azaguanine has not been studied in present paper. Further experiments on sporulation are being carried out in the view of multiplicity and regulation of methionine metabolism.

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Summary

DL-Ethionine, 8-azaguanine, and 5-bromouracil inhibited the sporulation but not mycelial growth of *Aspergillus niger*. On the other hand, 5-methyltryptophan and p-fluorophenylalanine inhibited both the sporulation and mycelial growth. Sporulation of a normal strain and a methionine requiring mutant of *Aspergillus niger* was markedly and irreversibly inhibited by the addition of excess L-methionine into growing or non growing medium at the conidiophore inductive stage. Other amino acids except L-lysine were not so effective on sporulation as methionine. L-Methionine in medium was rapidly incorporated into mycelium under growing or non growing condition. L-Methionine had little effect on the rate of endogenous respiration of normal strain, but had slight effect on that of the mutant starved methionine or preincubated with methionine. S-Adenosylmethionine was rapidly accumulated in hyphal cells of normal strain which are incubated in medium containing methionine and then decreased at a low level. In normal strain, 8-azaguanine promoted the accumulation of S-adenosylmethionine in hyphal cells during incubation. Under supplement of 8-azaguanine with methionine to culture medium, S-adenosylmethionine was accumulated at a high level over additive effect of each compound. Furthermore, it has been shown that the maximal activity of methionine adenosyl-transpherase *in vitro* was

recognized in the fraction obtained from sporeforming mycelium of normal strain.

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