Reversal of Ethionine Inhibition by Methionine during Slime Mold Development

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Dictyostelium discoideum, a cellular slime mold, has been shown by Filosa (1960) to produce abnormal fruiting bodies when grown on *Escherichia coli* in the presence of 1.2×10^{-3} M ethionine. The fruiting bodies obtained under those conditions were described as short, with thick stalks and elongated sori. At a concentration of 4.8×10^{-3} M no growth occurred. Starting from this initial observation we have attempted to answer the following questions: Which elements of development, such as morphogenesis or differentiation of spores and stalk cells, are affected at various concentrations of ethionine? Which phases of the life cycle are sensitive to ethionine? What is the mechanism of ethionine inhibition? Answers to these questions might possibly provide clues to the mechanism of development in these organisms, where an integrated mass of myxamoebae produces, in the absence of an external source of energy, a well-proportioned fruiting body composed of a cellulose-ensheathed stalk bearing a lobose sorus of spores.

MATERIALS AND METHODS

Myxamoebae of *Dictyostelium discoideum* NC-4(S2), a haploid strain, were grown at 24°C in the presence of *Escherichia coli* B/r on a medium containing 1% lactose, 1% peptone, and 1.5% Difco agar. After 44–46 hours, shortly before the cells reached the stationary growth phase, they were harvested with ice-cold Sörensen's phosphate buffer (0.016 M, pH 6.0), washed, and their concentration adjusted to $1.5–2.0 \times 10^8$ cells/ml. A 0.5-ml sample of cell suspension containing $0.75–1.0 \times 10^8$ cells was spread onto millipore filters according to the method of Sussman and Lovgren (1965). For this the millipore filters (48 mm diameter, black), resting on absorbent filter pads, were placed in plastic petri dishes (60 mm diameter). Prior to the addition of myxamoebae the pads were soaked with 1.5 ml of phosphate buffer containing per ml 0.67 mg of streptomycin and 0.13 mg of sodiumlaurylsulfate together with appropriate concentrations of the test solution. The sodiumlaurylsulfate appears to contribute to a more uniform development of the population and to enhanced effectiveness of at least some compounds, probably by increasing the cell permeability without having any obvious effect on normal development. All cultures were incubated at 24°C in the dark; in some experiments when the plates were kept at room temperature overnight, the temperature varied between 21° and 25°C. The plates were scored every 4 hours until no further developmental changes were observed. To test the effect of ethionine on growth and agglutination, the cells were grown on *E. coli* in shake cultures in the presence of the compound and the growth curves determined (Hohl and Raper, 1963), or washed cells were incubated in roller tubes and their pattern of agglutination was observed (Hohl and Raper, 1964). For the growth in shake cultures, *D. discoideum* V-12 was used.

RESULTS

Ethionine completely inhibits growth of *Dictyostelium* at a concentration of 3.0×10^{-2} M and shows no inhibitory effect at 1.0×10^{-3} M (Fig. 1). In no concentration, however, does it interfere with agglutination of myxamoebae as judged from the behavior of cells in roller tubes. Even at a concentration of 3.0×10^{-2} M there was no sign of inhibition either in the rate of agglutination or in the size of the agglutinates formed. This indicates that

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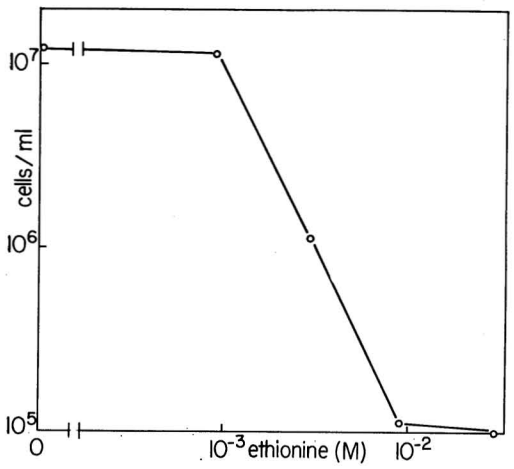


FIG. 1. Growth of *Dictyostelium discoideum* on *Escherichia coli* in the presence of various concentrations of ethionine. The ordinate indicates cells/ml at the end of the growth period (46 hours). The inoculum consisted of 1.0×10^5 myxamoebae/ml.

cell adherence or "stickiness" is not suppressed by ethionine. This result corroborates the experiments to be reported next, where it was found that even high concentrations of ethionine do not completely inhibit streaming and a type of massing of cells reminiscent of aggregation.

In order to test the effect of ethionine on the developmental stages per se, the myxamoebae were grown in the absence of ethionine first and then washed populations were subjected to ethionine as described under materials and methods. Figure 2 presents the effects of various concentrations of ethionine on the development

ETHIONINE CONCENTRATION (M)	DIFFERENTIATION SPORES	SHEATH	INTO STALK CELLS	TYPICAL FORMS	TYPE NO.
0	+	+	+	3mm	1
7.5×10^{-4}	+	+	+		2
1.5×10^{-3}	−	+	+		3
3.0×10^{-3}	−	−	+		4
6.0×10^{-3}	−	−	−		5
1.5×10^{-2}	−	−	−		6

FIG. 2. Influence of increasing concentrations of ethionine on the development of *Dictyostelium discoideum*. The results represent the final stages of development regularly reached after 24 to 30 hours.

of *D. discoideum*. Ethionine did not appreciably reduce the viability of the myxamoebae during the time of the experiment. Hence, the effects observed cannot be attributed to a partial killing of the cell population. A concentration of 1.5×10^{-2} M completely inhibits morphogenesis, although some streaming may occur resulting in the formation of vaguely defined clumps that usually disappear within 24 hours. At 6.0×10^{-3} M large, flat mounds of myxamoebae are formed out of which tiny papillae may protrude, rudimentary signs of induction of polarity and fruiting body formation. The large number of these small papillae per mound indicates further that the critical mass, i.e., the mass of cells capable of integrated behavior (Hohl and Raper, 1964), has been drastically reduced. At a concentration of 3.0×10^{-3} M the myxamoebae collect into large aggregates that split up to form many finger-like protrusions. These protrusions are made up typically of a heavy mass of cells at the base, a short, thick stalk-like structure oftentimes carrying at its apical end a lobose mass of cells somewhat resembling a sorus. Up to this stage all the structures are made up of roundish cells without signs of spore differentiation or production of a cellulose sheath. Some large vacuolated cells, however, are present and we interpret them as representing stalk cells. At a concentration of 1.5×10^{-3} M stalks are formed and are ensheathed in a smooth cellulose envelope. The outside of the sheath is often covered with masses of undifferentiated cells, thus making the structures as a whole appear distorted. No spores are found in these fruiting bodies, but clumps of undifferentiated cells may occur at the tip of the stalk. Because of the still heavy base and the stalk without sorus the whole structure at this level often resembles a bowling pin. At a concentration of 7.5×10^{-4} M the fruiting bodies, apart from occasional distorted forms, are normal in appearance though reduced in size. No apparent effect can be observed at lower levels of ethionine. For convenience the different levels of inhibition have been numbered from 1 to 6, as indicated in Figure 2. In general, spore differentiation is most sensitive to ethionine, followed by sheath production, stalk cell differentiation, and lastly morphogenetic move-

ment. Of developmental stages, spore formation is also the most sensitive to treatment with 2-mercaptoethanol, as shown by Gerisch (1961). Spore formation and normal morphogenesis are closely linked, as spores have been found only in normal sori, and normal-appearing sori always contained spores.

Whereas no morphogenesis takes place in the presence of 1.5×10^{-2} M ethionine, the simultaneous addition of methionine in increasing amounts leads to the formation of progressively more normal appearing fruiting bodies (Fig. 3). The structure of the resulting sorocarps follows the same pattern as described above for ethionine-treated populations. When the concentration of methionine approaches twice that of ethionine the fruiting bodies look normal but are somewhat smaller in size (equal to stage 2) compared with controls growing on methionine alone or on the buffer alone. If the experiment is repeated with 1/5 the concentration of ethionine and a correspondingly lowered amount of methionine the results remain unchanged. This demonstrates that the ratio of ethionine to methionine is the critical factor, rather than the absolute amounts of either compound. Ethionine clearly behaves like a competitive inhibitor of methionine.

Next, ethionine sensitivity at the various developmental stages (after cessation of vegetative growth) was determined. For this, populations were exposed to 1.5×10^{-2} M ethionine at selected points in their development for various periods of time. Some of the results are summarized in Figure 4. Before and after the treatment with ethionine the cells were kept on equimolar concentrations of methionine. This was done in order to assure a quick removal of any possible free ethionine in the internal amino acid pool (Wright and Anderson, 1960) that might obscure the results. In fact, it was found that when the cells were transferred (after treatment with ethionine) to buffer alone, instead of to buffer plus methionine, morphogenesis was permanently inhibited. This strongly indicates the presence of a rather large internal pool where the ethionine can persist for hours, unless it is exchanged for exogenous methionine. The main conclusions to be drawn from Figure 4 are: (1) the entire morphogenetic part of the life cycle up to the actual formation of the stalk is sensitive to ethionine, and (2) the inhibitory effect of ethionine is a gradual one, the damage becoming more severe the longer the cells are in contact with the substance. An important point is that even if ethionine is administered just prior to commencement of stalk production the inhibitory

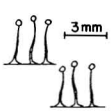
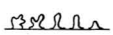
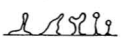
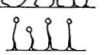
ETHIONINE CONC. (M)	METHIONINE CONC. (M)	TYPICAL FORMS	TYPE NO.
0	0		1
0	1.5×10^{-2}		1
1.5×10^{-2}	0		6
1.5×10^{-2}	0.75×10^{-2}		4
1.5×10^{-2}	1.5×10^{-2}		3,2
1.5×10^{-2}	3.0×10^{-2}		2,1

FIG. 3. Influence of ethionine and methionine, alone and in combination, on the development of *Dictyostelium discoideum*. The results represent the final stages of development regularly reached after 24 to 30 hours.

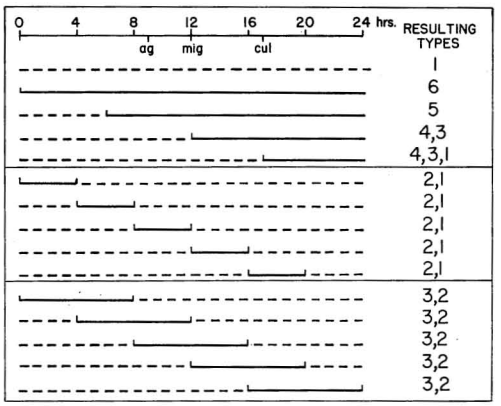


FIG. 4. Sensitivity of various stages of slime mold development to the inhibitory action of ethionine. The time scale indicates hours after deposition of the washed myxamoebae populations together with the onset of aggregation (*ag*), migration (*mig*), and culmination (*cul*) of the control population. *Solid lines* denote the presence of ethionine, *dotted lines* of methionine. The resulting types of sorocarps are numbered according to Figure 1. They represent the final levels of development reached under the respective conditions. The delay in time to reach these final stages with respect to the controls is approximately equal to the time the populations were exposed to ethionine.

effect is still very strong. Once the stalk is being formed the mass rises above the substrate and the further effects of ethionine are difficult to assess since the substances in the filter pad are no longer in direct contact with the cell mass. If ethionine is added for a certain period of time before aggregation, aggregation itself is also disturbed as described previously, i.e., only vaguely defined clumps form as the result of some streaming, or there is no reaction at all. Also, if ethionine is added after aggregation, then the proper polarization of the aggregated mass is disturbed and many small tips are formed as long as the contact with ethionine is maintained. The results of this experiment show that ethionine acts on several phases of the developmental sequence as well as on the vegetative growth. Most significantly, however, it acts directly on the last stage, culmination.

DISCUSSION

Ethionine interferes with various stages of the life cycle of *Dictyostelium,* such as vegetative growth, aggregation, and culmination. We have been able to show that the inhibition of the later stages of development is not necessarily a consequence of inhibition of earlier ones, since addition of ethionine as late as just prior to culmination is still inhibitory to a large extent.

Spore differentiation is most sensitive to the action of ethionine, followed by cellulose sheath formation, stalk cell differentiation, and finally, morphogenetic movement. A similar sequence of sensitivity has been observed by Gerisch (1961) in the case of mercaptoethanol. Ethionine, then, seems to be primarily an inhibitor of differentiation. In turn, several aspects of disturbed morphogenesis, such as lack of a sorus or production of a short bulky stalk, can be indirectly attributed to this effect. Moreover, how can the cell mass build a slender, evenly tapered stalk in the absence of any cellulose component for structural support?

We have established that ethionine acts as a competitive inhibitor of methionine. This conclusion is based on the observations that (1) methionine is able to reverse the inhibitory effect of ethionine when the two are added to the cultures simultaneously, and (2) the final product of development is a function of the ratio of the two components rather than of their absolute amounts. The proteins incorporating ethionine instead of methionine are rendered biologically inactive and, therefore, normal development cannot proceed. It may be imagined that the various disturbances effected by ethionine have a common cause, but this is unlikely since ethionine is probably incorporated into a variety of proteins normally containing methionine. One point deserves mentioning: the ethionine has to be in contact with the cell population for at least 4–8 hours to produce any persisting inhibitory effect. At least two possible explanations might account for this: (1) ethionine slows down protein synthesis as long as it is in contact with the cells, so that only a small amount of ethionine-containing material is formed; and (2) the protein turnover is high, so that the ethionine-containing proteins are rapidly broken down and replaced by the normal methionine-containing ones, as soon as the cells are switched from ethionine to methionine.

The fate of the ethionine in the cell population has not been directly traced. However, from the work of Wright and Anderson (1960) on methionine metabolism in *Dictyostelium* we are able to get some information directly applicable to our situation. These authors have shown that the endogenous "free" amino acid pool is a function of the stage of development only and is not influenced by exogenous methionine. However, exogenous methionine can exchange with endogenous methionine, and the extent of this exchange is a linear function of the exogenous methionine present. This means that increasing the external methionine concentration does not alter the size of the internal pool, but does increase the rate of exchange between exogenous and endogenous methionine. If we assume that ethionine behaves similarly in this respect, it then becomes clear that increasing the amount of ethionine in the medium does not simply add more ethionine to the existing endogenous amino acid pool, but results in a higher exchange of ethionine for methionine between this pool and the environment. In this way we have changed the ratio of methionine to ethionine, which is the essen-

tial determinant for subsequent developmental events.

The effectiveness of ethionine up to the point of actual stalk formation indicates that at least some of the proteins essential for normal development are formed right up to the point of culmination. Wright and Anderson (1960) have shown that certain ethanol-insoluble, methionine-containing proteins are produced at an increased rate during the stage of pre-culmination. It will be of interest to determine whether these proteins are enzymes involved in cellulose synthesis or if they are structural proteins responsible for cell polarization and differentiation. Evidence from experiments with colchicine in conjunction with electron microscopy indicates the occurrence of such a proteinaceous cytoskeleton in *Dictyostelium* (Hohl and George, 1966).

SUMMARY

Ethionine progressively inhibits the development of *Dictyostelium discoideum,* from a concentration of 7.5×10^{-4} M (which induces somewhat smaller but normal fruiting bodies) to a concentration of 1.5×10^{-2} M (which results in complete inhibition). Intermediate concentrations produce a variety of distorted forms. With increasing concentrations the inhibitory effect is first noticed in spore differentiation, then in cellulose sheath production, followed by stalk cell differentiation, and finally in morphogenetic movement.

Simultaneous addition of methionine reverses the effect of ethionine, the final result depending on the ratio of ethionine to methionine rather than on the absolute amounts of either substance administered. Ethionine exerts its effect at any time in the life cycle up to the actual formation of the stalk, the final appearance of the fruiting bodies being a function both of the stage at which the ethionine was applied and of the period of time the cultures were in contact with it. The results indicate that, first, ethionine acts as a competitive inhibitor of methionine, and, second, the protein or proteins incorporating ethionine and thereby rendered biologically inactive are being produced continuously up to the time of actual stalk formation.

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