Effects of the Self-Inhibitor of *Dictyostelium discoideum* on Spore Metabolism

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SUMMARY

The effects of the self-inhibitor, *N,N*-dimethylguanosine, on the metabolism of spores of *Dictyostelium discoideum* were investigated. It is concluded that since both self-inhibited and activated spores had similar initial Q_{O_2} values, the site of action of the self-inhibitor was not on respiratory metabolism. Incorporation of [^{14}C]glucose revealed that organic acids and amino acids accumulated in self-inhibited spores and that protein synthesis was inhibited. Succinic acid accumulated only when the activated self-inhibited spores became anaerobic so its accumulation was not directly related to self-inhibition. Phenylalanine, methionine and tryptophan were absent in dormant spores but appeared after heat-activation and accumulated in self-inhibited spores. The accumulation of amino acids was related to the inability of self-inhibited spores to synthesize protein. *In vivo* analysis showed that protein synthesis began early in spore germination and that the self-inhibitor immediately arrested this process when added to activated spores and slowed it when added to germinating spores. No effect of self-inhibitor on RNA synthesis during germination was detected. Thus, we conclude that the self-inhibitor, a methylated base of guanine, regulated spore germination by preventing the synthesis of protein necessary for subsequent development.

INTRODUCTION

In many fungi crowding of spores reduces the percentage of germination. This is known as self-inhibition or auto-inhibition; spores which exhibit self-inhibition fail to germinate when crowded, but do so readily when sparsely dispersed. A gradation from total inhibition to complete development can be obtained by serial dilution of such spores. Once the spores are freed of the inhibitor, development may continue as an irreversible process. Because self-inhibitors are restraints upon development which originate within the spore, they are considered among the constitutive mechanisms through which development is regulated in some fungal spores, as contrasted with exogenous mechanisms (Sussman & Halvorson, 1966).

Russell & Bonner (1960) demonstrated an inverse relationship between germination and spore concentration in *Dictyostelium mucoroides*, a cellular slime mould, and concluded that a self-inhibitor was responsible. Snyder & Ceccarini (1966) demonstrated the widespread occurrence of the phenomenon in the Acrasiales and Ceccarini & Cohen (1967) determined some of the properties of the inhibitor(s). The self-inhibitor of *D. discoideum* has been identified as 2-dimethylamino-6-oxypurineriboside (*N,N*-dimethylguanosine) by

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means of chromatographic, ultraviolet and high-resolution mass spectral analyses (Bacon, 1972; Bacon, Sussman & Paul, 1973).

One of the most characteristic and perhaps significant features of the germination of fungal spores is the increase in both general metabolic rate and synthesis of macromolecules (Staples, Syamananda, Kao & Block, 1962; Gottlieb, 1966; Van Etten, 1969; Holloman, 1970). These increases suggest that these processes are ones to study in relation to the mechanism of self-inhibition. Therefore, a study was begun to determine whether the self-inhibitor of *D. discoideum*, *N,N*-dimethylguanosine, exerts its effect by blocking respiratory metabolism or by controlling a part of macromolecular synthesis that is essential to spore development.

METHODS

Organism and production of spores. *Dictyostelium discoideum* NC-4H was obtained as lyophilized spores through the kindness of Dr K. B. Raper of the University of Wisconsin. For the production of spores, the slime mould was grown together with its food source, *Escherichia coli* B/r, on 100 ml of a nutrient agar medium (Cotter & Raper, 1968) in Pyrex baking trays (33 × 20.5 × 6.5 cm). One ml of a heat-shocked (45 °C for 35 min) spore suspension (0.01 mg dry wt/ml) was inoculated onto the trays together with 1 ml of bacteria (5×10^9 /ml) and 5 ml of sterile distilled water. The cultures were incubated at 22 °C under constant illumination from 40 W fluorescent light tubes. This procedure produced uniform growth and synchronous development, with mature sorocarps being formed in 4 days. Sorocarps were harvested after 7 days of growth by washing them from the agar with sterile distilled water. The sorocarp suspension was stirred by a magnetic stirrer for 30 min to remove the self-inhibitor and passed through Nytex nylon monofilament screen (93 µm) to separate stalks from spores. The resulting spore suspension was centrifuged (2000 g for 15 min), the spores resuspended in sterile distilled water and stirred an additional 15 min. This spore suspension was centrifuged as before and the water decanted. These two washings were adequate to dilute out the self-inhibitor. These spores were then separated from bacteria by repeated low speed centrifugations (2000 g for 15 min) in sterile distilled water until microscopic observation revealed that no bacteria were present. These self-inhibitor free spores were suspended finally in distilled water and frozen in a dry ice-ethyl alcohol mixture. Finally, the frozen spores were lyophilized and stored at 4 °C. The yield obtained was 0.2 to 0.4 mg (dry wt)/tray.

Source of self-inhibitor and radioisotopes. The self-inhibitor, *N,N*-dimethylguanosine, was extracted from spores and purified repeatedly by column and paper chromatography (Bacon, Sussman & Paul, 1973). The authentic compound used was synthesized by Dr G. B. Elion, Burroughs Wellcome and Co., Raleigh, North Carolina, U.S.A. The authentic compound was used either in addition to or in place of the naturally isolated compound but the results in either case were identical. D-[U-¹⁴C]glucose (200 mCi/mmole), L-[U-¹⁴C]-leucine (210 mCi/mmole), and [5-³H]uracil (2Ci/mmole) were purchased from International Chemical and Nuclear Corporation, Irvine, California, U.S.A.

Assessment of spore development and bioassay. The lyophilized spores were heat-shocked at 45 °C for 35 min in 50 ml flasks with 10 ml of liquid synthetic medium (Cotter & Raper, 1968) on a reciprocally shaking water bath. Then the spores were incubated at 22 °C in constant light, on a reciprocating shaker. In all experiments, each flask contained 0.01 mg spores (dry wt)/ml as higher concentrations resulted in poor germination due to a deficiency of oxygen.

The various stages of spore development were determined by examination of 0.1 ml of

Fig. 1. Procedure for fractionating spores of *Dictyostelium discoideum*.

spore suspension at $400\times$ magnification. At least 200 spores were counted and scored as dormant, germinated (swollen), or in stages of outgrowth. In some experiments results were expressed in terms of amount of emerged amoebae. Dormant spores, as defined here, are those able to germinate under favourable conditions of culture. Heat-shocked spores that have been restored to dormancy are called secondarily dormant spores. Germinating spores are those that have undergone swelling and a loss of heat-resistance. Outgrowth refers to the emergence of the amoebae from the spore. Activation is the breaking of dormancy, in this case by the application of a heat-shock.

When the self-inhibitor or any other inhibitory compound was assayed, activated spores were incubated in the presence of various amounts of the substance and at specific time intervals spores were removed and scored as described above. In all experiments in which the self-inhibitor was used, the concentration of inhibitor ($50\text{ }\mu\text{g/ml}$) was enough to cause 100% inhibition of the spore suspension (0.01 mg/ml).

Polarographic measurement of O_2 uptake. Oxygen uptake was measured at 25°C with a Clark-type oxygen electrode. This electrode was used in conjunction with an amplifier and a chart recorder (Maheshwari & Sussman, 1970). In determining the uptake of oxygen by germinating spores, samples were removed from the spore suspension, added to the oxygen

cuvette and a rate determined. This procedure was necessary since in the respirometer spores rapidly became anaerobic and did not continue development under such conditions. Results are expressed as Q_{O_2} (nmoles O_2 taken up/h/mg dry wt).

Fractionation of spores. Extracts were obtained by suspending spores or amoebae in 10 ml of distilled water and grinding with a pestle in a chilled mortar, using twice their weight of sand for 20 min. Microscopic examination showed that about 80 % of the spores were broken by this means. The spore suspension was centrifuged at 10000 g for 15 min to remove debris. The procedure used for fractioning spores into various classes of substances is illustrated in Fig. 1. The ethyl alcohol-formic acid residue was separated into protein, RNA and DNA by the method of Kennell (1967) with glass fibre discs (Whatman GF/A). The method of Maheshwari & Sussman (1971) was used to separate lipids, amino acids, organic acids and sugars, and the amino acids and organic acids were further separated by paper chromatography as described below.

Analytical procedures. Organic acids were resolved by the method of Stark, Goodban & Owens (1951). Amino acids were identified by the two-dimensional chromatographic method of Haworth & Heathcote (1969). Chemical tests were used to determine the level of methionine, tryptophan, and phenylalanine in spore extracts without prior chromatography. Methionine was determined by the method of Durell, Anderson & Cantoni (1957), tryptophan by the method of Spies & Chambers (1949) and phenylalanine by that of Udenfriend (1957). Protein was determined according to Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumen, fraction IV, as a standard. The orcinol method (Schneider, 1957) was used to determine RNA, and yeast RNA was used as a standard.

Enzyme assays. Extracts were obtained from spores as described previously, and cell fragments and whole spores were removed by centrifugation at 7000 g for 10 min at 4 °C. Specific activities are expressed as μ mole substrate transformed/min/mg protein.

Succinate dehydrogenase activity was determined spectrophotometrically at 420 nm according to Veeger, DerVartanian & Zeylemaker (1969). Succinate thiokinase activity was measured spectrophotometrically at 235 nm according to Cha (1969).

Labelled precursor assays. Spores were incubated in the presence or absence of the self-inhibitor with [U - ^{14}C]glucose (200 μ Ci/ml) as a non-specific precursor of cellular intermediates. At specific times the incubation was terminated by submerging the cells in a dry ice-ethyl alcohol mixture after which fractionation was carried out as described.

To measure protein and RNA synthesis, 10 ml samples of spores were incubated 10 min in the presence of an appropriate labelled precursor. Protein synthesis was determined with [U - ^{14}C]leucine (0.620 μ mole/ml) and RNA with [5 - 3H]uracil (2.39 μ mole/ml). When the effects of an inhibitor were to be measured, incubation in this substance was begun 15 min earlier so that the spores were exposed to the inhibitor before the addition of the labelled precursor. Incubation was stopped by the addition of 2 ml of 30 % trichloroacetic acid and the assay mixture was placed in a dry ice-ethyl alcohol mixture until analysis. For analysis the spores were transferred quantitatively to a prechilled mortar, ground as described earlier, and fractionated for protein and RNA as above. A Packard 'Tricarb' liquid scintillation spectrometer, model 3310 (Packard Instrument Co.) was used for all radioactivity determinations. Samples were counted in a scintillation fluid of the following composition: 2,5-diphenyloxazole, 40 g; 1,4-bis-2-(5-phenyloxazolyl)-benzene, 160 mg; dimethoxyethane, 400 ml; *p*-dioxane, 2400 ml; *p*-anisole, 400 ml. All results were corrected for zero-time incorporation and background.

Fig. 2. Germination kinetics of spores of *Dictyostelium discoideum* to which natural self-inhibitor was added 2.5 h after incubation, (●). Control spores (○).

RESULTS

Developmental kinetics. The kinetics of the germination of spores of *Dictyostelium discoideum* are shown in Fig. 2. These data also indicate that the self-inhibitor delays germination and outgrowth when added to swollen spores. Spore development is completely inhibited if the self-inhibitor is added before this time. Activation and germination are two separate processes because spores heat-shocked in the presence of the self-inhibitor would show 94 to 99 % outgrowth if they were washed free of the self-inhibitor immediately after activation. If activated spores were immediately frozen at -20°C , they remained activated; if they were chilled and stored at 4°C , dormancy was restored and these spores had to be reactivated before development would proceed. These observations are similar to those of Cotter & Raper (1968).

Respiratory responses. Activated spores had higher rates of respiration than dormant ones (Fig. 3). Self-inhibited spores started with rates that were similar to those of activated spores but, after about 2 h, these fell and by 4 h had reached the rates of dormant spores. No significance is attached to differences in the Q_{O_2} values of spores to which the inhibitor was added 1 h after activation and those activated in the presence of the inhibitor. Replicate experiments revealed that, whereas the overall pattern was similar, absolute Q_{O_2} values varied slightly from one experiment to another.

Respiratory rates continued to rise in those spores to which the self-inhibitor was added 2 h after activation and such spores also swelled. It is concluded from the above results that the self-inhibitor acted on a process occurring during the first 1.5 to 2 h after activation. Consequently, most experiments were performed within 1.5 h after heat-shock so that secondarily dormant spores were avoided.

The action of the self-inhibitor on the Q_{O_2} was compared with that of inhibitors which affected the electron transport system, Krebs cycle and glycolysis (Table 1). The results suggest that these pathways were active in the early stages of germination. The uncoupler, 2,4-dinitrophenol, increased the Q_{O_2} and higher concentration (10^{-3}M) killed the spores. The reason for the early insensitivity to malonate and fluoride is not known. The effect of

Fig. 3. Oxygen uptake of spores of *Dictyostelium discoideum*: controls, (○); self-inhibitor added 2.5 h after activation, (●); self-inhibitor added 1 h after activation, (■); self-inhibitor present during activation, (□); dormant spores, (△).

Table 1. *The effect of inhibitors on the Q_{O_2} of germinating spores of *Dictyostelium discoideum**

Spores were activated and incubated in liquid synthetic medium. At various time interval 1 ml samples (0.01 mg dry wt) were removed and added to the oxygen cuvette. Final concentrations of inhibitors used were: self-inhibitor (SI), 50 μ g/ml; 2,4-dinitrophenol (2,4-DNP), 0.0002 M; fluoride (Flu), 0.02 M; malonate (Mal), 0.08 M; azide, 0.002 M; and cyanide (Cya), 0.002 M. Inhibition is expressed as per cent of controls.

Min	Q_{O_2}		Inhibition (%)				
	Control	2,4-DNP	SI	Flu	Mal	Azide	Cya
0	1.7	1.9	0	0	0	100	100
15	2.4	3.2	0	89	99	100	100
30	3.6	4.1	0	91	100	100	100
45	4.2	6.0	0	90	100	100	100
60	5.3	8.2	0	94	100	100	100
90	7.2	14.0	91	99	100	100	100

the other substances may be explained either on the basis of greater participation of the Krebs cycle and glycolysis in the metabolism of later stages in germinating spores or to an increase in their permeability at this time.

Incorporation of [U - ^{14}C]glucose. A study was made of the effect of the self-inhibitor on the incorporation of ^{14}C into various spore fractions during a 1-h incubation period. The distribution of label in many fractions of inhibited spores (Table 2) was similar to that in the control spores but there are distinct differences in the incorporation into protein and amino

Table 2. ^{14}C in various fractions of spores of *Dictyostelium discoideum* after incubation in $[\text{U-}^{14}\text{C}]\text{glucose}$ in the presence and absence of the self-inhibitorSpores were activated and incubated in $[\text{U-}^{14}\text{C}]\text{glucose}$ for 1 h.

Fraction type	Radioactivity (c.p.m.)		Percentage of control
	Control	Inhibitor	
Total ^{14}C uptake	21960	19874	90
Total label	17694	16342	93
Amino acids	12095	13970	116
Organic acids	4635	4972	107
Free lipids	391	386	99
Protein	1422	110	8
RNA	0	0	(—)
DNA	0	0	(—)

Table 3. Effect of self-inhibitor of *Dictyostelium discoideum* spores on the levels of organic acids in spores incubated in $[\text{U-}^{14}\text{C}]\text{glucose}$

Values are given as % of controls.

Organic acids	Time after addition of self-inhibitor		
	Zero	30 min	60 min
Pyruvic acid	94	95	77
Oxaloacetate	72	94	73
α -Ketoglutarate	99	97	84
Citrate	84	97	80
Malate	92	94	80
Succinate	97	97	305
Fumarate	97	97	98

and organic acids. Thus, there was 92 % inhibition of incorporation of ^{14}C into protein and an increase in incorporation into the amino acid and organic acid fractions.

Since these data give no indication of the effect of the inhibitor on the incorporation into individual components within a fraction, the organic acid, amino acid and protein fraction were analysed in detail.

Organic acids. The effect of the inhibitor on organic acids is shown in Table 3 and it is apparent that the concentration of succinic acid was appreciably increased. Such an increase was not a result of the inhibition of succinate dehydrogenase since neither the natural nor the synthetic self-inhibitor affected the activities of this enzyme in extracts. Thus, the specific activities for controls and extracts with self-inhibitor were 0.067 and 0.063 respectively.

Succinic thiokinase activity was not detected in extracts. It should be pointed out that this enzyme is reported to be very unstable and it has not been reported from slime moulds.

Free amino acids. Methionine, phenylalanine and tryptophan are of special interest because they were completely absent from the dormant spore; otherwise, these and the 18 other amino acids tested always were present. Since these three amino acids are germinants (Cottor & Raper, 1966), their relationship to self-inhibition was examined. Uniformly labelled $[\text{U-}^{14}\text{C}]\text{glucose}$ was used to determine whether the amino acids were synthesized *de novo* or by the turnover of protein. The data in Fig. 4 not only indicate that these amino acids were synthesized when exogenous glucose was used as a carbon source but that in

Fig. 4. Synthesis of methionine (a); tryptophan (b); and phenylalanine (c) from [U- ^{14}C]glucose in spores of *Dictyostelium discoideum* incubated with (●) and without (○) self-inhibitor.

self-inhibited spores they accumulated to levels higher than in controls. It should be noted that there was a marked reduction in tryptophan in the controls during the beginning of outgrowth as opposed to the higher constant level in self-inhibited spores. These data indicate that activation resulted in the appearance of these amino acids in the pool and also that the self-inhibitor did not prevent their accumulation. Thus, these secondarily dormant spores were different from dormant ones in at least this respect.

Protein and RNA syntheses. The data in Table 2 show that there was a 92 % inhibition of ^{14}C -incorporation into protein during the first hour of spore germination, so this phenomenon was examined more closely. The kinetics of uracil and leucine incorporation into RNA and protein, respectively, are shown in Fig. 5. The rates of incorporation of these precursors suggest that protein synthesis began immediately after activation and continued for 2.5 h in the apparent absence of RNA synthesis. Interpretation of the evidence that protein synthesis early in development was independent of messenger RNA synthesis necessarily assumes that no internal pools of RNA precursors selectively depressed the uptake of exogenous precursors during this time and that the cells were permeable to the labelled precursors during the same period. We have no evidence on these points except that nucleosides like the self-inhibitor and other compounds did enter the spores very rapidly.

Cycloheximide (100 $\mu\text{g}/\text{ml}$) inhibited the incorporation of [^{14}C]leucine into protein by less than 50 %. Perhaps it was only at 1.5 h that the spores were permeable to the antibiotic. The use of higher concentrations (200 $\mu\text{g}/\text{ml}$) in a separate experiment produced identical results.

Fig. 5. Incorporation of [^{14}C]leucine, (●) and [^3H]uracil, (○) into protein and RNA respectively, by germinating spores of *Dictyostelium discoideum*.

The results of a short-term labelling experiment in which spores were incubated in the presence of the self-inhibitor are illustrated in Fig. 6. It is evident that such spores incorporated no [^{14}C]leucine into protein after this treatment, thereby reinforcing the previous conclusion that protein synthesis was required for germination to take place. This process was stopped completely after a short lag in spores which had germinated for 1 h whereas in swollen spores the process was inhibited initially but continued again after 5.5 h. These data are comparable with those in Fig. 2 in which outgrowth took longer when the same amount of self-inhibitor was added to germinating spores.

Incubation of spores with actinomycin D (100 $\mu\text{g}/\text{ml}$) caused a 35 % inhibition of uracil incorporation, but only germinating spores were sensitive to the antibiotic. Moreover, the rapid increase in leucine incorporation which occurred 5 h after activation was depressed by actinomycin D whereas the initial increase was unaffected (Fig. 7). These results are very similar to the data of Brambl & Van Etten (1970) with conidia of *Botryodiplodia theobromae*.

When spores were incubated in the presence of actinomycin D or cycloheximide, germination was not complete, stopping at the stage of swelling in both instances. Spores washed free of these inhibitors were capable of completing development so activation was not affected. Furthermore, the self-inhibitor, when added to spores during outgrowth, affected only the rate of protein synthesis and had no effect on RNA synthesis (Fig. 8). That the effects of the self-inhibitor under these conditions were overcome also is apparent from these data.

Fig. 6

Fig. 6. Inhibition of [^{14}C]leucine incorporation into protein of spores of *Dictyostelium discoideum* by the self-inhibitor. The self-inhibitor was added 1 h ($\square$), or 2.5 h ($\circ$) after activation and the controls ($\bullet$) had no inhibitor added.

Fig. 7

Fig. 7. Effect of actinomycin D (100 $\mu\text{g}/\text{ml}$) on the incorporation of [^{14}C]leucine into protein of the spores of *Dictyostelium discoideum*. Control spores ($\circ$) and spores to which actinomycin D was added immediately after activation ($\bullet$).

DISCUSSION

For the first 1.5 h after activation the pattern of oxygen uptake was not affected in either inhibited or control spores. In inhibitor-free spores this period of oxygen uptake was characterized by protein synthesis whereas self-inhibited spores were characterized by the absence of protein synthesis followed by a decline in the rate of oxygen uptake. It is unlikely that this decline was caused by exhaustion of substrate since these spores could be reactivated by removal of the inhibitor and patterns of oxygen uptake characteristic of activated spores recovered. Moreover, attempts to maintain oxidation at a steady rate by administering glucose or a mixture of three amino acids (phenylalanine, methionine and tryptophan, 10^{-2} M each) failed (unpublished data). Since oxygen uptake was similar in self-inhibited and activated spores during the early period of spore germination when protein synthesis was profoundly affected, we conclude that the self-inhibitor did not act directly on oxidative processes. Similar conclusions were reached by Williams & Allen (1967), Bush (1968) and Maheshwari & Sussman (1970) who found that respiration was not proportional to self-inhibition in uredospores of *Puccinia graminis* var. *tritici*.

High levels of succinic acid in self-inhibited spores prompted a study of this organic acid.

Fig. 8. Effect of self-inhibitor of *Dictyostelium discoideum* on protein and RNA synthesis in germinating spores. Self-inhibitor was added to spores 3.5 h after activation: [¹⁴C]leucine control, (○); [¹⁴C]leucine with self-inhibitor, (●); uracil incorporation control, (□); uracil with self-inhibitor, (■).

Such an increase suggests either more rapid synthesis in self-inhibited spores or slower withdrawal from the pool or both. Since the self-inhibitor had no influence on succinate dehydrogenase in extracts and the enzyme was present in inhibited spores at levels comparable to those in control spores, we conclude that it, at least, was not affected. According to Wright (1963), cellular slime moulds are facultative anaerobes in that they will grow and undergo binary fission anaerobically. Some facultative anaerobes possess a form of fermentation which results in the accumulation of succinate (Davis, Kornberg, Nagler, Miller & Mingioli, 1959; Hauber & Singer, 1967) and the data in Fig. 3 suggests that the self-inhibited spores became less capable of respiring and, perhaps, ferment glucose.

The total quantity of free amino acids was higher in self-inhibited spores than in inhibitor-free spores. This situation was to be expected because the turnover of most amino acids in the pool would have been slow in self-inhibited spores because protein synthesis was inhibited. It is important to recognize that the pool of free amino acids may not have been the only supply of amino acids for the synthesis of new protein molecules, for there exists the possibility that pre-existing proteins may have been degraded to their constituent amino acids which were then incorporated preferentially into the protein. The data obtained with labelled glucose do not allow us to conclude that all of the amino acids were synthesized *de novo*; what they do suggest is that the self-inhibitor did not interfere with the synthesis of such amino acids when exogenous glucose was used. According to Ceccarini (1967) glucose serves as the carbon source during the early stages of activation and trehalose during germination. The present data establish the existence of a means whereby the pool could be replenished in activated spores and that the self-inhibitor's action was not related to this aspect of metabolism.

The variation in free tryptophan content during development is interesting because it is the only amino acid tested that showed a distinct decrease during spore germination.

Krivanek & Krivanek (1959) in their study of amino acids in the cellular slime mould did not report the presence of this amino acid in either the hydrolysed or unhydrolysed tissue of any of the developmental stages they studied. It appears only in activated spores and such spores were not used in the Krivanek's study. Another curious finding is that tryptophan, along with phenylalanine and methionine, stimulated development in inhibitor-free spores (Cotter & Raper, 1966). Perhaps tryptophan is used for the *de novo* synthesis of germination-specific enzymes. Trehalase is one such enzyme because after outgrowth it is released into the medium (Ceccarini, 1967).

The kinetics of precursor incorporation suggest that the resumption of protein and RNA syntheses were distinct events in time. Protein synthesis appeared to be necessary for germination to begin and was inhibited 100 % by the self-inhibitor and only about 50 % by cycloheximide, presumably because the spores were impermeable to the antibiotic during the early stages of development. The fact that protein synthesis was begun immediately upon activation, but in the absence of detectable RNA synthesis, suggests that dormant spores contained stable mRNA. Also the fact that actinomycin D prevented the rapid synthesis of protein after 5 h (Fig. 7) is more evidence that dormant spores contained messenger RNA. Data on other fungal spores support the generality of this hypothesis (Ono, Kimura & Yanagita, 1966; Staples, App, McCarthy & Gerosa, 1966; Ramakrishnan & Staples, 1968; Hollomon, 1969, 1970).

The chemical structure of *N,N*-dimethylguanosine suggests a relationship to the process of protein synthesis, because the work of Conway & Lipmann (1964) demonstrated an absolute requirement for a guanine base during this process. Structural analogues of guanosine triphosphate have been shown to act as competitive inhibitors during protein synthesis (Hershey & Monroe, 1966; Remy, Engel, Dirheimer, Eber & Revel, 1970) and *N,N*-dimethylguanosine may be another very specific inhibitor. Although many free nucleosides have been discovered as products of microbial metabolism, and many are considered as inhibitors, a biological function for methylated bases is not known. This study suggests a function for one such compound: *N,N*-dimethylguanosine serves to regulate spore germination in *Dictyostelium discoideum*.

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