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The ethylene action in the development of cellular slime molds: an analogy to higher plants

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Summary. The cellular slime mold *Dictyostelium mucoroides*-7 (Dm 7) and its mutant (MF 1) exhibit sexual or asexual development depending upon culture conditions. During the sexual cycle macrocyst formation occurs, whereas sorocarps containing spores and stalk cells are asexually formed. As previously reported, the macrocyst formation is marked by the emergence of true zygotes, and is induced by a potent plant hormone, ethylene. The concentration of ethylene required for macrocyst induction was determined to establish the similarity of ethylene action between this organism and higher plants. Macrocysts are induced by low (1 µl/l) exogenous concentrations of ethylene. Higher concentrations (10–1,000 µl/l) also gave essentially the same inductive activity. Ethionine, an analogue of methionine, was found to inhibit zygote formation during sexual development through its interference with ethylene production by Dm 7 and MF 1 cells. In fact, the inhibitory effect of ethionine was mostly nullified by the application of ethylene, S-adenosyl-L-methionine, or 1-aminocyclopropane-1-carboxylic acid. Taken together these results suggest that both the effective concentration of ethylene and the pathway of ethylene biosynthesis in *D. mucoroides* may be similar to those in higher plants. Ethylene was also found to be produced in various species and strains of cellular slime molds, even during the asexual process. The possible functions of ethylene in the asexual development are discussed in relation to cell aggregation and differentiation.

Keywords: *Dictyostelium mucoroides*; Ethylene; Sexual development; Macrocyst; Sorocarp.

Abbreviations: SAM S-adenosyl-L-methionine; ACC 1-aminocyclopropane-1-carboxylic acid; AOA (aminooxy) acetic acid; BSS Bonner's salt solution; DAPI 4′,6-diamidino-2-phenylindole.

Introduction

The cellular slime mold *Dictyostelium mucoroides*-7 (Dm 7) and a mutant (MF 1) derived from it exhibit clear dimorphism in development on agar plates; sorocarp formation as an asexual process, and macrocyst formation as a sexual process (Blaskovics and Raper 1957). In sorocarp formation, a spore germinates to release an amoeba, which divides and increases in number while feeding on bacteria. Upon the exhaustion of the bacterial food supply, cells gather together, forming aggregation streams. The aggregated cells migrate as a slug-shaped mass. After the migration, a sorocarp consisting of a stalk with an apical mass of spores is constructed. In contrast to this asexual process, macrocyst formation is characterized by the formation of large aggregates of cells, which are subdivided into smaller masses (precysts). Each precyst is surrounded by a fibrillar sheath. At the center of each precyst there arises a cytophagic cell (giant cell), which is a true zygote formed by cell fusion and subsequent nuclear fusion (Amagai 1989). The cytophagic cell in turn engulfs all the other cells in the precyst. The enlarged cytophagic cell finally becomes surrounded by a thick wall to form the mature macrocyst (Blaskovics and Raper 1957, Filosa and Dengler 1972). The macrocyst thus formed germinates to release some amoebae, and to complete the life cycle. Induction of a high germination rate has been explored by Abe and Maeda (1986).

Two developmental modes were regulated by several environmental conditions such as light and water (Weinkauf and Filosa 1965, Filosa 1979). Dm 7 cells form sorocarps in the light and macrocysts in the dark, whereas MF 1 cells form macrocysts above a certain cell density irrespective of the light and dark. Under submerged conditions, only macrocysts are formed in

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both Dm 7 and MF 1 strains. These environmental conditions have been supposed to affect chemical circumstances in the cells, thus resulting in the choice of developmental pathways. Two substances, cAMP and ethylene, have been identified as the chemical regulators (Amagai 1984, Amagai and Filosa 1984); cAMP induces sorocarps and inhibits macrocyst formation, while ethylene shifts the developmental mode from sorocarp to macrocyst by inducing zygote formation (Amagai 1989).

Ethylene is a gaseous hormone which controls many physiological and developmental processes in higher plants, including sex expression, fruit ripening, senescence and responses to wounding. The threshold concentration of ethylene action in many higher plants is between 0.01 and 0.1 µl/l, with the saturation concentration of 10 to 100 µl/l and half maximal concentration of 0.1 to 1 µl/l (Abeles 1973). Ethylene is known to regulate the accumulation of specific mRNA, as well as the transcription rate of specific plant genes (Broglie et al. 1986) Ethylene biosynthesis in higher plants occurs through methionine, SAM and ACC intermediates (Adams and Yang 1979).

The effective concentration of ethylene in *D. mucoroides* development was studied by using an air-tight culture system to determine if the action of ethylene in *D. mucoroides* is basically similar to that in higher plants. The developmental mode of MF 1 cells has been shown by Filosa (1987) to be shifted from macrocyst to sorocarp formation by the addition of ethionine, an analogue of methionine, and the effect of ethinonine is completely canceled by methionine. Moreover, we (Amagai 1984, 1987) have reported that AOA, which blocks the conversion of SAM to ACC in the process of ethylene biosynthesis (Amrhein and Wenker 1979), inhibits macrocyst formation through decreasing ethylene production, and that its effect is canceled by ethylene application. Taken together these results strongly suggest that ethylene is synthesized from methionine through SAM and ACC in the cellular slime mold, as in higher plants. In the present work, this possibility was further examined. The amounts of ethylene produced by several species and strains of the cellular slime molds were also determined to estimate the possible significance of ethylene in various cellular functions.

Materials and methods

Organisms and cultivation

Wild type *Dictyostelium mucoroides*-7 (Dm 7) and a spontaneously occurring mutant, MF 1, isolated from Dm 7, were mainly used. For the estimation of ethylene production, in addition to the above two strains, *D. discoideum*-NC 4, -V 12 M 2, -KYH 13 (a mutant derived from NC 4) (Amagai et al. 1983), *D. mucoroides*-11, *D. purpureum*, *Polysphondylium pallidum*, *P. violaceum*, and *Acrasis rosea* were used. Spores of Dm 7 and MF 1 were allowed to germinate and grown with *Escherichia coli* B/r, by shake culture as previously described (Amagai and Filosa 1984). Vegetative cells at the density above 2.5×10^6 cells/ml were harvested and washed three times with BSS (Blaskovics and Raper 1957). For other species and strains, the two-membered cultures with *E. coli* on nutrient agar plates were adopted (Bonner 1947).

Development under an air-tight condition

The concentration of ethylene required for macrocyst formation and the effect of ethylene application were examined using cultures in air-tight containers. Two kinds of agar, Bacto-agar (Difco) and BiTek-agar (Difco) were used. For the determination of effective ethylene concentration, MF 1 cells were used. The cell suspension was placed at 5×10^5 cells/cm^2 on 2% plain agar (2 ml) in a 20 ml Erlenmeyer flask and excess water was removed after cells had settled on the agar surface for 10 min. The flask was tightly sealed with a silicone stopper after the agar surface was allowed to dry for 1–1.5 h. 4 ml of air or authentic ethylene gas diluted at various ratios was injected into the flask by a glass syringe inserted between the silicone stopper and the edge of the flask, following by incubation at 23 °C in the light. For the ethylene application to ethionine-treated cells, harvested Dm 7 or MF 1 cells were washed three times with 20 mM MES (pH 7.0) and placed on the 20 mM MES (pH 7.0)-agar containing various concentrations of ethionine. After the agar surface was allowed to dry, the flask was sealed with a silicone stopper, as described above. 4 ml of air or 1/200 diluted ethylene gas (1,000 µl/l, final concentration) was injected into the flask. Dm 7 cells were incubated at 23 °C at 1×10^6 cells/cm^2 in the dark, and MF 1 cells either at 5×10^5 cells/cm^2 in the dark or at 1×10^6 cells/cm^2 in the light. After 48 h incubation, the final developmental modes were observed under a dissecting microscope or a phase-contrast microscope.

Development under submerged conditions

The effect of ethionine on binucleate cell (zygote) formation was examined by submerging 1×10^7 cells of Dm 7 in 1 ml BSS containing streptomycin (200 µg/ml, final concentration) and various concentrations of ethionine in plastic dishes (Falcon, 3 cm diameter). After the cells had settled on the bottom of dishes for 30 min, the dishes were incubated for 5 h at 23 °C in the light, and then overnight at 4 °C to synchronize the development of cells. The culture dishes were re-shifted from 4 °C to 23 °C and allowed to develop. At 2 h intervals, cells were fixed in methanol and stained with DAPI, and the ratio of binucleate cells to total cells was determined under a fluorescence microscope, as previously described (Amagai 1989). In another experiment, SAM or ACC was applied into ethionine-containing dishes to examine whether the ethionine effect is canceled by these chemicals or compounds. Photographs were taken using a Kodak technical pan film.

Two facing culture for the bioassay of ethylene production

Ethylene production was monitored by slight modification (Iijima et al. 1991) of the original method (Weinkauf and Filosa 1965, Amagai and Filosa 1984). MF 1 cells were plated at a low density (1×10^5

cells/cm^2) as tester cells, and MF 1 or Dm 7 cells plated at a high density (1×10^6 cells/cm^2) on a 2 ml layer of 2% agar with or without 1.2 mM ethionine as helper cells (a source of ethylene production and release). The tester and helper cells were separately incubated for 3 h at 23 °C and overnight at 4 °C. After 2 h from temperature-shift to 23 °C, the two culture plates were faced together and incubated for 48 h in the light. The final developmental modes of the tester cells were observed under a dissecting microscope. Photographs were taken using a Kodak Panatomic-X film.

Determination of ethylene production by gas chromatography

Washed cells (1 ml) of various species and strains were separately put into a 20 ml Erlenmeyer flask and sealed tightly with a silicone stopper. After 48 h shaking at 100 rpm, the amounts of ethylene released from cells were determined by gas chromatography, as previously described (Amagai 1984). Under this shaking condition, all of the species and strains other than Dm 7 and MF 1 remained as unaggregated single cells at high densities above 1×10^7 cells/ml, while they formed cell aggregates at densities below 1×10^7 cells/ml. In the case of Dm 7 and MF 1 cells, formation of cell aggregates was noticed even at a quite high density (2×10^8 cells/ml), and the unaggregated state was accomplished by raising the shaking speed from 100 rpm to 150 rpm, as previously described (Amagai 1987).

Chemicals

DL-ethionine, SAM and ACC were purchased from Sigma Chemicals (St. Louis, U.S.A.). Ethylene gas was from Nishio Industrial Company (Tokyo, Japan).

Results

The concentrations of ethylene required for macrocyst formation

Although ethylene induces macrocyst formation as previously reported (Amagai 1984), its concentration required for the induction still remained to be elucidated. When 4 ml air was applied as a control, MF 1 cells (5×10^5 cells/cm^2) formed sorocarps, possibly because of the subthreshold concentration of ethylene in the air space. Exogenously added ethylene at 1 µl/l (final conc.) was found to induce macrocyst formation of MF 1 cells (data not shown), confirming essentially the previous data obtained by the use of a less air-tight culture system (Amagai 1984). Higher concentrations (10–1,000 µl/l) of exogenously added ethylene also gave essentially the same inductive effect. Incidentally, macrocysts were often formed without any ethylene application. Thus the threshold concentration of ethylene for macrocyst induction could be very low. When BiTek-agar (Difco) was used instead of Bacto-agar (Difco), only sorocarps were stably formed without ethylene application; a higher concentration of ethylene was needed for macrocyst induction, and macrocysts were induced by 10 µl/l ethylene in the five cases of nine independent experiments (56%).

Effect of ethionine on ethylene production

In higher plants, methionine is the substrate in ethylene biosynthesis (Lieberman and Mapson 1964) and therefore ethinonine, an analogue of methionine, inhibits ethylene production (Alix 1982) by forming S-adenosylethionine, which is a competitive inhibitor of ACC synthase (Yu et al. 1979). In *Dictyostelium mucoroides*, ethionine shifts the developmental mode from macrocyst to sorocarp formation, and this effect is nullified by the addition of methionine (Filosa 1987). To know if ethionine actually inhibits macrocyst formation by lowering ethylene production in *D. mucoroides* cells, the effect of ethionine on ethylene production was monitored using the two facing culture system. The dish on which tester MF 1 cells had been plated at a low cell density (1×10^5 cells/cm^2) was faced together with the dish on which helper MF 1 or Dm 7 cells had been plated at a high cell density (1×10^6 cells /cm^2) on 2% plain agar with or without 1.2 mM ethionine. When MF 1 cells were incubated without ethionine as helper cells, tester MF 1 cells formed macrocysts by a sufficient supply of ethylene from helper MF 1 cells that themselves formed macrocysts (Fig. 1 A and B). In the presence of 1.2 mM ethionine, however, tester MF 1 cells formed sorocarps and aggregates possibly because of a decreased amount of ethylene supply from the helper cells. The aggregates thus formed seemed to be an intermediate form between a macrocyst and a sorocarp. Ethionine-treated helper MF 1 cells themselves formed sorocarps but not macrocysts (Fig. 1 E and F). When Dm 7 cells were used as helper cells, essentially the same results were obtained, with the exception that the helper Dm 7 cells themselves formed sorocarps without ethionine as previously described (Amagai and Filosa 1984). That is, tester MF 1 cells formed aggregates when helper Dm 7 cells were allowed to develop without ethionine (Fig. 1 C and D). This might be due to the lower ethylene productivity of the helper Dm 7 cells, as compared with MF 1 cells. In the presence of 1.2 mM ethionine the developmental mode of tester MF 1 cells was completely shifted from aggregate to sorocarp. Under this condition, the helper Dm 7 cells themselves formed sorocarps (Fig. 1 G and H).

To examine whether such an ethionine effect is canceled by externally added ethylene, authentic ethylene gas was applied into the tightly sealed flask in which Dm 7 or MF 1 cells had been settled on the agar containing various concentrations of ethionine. Since sorocarp induction by ethionine was more conspicuous on agar containing 20 mM MES (pH 7.0) as compared with on

agar containing BSS (data not shown), 20 mM MES agar was used for this experiment. The effect of ethionine on the choice of developmental modes was found to be considerably affected by the cell density at the time-point of starvation of cells. When Dm 7 cells were harvested at the density above 2.5×10^6 cells/ml and plated at 1×10^6 cells/cm^2, the percentage of sorocarp formation in the presence of 1.0 to 1.2 mM ethionine was 88%, while in Dm 7 cells harvested at the density below 2.5×10^6 cells/ml and plated 1×10^6 cells/cm^2, the percentage was greatly reduced (22%). Thereupon, Dm 7 or MF 1 cells were harvested at the density above 2.5×10^6 cells/ml to monitor the effect of ethionine. The minimal concentrations of ethionine for inhibiting macrocyst formation were 0.8 mM for Dm 7 cells in the dark, 0.6 mM for MF 1 cells in the light, and 1.0 mM for MF 1 cells in the dark. When ethylene (1,000 µl/l, final conc.) was applied into the air-tight flask where Dm 7 cells had been settled on agar containing various concentrations ethionine, the ethionine effect was almost completely canceled, thus macrocyst formation being induced (Fig. 2 A). The effect of higher ethionine concentrations was scarcely canceled by ethylene application. Essentially the same results were obtained when MF 1 cells were allowed to develop either at 1×10^6 cells/cm^2 in the light or at 5×10^5 cells/cm^2 in the dark (Fig. 2 B and C). In both the light and dark, MF 1 cells formed macrocysts, but a two times higher density of cells was required for macrocyst formation in the light than in the dark. MF 1 cells incubated on Bacto-agar (Difco) in the dark showed a higher percentage of macrocyst induction by ethylene application as compared with the case for BiTek-agar (Fig. 2 D). Taken together these results indicate that ethionine inhibits macrocyst formation by lowering primarily ethylene production.

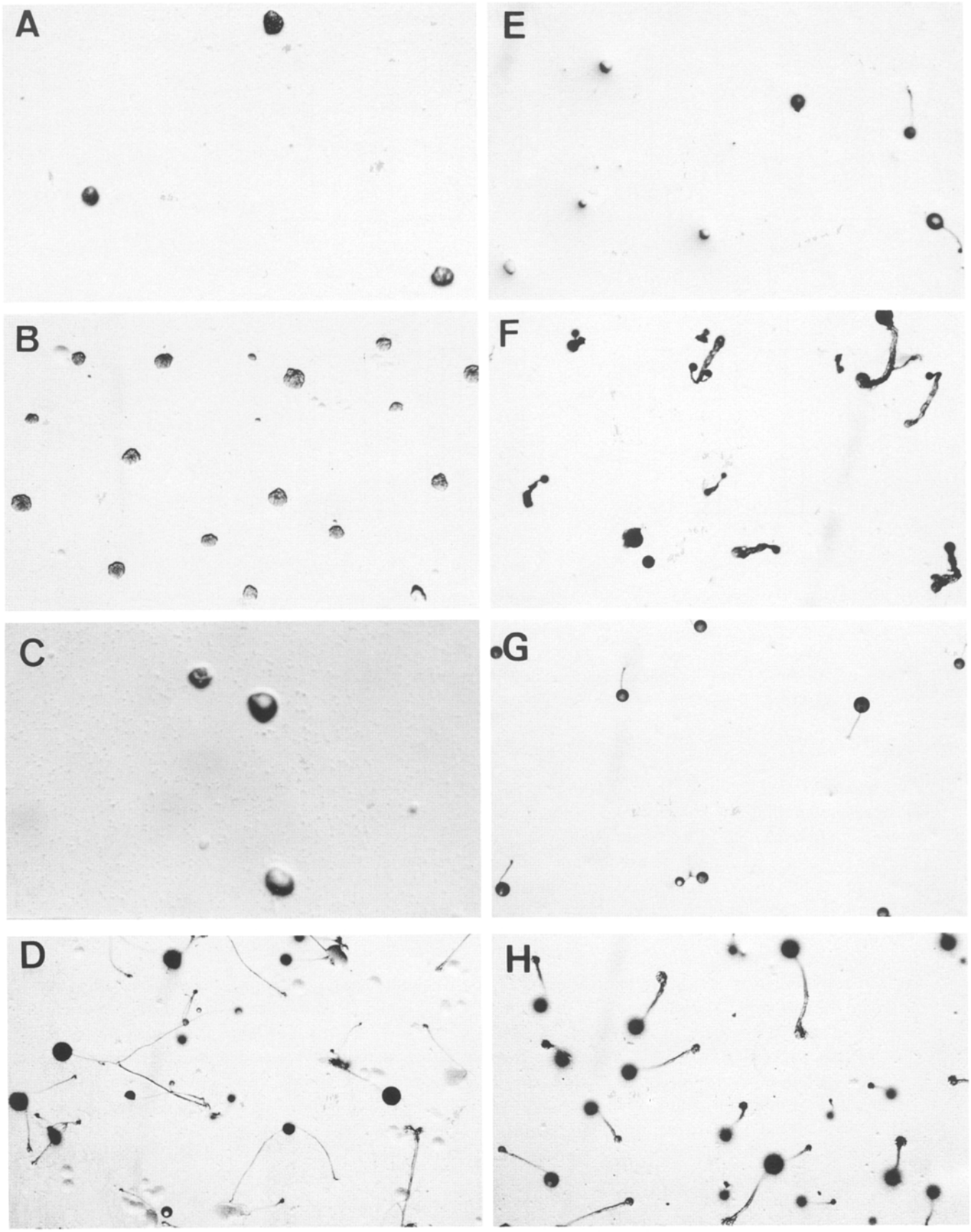
Inhibition of zygote formation by ethionine

Ethylene is known to induce the zygote which is formed by cell fusion and subsequent nuclear fusion (Amagai 1989). To confirm that inhibition of macrocyst formation by ethionine is due to a failure of cells to form zygotes, submerged-cultures of Dm 7 cells were adopted, followed by monitoring the formation of binucleate cells (zygotes). In the control without ethionine, Dm 7 cells began to form aggregation streams at 2 h after the cold (4 °C) treatment, formed aggregates at 8 h, and finally macrocysts even in the light. In the presence of 0.4, 0.8, and 1.6 mM ethionine, Dm 7 cells started forming aggregation streams with almost the same timing as a control, but the territory size of aggregation became much larger (Fig. 3 A and B). In addition, Dm 7 cells remained as large-sized aggregation streams for a prolonged period in the presence of 0.4 or 0.8 mM ethionine, thus a longer time was needed for the final development of the aggregates to macrocysts. Macrocyst formation was markedly inhibited by 1.6 mM ethionine. To determine the effective period of ethionine, a high concentration (3.6 mM) of ethionine was applied into submerged cultures at various developmental stages. When applied immediately after starvation, cells never formed macrocysts and remained as undifferentiated single cells, even after 2 days incubation. However, ethionine application after 2 h of incubation was no longer effective, thus cells formed macrocysts in spite of the presence of a high concentration of ethionine (data not shown). These results indicate that the effective period of ethionine is quite limited to the early developmental phase. The data shown in Fig. 4 indicate that the formation of binucleate cells is inhibited by ethionine in a dose-dependent manner.

Nullification of the inhibitory effect of ethionine by SAM or ACC

As ethionine inhibited binucleate cell formation by decreasing ethlyene production, methionine may be a key substrate for ethylene biosynthesis in *D. mucoroides*. In this connection, it is of interest to know if ethylene

Fig. 1. Effect of ethionine on the ethylene release from helper cells. A high density of helper MF 1 or Dm 7 cells (1×10^6/cm^2) were plated on the lower agar dish containing various concentrations of ethionine. The agar dish on which MF 1 cells had been plated as tester cells at a low density (1×10^5 cells/cm^2) was faced together with the lower dish and sealed tightly, to monitor the effect of ethylene released from the helper cells during the course (48 h) of development at 23 °C. Without ethionine, tester MF 1 cells incubated with helper MF 1 cells formed large clusters of macrocysts (**A**), and the helper MF 1 cells themselves also formed clusters of macrocysts (**B**). In the presence of 1.2 mM ethionine, macrocyst formation by tester MF 1 cells was completely inhibited, thus sorocarps and undifferentiated aggregates were formed (**E**). In this case, helper MF 1 cells themselves developed to sorocarps (**F**). When Dm 7 cells were used as helper cells instead of MF 1 cells and incubated in the absence of ethionine, tester MF 1 cells formed undifferentiated aggregates and a small number of macrocysts (**C**), and helper Dm 7 cells formed sorocarps (**D**). In the presence of 1.2 mM ethionine, both the tester MF 1 cells (**G**) and the helper Dm 7 cells (**H**) formed sorocarps. Magnifications: A, C, × 30; E, × 24; B, D, F–H, × 15


A
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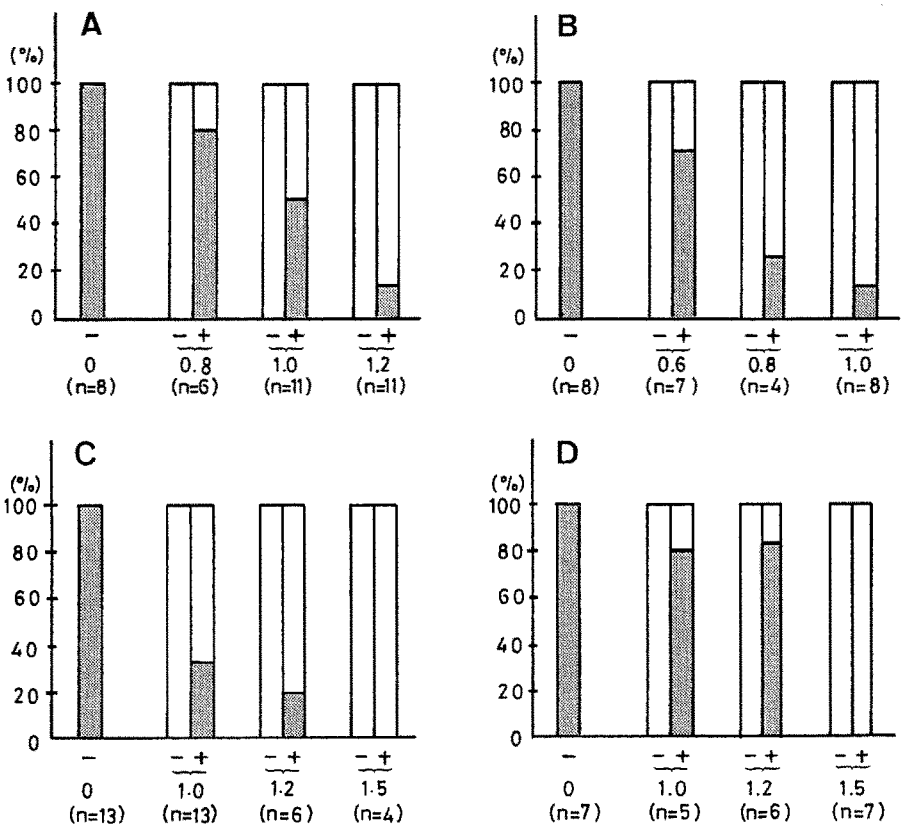

Fig. 2. Nullification of ethinonine effect by ethylene. Harvested Dm 7 or MF 1 cells were allowed to develop on 20 mM MES agar containing ethionine at the designated concentrations in the axis of abscissa, with (+) or without (−) ethylene (1,000 µl/l final conc.) application. **A** Dm 7 cells were incubated for 48 h at $1 \times 10^6/cm^2$ on BiTek-agar (Difco) in the dark. **B** MF 1 cells ($1 \times 10^6/cm^2$) in the light. **C** MF 1 cells ($5 \times 10^5/cm^2$) in the dark. **D** MF 1 cells ($5 \times 10^5/cm^2$) on Bacto-agar (Difco) in the dark. The final developmental modes were observed under a dissecting microscope. The precentage indicated on the ordinate represents the ratio of number of plates showing the designated developmental modes. ■ Macrocyst; □ sorocarps. *n* Number of separate experiments

is synthesized from methionine through SAM and ACC in *D. mucoroides*, as the case for higher plants. Immediately after the cold (4 °C) treatment, SAM (1 or 2 mM, final conc.) was added into the submerged culture containing 1.6 mM ethionine. As a result, large aggregation streams as observed in ethionine-treated cells were never formed, and relatively small-sized aggregates thus formed eventually developed to macrocysts in response to the SAM application (Fig. 3 C). Actually, the formation of binucleate cells in the presence of ethionine was greatly recovered by SAM (Fig. 5), thus suggesting that SAM may be an intermediate of ethylene biosynthesis in *D. mucoroides*. In the presence of ethionine and SAM, macrocyst formation was delayed by several hours. Thus binucleate cells seemed to increase in number with a slower rate for a longer period and finally reach to the same ratio as a control. When 1 mM ACC was added into the submerged culture at the same time as the addition of 2.5 mM ethionine, the ethionine effect was completely canceled and macrocysts were formed even in the presence of such a high concentration of ethionine, as shown in Fig. 6.

Ethylene production in various species and strains of cellular slime molds

The ethylene production by *D. discoideum* cells (Bonner 1973) and *D. mucoroides* cells (Amagai 1984) has been already reported. The present gas-chromatographical study showed that the other species and strains of cellular slime molds also produced and released ethylene, though the amounts of ethylene released varied considerably from strain to strain as well as from species to species (Table 1). In all of the species and strains examined, there arose a peak at the same retention time as authentic ethylene and the peak was completely vanished by passing the gas through mercury perchlorate

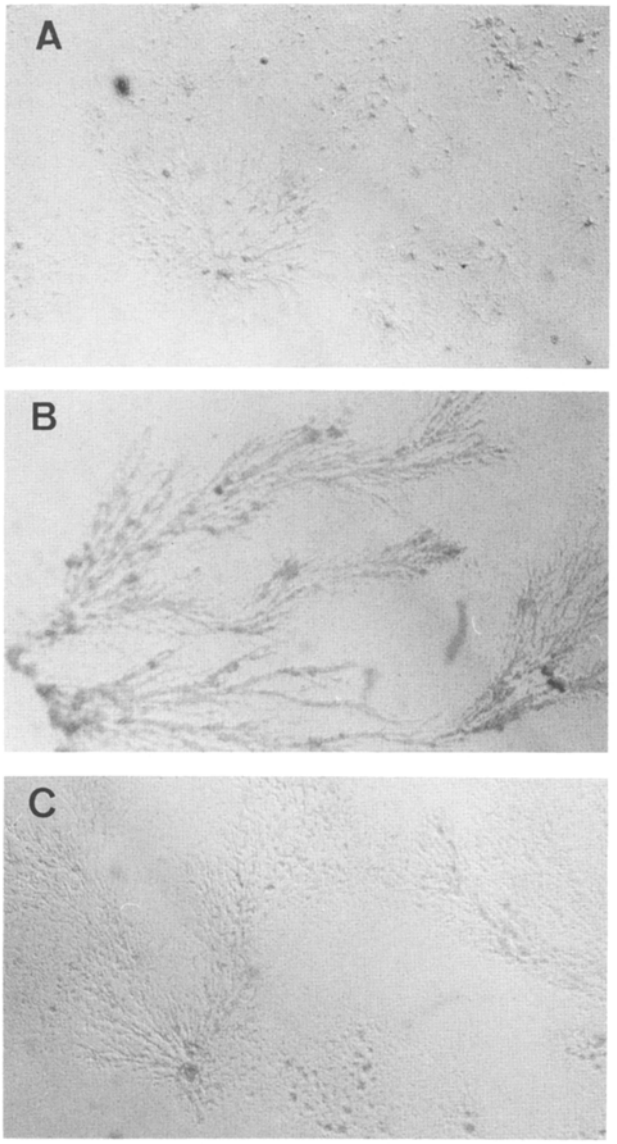

Fig. 3. Effects of ethionine and SAM on the territory size of cell aggregation. After temperature-shift from 4 °C to 23 °C, harvested Dm 7 cells were incubated for 8 h under submerged conditions with and without ethionine (plus SAM) application. **A** Normal-sized aggregation streams formed by Dm 7 cells without ethionine application. **B** Large-sized aggregation streams formed by Dm 7 cells in the presence of 1.6 mM ethionine. **C** The enlargement of the territory size by ethionine was completely prohibited by co-application of 2 mM SAM, thus giving rise to the normal-sized aggregation territory. Magnification: × 32

solution. *D. discoideum-NC-4, Polysphondylium violaceum*, and *Acrasis rosea* seemed to release particularly large amounts of ethylene. The release of ethylene was higher in unaggregated single cells than in somewhat aggregated (differentiating) cells, as shown in Table 1.

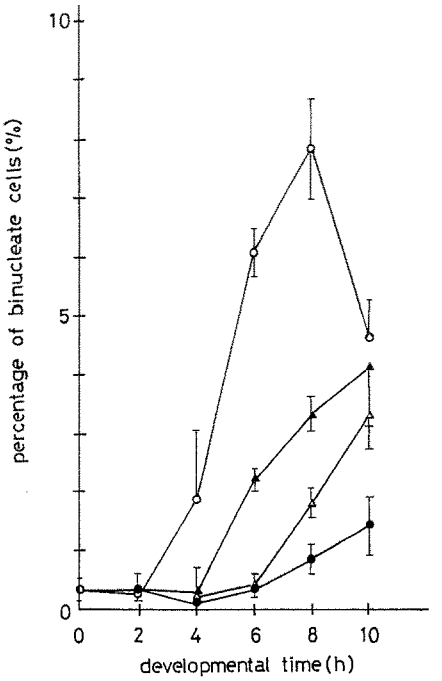

Fig. 4. Inhibition of zygote formation by ethionine. Harvested Dm 7 cells were incubated in 1 ml BSS containing 0 mM (○), 0.4 mM (▲), 0.8 mM (△), and 1.6 mM (●) ethionine. Developmental times refer to the times in hours after the shift from 4 °C to 23 °C. The percentage of binucleate cell formation was determined by DAPI-staining and used as an index of zygote formation. Bars indicate standard deviations of three independent experiments

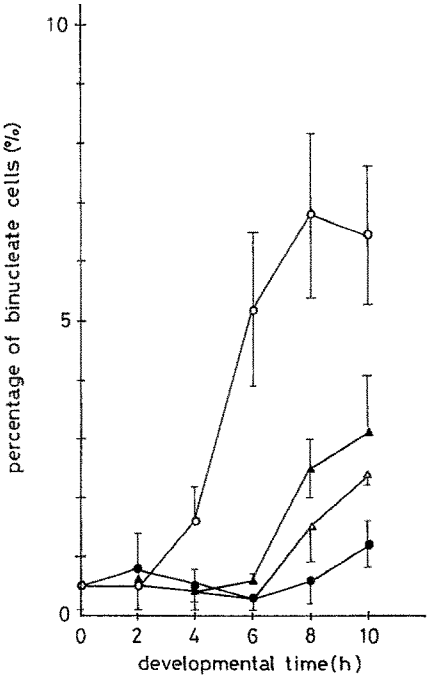

Fig. 5. Recovery of zygote formation in ethionine-treated cells by SAM. Harvested Dm 7 cells were incubated in 1 ml BSS without any addition (○), with 1.6 mM ethionine (●), with 1.6 mM ethionine plus 1 mM SAM (△), and with 1.6 mM ethionine plus 2 mM SAM (▲). Bars indicate standard deviations of three independent experiments

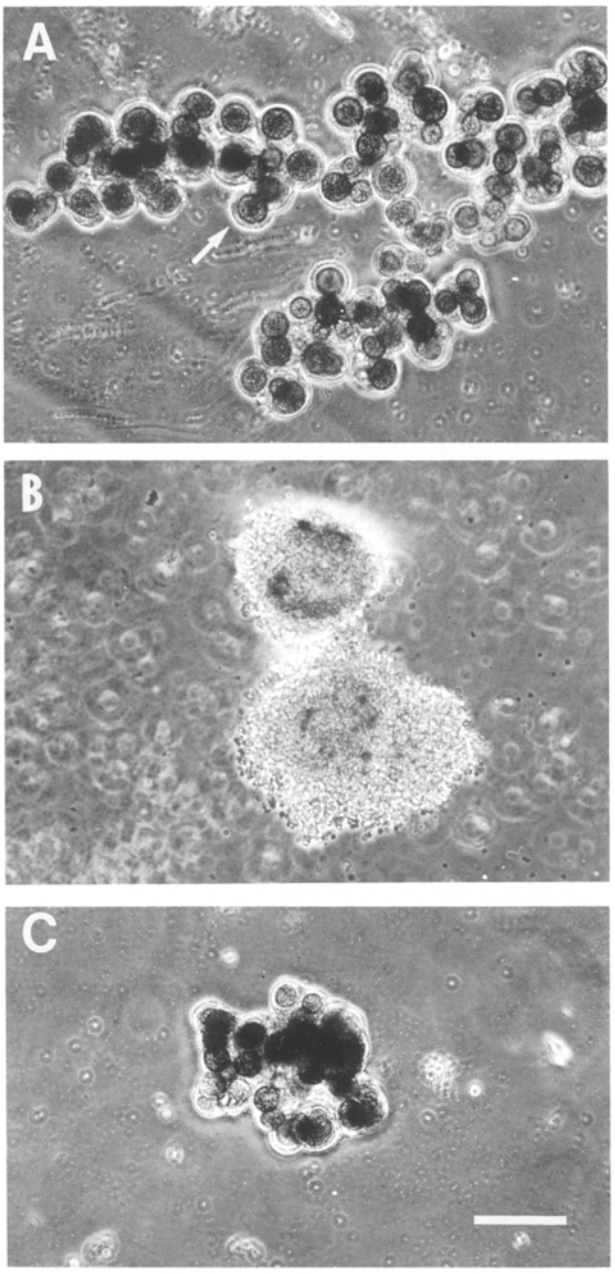

Fig. 6. Nullification of ethionine effect by ACC. Harvested Dm 7 cells were incubated for 48 h under submerged conditions with and without ethionine (plus ACC) application. **A** Clusters of macrocysts formed in 1 ml BSS. An arrow indicates a mature macrocyst surrounded by a thick wall. **B** Loose aggregates formed in 1 ml BSS containing 2.5 mM ethionine. **C** A cluster of macrocysts formed in 1 ml BSS containing 2.5 mM ethionine and 1 mM ACC. Bar: 100 μm

Table 1. Ethylene production in various species and strains of cellular slime molds

Species (strains)	Cell density (cells/ml)	Ethylene amount per 2×10^8 cells means ± SD ($\times 10^{-2}$ μl/l)	Final developmental modes
Dictyostelium mucoroides			
7	2×10^8	1.7 ± 1.0 (6)	MC
	2×10^{8a}	5.2 ± 0.9 (7)	US
MF 1	2×10^8	2.0 ± 0.5 (7)	MC
	2×10^{8a}	6.1 ± 0.9 (6)	US
11	1×10^8	3.8 ± 0.7 (7)	SC
D. discoideum			
NC 4	5×10^7	16.0 ± 8.6 (6)	SC
V 12 M 2	5×10^7	5.1 ± 2.7 (8)	SC
	2×10^8	9.0 (2)	US
KYH 13	5×10^7	2.8 ± 1.3 (6)	SC
	2×10^8	9.3 (2)	US
D. purpureum	5×10^7	2.5 ± 1.9 (5)	SC
	2×10^8	2.7 (1)	US
Polysphondylium pallidum	1×10^8	0.9 ± 0.7 (5)	SC
	2×10^8	3.5 (1)	US
P. violaceum	5×10^7	0.7 ± 0.5 (3)	SC
	2×10^8	24 (1)	US
Acrasis rosea	2×10^6	51 ± 36 (6)	MIC

Vegetative cells of various species and strains were suspended in 1 ml BSS in a 20 ml Erlenmeyer flask and shaken at 100 rpm for 48 h under air-tight conditions. The amount of ethylene accumulated in the culture flask was determined by gas-chromatography. During 48 h shaking cultures of *D. mucoroides*-7 (Dm 7) and MF 1, many macrocysts were formed in the medium. However, Dm 7 and MF 1 cells never aggregated and remained as undifferentiated single cells, when the shaking speed was raised from 100 to 150 rpm. *Acrasis rosea* formed microcysts during the shaking culture. The other species showed a tendency to form sorocarps at the interface between the culture medium and the air, particularly during cultures at low densities ($0.5–1.0 \times 10^8$ cells/ml). At a high density around 2.0×10^8 cells/ml, however, almost all of the cells remained as undifferentiated single cells

[a] The shaking speed was raised from 100 to 150 rpm

In the parentheses, the number of separate experiments

MC Macrocysts, *US* undifferentiated single cells, *SC* sorocarps, *MIC* microcysts

Discussion

The present results obtained using an air-tight culture system provide evidence that the sexual process (macrocyst formation) in *D. mucoroides* is induced by a low concentration (1 μl/l) of ethylene, basically confirming the previous finding attained by the use of a less air-tight system (Amagai 1984). Without ethylene application, macrocysts were sometimes formed on Bacto-agar (Difco), but only sorocarps were always formed on BiTek-agar (Difco). Thus a higher concentration of ethylene must be required for macrocyst formation on BiTek-agar. There are some differences between the two kinds of agar. For example, the surface

of Bacto-agar seemed to be more moist, as compared with the surface of BiTek-agar. In general, agar contains various ions including Ca^{2+} that is essential for macrocyst formation (Chagla et al. 1980, Szabo et al. 1982). One can imagine that the ionic constitution is dissimilar between the two kinds of agar used, thus giving somewhat different results. These differences between the two agars may affect ethylene and cAMP production within cells. Although the functional concentration of ethylene was changable by environmental conditions, the effective concentration of ethylene for macrocyst formation seems to be within the same range as that in higher plants.

As reported by Filosa (1987), the developmental mode of MF 1 cells was changed by ethionine from macrocyst to sorocarp formation (Fig. 1). Ethionine is the analogue of methionine and its inhibitory effect on macrocyst formation is nullified by methionine (Filosa 1987). In addition, it is clear from the present work that the effect of ethionine is almost completely canceled by the addition of ethylene, SAM, or ACC (Figs. 2, 5, and 6). Accordingly, it is quite likely that in *D. mucoroides* ethylene is synthesized from methionine through SAM and ACC, as the case for higher plants. Although in stationally grown *Pencillium digitatum* ethylene is produced from glutamate of α-ketoglutamate (Chou and Yang 1973), methionine is a precursor for ethylene production in shake culture (Chalutz et al. 1977). Thus the biosynthetic pathway of ethylene in shake-cultured *P. digitatum* is similar to that in slime mold cells. The effect of higher ethionine concentrations was scarcely canceled by ethylene application. This seems to indicate that ethionine also may be involved in the formation of abnormal proteins, thus inhibiting the progress of sexual development. It is of interest to note that the effective period of ethionine as an inhibitor of macrocyst formation is very limited to the early developmental stage. The addition of ethionine to cells after 2 h starvation no longer had an inhibitory effect on macrocyst formation. This seems to indicate that methionine or protein synthesis is not necessarily required for macrocyst formation by 2 h starved cells. As described in Results, the inhibitory efficiency of ethionine on macrocyst formation varied depending on the cell densities at the time point of starvation; Dm 7 cells starved at the late exponential or stationary growth phase were more sensitive to the ethionine addition than those starved at the early exponential growth phase. This seems to reflect that the nutrients including methionine may be consumed by growing cells. Various species of cellular slime molds were found to produce and release ethylene, though the amounts of ethylene released varied greatly from species to species. Considering the fact that the ethylene release is naturally observed even in the asexual process including sorocarp formation, ethylene might be involved in developmental events other than zygote formation. In this connection, it has been shown that the proportion of prespore cells in *D. mucoroides* slugs is considerably reduced by ethionine, thus resulting in the formation of sorocarps with very thick stalks (Filosa 1987). Based on this observation, Filosa (1987) has raised two possibilities about the ethionine effect on cell type proportioning: (1) The inhibition of the methylation of nucleic acids, proteins, and phospholipids, and/or (2) the inhibition of the ethylene biosynthesis. The results presented here strongly suggested the importance of ethylene in differentiation of the prestalk/prespore (or stalk/spore) cells, thus being involved in the regulation of cell type proportioning. In addition, the territory size of cell aggregation was also found to be greatly affected by ethionine. Therefore, it is possible that ethylene may be implicated for the cAMP-signalling system, and in its turn for the determination of the aggregation size. We have now suggested in the cellular slime mold that both functional concentration of ethylene and the process of ethylene biosynthesis are similar to those in higher plants. Since the development of cellular slime molds is an excellent system to study the regulatory mechanisms of growth and differentiation at the cellular and molecular levels, further analysis of the ethylene function using this organism will provide valuable information about the action mechanism of ethylene in various biological events, particularly at the cellular level.

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