The ribonucleic acids in dormant and germination spores of the cellular slime mold Dictyostelium discoideum.

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS REÇUE
THE RIBONUCLEIC ACIDS IN DORMANT AND GERMINATION SPORES OF
THE CELLULAR SLIME MOLD DICTYOSTELIUM DISCOIDEUM

by

John Edward Hamer

A Thesis
submitted to the Faculty of Graduate Studies
through the Department of Biology
in Partial Fulfillment of
the requirements for the
Degree of Master of Science at
the University of Windsor

Windsor, Ontario; Canada
1982
DEDICATION

This thesis is dedicated to my father, Alexander, and my late mother, Francis Clara; who saw education, not as a right but as a privilege to be treasured.
ABSTRACT

A study was initiated on the ribonucleic acids of dormant and germinating spores of B. discoideum. An analysis of the ribonucleic (RNA) acids of dormant spores revealed a full complement of eukaryotic cellular RNAs including 5.8s and 5.0s ribosomal associated RNA, 4.0s transfer RNAs (tRNA), and messenger RNA. The transfer RNAs were purified from dormant spores and vegetative amoebae and the two preparations were compared. The following results were obtained: (1) the charging levels for spore tRNA and amoeba tRNA were found to be similar for twelve amino acids; (2) periodate oxidation demonstrated that 20% of the spore tRNAs were 'precharged' as compared to 46% in the vegetative cell; (3), using a similar technique the tRNAs for tryptophan, phenylalanine and methionine were found to be 'precharged' to the same extent in both spore and amoeba tRNA preparations.

RNA synthesis initiates approximately one hour after activation and the rate of RNA synthesis increases non-coordinate during germination. Ribosomal and ribosomal associated RNAs undergo generalized increases in their rate of synthesis. Transfer RNAs are synthesized after the second hour of germination and do not appear to become methylated during germination. Polyadenylate
containing RNA is synthesized maximally during the transition from late swelling of early emergence, accounting for approximately 10% of the total amount of RNA synthesis. Additionally, it appears that dormant spores contain all of the ribonucleotide precursors essential for RNA synthesis.

A survey of RNA synthesis inhibitors demonstrated that RNA synthesis is only required for the emergence stage of germination. Two inhibitors which proved useful were the mutagen 4-nitroquinoline 1-oxide and the application of ultraviolet light. Using UV light I have demonstrated that: (1), RNA synthesis is abruptly terminated following the application of UV light with the accumulation of prematurely terminated transcripts; (2), all species of RNA except poly(A)+ RNA, remain stable in the irradiated swollen spore; (3), the poly(A)+ RNA synthesized during germination decays with a half-life of 15 minutes. This latter point is supported by studies on the in vivo decay of protein synthesis following the application of ultraviolet light.
ACKNOWLEDGEMENT

I wish to first express my appreciation to the students of Dr. Cotter's laboratory, both past and present, for their encouragement and support in this work. I would like to acknowledge the students of Dr. Pillay's laboratory for their help and advice with the work on transfer RNA. I would also like to acknowledge Dr. James L. Van Etten and Dr. James S. Lovett for their advice and suggestions on the extraction and analysis of ribonucleic acids from fungal spores.

Finally, I would like to acknowledge and express my deepest appreciation to Dr. David A. Cotter, whose personal concern and enthusiasm made this project challenging, rewarding and enjoyable.
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INTRODUCTION

Part I. General Aspects of the Organism Dictyostelium

Dictyostelium discoideum is a member of the family Dictyosteliaceae in the order Acraiales. This group of organisms is commonly referred to as the cellular slime molds. While the first species of this genus, Dictyostelium mucoroides was described in 1869 by the German mycologist Oskar Brefeld, the discovery of D. discoideum was not made until 1935 by Kenneth B. Raper. The organism was isolated from decaying forest litter in the soils of North Carolina (Raper, 1935). While the organism obviously plays a role in the economy of nature, its major significance to man has been as a laboratory tool for the study of morphogenesis and cellular differentiation.

Several characteristics have contributed to D. discoideum's employment as a laboratory model. The organism can be easily cultivated on media that will support the growth of the common bacterium, Escherichia coli, on which the slime mold amoebae feed. Alternately, axenic mutants have been described that will grow in liquid culture in the absence of bacteria (Sussman and Sussman, 1967; Watts and Ashworth, 1970; Loomis, 1971). The relatively small genome as well as the haploid nature of the organism have facilitated classical as well as molecular genetic studies.
In the wild and on petri dishes, slime mold amoebae feed on bacteria; upon the depletion of food in the local environment a unique and characteristic series of morphological events occur. The cells first pass through interphase which is a period of differentiation, (Bonner, 1967) and then they aggregate to form a multicellular pseudoplasmodium (grex or slug). The pseudoplasmodium migrates for a period and culminates to form the fruiting body bearing an apical soti containing dormant spores (see Fig. 1.)

Aggregation is mediated by a few autonomous cells which release short, periodic pulses of an 'acrasin', identified as cAMP (Konijn et al., 1968). This pulsing of cAMP in turn triggers neighbouring cells to begin propagating cAMP in a similar pulse-like manner, and within two minutes a chemotactic response is initiated. The cells aggregate by typical amoeboid movement and collect in large streaming patterns to form groups containing up to 10^5 cells. These aggregates become integrated by deposition of a surface sheath which covers the whole mound. A morphological tip is formed at the center of the mound which serves as an organizer for pseudoplasmodium formation.

The pseudoplasmodium is the migratory stage of the life cycle. It is phototactic to light sources of extremely low intensity and thermotactic to differences of 0.0005°C
Figure 1. The life cycle of *Dictyostelium discoideum*
(Bonner, 1950). Cells at the anterior one third of the slug are committed to stalk cell differentiation and are termed 'prestalk', while the posterior two thirds of the slug are termed 'prespore' and are committed to form dormant spores. These two cell types are distinguishable by histochemical staining and gradient density centrifugation. If the slug encounters additional bacteria the cells will dedifferentiate and return to their amoeboid character, however unless bacteria are found the slug stops migrating and culmination ensues.

Culmination initiates when migratory behaviour ceases and the anterior tip moves vertically. At this point cells near the tip of the mass begin to synthesize increased amounts of cellulose forming a sheath within which the cells vacuolize to form the stalk. The prespore cells are drawn upwards by the expanding stalk and the peripheral cells begin to encapsulate and form the apical sorus, consisting of dormant spores. The dormant spores are surrounded by a viscous extracellular matrix consisting of numerous proteins, carbohydrates, and germination inhibition factors. The germination of the dormant spores completes and reinitiates the entire developmental program. An extensive review of the organism has been prepared by Dr. William F. Loomis (1975).
Part II. Spores and Spore Germination in Dictyostelium discoideum.

The mature dormant spores of *D. discoideum* NC-4 are capsule shaped, 6 to 9 um long and 2.5 to 3.5 um in diameter (Raper, 1935; Bonner, 1967). Typically, spores are resistant to mild heating, freezing, drying and other adverse environmental conditions (Cotter and Raper, 1968). Uniquely however they are extremely sensitive to ultraviolet irradiation while vegetative amoebae are resistant (Liverant and Pierre de Silva, 1976; Cotter, 1973; Demps, 1981). The dormant spores are constitutively dormant as defined by Sussman (1976) and thus exist in a hypometabolic state until a suitable activation stimulus is applied. The four developmentally recognized stages in *D. discoideum* germination are activation, post-activation lag, swelling and emergence (Cotter, 1975).

Activation is defined by Sussman (1976) as any process that increases the rate of germination. Cotter and co-workers (see Cotter, 1981 for review) have developed a large variety of activation techniques which induce synchronous germination. Prior to the application of any activation technique, spores must be washed in 0.01 M phosphate buffer, pH 6.5, to remove the germination inhibitor discadenine (Abe *et al.*, 1976). The presence of this inhibitor in even minute concentrations will severely retard germination. The activation techniques
include the following: 1% peptone for 30 min (Cotter and Raper, 1966); heat at 45°C for 30 min (Cotter and Raper, 1968ab); 20% dimethyl sulfoxide (DMSO) for 30 min (Cotter et al., 1976); 8 M urea for 30 min (Cotter and O'Connell, 1976); 3 M ethylene glycol for 60 min (Cotter, 1977); 6 M guanidine HCl for 60 min, 2 M dimethylurea for 60 min, and 2 M tetramethylurea for 30 min (Cotter, 1979). There have been relatively few changes that have been shown to occur during activation. In a freeze-fracture study of spore plasma membranes, Hohl et al. (1978) found that at the end of heat-activation the distribution of spore plasma membrane particles between the two fractured faces (protoplasmic face (PF) and exoplasmic face (EF)) had changed. Prior to activation the particle ratio of PF/EF was 1:1. Following activation this was increased to 9:1. Although transmission electron microscopy and light microscopy have failed to show changes with heat activation (Cotter and Raper, 1966; Cotter et al., 1969; Cotter and George, 1975) calculations of enthalpy changes have led to the proposal of a thermodynamic model for spore activation as well as a hypothetical site for activation (Cotter, 1975; 1981).

The time between the removal of the activating agent or stimulus and the first sign of spore swelling is defined as the postactivation lag. This stage can be further divided into an early and late lag stage. Activation procedures carried out in excess result in spore death (Cotter and George,
1975) and generally the more severe the activation procedure, the longer the post-activation lag. During this stage, activated spores are extremely sensitive to environmental changes and thus alteration in either pH, osmotic pressure, ionic strength, temperature, or interference in O2 uptake will deactivate the spores (see Cotter, 1981). Studies involving activating and deactivating agents have elucidated a 'fail-safe' point for spore swelling (Cotter et al., 1979). Interestingly, spores may be held in the lag stage of germination indefinitely by the addition of DMSO to a final concentration of 5% (v/v). Removal of the DMSO results in rapid and synchronous swelling (Cotter et al., 1976).

The swelling stage of germination can be further subdivided on the basis of spore morphology into early, middle, and late stages. Early swelling is characterized by the appearance of a localized, lateral protuberance on the spore wall. Middle swelling initiates as the protuberance widens to form a rounded swollen spore which appears phase dark under the microscope. The latter state is characterized by the appearance of granules and one or more contractile vacuoles as the spore continues to widen and swell (Cotter and Raper, 1966, 1968ab; Cotter, 1975). Swelling culminates with the splitting of the inner spore walls (Cotter et al., 1969; Hemmes et al., 1972).

The final stage of germination is characterized by a splitting of the innermost spore wall, and the emergence
of a single, phase dark, myxamoebae (Cotter et al., 1969; Hemmes et al., 1972). Lipoidal bodies as well as proteinaceous crystals are degraded during this stage so that the true vegetative amoebae is devoid of these structures (Cotter et al., 1969; Gregg and Badman, 1970).

The optimal germination conditions for the wildtype strain, NC-4H, are as follows. A temperature of 23.5°C and a spore concentration not exceeding 2 x 10^7 spores/ml. While a number of buffers or distilled water could be used, germination in our lab is routinely done in 10 mM phosphate buffer, pH 6.5. In order to obtain good aeration of the germinating culture a high surface to volume ratio must be maintained with continuous shaking (Cotter and Raper, 1968ab). When one-day-old spores are heat activated and germinated as described above, greater than 95% of the spore population will synchronously release myxamoebae by 5 hours. Under these conditions post-activation lag lasts for 1 hour, swelling occurs between 1-2.5 hours and amoebae are released shortly after 2.5 hours (see Figure 2).

Two mutants of D. discoideum have been isolated which do not require an external activation treatment. These have been designated SG1 and SG2 for their spontaneous germination phenotype (Cotter and Dahlberg, 1977). Several interesting characteristics of these mutants may prove useful in future studies. Since these mutants arose spontaneously, and were not induced by mutagenesis, they
Fig. 2.

The kinetics of heat-activated spore germination in the cellular slime mold *Dictyostelium discoideum*. Mature two-day-old spores were heat-activated at 45°C for 30 minutes, and germinated as described in the Materials and Methods.

Symbols:

- (○) percent spore swelling
- (●) percent myxamoebae emergence
may be the products of a single mutation. Additionally, they exhibit temporal and quantitative shifts in the patterns of expression of certain developmentally regulated enzymes (Chan and Cotter, 1982). However extensive biochemical and genetic analysis remains to be done.

Part III. RNA Metabolism During Growth and Development of D. discoideum.

*Dictyostelium discoideum* is a typical metazoan cell containing all of the major classes of RNA; ribosomal RNA, messenger RNA, and transfer RNA. The desire to use *D. discoideum* as a laboratory model for studies on morphogenesis and the dogma that development is mediated through differential gene action, has prompted intense studies on the regulation of ribonucleic acid metabolism in this organism. At the present time there are no reviews available on this area except a very brief description by Loomis (1975). Thus I have relied extensively on original papers published after this point. In order to fully understand RNA metabolism in *Dictyostelium* a brief description of the genome is in order, since it distinguishes this organism from all other fungi.

The seven chromosomes of *D. discoideum* contain $3.6 \times 10^{10}$ daltons or about $5.4 \times 10^{7}$ nucleotide pairs (Sussman and Rayner, 1971) of DNA. The DNA has a buoyant density
of 1.683 gm/cm$^3$ and a $T_m$ of 78.8 C indicating a base composition of 23% G+C (Firtel and Bonner, 1972; Leach and Ashworth, 1972). Uniquely, the mitochondrial DNA accounts for between 28-40% of the total cellular DNA (Sussman and Rayner, 1971; Firtel and Bonner, 1972).

The genome of most fungi is characterized by the presence of low amounts of repetitive DNA which is usually localized rather than interspersed (personal communication, W.E. Timberlake) as in higher eukaryotes. In the majority of cases this repetitive element contains the ribosomal RNA genes. In Dictyostelium a third of the genome is made up of repetitive sequences of different lengths interspersed with unique or single copy DNA (Firtel and Bonner, 1972). In this way the slime mold genome is unique from other fungi. At present the exact function of these repetitive elements is unknown, however recent evidence suggests that these sequences may serve as regulatory signals by punctuating the single copy coding regions. Firtel et al., (1979) has shown that actin genes in Dictyostelium are literally embedded in almost pure A-T rich DNA.

A. Ribosomal RNA

The ribosomal RNA genes are located in a 38,000 base pair repeating unit reiterated 200-fold and arranged as a nonchromosomal palindromic dimer (Cockburn et al., 1978; Maizels, 1976). The 25s and 17s rRNA genes are separated
by a short spacer region and the 5.8S rRNA gene. The genes are transcribed by an α-aminitin insensitive DNA-dependent RNA polymerase (420,000 mw) designated polymerase I (Pong and Loomis, 1973a). Transcription results in a 36S precursor which shortens to a 19S precursor in the nuclei (Cocucci and Sussman, 1970; Mizukami and Iwabuchi, 1972; Firtel and Lodish, 1973).

Studies on RNA synthesis were prompted by the finding that upon the initiation of development the RNA content of growing cells decreased from 1.0 mg RNA/10^8 cells to 0.4 mg RNA/10^8 cells in developing cells. This was found to result from the unbalanced turnover of rRNA (White and Sussman, 1961; Cocucci and Sussman, 1970). Cocucci and Sussman showed that the turnover of rRNA was due to the degradation of existing ribosomes and the resynthesis of new ribosomes, which according to their data were incorporated into polysomes. The remaining vegetative ribosomes were either degraded or preferentially accumulated as 80S monosomes. These workers hypothesized that the new ribosomes were somehow different from vegetative ribosomes and thus may play a role in regulating stage specific protein synthesis at the level of translation. They also hypothesized that vegetative ribosomes were rendered non-functional at the initiation of development and accumulated as 80S monosomes.

In an attempt to define the differences in rRNA
metabolism in growing and developing cells, oligonucleotide maps and primary sequence data for the 25s, 17s and 5s RNA's was obtained (Batts-Young et al., 1980). This work concluded that the 5s rRNA sequence was unchanged and that no detectable changes could be observed in the 25s and 17s RNA in developing cells and in their vegetative counterparts. Although this work did not address itself to limited modifications such as methylation, it appears to rule out the idea of novel rRNA expression during development. In a related piece of work from the same laboratory, an extensive investigation was made into rRNA synthesis and degradation during cell differentiation (Mangiarotti et al., 1981). By using double labelling techniques these workers showed that the same fraction of old and new 60s ribosomal subunits, and old and new 40s subunits are found in polysomes during development. This contradicts earlier findings of Cocucci and Sussman (1961) and concludes that ribosomes synthesized during growth and differentiation are functionally indistinguishable. They also concluded that the rate of synthesis of rRNA during development is much slower than in growing cells and thus the replacement of vegetative stage ribosomes with those synthesized during differentiation is a slow and incomplete process. This conclusion is also at variance with Cocucci and Sussman (1970) who claimed that the majority of polysomes present during development contained new ribosomes. Mangiarotti
et al., also found that the developmental ribosomes and vegetative ribosomes were degraded to the same extent. Finally they concluded that the degradation of ribosomes is an all or nothing process. The accumulated 80s monosomes present in developing cells are not nonfunctional but can resume protein synthesis if growth conditions are restored. Thus from the body of evidence to date it appears unlikely that ribosomes synthesized during differentiation are essential for the translation of developmentally regulated mRNAs. One can question then, why does the slime mold expend energy synthesizing new ribosomes during differentiation while at the same time it degrades a pool of functional ribosomes? Loomis (1975) has suggested that D. discoideum may lack control mechanisms for regulating rRNA and ribosome synthesis. Recently, Ramagopal and Ennis, (1981) have demonstrated small but distinct changes in ribosomal proteins isolated from the ribosomes of growing and differentiating cells. Whether these changes have a significant regulatory role remains to be seen.

B. Messenger RNA

One of the most interesting features concerning mRNA metabolism was the discovery of a relatively conserved but detectable heterogenous nuclear RNA species (hnRNA) (Firtel et al., 1972). This distinguishes D. discoideum from other metazoans such as yeast and the filamentous fungi which
and thus is foundational for establishing *Dictyostelium* as a model for studying gene regulation in higher eukaryotes.

The messenger RNA (mRNA) of *Dictyostelium* is typically eukaryotic. It is transcribed from the DNA as a 500,000 dalton hnRNA species by an α-aminitin sensitive, DNA dependent RNA polymerase. The hnRNA is 70% transcribed from unique single copy regions on the DNA while 25% is transcribed from repetitive regions present in several hundred copies per genome (Firtel and Lodish, 1973). This repetitive region appears at the 5' end and is not conserved during processing. Another unique feature of the hnRNA molecule is the presence of a transcribed oligo (dT) region of about 25 base pairs (Jacobsen et al., 1974). This region is conserved during processing and is localized near the 3' end of the molecule.

As is typical of eukaryotic mRNA the addition of a 3' poly (A) 'tail' and a 7-methyl-guanosine 'cap' completes the processing. The poly(A) 'tail' initially has a modal size length of about 110 to 115 nucleotides which shortens with ages to about 60 to 65 nucleotides (Palatnik et al., 1979). Palatnik and coworkers have extensively examined the poly(A) metabolism and the role of the poly(A) tail. They conclude that it is unclear whether it has a significant regulatory role (Palatnik et al., 1980). It does not play a role in the stability of the mRNA population as a whole, and does
not appear to be required for protein synthesis. Studies on protein synthesis have shown that apart from histones, poly(A)-RNA codes for only a small group of proteins, none of which are unique (Lodish et al., 1974; Palatnik et al., 1979). The 7-methyl-guanosine cap region is slightly different than the cap region of other eukaryotes. The cap structure lacks a 2'-0'-methylated nucleoside reported in mammalian cells and 6-methyladenosine found in other eukaryotes (Dottin R.P., et al., 1976. 'Abstracts of papers presented at the meeting on Dictyostelium', Cold Spring Harbour, pp. 3).

Several approaches have been made to analyse the pattern of gene expression during growth and development. Initial studies involved the analysis of vegetative and stage-specific enzymes as biochemical markers of differentiation (see Loomis, 1975 for review). Many of these enzymes appeared to be under transcriptional control. Later work employed the use of two-dimensional gels as a means to estimate the number of genes required for differentiation and growth, and to define periods of translational and transcriptional control (Alton and Lodish, 1977a). However both these procedures provided information on only enzymatically detectable or abundant proteins. Recently, the ability to hybridize mRNA to its copied DNA as well as RNA-DNA excess hybridization have provided a direct and sensitive estimation of the transcriptional patterns under-
lying growth and differentiation.

In the vegetative cells the complexity of the nuclear, polysomal and whole cytoplasmic polyadenylate RNA has been examined employing both kinetic cDNA–RNA hybridization and saturation single copy DNA–RNA hybridization (Blumberg and Lodish, 1980a). Polyadenylate RNA comprised about 82% of the cellular mRNA and was divided into three abundance classes. There are approximately 40 abundant species (1000 copies/cell); 500 moderately abundant species (> 150 copies/cell) and a remaining 3000–4000 complex RNA species (10–15 copies/cell). The majority of these species can be found on polysomes. There also appears to be an additional 9000 transcripts found in the nucleus, however these appear to be transferred poorly, if at all, to the cytoplasm. Therefore, while D. discoideum has a coding capacity of about 30,000 average size transcripts, it appears to require between 4000–5000 genes to grow, or about 19% of its total coding capacity (Blumberg and Lodish, 1980a). Interestingly, this almost equals the number of proteins in growing cells detected by two-dimensional gels (Alton and Lodish, 1977b).

Studies on developing cells employing hybridization techniques have lent support to previous studies utilizing polyacrylamide gel electrophoresis (Alton and Lodish, 1977a). There appears to be only one time period during the developmental program when the initiation of expression of a
significant number of genes occurs. This point occurs midway through the developmental cycle at a time coincident with tip formation and multicellularity, just prior to the onset of cellular differentiation. There is the coordinate expression and appearance in the cytoplasm of an additional 100 to 150, moderately abundant genes (80-100 copies/cell) and 3000 genes of a complex class expressed at about 5 copies per cell (Blumberg and Lodish, 1980b). These transcripts represent 31 to 32% of the mass of the polyadenylated RNA in the cell during late aggregation and culmination stages. The remaining 68 to 69% of the transcripts in late developing cells are composed of 5000 different RNA species which were expressed during growth and early development. Both these transcripts sets can be found in polyribosomal complexes.

From the above experiments Blumberg and Lodish estimated that approximately 3000 genes are required for development. Genetic estimates involving a comparison of the mutational target size for several structural genes with the frequency of occurrence of mutants which are deficient in aggregation or exhibit aberrant developmental morphology, suggest that only 300 genes are required (Loomis, 1978). Discrepancies in these two numbers at first appear large, however, genetic analysis would not score genes that have subtle or lethal effects on development. While hybridization would score genes that while expressed during development, they are not
obligatory (e.g. beta-glucosidase and alpha-mannosidase).

Mangiarotti et al., have probed a lambda library of cloned genomic *D. discoideum* DNA with developmentally expressed transcripts using hybridization competition (Mangiarotti et al., 1981). Phage genomes containing 8 kb inserts of *Dictyostelium* DNA were fixed to nitrocellulose filters and were hybridized with a small amount of labelled mRNA isolated from developing cells. The hybridization was preformed in the presence of large excesses of 'cold' vegetative mRNA which competed with any labelled vegetative sequence present in the developmental mRNA preparation. Thus autoradiographic signals would only develop in the presence of a developmental specific sequence. In this way several clones carrying developmentally expressed sequences were characterized. The result showed that while some developmental genes appeared clustered, others were interspersed with vegetative sequences.

While studies on mRNA synthesis have shed light on the genetic requirement for development, studies on the role of cAMP in RNA metabolism have led to mechanistic models for the regulation of developmental gene expression.

In one study that was similar to the work of Blumberg and Lodish (1980), Williams and Lloyd (1979) prepared DNA copies from poly(A)+RNA sequences isolated during development. Their work reported similar results to Alton and Lodish (1977) and Blumberg and Lodish (1980b); that is, new gene expression
during development was largely confined to the stage of cell-cell contact and aggregation. In particular they found one poly(A)+RNA segment that was almost completely absent from vegetative cells but increased dramatically by the fourth hour of development, accounting for about 2% of the total poly(A)+RNA concentration. After this point the concentration of this sequence dropped during the next 4 hours of development. The genomic DNA of this poly(A)+RNA segment was cloned into a plasmid pMB 9 and termed pDd 812 (Williams and Lloyd, 1979).

Subsequent analysis of this cloned sequence showed that pDd 812 was an mRNA sequence which coded for the synthesis of one major, 33,000 dalton protein and a minor 31,000 dalton protein. Transcription of this sequence in isolated nuclei demonstrated changes in the level of transcription analogous to observed cytoplasmic changes (Williams et al., 1980). Recent work has demonstrated that this plasmid, pDd 812, actually encodes a lectin known as discoidin Ia and part of another lectin known as discoidin Ib. These lectins are believed to have a role in cell-cell recognition and the establishment of cell contacts. The addition of exogenous cAMP to developing cells causes a depression in the levels of discoidin I mRNA. Using a transcription system in isolated nuclei, Williams and coworkers found that this depression of discoidin I mRNA was due to a decrease in the level of
transcription in response to cAMP. They suggested increases in the intracellular cAMP concentration act at the level of transcription to control gene expression during development. They supported this idea by observing that when cells were exposed to exogenous cAMP poly(A)+RNA sequences which were normally expressed late in development, were prematurely synthesized. A slightly different role for cAMP has been presented by Dr. Harvey F. Lodish and co-workers.

Alton and Lodish (1977ab) and Blumberg and Lodish (1980ab), agree that new gene expression is confined to the stage of cell-cell aggregation. Alton and Lodish (1977b) have shown that cell-cell contact is a prerequisite for the expression of these early genes. Disruption of cell-cell contact results in a rapid cessation of synthesis of post-aggregation stage proteins (Okamoto and Takeuchi, 1976; Newell et al., 1971, 1972; Alton and Lodish, 1977b; Landfear and Lodish, 1980). Furthermore cAMP appears to be directly involved in controlling the synthesis of aggregation stage proteins (Landfear and Lodish, 1980). Specifically, upon the disruption of cell-cell contacts, aggregation-dependent poly(A)+RNA sequences decay with a half-life of about 15-20 minutes, while early and late poly(A)+RNA sequences that are also expressed at stages other than aggregation, decay with a half-life of about four hours (Chung et al., 1981; Margolskee and Lodish, 1980).
The addition of cAMP to disaggregated cells appears to restore the level of most late mRNAs within 3 hours. The authors conclude that cAMP stimulates the synthesis of these mRNAs and serves to stabilize them in the cytoplasm. This effect can only be found in disaggregated cells and not in suspension starved amoebae. Chung and coworkers suggest that the regulation of gene expression in development may be via mRNA stability and thus provides flexibility to the developmental program of Dictyostelium discoideum.

C. Transfer RNA

Studies on transfer RNA (tRNA) in growing and developing cells have only recently been initiated. Palatnik and Katz (1976) compared tRNA from growing and developing cells, charging them with 17 amino acids and using homologous synthetases. While the levels of charging were the same, some minor reproducible peak displacements were noted during reverse phase chromatography. The authors concluded by stating that there was no change in the transcription of tRNA genes during changes from growth to development. However they observed that some possible post-translational modifications may have occurred.

Dingermann et al., (1977) looked for changes in modified bases of tRNAs during development. Only the ribothymidine (rT) content appeared to change, from 0.9 mol% in vegetative
cells, to 0.5 mol% in developing cells, with spore tRNA being 0.4 mol% in rT. The remaining modified bases are unchanged in their molar content during development. These authors speculate that the decrease in rT content is due to the stage specific synthesis of an inhibitor of tRNA (uvacil 5)'methyltransferase, however no additional evidence is available to support this conclusion.

Recently, Dingermann (1979) has provided evidence that the decrease in rT occurs at position 54 in loop IV and occurs as a result of the synthesis of a new tRNA species with uridine in the place of rT. This new tRNA has an electrophoretic mobility similar to tRNA$^{\text{ASN}}$. Interestingly, this new tRNA species is synthesized just prior to aggregation - a time now known to be coincidental with novel developmental gene expression (Blumberg and Lodish, 1980b). This 'developmental tRNA' could also be extracted from polysomes. As development continued they also found that tRNAs accumulated with 3'-O-methyl-ribothymidine in the place of rT. Thus these tRNAs are distributed in position 54 modification classes B, C and D as described and defined by Roe et al., (1976). Dingermann describes four major observations that support the idea that modifications at position 54 in tRNA may have a regulatory function in protein synthesis (Dingermann et al., 1979).

The most recent evidence for the role of tRNA during development came from Dingermann et al., which have shown
that after the onset of development there is a marked reduction in the aminoacylation of tRNA\textsuperscript{ASN}, (occurrence of uncharged tRNA\textsubscript{ASN} with guanine in place of queine during the onset of development in \textit{Dictyostelium discoideum}: Possible role in translational control mechanisms. Abstracts on Gene Expression and Membrane Changes in Cellular Slime Molds, Tutzing, W. Germany. 1981). One major tRNA\textsuperscript{ASN} isoacceptor appears to be completely unacylated, where as 10 other tRNA species were acylated. These tRNA isoacceptors differed with respects to the first position of the anticodon. Transfer RNA\textsubscript{2ASN} had the hypermodified nucleoside queine (Q), while tRNA\textsubscript{3ASN} had the guanine nucleotide. Thus, tRNA may function as a regulatory molecule in initiating development. Deacylated tRNA may act as an inducer of a pleiotropic response in eukaryotes in much the same way as they are involved in the stringent response in bacteria (Herschko et al., 1971). It should be noted here that both the developmental process in \textit{Dictyostelium} and the stringent response in bacteria are initiated by amino acid deprivation. Bergmann and Lodish (1979) have suggested that a transient limitation in aminoacylation of a particular tRNA isoacceptor might be involved in the inhibition of initiation in specific mRNAs. Whether this model is applicable to the initiation of development in the cellular slime mold is as yet unknown.
Part IV. RNA synthesis during spore germination in *Dictyostelium discoideum*.

One of the major problems of developmental biology is to distinguish between true differentiation phenomena and normal growth processes. The dormant spores of the cellular slime mold *Dictyostelium discoideum* differentiate to form free-living amoebae in the absence of exogenous nutrients and without an apparent increase in dry weight or protein content. Thus spore germination may be a useful paradigm for studies on cellular differentiation.

Early studies on macromolecular synthesis during spore germination demonstrated a distinct requirement for protein synthesis to allow the emergence of myxamoebae from swollen spores (Cotter and Raper, 1968; 1970). However studies on RNA synthesis were not as successful. Inhibitors of RNA synthesis such as actinomycin D, did not inhibit germination even at high concentrations (Cotter and Raper, 1966). Bacon and Sussman (1973) found only a 35% inhibition of RNA synthesis with actinomycin D.

Later work reported that 600 μg/ml of actinomycin D suppressed rRNA synthesis while allowing normal germination (Yagura and Iwabuchi, 1976). In addition, Giri and Ennis (1977) reported on the effects of several RNA synthesis inhibitors including actinomycin D, daunomycin, thiolutin and lomofungin. They concluded that spores were impermeable
to actinomycin D, even at high concentrations, and that permeability increased during germination. Thiolutin appeared to block spore swelling, emergence and RNA synthesis. These results implied that RNA synthesis was required for spore swelling. Recently Ennis (1981) has demonstrated that spore swelling can occur in the absence of detectable RNA synthesis when spores are treated with the anthracycline antibiotic, nogalamycin, at 300 µg/ml in 95% ethanol. However, minute concentrations of ethanol will drastically alter germination kinetics (this thesis) and nogalamycin has been reported to cause uncoupling of oxidative phosphorylation in rat liver mitochondria (Reusser and Bhuyan, 1967).

The synthesis of RNA during spore germination in D. discoideum has been well documented (Bacon and Sussman, 1973; Giri and Ennis, 1977; 1978; Ennis, 1981). Dormant spores have been shown to contain a translatable preformed mRNA population as well as abundant ribosomes, however polysomes are not detected until the first hour following activation (Giri and Ennis, 1977). RNA synthesis initiates at about the same time and increases significantly with the emergence of myxamoebae. Continuous labelling studies have demonstrated that all species of RNA are actively synthesized (Giri and Ennis, 1978).

Significant changes in the pattern of gene expression have been noted during Dictyostelium discoideum spore germination (Dowbenko and Ennis, 1980). Studies on actin
mRNA synthesis have demonstrated a 300-fold increase in actin mRNA (McCleod et al., 1980) however a role (if any) for actin during spore germination is unclear. Dowbenko and Ennis (1980) have studied the in vivo and in vitro protein products during spore germination on two-dimensional gels in a similar manner as Alton and Lodish (1977). They noted only a few stage-specific proteins and that many proteins had short distinct periods of synthesis. Overall, spore germination appeared to be under transcriptional control. It should be noted that the synthesis of a few stage-specific proteins at varying times during germination is markedly different from the pattern of gene expression observed during multicellular development (Alton and Lodish, 1977).

Part V. Purpose of this study

Labelling studies on D. discoideum spore germination have been done employing the strain B mutant of D. discoideum (Giri and Ennis, 1978). This strain has an altered spore morphology as well as a perturbed response to various activating agents (Cotter and Raper, 1968b). Additionally only continuous labelling studies have been done at times that do not correspond to the previously mentioned developmental stages of spore germination. Thus, one purpose of this study was to define the RNA content of dormant spores and to clarify the periods of RNA synthesis.
as they relate to the four developmental stages.

Dormant spores of *D. discoideum* are uniquely more sensitive to ultraviolet light irradiation than their vegetative counterpart (Gilles et al., 1976). However, the physiological effects of this sensitivity have only recently been investigated (Demsar and Gotter, 1981). A second purpose of this study was to observe and define the effects of UV light on RNA synthesis during *D. discoideum* spore germination, and to compare its effects to other inhibitors.
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MATERIALS AND METHODS


   A. Strains and Media

   Throughout this study two haploid strains of Dictyostelium were used: the wild type strain NC4 Raper and an axenic mutant designated A3. The wild type strain was grown in conjunction with Escherichia coli B/r on glucose-salts agar plates (Adams, 1959). This medium consisted of: 1.0 g NH₄Cl, 0.13 g MgSO₄, 3.0 g KH₂PO₄, 6.0 g Na₂HPO₄, 20 g Bacto agar (Difco), and 0.4 g glucose in one liter of distilled water. The medium was dispensed into 100 x 15 mm petri dishes after autoclaving. The axenic mutant, A3, was grown in HL-5 medium (Loomis, 1975). This medium consisted of: 10 g bacto peptone (Difco), 5 g yeast extract (Difco), 10 g glucose in 10 mM phosphate buffer (pH 6.5).

   B. Preparation of spores

   Spores of D. discoideum were transferred from a stock plate to 20-200 ml of sterile distilled water. Several loopfuls of E. coli B/r were added and the suspension shaken lightly to disperse the inoculum. Between 1-2 ml of this inoculum was transferred to
glucose-salts plates which were subsequently incubated at 23.5°C. After 24 hours, the plates were agitated to disperse the inoculum in order to allow synchronous growth and development. A lawn of fully developed sorocarps formed within 4-5 days. Sorocarps were aged 1-3 days in order to insure that all spores had entered constitutive dormancy.

Mature dormant spores were harvested by passing a microscope slide 2-3 mm above the agar surface several times across each petri dish. After each passage the spores and extracellular materials were collected in 5-15 ml of double distilled, sterile water. When all the plates had been harvested the spores were washed free of the extracellular material by low speed centrifugation at room temperature in an IEC clinical centrifuge at a setting of 6. The pelleted spores were then resuspended in 5 ml of 10 mM phosphate buffer, (PB) pH 6.5, and recentrifuged as before. After an additional washing in PB, dormant spores were either germinated immediately or frozen at -20°C. Freezing was found to have no effect on the germination kinetics. For larger preparations of spores (i.e. > 50 plates) additional washings in larger volumes of PB were required.
C. Preparation of vegetative amoebae

Vegetative amoebae were prepared in two ways. Spores of the axenic mutant AX3 were aseptically transferred into 50 ml of HL-5 medium held in a 250 ml Erlenmeyer flask. The amoebae of AX3 grow with a doubling time of approximately 10 hours and reach a stationary phase concentration of $2 \times 10^6$ amoebae/ml of medium in about 5 days. An inoculum of 0.1 ml was used to serially transfer this stock culture from which larger volumes of amoebae were generated when needed. Amoebae were harvested by centrifugation and used immediately for preparing enzyme fractions or RNA.

Alternately, MC4 spores in the presence of E. coli B/r were placed on 14 cm glass plates containing glucose-salts agar. These plates were kept wet by adding 4 ml of sterile, distilled water to deter aggregation. After 3-4 days the amoebae and bacteria were washed from the plates with PB into a large beaker. The amoebae were collected and washed free from the bacteria by repeated washings with PB and low speed centrifugation at room temperature. Amoebae prepared in this way were used immediately to prepare appropriate enzyme fractions.

D. Germination of dormant spores

Throughout this study both physical and chemical methods were used to activate dormant spores. Heat-
activation was performed by suspending dormant spores in 5 ml of PB in 15 ml conical centrifuge tubes. The tubes were placed in a Braun model waterbath at 45°C for 30 minutes. When activating particularly large batches of spores (i.e., > 20 plates) it is necessary to vortex the suspension periodically to prevent excessive clumping. Following activation the spores were diluted to approximately 1.0 x 10^7 spores/ml in PB and placed in an appropriately sized Erlenmeyer flask with a loose-fitting cap to allow adequate aeration. The suspension was gently shaken at 23.5°C to allow germination to proceed (Cotter and Raper, 1966; 1968ab).

When small germination volumes were desired (i.e., < 5 ml) dilute spore preparations were dispensed into 10 x 1 cm test tubes containing small magnetic stirring bars. The tubes were placed in a Thermomix waterbath (Canlab) held at 23.5°C which contained a submersed (Tri-R) magnetic stirrer.

Chemical activation was performed using 20% dimethyl sulfoxide (DMSO) for 30 minutes (Cotter et al., 1976). Spores were suspended in 5-10 ml of 20% DMSO (v/v) in PB for 30 minutes at 23.5°C. Following activation, the chemicals were removed by filtration through a 1.2 um filter (Millipore) and the spores were washed twice in PB. The spores were diluted to a final germination concentration of 1.0 x 10^7 spores/ml. All spore
concentrations were determined using a hemocytometer.

Spore germination was monitored by phase contrast microscopy using a Zeiss Lena microscope at 320X. A drop of the spore suspension was placed on a microscope slide and a coverslip was carefully placed over the drop. The slide was mounted and the percent germination was determined by counting the first 200 objects and scoring them as: dormant spores, swollen spores, and/or emerged myxamoebae.

E. Oxygen uptake during spore germination

Cumulative oxygen uptake was measured as previously described (Garnish, 1978; Demsar, 1981) in a YSI model 53 oxygen monitor coupled to a Haake model 53 circulator. Activated spores were suspended in fully aerated PB at a concentration of 3-5 x 10^6 spores/ml. Cumulative oxygen uptake was either read directly from the oxygen monitor at 10 minute intervals, or from a Linear chart recorder coupled to the oxygen monitor. When the percent saturation fell below 60% the probes were removed and the spore suspensions were aerated back to 100%.
2. Radiolabelling of Germinating Spores

A: \(^3\)H-uracil labelling.

Dormant spores, activated either by a heat shock or chemicals, were germinated as described above. Continuous labelling with \(^3\)H-uracil was performed as previously described (Yagura and Iwabuchi, 1976; Cotter et al., 1979). The labelled precursor was used at a concentration of 4 \(\mu\)Ci/ml (sp. act. 25 Ci/mmol).

Streptomycin sulphate (Sigma Chem. Co.) was added to a final concentration of 25 \(\mu\)g/ml. Triplicate samples (0.2 ml aliquots) were removed every half hour during the five hour germination period and injected into 2 ml of 10% trichloroacetic acid (TCA) (v/v) and held on ice. Precipitated material was collected on 24 mm glass fiber filters (Whatman GF/A) and washed with six 2 ml aliquots of cold 5% TCA. The presence or absence of cold uracil (1 mmole) in the TCA wash did not affect the final counts. All filters were air-dried and the radioactivity was measured in a Beckman scintillation counter using 5 ml of Scintisol (Isolab) as the cocktail. All counts were normalized to a spore concentration of 1.0 \(\times\)\(10^7\) spores/ml.

When RNA was to be extracted, the spores were labelled with 3 to 10 \(\mu\)Ci of \(^3\)H-uracil. After incubation for specific time intervals, the spores were harvested by centrifugation and their RNA was immediately extracted.
B. Radiolabelling of spores with $^3$H-amino acids.

Methylation of certain RNA species during germination was studied using $^3$H-methyl methionine at 10 uCi/ml (sp. act. 50 Ci/mmol). Germinating spores were labelled for 4 hours in the presence of the isotope and streptomycin sulphate (25 ug/ml). Labelled spores were harvested by centrifugation and the RNA was immediately extracted.

$^3$H-leucine was used to determine the general protein synthetic rate as described by Demsar et al., (manuscript in preparation). Dormant spores were activated and germinated in a six ml volume in 30 ml corex tubes. The final concentration of the label was 5 uCi/ml (sp. act. 50 Ci/mmol). Streptomycin sulphate was added to a final concentration of 25 ug/ml. At regular intervals, triplicate 200 ul aliquots were removed to 2 ml of 10% TCA held at 90°C. After 20 minutes the tubes were removed to ice and the precipitates were collected on glass fiber discs (Whatman PG/A). Each sample was washed with three 5 ml volumes of cold 5% TCA, dried and counted as described above.

3. Irradiation procedures

All irradiation procedures were performed according to the methods of Densar and Corcer (1981). In all experiments a General Electric germicidal lamp (254 nm) was used at a distance of 20 cm. The lamp was calibrated
according to the procedure of Densar (1981) and delivered a fluence of \(1.7 \text{ J/m}^2/\text{sec}\). For most experiments involving approximately 20 plates of spores, the following procedure was used.

Mature, two-day-old spores (approx. \(10^8\)) were suspended in 60 ml of phosphate buffer and hand shaken for 2 minutes to remove clumps. Occasionally it was necessary to mildly heat the suspension at 45°C for 1 minute to remove larger aggregates. This treatment had no effect on the later germination of the spores. The suspension was divided into two 30 ml aliquots and added to two 25 cm glass petri dishes. The petri dishes were placed under the UV lamp at a distance of 20 cm and irradiated for a period of 2.5 minutes. This resulted in a total UV fluence of 250 J/m². During irradiation the plates were swirled in order to ensure complete and uniform exposure. Following irradiation the spores were collected at room temperature by centrifugation.

For larger preparations (i.e., > 50 plates), dormant spores were suspended in 100 ml of PB and shaken vigorously as described above. The suspension was poured into a pyrex glass baking dish (6" x 12") and irradiated for 2.5 minutes. During the irradiation the spores were swirled in order to insure a uniform dosage of ultraviolet light.
4. Preparation and use of RNA synthesis inhibitors.

Inhibitors were prepared in stock solutions, either in PB or using a combination of solvents. All inhibitors were rendered soluble and were not stored longer than a month before fresh stocks were made. All inhibitors were stored in the dark at 4°C, and direct lighting was avoided when possible, during experimentation.

Actinomycin D (P.L. Biochemicals Ltd.) and daunomycin (P.L. Biochemicals Ltd.) were prepared in PB as concentrations of 1.25 mg/ml and 500 μg/ml respectively.

Nogalamyins U12, 244, U15, 167, and U20, 661 (Upjohn Co.) were rendered soluble according to the methods of Ennis (1981a). Stock solutions of these inhibitors were prepared at concentrations of 30 mg/ml in 95% ethanol. The inhibitor solutions were diluted to 300 μg/ml and thus contained 0.95% ethanol in all experiments.

Thiolutin (Phiezer Co.) was prepared as described by Giri and Ennis (1976). A stock solution of 1 mg/ml was prepared in 10% dimethyl sulfoxide (DMSO).

Consequently control spores were suspended in 1.0% DMSO whenever this inhibitor was used.

The drug 4-nitroquinoline-1-oxide was prepared at a concentration of 0.1 mg/ml in PB. A brief heating at 45°C was required to fully solublize the inhibitor. This did not seem to affect the inhibitor.
S. Extraction of Ribonucleic Acids from Dormant and Germinating Spores.

A. Total cellular RNA.

The RNA of dormant, swollen or emerged amoebae was extracted by a modification of the procedure of Lovett and Leaver (1969). Spores were combined with 5 ml of 6% (w/v) p-aminosalicylic acid, sodium salt; 0.01 M tris (pH 7.5) and 1% diethyl oxydiformate (Eastman Chem. Co.) and broken by three passages through a French pressure cell at 20,000 psi at 4°C. The lysate from the final passage was collected in 3 ml of 80% phenol containing 0.01% 8-hydroxyquinoline. The suspension was shaken for a few seconds and 3 ml of chloroform/isoamyl alcohol (24:1) was added and the shaking continued for an additional minute. After centrifugation to separate the phases, the aqueous phase was re-extracted three more times using fresh phenol followed by the chloroform/isoamyl alcohol mixture. RNA was precipitated from the final aqueous phase by the addition of 2.5 volumes of ice cold 95% ethanol and a tenth volume of 1.0 M sodium acetate followed by storage at -20°C for at least 3 hours. The precipitate was collected by centrifugation at 23,000 x g for 20 minutes and the pellet was dried in a vacuum desiccator and redissolved in sterile distilled water. The A$_{260}$/A$_{280}$ ratio was routinely in excess of 2.0. A spore preparation of 2 x 10$^8$ spores yielded between 0.3 and 0.5 milligrams of total RNA.
B. Fractionation of poly(A)+ RNA.

The fractionation of poly(A)+ RNA was performed by a modification of the procedure of Giri and Ennis (1977). A 1.5 ml bed volume of oligo(dT) cellulose, type 7 (P.L. Biochemicals) was prepared and equilibrated with 0.01 M tris-HCl (equimolar tris base and tris-HCl), pH 7.5, 0.1 M NaCl, and 0.2% sodium dodecyl sulphate (SDS). The RNA was heat treated at 60 C for 4 minutes to remove any preformed aggregates. Following the heat treatment the RNA was made 0.01 M with respect to tris-HCl, pH 7.5 and 0.2% SDS. The sample was made 0.1 M with NaCl and rechromatographed as described above. This process was repeated once more and the poly(A)+ RNA was eluted. Duplicate samples were taken from the high salt wash and the eluted poly(A)+ RNA samples and counted in 5 ml of Scintisol (Isolab).

C. Extraction of transfer RNA.

Transfer RNA was extracted from dormant spores and vegetative amoebae by a modification of the procedure of Palatnik et al., (1977). Dormant spores from 1000 plates were harvested and washed extensively. Spores were suspended in 30 ml of TMS buffer (0.01 M tris-HCl, pH 7.5, 10 mM MgCl2, and 2 mM Na2S2O3) and broken by four passages through a French pressure cell at 20,000 psi.
After the final passage the lysate was collected in an equal volume of TMS saturated phenol and shaken vigorously in the cold for 1 hour. The resulting suspension was centrifuged at 23,000 x g for 30 minutes. The aqueous phase was withdrawn and a 1/50th volume of 4 M NaCl was added to give a final concentration of 0.1 M NaCl. The RNA was precipitated by the addition of 2.5 volumes of 95% ethanol and storage at -20°C overnight.

If the RNA was to be decylated, the pellet was resuspended in 1.8 M tris-ace	
tate, pH 8.0 and held at 35°C for 45 minutes. The preparation was then made 2.0 M with respect to NaCl and the large molecular weight RNA was removed by incubation at 0°C for two hours followed by centrifugation at 23,000 x g and the pellet discarded. The soluble RNA was collected by the addition of 2.5 volumes of ethanol and storage at -20°C for at least 3 hours. The pellet was collected by centrifugation, dried and resuspended in TMS buffer and dialysed to remove additional NaCl. Following dialysis the preparation was made 0.02 M NaCl in TMS and was passed over a DEAE-52 cellulose ion-exchange column (10 x 0.6 cm) previously equilibrated with 0.02 M NaCl/TMS. The column was then washed with 0.1 M NaCl/TMS until the A_260 of the column effluent was below 0.05. The tRNA was then eluted by the addition of 1.0 M NaCl/TMS and
those fractions with an $A_{254}$ greater than 1.0 were pooled and the RNA was precipitated by the addition of 95% ethanol and stored at -20°C overnight. The RNA was collected by centrifugation, dried and resuspended in sterile distilled water. The $A_{260}/A_{280}$ ratio was routinely in excess of 2.0 and 1 mg of RNA was assumed to have 24 OD units.

Transfer RNAs isolated in this way were between 70-80% pure as judged by 10% polyacrylamide gel electrophoresis, the major contaminants being 17s rRNA and 5.8s RNA.

6. Preparation of Aminoacyl-tRNA Synthetase.

All procedures for the extraction of aminoacyl-tRNA synthetases (synthetases) were performed at 4°C unless otherwise stated. The methods were identical to the procedure of Palatnick et al., (1977).

Vegetative cells were harvested by centrifugation and washed once in 10 mM tris/HCl, pH 7.5, 10 mM MgCl₂, 10% glycerol, 14 mM mercaptoethanol, and resuspended in about 20 ml of the same buffer. Cells were broken by two passages through a French pressure cell at 20,000 psi and centrifuged at 37,000 x g for 30 minutes. The supernatant was removed, transferred to an ultracentrifuge tube and the tube was spun at 105,000 x g for 1 hour. The supernatant was removed and a quarter
volume of 1.0 M KCl was added to give a final concentration of 0.2 M KCl. The extract was then passed onto a DEAE cellulose column (Whatman DE 52, 6 ml bed volume/gram of cells) which had previously been equilibrated at 4 C with 50 mM tris-HCl, pH 7.5, 0.2 M KCl, 10 mM MgCl₂, 10% glycerol and 14 mM mercaptoethanol. The column was then eluted directly with the same buffer. Fractions containing an A₂₈₀/A₂₆₀ of less than 1.2 were pooled. The pooled fractions were concentrated and free amino acids were removed by vacuum dialysis against two changes of synthetase buffer, the second dialysis being performed in 50% glycerol.

Synthetase preparations were stored in 50 ul aliquots until use. Prepared in this way, they were stable for at least six months. Preparations were stored at concentrations between 20-30 mg/ml of protein.

7. Extraction and Analysis of Nucleoside 5'-triphosphates.

Spores were suspended in 5 ml of 1.0 N perchloric acid and broken by three passages through a French pressure cell at 20,000 psi. The lysate was centrifuged at 7000 x g for 20 minutes and the pellet was reextracted with 2.5 ml of 0.5 N perchloric acid and centrifuged as before. After centrifugation the pellet was discarded and the soluble fractions were combined and the A₂₆₀/A₂₈₀ ratio was measured and recorded along with the final volume of the sample.
A 0.5 N solution of alamine 336 (General Mills LTD) was prepared in freon and washed once with an equal volume of 0.01 N NaOH and three times with an equal volume of 0.1 N NaCl. The freon/alamine preparation was filtered through Whatman No. 1 paper and stored in a dark bottle. Before use, the preparation was filtered to remove any particulate matter. The acid soluble fraction was neutralized by extraction with approximately 15 ml of the freon/alamine mixture. The solution was separated into an organic and aqueous phase by centrifugation at 1000 x g. The aqueous phase was withdrawn and the pH, volume, and A_{260}/A_{280} ratio was determined. The aqueous phase was concentrated by roto-evaporation to approximately 1 ml volume and clarified by centrifugation at 7000 x g. The final volume and A_{260} reading were determined and the preparation was stored at -20 °C until chromatographic procedures could be performed.

Nucleoside 5'-triphosphates were analyzed by HPLC on an Ultrasil®-Ax ion exchange column (Flow Laboratories Ltd.) coupled to a Beckman Gradient Liquid Chromatograph model 334. The chromatography program was as follows: chart speed 1.0 cm/minute; flow rate 1.0 ml/minute; %B was 5% for 1 minute and then increased isocratically to 100% in 30 minutes; B was 1.0 M phosphate buffer, pH 5.6. Samples were chromatographed at two separate sensitivities and the area under the peaks was determined for each sample. Standards were run under identical conditions in order to identify the various
peaks. A conversion factor from mm$^2$ to optical density units was kindly supplied by Dr. A.H. Warner. All calculations were normalized to O.D. units at 254 nm/10$^7$ spores.

8. **Electrophoresis of Ribonucleic Acids.**

RNA from dormant and germinating spores was fractionated on gels containing various concentrations of agarose and/or polyacrylamide. All gels were run in borosilicate glass tubes (0.4 cm x 10 cm) that had been silanized in dichloro-dimethylsilane (Sigma Chem. Co.). Electrophoresis was performed at room temperature in 0.04 M tris, 0.033 M sodium acetate, pH 7.2, 0.001 M sodium EDTA and 0.2% SDS (Bishop et al., 1967). Gels were prerun for 4 hours at 1 mA/gel and the samples were applied in 10% sucrose and run at room temperature for 4-5 hours at 4 mA/gel. Following electrophoresis the gels were fixed in cold 5% TCA for one hour and scanned in a Gilford model linear transport system coupled to a Gilford model 250 spectrophotometer.

Labelled RNA preparations were analysed on 1.7% agarose/acylamide gels prepared in the following way. A total of 0.23 g of agarose was dissolved in 16 ml of distilled water by bringing the solution to a boil and placing it in a 60 C waterbath. Acrylamide solution was prepared by mixing 3.4 ml of acrylamide/bis-acrylamide stock solution (30 g acrylamide 0.8 g bis-acrylamide) with 4.0 ml of 10x electrophoresis buffer, 15.9 ml of distilled water, and .02 ml of TEMED. This
mixture was also placed in a 60 C waterbath. Silanized borosilicate glass tubes were sealed at one end with three layers of Parafilm® and aligned vertically in a gel pouring rack (Biorad Ltd.). The solutions were mixed by placing 14 ml of the agarose solution into the acrylamide solution and stirring them vigorously with a glass rod. Ammonium persulphate was added (0.1 ml) and the tubes were filled to overflowing. After the gels had hardened the agarose was cut to a length of about 8.0 cm with a razor blade. Ten percent acrylamide gels were prepared according to the method of Laemmeli (1965).

Following electrophoresis, fixing, and scanning, the gels were sliced into 2 mm slices using a gel slicer (Biorad Ltd.). The slices were incubated in 0.5 ml of 90% NCS (Amersham) for 24 hours, and counted in 10 ml of Scintisol (Isolab). Alternately gel slices in 90% NCS were incubated at 50 C for 2 hours, cooled and counted as described above. In both cases a dark incubation for about 12 hours (overnight) was necessary to remove chemiluminescence. Agarose/acrylamide gels of 3% concentration were prepared according to the procedure of Knight and Van Etten (1976).

8. Poly(A)+ RNA Determinations.
A. Poly(U) filter binding assay.

Newly synthesized, labelled, poly(A) containing RNA was assayed by a modification of the procedure of Sheldon et al.
Glass fibre discs (Whatman GF/A) were prepared by adding a total of 0.3 ml of poly(U) solution (1 mg/ml in distilled water) to each filter. Filters were dried and irradiated for 2.5 minutes on each side at a distance of 22 cm with a 30 W germicidal lamp (UV). Filters were washed with distilled water, dried and stored at -20°C.

Labelled RNA preparations were suspended in 5 ml of binding buffer (0.12 M NaCl, 0.002 M EDTA, 0.5% SDS, 0.03 M tris-HCl, pH 7.5). Poly(U) impregnated filters were washed with 15 ml of binding buffer before use. The RNA samples were applied to the filter held on a support, and filtered slowly at approximately 0.5 ml/min. The filters were washed with an additional 15 ml of binding buffer followed by treatment with 10 ml of ice cold 10% TCA and 5 ml of cold 95% ethanol. The filters were dried and counted as described previously. Non-specific binding was corrected by filtering RNA preparations through filters not impregnated with poly(U) under identical conditions, and subtracting the amount of label bound from the values obtained for impregnated filters.

B. Poly(A-U) hybridization.

3H-poly(U) hybridization was performed as described by Bishop et al., (1974). The 3H-poly(U) was purchased from Amersham Co. (sp. act. 500 Ci/m mole). RNA concentrations of 0.5, 1.0, and 2.0 A260 units were hybridized in duplicate samples in a reaction volume of 0.25 ml containing 0.05 uCi
of $^3$H-poly(U) and 2x SSC buffer (1x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Prior to this an entire range of RNA concentrations were hybridized in order to establish saturation points for the various preparations of RNA. All RNA preparations were heated at 60°C for 5 minutes to remove any preformed aggregates. RNA preparations from amoebae saturated much faster than RNA from other germination stages and thus RNA concentrations of 0.05 and 0.1 A$_{260}$ units had to be used in the subsequent assays.

Reaction mixtures were incubated at 45°C for 12-15 hours and the reaction was terminated by the addition of 2.5 ml of cold 2x SSC buffer to each tube and 0.1 ml of 1 mg/ml of RNase (Sigma Chem. Co.). The tubes were incubated at 23°C for 15 minutes and the RNase resistant hybrids were precipitated by the addition of 0.7 ml of cold 30% TCA (w/v) and 0.025 ml of BSA (10 mg/ml) followed by one hour incubation at 5°C. The precipitate was collected by filtration on glass fiber discs (Whatman GF/A) and washed with 5% TCA followed by ether/alcohol (1:1). The filters were allowed to air dry and they were counted as previously described.

The amount of poly(A) hybridized was determined knowing the specific activity of the $^3$H-poly(U), the molecular weight of AMP and assuming 100% hybridization (i.e., moles of poly(U) hybridized is equal to the moles of poly(A) hybridized).
9. Determination Poly(A)+ RNA.

The decay of poly(A)+ RNA was assayed in two different ways. Spores, germinating in the presence of $^3$H-uracil, were irradiated 2.25 hours into germination and the RNA was extracted at various times after irradiation. The amount of label in the poly(A)+ RNA fraction was determined using the poly(U) filter binding assay (see section 8a Materials and Methods).

Alternately, in order that we could access the decay of protein synthesis following irradiation, spores were pulse labelled following irradiation. Spores were allowed to germinate until greater than 95% of the spores were swollen (approximately 2.25 hours into germination). At this time an aliquot of 0.5 ml was removed and pulse labelled for five minutes with 20 uCi/ml of $^3$H-leucine (50 Ci/m mole). At 2.5 minutes into the above pulse period the unlabelled batch culture was irradiated as described above for 2.5 minutes and an aliquot of 0.5 ml was immediately removed and pulse labelled for five minutes in an identical manner. Five minute pulses were made at various intervals following irradiation. Following each pulse period duplicate 0.2 ml aliquot were pipetted into hot (90 C) TCA and incubated for 20 minutes. Precipitates were collected and counted as previously described.
Aminoacylation Assays for tRNAs.

A. Charge ratio determination.

Charge ratio determinations were done according to the procedure of Brock (1966). Two hundred plates of spores were harvested and the tRNA was extracted as previously outlined, except the deacylation step was omitted. The RNA preparation was dissolved in sterile distilled water and divided into two 1 ml samples. To one sample 0.1 ml of 0.1 M periodate was added and the sample incubated at 25°C for 50 minutes in the dark. To the untreated sample an equal amount of sterile distilled water was added. Excess periodate was destroyed by adding a three fold excess concentration of glucose to both samples and incubating them for 5 minutes at room temperature. The samples were dialysed overnight against sterile distilled water at 4°C.

Following dialysis both samples were made 0.1 M with tris-HCl (pH 8.8) and were deacylated at 37°C for two hours to remove amino acids. The preparations were then made 0.1 M with respect to NaCl and the RNA was collected by ethanol precipitation.

The RNA preparations were charged using a ¹⁴C-amino acid mixture (New England Nuclear, 0.05 mCi), and individual amino acids as will be described.
B. Charging of dormant spore and amoeba tRNA.

Aminoacylation assays were carried out according to the procedures described by Palatnick et al., (1977). Final reaction mixtures of 0.5 ml contained 0.5–1.0 A_{260} units of spore or vegetative tRNA, 200–300 μg of the enzyme preparation, appropriate amounts of ATP and MgCl₂, 1.0 μCi of ¹⁴C-amino acid or 20 μCi of ³H-amino acid, 50 μM concentration of the other 19 unlabelled amino acids and 100 mM sodium cacodylate, pH 7.5. Reaction times as well as optimal ATP and MgCl₂ concentrations were obtained directly from the work of Palatnick et al., (1977). All reactions were terminated by the addition of 0.5 ml of ice cold 10% TCA. The tubes were allowed to stand on ice for twenty minutes and the precipitates were collected by vacuum filtration on glass fiber filters (Whatman GF/A). The filters were washed with approximately 10 ml of ice cold 5% TCA. The filters were dried under a heat lamp and counted in 5 ml of Scintisol (Isolab).
RESULTS

Part I. Ribonucleic Acids in Dormant Spores

A wide variety of techniques are available for the extraction and isolation of RNA from fungal spores. To extract RNA from dormant spores of Dictyostelium discoideum several procedures were utilized. The method of Giri and Ennis (1977) and the ammonium carbonate method of Freer et al. (1977) produced either limited or irreproducible results. One of the most widely used procedures is the one described by Lovett and Leaver (1969). The original procedure of Lovett and Leaver (1969) called for extraction of RNA at elevated temperatures. However, substantially more poly(A)+ RNA was extracted when using the modification described by Timberlake (1980). This involved the addition of an equal volume of chloroform/isoamyl alcohol (24:1) to the phenol extract before separating the phases. Following centrifugation the aqueous phase is reextracted twice with phenol and the chloroform/isoamyl alcohol mixture. Extractions performed in this way were largely free of contaminating protein or DNA. Additionally, this procedure was easily performed when spores were broken in a French pressure cell, while procedures that employed
Fig. 3.

Electrophoretic patterns of large rRNAs from myoblasts, slime mold spores, and *Escherichia coli*. RNA was extracted from slime mold spores according to the procedure outlined in the Materials and Methods. *E. coli* rRNA was purchased from Miles Laboratory Ltd. Myoblast RNA was kindly supplied by the laboratory of Dr. Michael Dufresne. The RNAs were electrophoresed on 1.7% agarose/acrylamide gels.
sodium doedecyl sulfate (SDS) in the extraction buffer produced considerable frothing, and made extractions messy.

A. Separation and identification of spore RNA

Dormant spores contained between 0.3 to 0.5 mg of RNA per $10^8$ spores. Figure 3 shows the electrophoresis of total RNA extracted from two-day-old spores. A total of 0.2 A$_{260}$ units were electrophoresed on 1.7% agarose/acylamide gels. RNA from E. coli and myoblasts were used initially as 23S, 16S and 28S, 18S, markers. This preformed 25S and 17S rRNA comprised the majority of the extractable spore RNA. In investigations where labelling experiments were performed, 1.7% gels were used in order to detect the presence of any high molecular weight precursors. The 3% gels were used when only structural information was required. For all experiments in which the extraction of RNA was performed, the presence of intact rRNA was used as an indicator of a lack of nuclease activity during extraction. Extractions from germinating spores (i.e. swollen) occasionally yielded substantially degraded rRNA. Whether this was due to a transient increase in ribonuclease activity is unknown.

The electrophoretic detection of low molecular weight ribosomal associated RNA is shown in Figure 4.
Fig. 4.

Electrophoretic profile of low molecular weight RNAs from the dormant spores of the cellular slime mold *Dictyostelium discoideum* resolved on 10% polyacrylamide gels. RNA was extracted from dormant spores and electrophoresis was carried out as described in the Materials and Methods. Commercially prepared yeast tRNA was used to mark the 4s peak.
In all experiments 10% polyacrylamide gels containing SDS were used together with a 1.7% stacking gel. The stacking gel was necessary to remove all large molecular weight RNA species, which may have interfered with the electrophoresis of the low molecular weight RNA. The position of the 4s RNA was determined using yeast tRNA as a standard.

Although the positions of the 5.0s and 5.8s RNA's were not marked, this electrophoretic pattern appears to be common for a number of other fungi (Van Etten, 1976). These RNA species were not resolved unless the RNA preparation was heat treated at 60 C for 5 minutes in order to dissociate any preformed aggregates.

B. Isolation and quantitative analysis of preformed poly(A)+ RNA from the dormant spore.

The presence of a preformed mRNA population in the dormant spores of the cellular slime mold Dictyostelium discoideum has been well documented (Yagura and Iwabuchi, 1976; Giff and Ennis, 1977; 1978; Dowbenko and Ennis, 1980; MacLeod et al., 1980). Thus it was not within the scope of this project to extensively analyze this mRNA component.

In order to estimate the amount of poly(A)+ RNA in the spore, total cellular RNA was hybridized to 3H-poly uridylic acid (poly U). The 3H-poly(U) hybridizes to
the 3' polyadenylic acid tails of the poly(A)+ RNA to form RNase resistant hybrids. Following a suitable period of hybridization and treatment with ribonuclease, the radioactive hybrids can be precipitated on glass fibre filters and the radioactivity determined. By knowing: the length (i.e., number of adenylic acid residues) of the poly(A) region; the specific activity of the 3H-poly(U); the size of the coding region (length of an average size transcript); and assuming total hybridization, the amount of poly(A)+RNA in the dormant spore can be determined from the relationship:

\[
\frac{\text{amount of poly(A)/amount of RNA}}{\text{amount of poly(A)+RNA/amount of RNA}} = \frac{\text{average mw of poly(A)}}{\text{average mw of poly(A)+RNA}}
\]

Since Dicystostelium has a 3' poly(A) tail length of 100 nucleotides and an average mRNA length of 1200 bases; this equation simplifies to:

\[
\frac{\text{amt of poly(A)+RNA/amt of RNA}}{\text{(amt of poly(A)/(amt of RNA)(390,000) \times (329)(100)}}
\]

assuming an average nucleotide molecular weight of 329.

Table 1 gives an example of five such calculations made from two separate preparations of total cellular RNA. Spores contain approximately 3 µg of poly(A)+ RNA per
<table>
<thead>
<tr>
<th>2.73</th>
<th>0.23</th>
<th>5.87</th>
<th>7.35</th>
<th>39.13</th>
<th>29.64</th>
<th>0.04</th>
<th>6.41</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.95</td>
<td>0.25</td>
<td>3.17</td>
<td>3.17</td>
<td>15.84</td>
<td>3.17</td>
<td>2.45</td>
<td>9.24</td>
</tr>
<tr>
<td>2.49</td>
<td>11.2</td>
<td>6.25</td>
<td>5.25</td>
<td>26.01</td>
<td>26.01</td>
<td>0.04</td>
<td>3.96</td>
</tr>
<tr>
<td>2.95</td>
<td>0.25</td>
<td>3.17</td>
<td>3.17</td>
<td>15.68</td>
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<td>2.80</td>
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<td>7.00</td>
<td>7.00</td>
<td>1.55</td>
<td>1.55</td>
<td>2.73</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Table 1. Quantitation of the polypeptide acid containing RHN in the document specified by the cell extract with dual electrophoresis.
Fig. 5.
Saturation kinetics of $^3$H-poly(U) hybridization performed using dormant spore RNA. Increasing concentrations of spore RNA were added to a constant amount of $^3$H-poly(U) and hybridization was carried out as described in the Materials and Methods.
milligram of total RNA. Previous calculations by the laboratory of Dr. Richard Firtel place this value at 4 ug (Macleod et al., 1980) however slightly different hybridization procedures were employed.

Prior to experiments of this sort the saturation kinetics for the amount of $^3$H-poly(U) had to be determined for the various RNA preparations used in this section and in section III E. Figure 5 shows a hybridization to saturation experiment with increasing amounts of total cellular RNA. This experiment insures that the amount of $^3$H-poly(U) does not limit the reaction.

Since poly(A)+ RNA comprises approximately 85% of the total mRNA population in Dictyostelium discoideum, this method provides a good estimate of the amount of mRNA in the dormant spore.

C. Extraction of transfer RNA from dormant spores.

Several procedures and variations were attempted in order to purify tRNAs from dormant spores. The method of Palatnik et al., (1977) for extracting tRNA from developing cells of D. discoideum yielded tRNA preparations heavily contaminated by rRNA. Hence a high salt precipitation step was added in order to remove some of the contaminating rRNA. Figure 6 shows an electrophoretic profile of a typical purified tRNA preparation. From peak height determinations a greater than 70% purity can be
Fig. 6.

Electrophoresis of purified dormant spore tRNA.
Transfer RNAs were extracted from dormant spores as outlined in the Materials and Methods. Purified preparations were routinely checked on 3-10% "stacked" gels. The preparation in this figure is approximately 70% pure from peak height determinations.
obtained, which was substantially greater than the 50-60% obtained by Palatnik et al., (1977). The contaminating components appear to be large tRNA and 5s RNA.

D. Aminoacylation of dormant spores and vegetative amoebae.

Dormant spores and vegetative amoebae tRNA were charged with homologous vegetative synthetases as outlined in the Materials and Methods section. Table 2 demonstrates that, of the twelve amino acids tested, dormant spores contained tRNA that could be aminoacylated (charged) to some extent. While the levels of aminoacylation were not optimal compared to previously published data, the ratio of vegetative tRNA aminoacylation to dormant spore tRNA aminoacylation only differed greatly in the case of the amino acid valine. The lack of optimal charging levels may be directly attributable to the synthetase preparation, which varied greatly in activity from one extract to the next. Additionally, synthetases were prepared from amoebae grown in conjunction with bacteria and thus high enough concentrations of synthetase preparations were difficult to obtain.

Finally, it appeared that some synthetase extracts may have been contaminated by ribonucleases since occasionally no charging of tRNA took place whatsoever. Kinetic studies with these preparations demonstrated that reaction plateaus could not be maintained. At no time, however,
Table 2. Acceptance of amino acids by spores and amoebae
transfer RNA preparations<sup>a</sup>.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>pmol amino acid accepted/A&lt;sub&gt;260&lt;/sub&gt; unit of Spore tRNA</th>
<th>pmol amino acid accepted/A&lt;sub&gt;260&lt;/sub&gt; unit of Amoeba tRNA</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>26.9</td>
<td>21.3</td>
<td>0.79</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>22.4</td>
<td>11.1</td>
<td>0.51</td>
</tr>
<tr>
<td>Proline</td>
<td>37.5</td>
<td>24.9</td>
<td>0.66</td>
</tr>
<tr>
<td>Serine</td>
<td>22.0</td>
<td>27.2</td>
<td>1.20</td>
</tr>
<tr>
<td>Threonine</td>
<td>20.7</td>
<td>25.4</td>
<td>1.24</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>10.3</td>
<td>16.1</td>
<td>1.56</td>
</tr>
<tr>
<td>Histidine</td>
<td>16.4</td>
<td>18.2</td>
<td>1.11</td>
</tr>
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<td>Glutamic acid</td>
<td>18.2</td>
<td>17.4</td>
<td>0.95</td>
</tr>
<tr>
<td>Alanine</td>
<td>15.4</td>
<td>9.8</td>
<td>0.63</td>
</tr>
<tr>
<td>Valine</td>
<td>11.6</td>
<td>28.5</td>
<td>2.45</td>
</tr>
<tr>
<td>Lysine</td>
<td>21.3</td>
<td>31.5</td>
<td>1.48</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>21.6</td>
<td>29.1</td>
<td>1.35</td>
</tr>
</tbody>
</table>

<sup>a</sup> All preparations were charged with homologous synthetase prepared from vegetative cells. Reactions were carried out as described in the Materials and Methods.
did the synthetase preparation have differential effects on spore or vegetative tRNA. Similar problems in synthetase preparations were noted by Palatnik et al. (1977) who observed the presence of some nondialysable factor which inhibited both the rate and extent of the aminoacylation reaction. Further purification of the synthetase extract on Sephadex G150 did not increase the activity. Regardless of this problem it can be concluded that dormant spores possess tRNAs that are functional to the extent that they can be charged with an amino acid. This however, does not reflect on their ability to bind to ribosomes and participate in peptide chain elongation.

E. Charge ratio determinations.

Early experiments on *Dictyostelium discoideum* spore germination have demonstrated that a mixture of phenylalanine, methionine, and tryptophan will activate dormant spores and induce synchronous germination (Cotter and Raper, 1966). Bacon and Sussman (1973) demonstrated that these three amino acids were absent from the free amino acid pool in the dormant spores. Thus the absence of these three amino acids may serve to initiate and maintain dormancy by blocking macromolecular synthesis via a stringent response type mechanism. It is therefore conceivable that an initial step in germination
may be a derepression of the stringent response as pool levels of these amino acids are restored as well, as the charge ratios (i.e., percentage of the tRNA charged) of their cognate tRNAs. This process may prime or turn on cellular protein synthesis.

Evidence of such a mechanism would include the presence of tRNA\textsubscript{phe}, and/or tRNA\textsubscript{met}, and/or tRNA\textsubscript{trp} in an uncharged or deacylated state in the dormant spore. Simple charging experiments are not sufficient to provide support for this idea since during the isolation procedure the tRNA are routinely stripped of their amino acids. Section 11 in the Materials and Methods outlines the procedure for the oxidation of uncharged tRNA with periodate. The percentage of the difference between the charging levels (cpm/ug of tRNA) of unoxidized tRNA and oxidized tRNA prepared from the same batch of spores, gives the percentage of the tRNA that are charged for a particular amino acid.

Table 3 gives an estimate of the overall charge ratio in dormant spores and vegetative amoebae. This was done by charging the tRNA\textsuperscript{+} with \textsuperscript{14}C-labelled protein hydrolysate. Each 0.5 ml reaction mixture contained 10 mM ATP, 10 mM MgCl\textsubscript{2}, 0.5 A\textsubscript{260} unit of tRNA, 200 ug of enzyme, 0.1 M \textit{tris}-HCl, pH 7.5, and 1 uc\textsubscript{1} of \textsuperscript{14}C-algal protein hydrolysate. The reaction was terminated and counted as described in the Materials and Methods section.
Table 3. Comparison of charge ratios in dormant spores and vegetative amoebae of *Dictyostelium discoideum*

<table>
<thead>
<tr>
<th>tRNA Preparation</th>
<th>Activity (CPM/ug tRNA)</th>
<th>Percent Charged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control spore tRNA</td>
<td>1939.8</td>
<td>20.8</td>
</tr>
<tr>
<td>Oxidized spore tRNA</td>
<td>402.9</td>
<td></td>
</tr>
<tr>
<td>Control vegetative tRNA</td>
<td>1670.1</td>
<td>45.8</td>
</tr>
<tr>
<td>Oxidized vegetative tRNA</td>
<td>764.5</td>
<td></td>
</tr>
</tbody>
</table>
Dormant spores appear to have low charge ratios when compared to the vegetative amoebae. In contrast the zoospores of *Blastocladiella emersonii* have a high charge ratio of 87% when compared to the vegetative plant, 45% (Schmoyer and Lovett, 1967). The fungus spore *Botryodiplodia theobromae* also has a high charge ratio (Van Etten et al., 1969). Thus the low charge ratio in *D. discoideum* spores appears to be unique among a few of the fungal spores examined. This low charge ratio is consistent with the idea that uncharged tRNA may play some role in the repression of cellular protein synthesis. However specific amino acids would need to be examined.

Table 4 presents the charge ratio for the amino acids leucine and the 'germination requiring' amino acids, phenylalanine, methionine and tryptophan. The amino acid leucine is present in the free amino acid pool of the dormant spore and the majority of its cognate tRNA appear to be charged. Methionine and phenylalanine do not occur in the soluble amino acid pool according to Bacon and Sussman (1973). Recently, Ennis (1981b) using an amino acid analyser, demonstrated that in fact phenylalanine and methionine do occur in the free amino acid pool. Table 4 demonstrates charged tRNAs for phenylalanine and methionine do occur at similar percentages of 39.7% and 11.0% respectively.
Table 4. Charge ratio determinations for specific amino acids in dormant spores and vegetative amoebae of Dictyostelium discoideum.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>tRNA preparation</th>
<th>Activity (CPM/ug tRNA)</th>
<th>Charge Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>con-veg-tRNA</td>
<td>6886.3</td>
<td>54.8</td>
</tr>
<tr>
<td></td>
<td>oxid-veg-tRNA</td>
<td>3770.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>con-spo-tRNA</td>
<td>6906.0</td>
<td>95.2</td>
</tr>
<tr>
<td></td>
<td>oxid-spo-tRNA</td>
<td>6576.4</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>con-veg-tRNA</td>
<td>3073.8</td>
<td>25.9</td>
</tr>
<tr>
<td></td>
<td>oxid-veg-tRNA</td>
<td>798.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>con-spo-tRNA</td>
<td>3107.4</td>
<td>39.7</td>
</tr>
<tr>
<td></td>
<td>oxid-spo-tRNA</td>
<td>1232.3</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>con-veg-tRNA</td>
<td>707.7</td>
<td>51.0</td>
</tr>
<tr>
<td></td>
<td>oxid-veg-tRNA</td>
<td>361.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>con-spo-tRNA</td>
<td>301.1</td>
<td>41.0</td>
</tr>
<tr>
<td></td>
<td>oxid-spo-tRNA</td>
<td>123.2</td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>con-veg-tRNA</td>
<td>1091.1</td>
<td>53.0</td>
</tr>
<tr>
<td></td>
<td>oxid-veg-tRNA</td>
<td>578.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>con-spo-tRNA</td>
<td>382.8</td>
<td>34.1</td>
</tr>
<tr>
<td></td>
<td>oxid-spo-tRNA</td>
<td>130.5</td>
<td></td>
</tr>
</tbody>
</table>

*a Abbreviations: control (con), oxidized (oxid), vegetative (veg), spore (spo)
Ennis (1981b) was not able to detect the presence of free tryptophan using an amino acid analyser. Experiments in our lab using thin-layer chromatography support this result (data not shown). However the results in Table 4 clearly show that 34.1% of the tryptophan tRNAs are charged in the dormant spore. The findings that all three of the 'germination promoting' amino acids are precharged on their respective tRNAs, sheds doubt on the role of unacylated tRNA in maintaining dormancy. However the finding that the overall charge ratio of amoebae is twice that of the dormant spores, suggests that during germination, the charge ratio may increase. Some preliminary results from our lab suggest that proteins may be metabolized during germination in order to liberate free amino acids for protein synthesis (D. Jackson, unpublished report).

Part II. RNA Synthesis During D. discoideum Spore Germination.

A. Incorporation of $^3$H-uracil during spore germination.

Germination in many fungal spores is characterized by rapid increases in the rates of RNA and protein synthesis when dormant spores are placed under germination promoting conditions. In general, dormant spores of many fungi are permeable to precursors of macromolecular
Fig. 7.

Incorporation of $^3$H-uracil during spore germination of *Dictyostelium discoideum*. Heat-activated spores were germinated in the presence of $^3$H-uracil and the incorporation of the radioisotope into a TCA insoluble fraction was monitored as outlined in the Materials and Methods.

Symbols:

- ($\Delta$) incorporation of $^3$H-uracil per $10^7$ spores
- (O) percent spore swelling
- (●) percent myxamoebae emergence
synthesis. The incorporation of these radio-labelled precursors into a TCA precipitable fraction is at least a preliminary indicator of macromolecular synthesis.

Heat-activated spores of *H. discoides* were continuously labelled with $^3$H-uracil during germination. The increase in labelled trichloroacetic acid insoluble material is shown in Figure 7. As previously reported by other researchers, RNA synthesis initiates during early swelling and increases dramatically throughout emergence (Yagura and Iwabuchi, 1976; Giri and Ennis, 1977; 1978; Tisa, 1979; Densar, 1981).

B. Pulse-Labelling of high molecular weight ribosomal RNA during spore germination.

Germinating spores were pulse-labelled at discreet stage specific intervals from 0-1 hour (post-activation lag stage); 1-2 hours (swelling stage); 2-3 hours (late swelling-early emergence); and 3-4 hours (emergence stage). The RNA was immediately extracted from the germinating spores as described in the Materials and Methods section. Following electrophoresis, the gels were scanned, sliced and counted. Figure 8 shows the pattern of incorporation into the large rRNA. While there may be some synthesis during the 0-1 hour pulse, the majority of the 25s and 17s rRNA is synthesized during the other pulse periods. The
Fig. 8.

Pulse labelling of high molecular weight rRNA during spore germination. The following figures depict the incorporation of $^3$H-uracil into high molecular weight rRNA during spore germination. Germinating spores were pulse labelled with $^3$H-uracil and RNA was immediately extracted and profiled on 1.7% agarose/acrylamide gels. A total of 0.2 A$_{260}$ units of RNA were added per gel. Following electrophoresis the gels were fixed, scanned, sliced and counted as described in the Materials and Methods. The solid line indicates the extinction of 260 nm.

Symbols:

(●) incorporation of $^3$H-uracil
greatest amount of rRNA synthesis occurs during the 3-4 hour pulse. This pulse time corresponds to the emergence of the myxamoebae. At the end of the last pulse time there was 85% emergence.

This data supports the results of Giri and Ennis (1978) who, using continuous labelling, found that the majority of the rRNA was synthesized late in germination. Since D. discoideum synthesizes rRNA at all stages of growth and development (see Loomis, 1975; also Introduction to this thesis) it appears doubtful that rRNA synthesis is an obligatory step in spore germination.

C. Pulse-labelling of low molecular weight RNA during germination

The pulse-labelled RNA preparations described above were also used to investigate the timing of low molecular weight RNA synthesis. Previous studies employing continuous labelling and sucrose density gradients did not resolve the low molecular weight RNAs. Instead, the 5.0, 5.8 and 4s RNAs sedimented together as one heterogeneous peak (Giri and Ennis, 1978). From the data in Figure 9, it can be seen that low molecular weight RNA synthesis (5.0s and 5.8s) initiated at about the same time as 25s and 17s RNA. Twice the number of optical density units had to be added to the gel in order to visualize the peaks in the gel scanner. Thus, actual
Fig. 9.

Pulse labelling of low molecular weight RNA. The following figures diagram the incorporation of $^3$H-uracil during 1 hour pulse periods during the germination of D. discoideum spores. The same RNA preparations which were used to generate the data in Fig. 7. were used here. A total of 0.4 AU units was added per gel. The 10% gels were electrophoresed, fixed, scanned, sliced, and counted as described in the Materials and Methods. The solid line indicates the extinction at 260 nm.

Symbols:

- (●) incorporation of $^3$H-uracil
counts per minute (CPM) would be half the number of counts shown in these figures.

Previous data from Giri and Ennis (1978) show that tRNA is made during the first hour of germination. Using the finer resolving capacity of polyacrylamide gels, Figure 9 clearly shows that tRNA synthesis (4s RNA) initiated during late swelling-early emergence, but that the majority of the tRNA was synthesized late in germination during the emergence stage of germination.

D. Labelling of poly(A) + RNA during spore germination.

The same labelled RNA preparations which were used to generate Figures 8 and 9 were analysed by poly(U) filter hybridization. Glass fiber filters were impregnated with polyuridylic acid residues, as outlined in the Materials and Methods. The amount of label bound to a poly(U) impregnated filter was adjusted for background binding by subtracting the amount of label bound to an unimpregnated glass fiber filter. The amount of label bound in each RNA preparation from each pulse period, and the total percent incorporation into a poly(A) + RNA fraction, is shown in Table 5.

During the post-activation lag stage, little poly(A) + RNA is synthesized. As swelling ensues, approximately 3% of the $^3$H-uracil accumulates as poly(A) + RNA. The largest amount of poly(A) + RNA synthesized
Table 5. Incorporation of $^3$H-uracil into poly(A)$^+$ RNA during pulse labelling of germinating Dictyostelium discoideum spores$^a$

<table>
<thead>
<tr>
<th>Pulse Time</th>
<th>CPM added</th>
<th>CPM bound$^b$</th>
<th>% Poly(A)$^+$ RNA synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1 hr</td>
<td>4316.0</td>
<td>7.0</td>
<td>0.1</td>
</tr>
<tr>
<td>1-2 hr</td>
<td>9812.0</td>
<td>292.9</td>
<td>2.9</td>
</tr>
<tr>
<td>2-3 hr</td>
<td>24,220.4</td>
<td>2527.9</td>
<td>10.4</td>
</tr>
<tr>
<td>3-4</td>
<td>29,371.6</td>
<td>1777.9</td>
<td>6.0</td>
</tr>
</tbody>
</table>

$^a$ The level of poly(A)$^+$ RNA was assayed using a poly(U) impregnated filter assay as outlined in the Materials and Methods.

$^b$ The number of counts bound was determined by subtracting the amount of label bound to an unimpregnated filter from the number of counts bound to a poly(U) impregnated filter. Each value is the average of three such runs.
occurred during the transition from late swelling to early emergence. (2-3 hour pulse) where 10% of the labelled RNA appeared to be poly(A) containing. These data give at least a minimal approximation of the amount of messenger RNA synthesized during germination. Work by Blijdorp and Lodish (1980a) have shown that in the vegetative cell, whole cytoplasmic poly(A)+ RNA has a similar complexity as the polysomal poly(A)+ RNA and total mRNA.

In stark contrast to this work is the data of Giri and Ennis (1978). Their results show that throughout germination, spores synthesize between 40-50% of all their RNA as poly(A) containing RNA. Their results were arrived at by continuously labelling DMSO activated spores with $^3$H-uracil and fractioning the RNA on oligo-dT cellulose columns. Attempts to repeat this work using DMSO activation and oligo-dT cellulose chromatography failed; Spores which were activated by DMSO and heat synthesized similar levels of poly(A)+ RNA when the preparations were fractionated on oligo-dT cellulose (data not shown).

The results of Giri and Ennis are markedly different from the results reported for a number of other spores, *Rhizopus stolonifer* (Roheim et al., 1974); *Botryodiplodia theobromae* (Knight and Van Etten, 1975a) and recently *Allomyces macrogynus* (Smith and Burke, 1980). It also is doubtful that this difference is due to differences in the germination of strain B, used by Giri and Ennis, and the wildtype strain NC4, used in this study. The
discrepancies between these two sets of data await further verification.

E. Methylation of RNA during germination

The methylation of transfer RNA and ribosomal RNA has been reported for a number of fungi including *Botryodiplodia theobromae* (Knight and Van Etten, 1976a) and *Rhizopus stolonifer* (Roheim et al., 1974). In at least two cases it has been reported that preexisting tRNAs in dormant spores may be under methylated (Wong et al., 1971; Knight and Van Etten, 1976b).

In order to ascertain whether preexisting tRNAs in dormant spores of *D. discoideum* require methylation, germinating spores were pulse-labelled with $^3$H-methylmethionine.

Initially, germinating spores were pulse labelled at one hour intervals and the RNA was extracted and electrophoresed on 10% polyacrylamide gels. Analysis of the 4s peak failed to show any incorporation (data not shown). The isotopé concentration was doubled, and the experiment repeated, but without success. Finally, using a high isotope concentration, germinating spores were labelled continuously for four hours (85% emergence), the RNA was extracted and analysed on 10% gels. Figure 10 demonstrates that no incorporation of any form took place.
Fig. 10.

Incorporation of $^3$H-methyl-methionine during D. discoideum spore germination. Germinating spores were continuously labelled in the presence of $^3$H-methyl-methionine for four hours. The RNA was extracted and profiled on 1.7% and 10% gels as outlined in the Materials and Methods. (A), incorporation into large rRNA; (B), incorporation into low molecular weight RNA. The solid line indicates the extinction of 260 nm.

Symbols:

($\bullet$) incorporation of $^3$H-methyl-methionine
An obvious explanation for this data is that methionine uptake is somehow inhibited. However it has been demonstrated in our laboratory and others, that radiolabelled methionine can be incorporated into protein with relatively short labelling periods during spore germination (Dowbenko and Ennis, 1980; Tisa, 1979). Additionally, transfer RNAs of *Dictyostelium* contain methylated bases (Dingermann et al., 1977).

Another explanation is that the methylation of newly synthesized tRNA may occur later during vegetative growth and not during the measured time course of germination. Since spores are germinated under starvation conditions, methionine may be preferentially utilized in protein synthesis due to depleted amino acid pools, and may not be available for methylation. One way of testing this idea would be to germinate spores under high nutrient conditions and observe for methylation. Current axenic strains of *Dictyostelium* do not germinate well, and work is underway to enrich for a spontaneously germinating axenic strain.

These data however are consistent with the idea that the tRNA in the dormant spore are fully functional and do not require methylation, unlike the tRNAs in some other fungal species.
Inhibitors of ribonucleic acid synthesis and their effects on spore germination in D. discoideum.

At the outset of this investigation, the literature contained only one study on the effects of various RNA synthesis inhibitors on spore germination in the cellular slime mold. Giri and Ennis (1977), studied the effects of four inhibitors and were unable to conclude whether or not there was a requirement for RNA synthesis during germination. Recently, it was reported by Ennis (1981a) that the anthracycline antibiotic, nogalamycin, blocked RNA synthesis by 88% and prevented myxamoebae emergence at concentrations of 300 mg/ml.

Unpublished observations by Cotter showed the following: (1) a number of the classical RNA synthesis inhibitors which are generally used at high concentrations had effects on spore metabolism unrelated to RNA synthesis and (2) other inhibitors exist which are active at low concentration and block only the myxamoebae emergence stage of germination when suppressing RNA synthesis. Recent work by Demsar (1981) demonstrated that ultraviolet light (UV) had a drastic effect on myxamoebae emergence and inhibited the incorporation of ³H-uracil. Thus a survey of RNA synthesis inhibitors seemed necessary.

An ideal inhibitor for use in studies during spore
Table 6. The effects of various DNA synthesis inhibitors on *Dictyostelium discoideum* spore germination and the incorporation of ³H-uracil.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Percentage of spores</th>
<th></th>
<th>Percent Inhibition of Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dormant</td>
<td>Swollen</td>
<td>Emerged</td>
</tr>
<tr>
<td>Actinomycin D (125 µg/ml)</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Daunomycin (250 µg/ml)</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Daunomycin &amp; Actinomycin D</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thiolutin (100 µg/ml)</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nogalomycin U20,661 (300 µg/ml)</td>
<td>50</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>Nogalomycin U12,241 (300 µg/ml)</td>
<td>15</td>
<td>85</td>
<td>0</td>
</tr>
<tr>
<td>Nogalomycin U15,167 (300 µg/ml)</td>
<td>30</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>Ultraviolet light (250 J/m²)</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>4-nitroquinoline 1-oxide (5 µg/ml)</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
germination would have the following characteristics:

(1) inhibit RNA synthesis completely, (2) have limited adverse effects on other aspects of spore metabolism, (3) permeate the spore at any stage in germination, (4) be readily soluble in the germination medium (10 mM phosphate buffer, pH 6.5), and (5) be effective at low concentrations. With the above criteria in mind, a number of inhibitors were examined for their effects on germination kinetics and RNA synthesis. RNA synthesis was monitored by the incorporation of $^3$H-uracil into a cold TCA insoluble fraction as outlined in the Materials and Methods. Table 6 presents results obtained with nine inhibitors.

**Actinomycin D.**

Actinomycin D is a chromopeptide, which binds to guanosine residues on the DNA template, and has been used as an inhibitor of RNA synthesis in studies involving *Dictyostelium* (for review see Loomis, 1975). Table 6 shows a 30% inhibition of RNA synthesis and no effect on spore germination. These results are in accordance with earlier observations (Cotter and Raper, 1966; Bacon and Sussman, 1973; Giri and Ennis, 1976).

**Daunomycin**

Daunomycin has been used extensively together with
actinomycin D for studies on RNA synthesis in the vegetative cell of *Dictyostelium discoideum* (Margolskee and Lodish, 1980ab). It is an anthracycline antibiotic, and probably binds with DNA phosphate groups (Inagaki and Kageyama, 1970). Together with actinomycin D there was a 94% inhibition of RNA synthesis and a complete blockage of spore germination. On its own daunomycin produced virtually the same effect. Under phase-contrast microscopy, daunomycin treated spores possess vacuoles and appear almost phase dark. A condition termed 'microswollen', since lateral protuberances could not be observed.

Figure 11 shows the effect of daunomycin on cumulative oxygen uptake during spore germination. There is an inhibition of $O_2$ uptake before the end of the post-activation lag stage (1 hour) suggesting that daunomycin may interfere with $O_2$ uptake before swelling can occur. An interference with $O_2$ uptake before the onset of swelling will block swelling and eventually deactivate a majority of the spore population. Thus the effect of daunomycin on spore germination may not be directly attributable to a blockage of RNA synthesis.

**Thiolutin**

Thiolutin has been shown to be a powerful inhibitor of RNA synthesis in yeast cells (Tipper, 1973). Table 6
Fig. 11.
The effects of daunomycin on cumulative oxygen uptake during spore germination in *Dictyostelium discoideum*. Heat-activated spores were treated with 250 ug/ml of daunomycin and control spores were treated to an equal volume of phosphate buffer. Cumulative oxygen uptake was monitored on a YSI model oxygen meter as outlined in the Materials and Methods.

Symbols:

(●) oxygen uptake in spores germinating in the presence of daunomycin (250 ug/ml)

(○) oxygen uptake in control spores
shows that thiolutin did block the incorporation of radiolabelled uracil by 98% and also blocked spore swelling. This confirms earlier reports of Giri and Ennis (1977).

The mode of action of thiolutin is unknown. There are two major disadvantages to the use of thiolutin as a RNA synthesis inhibitor for studies during D. discoideum spore germination. First, thiolutin is highly insoluble in aqueous buffer, and thus 20% DMSO must be used to prepare stock solutions. Dilute concentrations of DMSO tend to slow down spore germination.

Secondly, Cotter (unpublished observations) has shown that thiolutin reduces spore ATP levels and deactivates the spores. Figure 12 shows the effect of thiolutin on oxygen uptake in germinating spores. The drug seems to act very early, suppressing oxygen uptake before the end of the post-activation lag stage. This lack of oxygen uptake may serve to deactivate the spore population.

Nogalomycins

Nogalomycins U12,231, U15,167, and U20,661 are a group of anthracycline antibiotics which intercalate with the DNA template and are strong inhibitors of DNA-directed RNA synthesis (Bhuyan, 1967). Nogalamycin has recently been used by Ennis (1981a) to determine the half-lives of mRNA in vegetatively growing amoebae, and
Fig. 12.
The effects of thiolutin on oxygen uptake during spore germination. Using the procedures outlined in the Materials and Methods, cumulative oxygen uptake was measured in spores treated with thiolutin (100 ug/ml) and control spores.

Symbols:

(●) oxygen uptake in thiolutin treated spores

(○) oxygen uptake in control spores
to demonstrate a requirement for RNA synthesis during spore germination. Ennis observed an 88% inhibition of RNA synthesis during germination, and found that a final concentration of 0.95% ethanol in the control and nogalamycin treated spores did not affect germination.

The results from experiments employing nogalamycin to inhibit $^3$H-uracil incorporation are presented in Table 6. The results agree with Ennis; i.e., nogalamycin U12,241 and U15,167 caused respectively a 94% and a 86.7% inhibition of $^3$H-uracil incorporation. Additionally, the majority of the spores reached the swelling stage of germination and emerging amoebae were not observed.

While nogalamycin appeared to be useful, two observations deterred the further use of the drug. First, was the rather high concentrations of the antibiotic that were required to block RNA synthesis. This suggests that the drug may be having secondary effects. Bhyuan and Reusser (1967) observed that nogalamycin can cause uncoupling of oxidative phosphorylation in rat liver mitochondrial extracts.

Secondly, there were rather drastic alterations in the spore germination kinetics. Figure 13 shows the germination of spores treated with the three nogalamycins. With all three nogalamycins, the swelling stage of germination was extended and incomplete. A significant percentage of the spores remained dormant. This effect
Fig. 13.

Germination kinetics of spores treated with Nogalomycins, U20,661, U12,241, and U15,167. The inhibitors were prepared in stock solutions of 30 mg/ml in 95\% ethanol. The inhibitors were added to activated spores at a concentration of 300 \mu g/ml. Control spores were treated with an equivalent amount of ethanol.

Symbols:

(Δ,▲) percent swelling and emergence of spores treated with U20,661

(○) percent swelling of spores treated with U15,167

(□) percent swelling of spores treated with U12,241

(○,●) percent swelling and emergence of control spores
was probably due to the presence of ethanol. Figure 14 shows the effect of 0.95% ethanol on spore germination kinetics. Spores treated with ethanol after heat activation were considerably slower during swelling and germinated to much lower percentages. An additional study by another lab has shown drastic effects of ethanol on spore germination and vegetative growth of amoebae (Hase, 1980).

Ultraviolet light

The effects of ultraviolet (UV) light on germinating spores of *Dictyostelium discoideum* has been recently well characterized (Demsar, 1981). The dormant spores of *D. discoideum* are considerably more sensitive to UV light than their vegetative counterparts. This may be due to a lack of active UV repair enzymes in dormant spores (Hashimoto and Wada, 1980). This phenomena can be exploited for the purpose of studying RNA metabolism. The exposure of germinating spores to UV light at a total fluence of 250 J/m² blocked the incorporation of $^3$H-uracil by 94%. Ultraviolet light irradiated spores would activate and swell at a rate comparable to control spores. UV irradiation does not affect oxygen uptake except after the point of maximal swelling (Demsar and Cotter, 1981), and the mode of action of ultraviolet light on eukaryotic cells is well defined. Problems of
Fig. 14.
The effects of 0.95% ethanol on spore germination in *Dictostelium discoideum*. Activated spores were made 0.95% (v/v) with respect to ethanol. Control spores were treated with an equivalent volume of phosphate buffer.

Symbols:

(Δ ▲) swelling and emergence of spores in the presence of 0.95% ethanol

(○ ●) control spore swelling and emergence
Fig. 15.

Kinetics of $^3$H-uracil incorporation in 4-NQO treated spores and control spores. Activated control and 4-NQO treated spores were germinated in the presence of $^3$H-uracil and the incorporation of radioisotope into a TCA insoluble fraction was monitored as described in the Materials and Methods. The inhibitor was used at a concentration of 5 ug/ml.

Symbols:

○ control spore incorporation

○ 4NQO treated spores
solubility and lack of specificity (inherent with the use of antibiotics) are conviently circumvented. Thus the effects of UV light on RNA synthesis were examined to a greater degree in the following section.

4-Nitroquinoline 1-oxide

The drug 4-nitroquinoline 1-oxide (4NQO) has been classified as a 'UV like' mutagen because of its mode of action on eukaryotic cells and the enzyme systems that repair 4NQO-induce damage. The mode of action of 4NQO has been well characterized and requires a metabolic activation. The compound is first reduced to a 4-hydroxyaminoquinoline 1-oxide (4HAQ) which then becomes aminoaacylated by some aminoaacyl-tRNA synthetase. In this form it reacts with the DNA template to form stable purine-4NQO adducts (for review see Ikenaga et al., 1981).

At concentrations of 5ug/ml, 4NQO was capable of reducing 3H-uracil incorporation by 98.2% during spore germination (see Figure 15). Germinating spores treated with 4NQO were able to swell but did not release myxamoebae. Figure 16 shows a dosage response curve of spores to 4NQO. A concentration of 2.5 ug/ml was capable of blocking emergence after five hours, however 5 ug/ml was required to block emergence for over a 24 hour period. This is the lowest concentration of any chemical inhibitor used to block D. discoideum spore
Germination of *Dictyostelium discoideum* spores in the presence of dilute concentrations of 4-NQO. Activated spores were suspended in varying concentrations of 4-NQO and allowed to germinate at 23.5°C. Germination was scored after 5 hours and 24 hours as outlined in the Materials and Methods.

Symbols:

- (○) percent germination after five hours
- (●) percent germination after 24 hours
germination.

Studies on $O_2$ uptake were analogous to findings established for UV irradiation. Figure 17 shows that only a stage specific inhibition of $O_2$ uptake occurs at mid-swelling when control spores begin to release amoebae. Additionally, Figure 18 shows that 4NQO had no effect on the kinetics of spore germination other than to block the specific release of myxamoebae.

In order to obtain a preliminary estimate on the time of action of 4NQO, spores were activated in the presence of 4NQO and samples were removed, washed free of the drug, and allowed to germinate. Table 7 shows the result of this experiment. It can be observed that when spores are activated in the presence of 4NQO and immediately washed free of the drug there is a 85% inhibition of emergence (i.e. only 15% amoebae were observed after five hours). Following activation, 15 minutes additional incubation in the drug is sufficient to block all emergence. These data suggest that the drug is able to permeate the spore very early in the germination process, and affect a complete inhibition of myxamoebae emergence. This contrasts with other drugs such as actinomycin D which only seem to permeate the spore at later stages in germination. It should be noted that the drug was completely irreversible in its inhibition of myxamoebae emergence; i.e. it resembles
Fig. 17.

Oxygen uptake in spores treated with 4-NQO. Spores treated with 4-NQO were placed in a YSI oxygen monitor assembly and the cumulative oxygen uptake was monitored as described in the Materials and Methods section.

Symbols:

- (●) oxygen uptake in 4NQO treated spores
- (○) oxygen uptake in control spores
Table 7. Removal of 4-nitroquinoline 1-oxide following activation of dormant Dictyostelium discoideum spores

<table>
<thead>
<tr>
<th>Time of removal of 4-nitroquinoline 1-oxide (min. after activation)</th>
<th>Final percent germination after five hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dormant</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>30</td>
<td>13</td>
</tr>
<tr>
<td>60</td>
<td>9</td>
</tr>
<tr>
<td>90</td>
<td>15</td>
</tr>
<tr>
<td>120</td>
<td>3</td>
</tr>
<tr>
<td>control</td>
<td>0</td>
</tr>
</tbody>
</table>

Superscript a: Spores were activated in 20% DMSO for 30 min. Spores were washed free of DMSO and suspended in 10 mM phosphate buffer at a concentration of 10⁶ spores/ml. 4NQO was added to a final concentration of 5μg/ml at time zero. At designated intervals 2 ml of the spore suspension was removed and washed free of the drug by centrifugation. Washed spores were resuspended in phosphate buffer and allowed to germinate. Germination was scored at the end of five hours.

Superscript b: For the time zero sample spores were activated in the presence of 4NQO. Following activation the spores were washed twice in phosphate buffer and allowed to germinate.
Fig. 18.

Germination kinetics of spores treated with 5 μg/ml of 4-NQO. Activated spores were treated with 5 μg/ml of 4-NQO and germination was monitored as described in the Materials and Methods section.

Symbols:

(○, ●) control spore swelling and emergence

(△) swelling of spores treated with 4-NQO
the effect of ultraviolet irradiation. It follows since dormant spores lack UV repair enzymes, they may also lack the ability to repair 4NQO induced damage.

An additional feature, which also suggests that 4NQO was acting as a 'UV mimic' was the shape of the amoebae in cultures that were treated with the drug late in the germination process. When germinating spores are irradiated during late swelling, the surviving amoebae are round, do not appear to have pseudopods, and do not clump in phosphate buffer. On the other hand control amoebae have the characteristic amoeboid shape, pseudo- and filopodia, and tend to clump quite readily. When late swelling spores are treated with 4NQO, they release myxamoebae which take on the rounded, singular appearance of UV irradiated amoebae. 4NQO was also soluble in phosphate buffer and thus appeared to be a good inhibitor of spore germination in D. discoideum.

In order to determine if 4NQO could be used to terminate RNA synthesis during germination, spores were allowed to germinate to a point near maximal swelling (approximately 2.25 hours). At this time the batch culture was split and one culture was made 5 ug/ml in 4NQO and an equal volume of phosphate buffer was added to the control culture. After five minutes of incubation, 3H-uracil (5 uCi/ml) was added to each culture, and germination was allowed to continue for
Fig. 19.

Inhibition of $^3$H-uracil incorporation following treatment with 4-NQO. A batch of late germinating spores was treated with 4-NQO for five minutes. $^3$H-uracil was then added and spores allowed to germinate for an additional hour. The RNA was extracted and profiled on 1.7% gels as described previously. (A), incorporation into the control culture; (B), incorporation into the 4-NQO culture. The solid line indicates the extinction at 260 nm.

Symbols:

(●) incorporation of $^3$H-uracil
an additional hour. The cultures were chilled briefly on ice and the RNA was extracted immediately. The RNA was profiled on 1.7% and 10% gels. The incorporation into various peaks was determined. Figure 19 shows approximately a 50% inhibition of $^3$H-uracil incorporation into the 25s and 17s rRNAs. Figure 20 shows that a similar level of inhibition was noted for the 5s and the 5.8s ribosomal associated RNAs. Interestingly, there did not appear to be decrease in tRNA synthesis. However, additional counts in the 4s peak may have been due to the accumulation of labelled 4NQO terminated transcripts. Since the mode of action of 4NQO is not central to this investigation this phenomena was not further investigated.

The fact that only a fifty percent inhibition of RNA synthesis was observed may have been due to the time required for the drugs activation. When added early in germination there is sufficient time before the onset of RNA synthesis for the drug to take action. However, when the drug is added during a time of rapid RNA synthesis the time required for activation may be sufficiently long enough to allow some RNA synthesis.

While 4-NQO appeared to possess some desirable characteristics for studies on spore germination (i.e. solubility, low concentration, etc.) the fact that only a fifty percent reduction in RNA synthesis is
Fig. 20.

Inhibition of $^3$H-uracil incorporation into low molecular weight RNA. The same RNA preparations used to generate the data in Fig. 17 were profiled on 10% gels. (A), incorporation into the control culture; (B), incorporation into the 4-NQ0 treated culture. The solid line indicates the extinction at 260 nm.

Symbols:

(●) incorporation of $^3$H-uracil
observed, precluded the use of this drug in further studies. 4-NQO would not be suitable for studies on mRNA half-lives during germination, since all RNA synthesis has to be abruptly stopped.

Part III. Termination of RNA Synthesis by Ultraviolet Light During D. discoideum Spore Germination

A. Germination in UV irradiated spores.

Unlike a number of inhibitors UV light does not alter the early kinetics of D. discoideum spore germination. Figure 21 demonstrates the stage specific effects of UV irradiation on D. discoideum spore germination. Control spores germinate in five hours following activation, while UV irradiated spores swell but remain irreversibly blocked at the swelling stage of germination. The blockage of emergence has been found to occur in spores irradiated any time prior to 2 hours into germination; after this point is reached spores become immune to irradiation and become committed to release amoebae (Demsar and Cotter, 1981).

B. Incorporation of $^{3}$H-uracil

The kinetics of incorporation of $^{3}$H-uracil into a cold TCA precipitable fraction during germination of control and irradiated spores is shown in Figure 22.
Fig. 21.

Effect of UV light on heat-induced spore germination

Dormant spores were prepared as described in the Materials and Methods section. A portion of the dormant spores received a total fluence of 250 J/m² (vertical arrow). Control and UV irradiated spores were heat-activated and subsequently incubated at room temperature for 5 hours.

Symbols:

(■) the heat-shocking period
(○) percent swelling of control spores
(△) percent swelling of UV irradiated spores
(●) percent myxamoebae emergence from control spores
(▲) percent myxamoebae emergence from UV irradiated spores
Fig. 22.

Incorporation of $^3$H-uracil during spore germination.

Symbols:

- (●) control incorporation
- (○) incorporation into UV irradiated spores

All points were counted in triplicate with a counting error of 0.1%.
As shown by other researchers, incorporation begins shortly after the first hour and increases with the emergence of myxamoebae (Yagura and Iwabuchi, 1976; Giri and Ennis, 1977). Irradiated spores incorporate less than 10% of the label incorporated by the control spores and remain irreversibly blocked at the swelling stage of germination. This may be due to the presence of damaged DNA templates which terminate transcription prematurely, allowing for the synthesis of short non-functional transcripts (Fukuyama et al., 1967). A detailed report on the effects of UV irradiation on protein synthesis has recently been submitted and will be presented elsewhere (Demsar et al., submitted).

C. **UV irradiation during RNA synthesis**

To demonstrate the effects of UV irradiation on RNA synthesis during spore germination, spores germinating in the presence of $^3$H-uracil were irradiated at various times during the germination process and the amount of label incorporated at the end of five hours was measured. Only a small amount of label was accumulated in spores subjected to irradiation during germination (Figure 23). Spores that were irradiated later in the germination process seemed to accumulate greater amounts of label than spores irradiated earlier (i.e. before 2.5 hours). This may be due to the fact that the rate of RNA synthesis
Fig. 23.

UV light-induced termination of RNA synthesis during heat-induced spore germination. Incorporation of $^3$H-uracil was measured at the time of irradiation and following several hours of incubation.

**Symbols:**

- (■) incorporation in the control spores
- (△) incorporation at the time of irradiation
- (▲) incorporation of irradiated cultures following several hours of additional incubation
- (●) percent emerged myxamoebae in the control

**NOTE:** the small loss of label in the irradiated spores results from the additional manipulations required to prepare these samples.
greatly increases after 2.5 hours. This would allow for a greater accumulation of terminated transcription products.

This data would suggest that RNA synthesis is abruptly terminated rather than slowed down, and thus UV may be used to study the synthesis of specific transcripts at discreet intervals during germination.

As a preliminary test of these ideas, spores were allowed to germinate until maximal swelling was reached. The culture was split in half and one half received a total fluence of 250 J/m² of UV light. A total of 10 μCi/ml of ³H-uracil was added to each culture, and they were both allowed to germinate for an additional hour. The RNA was extracted and profiled on 1.7% and 10% gels. The gels were fixed, scanned and the radioactivity in the various peaks determined as outlined in the Materials and Methods. The data of Figure 24 show that UV abruptly terminated the synthesis of the two large rRNAs, while in the control culture these species were actively synthesized. Additionally no degradation of the pre-existing rRNAs appeared to occur.

Figure 25 demonstrates the inhibition of ³H-uracil incorporation into low molecular weight RNA. The control actively synthesized all three RNA species. In the irradiated culture there was no observable synthesis
Fig. 24.

Inhibition of incorporation of $^3$H-uracil following irradiation by ultraviolet light. A late germinating batch of spores was split in two and one half received a dose of UV radiation. $^3$H-uracil was added to both cultures and germination continued for an additional hour. The RNA was extracted and electrophoresed on 1.7% gels as outlined in the Materials and Methods. (A), incorporation into the irradiated culture; (B), incorporation into the control culture. The solid line indicates the extinction at 260 nm.

Symbols:

(●) incorporation of $^3$H-uracil
Fig. 25.
Inhibition of $^3$H-uracil incorporation into low molecular weight RNA following UV irradiation. The same RNA preparations which were used to generate the data in Fig. 22. were profiled on 10% polyacrylamide gels as outlined in the Materials and Methods. (A), incorporation into the irradiated culture, (B), incorporation into the control culture. The solid line indicates the extinction at 260 nm.

Symbols:

$\bullet$ $^3$H-uracil incorporation
of the 5s and 5.8s RNAs. However, a large amount of label appeared to accumulate in the vicinity of the 4s RNA. This level of incorporation (in excess of 40,000 cpm) appeared to represent a heterogeneous population of low molecular weight RNAs. This finding is consistent with the presence of shortened UV-induced transcripts in UV-irradiated spores.

D. Polycrylamide Gel Electrophoresis

Total cellular RNA preparations were analysed on 3% agarose acrylamide and 10% acrylamide gels to determine if UV irradiation had any effect on preformed RNA species and to determine which RNA species, if any, were limiting the germination in the UV blocked spore. Figure 26 represents the electrophoresis of total cellular RNA on 3% agarose/acrylamide gels. Control spores before activation and germinated spores (myxamoebae) five hours after activation show the same pattern of 17s and 25s rRNAs. This pattern is also present at swelling (data not shown) and thus does not appear to change during the germination process. UV irradiation does not appear to significantly affect this distribution even after five hours of incubation.

Electrophoresis of total cellular RNA in 'stacked' 10% polyacrylamide gels gives the relative distribution of 5.8s from the 5.0s and 4s RNAs as shown in Figure 27.
Fig. 26.

3% agarose/acylamide gel electrophoresis of total cellular RNAs. All samples were prepared in 20% sucrose and electrophoresis was carried out for 2½ hours at room temperature. The gels were fixed in 5% TCA and scanned at 254 nm. Arrows depict the position of Escherichia coli 16S and 23S rRNAs as reference.

Symbols:

(a) control dormant spores
(b) UV-irradiated dormant spores
(c) emerged myxamoebae five hours after activation
(d) UV-blocked swollen spores five hours after activation
Fig. 27.
10% polyacrylamide gel electrophoresis of total cellular RNA. All gels contained a 3% stacking gel to remove large molecular weight species. RNA samples were heated at 60°C for 5 minutes to remove aggregates and layered on the gel in 20% sucrose.

Symbols:
(a) control dormant spores
(b) UV irradiated dormant spores
(c) emerged myxamoebae 5 hours after activation
(d) UV-blocked swollen spores 5 hours after activation.

I, II, and III represent the 4S, 5S, and 5.8S RNAs.
The RNA sample must be heat treated at 60°C for 5 minutes in order to resolve the 5.5s from the 5.0s RNA (Knight and Van Etten, 1976a). This pattern has been reported in a number of other fungal systems (Van Etten et al., 1976). As with the large molecular weight RNAs there appears to be no significant change in the pattern of distribution of the low molecular weight RNA species during germination. Note that the baseline shift in the irradiated sample after five hours may indicate the presence of prematurely terminated transcripts. Thus in irradiated swollen spores the high molecular weight rRNA, the low molecular weight ribosomal associated RNA and the tRNA do not appear to be limiting the germination process. These data appear to rule out the idea that UV irradiation may cause a random degradation of cellular RNAs. Additionally, the use of irradiated (UV-killed) bacteria as a heterologous transcription and translation system seems to suggest that the transcriptional and translational apparatus of cells (i.e. polymerases, ribosomes, tRNA, factors etc.) are unaffected by irradiation.

E. Poly(A-U) Hybridization

In order to determine if irradiation leads to a loss of poly(A)+RNA, poly(A-U) hybridization was performed. Total cellular RNA from dormant, swollen and five-hour germinated spores was hybridized in
increasing amounts to a constant amount of $^3$H-poly(U).

The amount of poly(A) was calculated from the specific activity of the poly(U) assuming total hybridization, and a 3' poly(A) 'tail' region of 100 bases. The amount of poly(A) per milligram of RNA which accumulated during germination of control and UV irradiated spores is diagrammed in Figure 28. Irradiated spores undergo a dramatic decrease in the amount of poly(A) containing RNA during germination when compared to control spores which show an approximate 2-3 fold increase. The same preparations which were used for electrophoretic analysis (Figures 26 and 27) were used in the poly(A) assays, and each value is the average of at least three separate assays.

If the level of poly(A) is indicative of the poly (A)+ RNA levels, then the loss of the preformed poly(A)+ RNA may be explained in a number of ways. The fact that normal swelling occurs in irradiated spores may suggest that this decay is a reflection of normal mRNA turnover via translation since swollen spores do contain unique and discreet proteins (Dowbenko and Ennis, 1980). Also the fact that irradiated spores continue to incorporate $^3$H-leucine into a hot TCA precipitable fraction, is also consistent with this idea (see Figure 30; also Demaar et al., manuscript in preparation).
Fig. 28.
Poly(U) hybridization of total cellular RNA. Dormant spores were irradiated and germinated; RNA was extracted at various times during germination and hybridized with $^{3}H$-poly(U) according to the Materials and Methods section.

Symbols:
- control spores
- UV irradiated spores

(A) dormant

(B) 2.5 hours after activation

(C) 5 hours after activation
Alternately the loss in poly(A) content is inconsistent with the idea of random nuclease digestion since all the other RNA species remain intact following irradiation. However, since mechanistic models for poly(A)+ RNA decay are lacking or at least limited in their scope, further work is necessary for a clearer understanding of this phenomena.

F. Determination of Poly(A)+ RNA decay in germinating spores of D. discoideum

Ultraviolet light irradiation may provide a convenient means of terminating RNA synthesis during spore germination which facilitates the measurement of mRNA half-lives. The results of such a determination on germinating spores is presented in Figure 29. Spores were irradiated at 2.25 hours into germination (95% swollen). This time corresponds to a 'competence point for emergence' (Demsar and Cotter, 1981; also this thesis). The level of labelled poly(A)+ RNA was measured using the poly(U) filter binding assay of Sheldon et al., (1972); this method has been used by other researchers to determine the half-life of vegetative cell mRNA (Palatnick et al., 1980). The decay curve appears complex (Figure 29) and resolves into possibly two components. A short lived component which has a half-life of approximately fifteen minutes and a much more stable component with a half-life
Fig. 29.

Poly(A)+ RNA decay after termination of RNA synthesis with ultraviolet light treatment. Dormant spores were activated and germinated in the presence of $^3\text{H}$-uracil. At exactly 2.25 hours into germination the culture was irradiated with a total UV fluence of 250 J/m$^2$ and the RNA was extracted at various intervals. The level of poly(A)+ RNA was assayed as described in the Materials and Methods section. The average values from three separate experiments is shown.

Symbol:

( ) amount of label bound on a poly(U) impregnated filter minus the amount of label bound to a filter not impregnated with poly(U)
longer than the germination sequence. Half-life determinations for poly(A) containing RNA have been obtained for the vegetative growth stage and the developmental stage (Margolskee and Lodish, 1980ab; Palatnik et al., 1980; Ennis, 1981). These studies have involved an analysis of both the structural and functional decay of the poly(A)+ RNA. Although Figure 29 represents a structural decay; this has been found to correlate well with the functional decay of the poly(A)+ RNA (Palatnic, et al., 1980).

The same preparations of RNA which were used to generate the data in Figure 29 were fractionated on 1.7% agarose/acrylamide gels as described in the Materials and Methods. The 25s and 17s rRNA bands were cut out and the amount of label in the two fractions determined. Table 8 shows that little or no degradation of 25s and 17s rRNA occurred. This is consistent with the idea of a specific poly(A)+ RNA decay.

G. Pulse-labelling irradiated spores with 3H-leucine

In order to substantiate these above findings, the decay of protein synthesis following irradiation was determined in vivo. This was done by allowing spores to germinate to maximal swelling, irradiating the culture and pulse labelling it at five minute intervals with high concentrations of 3H-leucine. From the results
<table>
<thead>
<tr>
<th>Time after irradiation (min)</th>
<th>25s rRNA (CPM/A_{260} unit)</th>
<th>17s rRNA (CPM/A_{260} unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>48,326</td>
<td>11,995</td>
</tr>
<tr>
<td>15</td>
<td>12,668</td>
<td>8,983</td>
</tr>
<tr>
<td>105</td>
<td>12,715</td>
<td>8,099</td>
</tr>
<tr>
<td>165</td>
<td>12,917</td>
<td>10,413</td>
</tr>
</tbody>
</table>
Fig. 30.
Decay of protein synthesis following irradiation with UV light. Germinating spores were pulse labelled with $^3$H-leucine for five minute intervals following the application of ultraviolet light. Labelling and counting procedures are outlined in the Materials and Methods. The arrow depicts the time of irradiation.
in Figure 30 there does not appear to be a detectable long lived translating component. Protein synthesis increases during the first two pulse periods following irradiation but rapidly decays thereafter. Thus, it is observed that incorporation during the pulse periods stops about fifteen minutes after irradiation. These results are consistent with the presence of a rapidly decaying mRNA population in these irradiated spores which appears to be mediated via translation.

Part IV. Analysis of Acid Soluble Nucleotides in Dormant and Germinating D. discoideum Spores.

A. Extraction and analysis

In order to determine if dormant spores were lacking essential nucleotide-triphosphates required for RNA synthesis, acid soluble nucleotides (ASN) of dormant and post-activated spores (i.e. 1 hour after activation) were analysed. Since dormant spores can germinate in the absence of nutrients, and RNA synthesis does not occur until 1 hour after activation, the post-activation lag stage may be a time when nucleotide-triphosphates are generated. This could occur by the phosphorylation of a pre-existing nucleoside pool, or by the biosynthesis of these precursors from a stored carbon source.

An initial extract was prepared by suspending spores in 1.0 M perchloric acid. However, little or no
nucleotides were extracted. Dormant spores broken in 1.0 M perchloric acid in a French press yielded better results. A combination of freon and amine was used to neutralize the nucleotide extract before chromatography. The final procedure is outlined in the Materials and Methods.

While a number of procedures were available for fractionating nucleotides, high performance liquid chromatography (HPLC) was the most sensitive and rapid of all the available techniques. HPLC was performed using the apparatus and procedures outlined in the Materials and Methods. Figure 3lab shows a chromatographic profile as ASN extracted from dormant spores and vegetative amoebae. Peaks were identified by the position of known nucleotide-mon-di- and triphosphates. Well resolved peaks were obtained with retention times less than 25 minutes and duplicate runs at varying spectrophotometric sensitivities could easily be run. Thus HPLC may have a wide range of applications for employment in studies on D. discoideum spore germination.

B. Nucleotide-triphosphate levels in the dormant and post-activated spore of D. discoideum

ASN from dormant and post-activated spores were extracted and analysed by HPLC. Uridine, adenine, guanine and cytosine triphosphates retention times were obtained
Fig. 31.
High performance liquid chromatography of acid soluble nucleotides from dormant spores and vegetative amoebae. Acid soluble nucleotide extractions and HPLC analysis were performed as described in the Materials and Methods. Various nucleotides were identified by chromatography of appropriate standards. (A), dormant spore; (B), vegetative (emerged) amoebae.
from running standards. The area under the appropriate peaks was determined. Each sample was chromatographed twice at two different sensitivities, resulting in a total of four chromatographic profiles per sample. A conversion factor was used to convert the calculated areas to optical density units and the final values were adjusted to O.D.\textsubscript{254}/10\textsuperscript{7} spores.

Table 9 shows the level of uridine, adenine, guanine and cytosine-5'-triphosphates, in dormant and post-activated stage spores. The observation that germinated amoebae (which actively synthesize RNA) had lower pool levels than the dormant spore, suggests that the dormant spore has RNA precursor levels that could support RNA synthesis. Thus the repression of RNA synthesis for approximately one hour after activation must be mediated by some other mechanism than simple precursor availability. An obvious mechanism would be the absence of active RNA polymerases. This mechanism is under current study.

C. Acid soluble nucleotide changes during germination

Since the extraction and analysis of ASN from dormant spores was relatively simple and quick, a study of changes associated with germination was facilitated.
Table 9. Nucleoside 5'-triphosphate levels in Dictyostelium discoideum spores

<table>
<thead>
<tr>
<th></th>
<th>ATP</th>
<th>GTP</th>
<th>CTP</th>
<th>UTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dormant spores</td>
<td>2.00</td>
<td>0.30</td>
<td>0.02</td>
<td>0.13</td>
</tr>
<tr>
<td>Post-activated spores</td>
<td>2.30</td>
<td>0.47</td>
<td>0.01</td>
<td>0.14</td>
</tr>
</tbody>
</table>

* Nucleotides in the spore extracts were separated by HPLC and the area under the appropriate peaks was calculated. Duplicate scans were performed using two different sensitivities at 0.16 AUFS and 0.8 AUFS. Results are expressed as:

  \[ \text{O.D. units} \times 10^{-2} \text{ per } 10^7 \text{ spores} \]

* Post-activated spores are those harvested just before the onset of swelling, one hour after activation. This time corresponds to the initiation of RNA synthesis.
Figure 32 shows an overall decrease in the level of extractable acid soluble nucleotides during germination. Thus, it appears that the dormant spore may serve as a reservoir of ASN required for germination, and may account in part for the spore's ability to germinate in nutrient depleted environments.

Figure 33 shows the overall changes associated with adenosine mono-, di-, and triphosphates. ATP levels drop nine-fold during the second hour of germination. This decrease is the most dramatic change of any nucleoside during germination. Additionally, there were smaller, less dramatic decreases in the ADP and AMP levels. From the data obtained, it appeared that ATP comprised roughly 25% of the extractable acid soluble nucleotides. The drop in ATP levels occurs at a time when protein synthesis and RNA synthesis begin, and when swelling occurs. Spore swelling has been shown to be an energy requiring process. A more detailed analysis of ATP and its relationship to other energy requiring processes during spore germination will be presented elsewhere (D.P. Jackson manuscript in preparation).

From the chromatographs obtained in the above experiment, changes in the GDP and GTP levels could be obtained. While GDP remains unchanged during germination, GTP increases slightly during the first hour and then drops 3 fold during swelling. This decrease occurs at
Fig. 32.
Acid soluble nucleotide levels during germination of D. discoideum spores. The bar graph indicates the amount of acid soluble $A_{254}$ units extracted at various hours of germination. The germination curve is stylized.

Symbols:

(○,●) percent spore swelling and myxamoebae emergence
Fig. 33.

Changes in the adenine nucleotide mono-, di- and triphosphates. The Materials and Methods detail the quantitation of ATP, ADP and AMP levels from HPLC profiles.

Symbols:

- (●) adenine triphosphate levels
- (○) adenine diphosphate levels
- (□) adenine monophosphate levels
Fig. 34.

Guanosine di-, and triphosphate levels during spore germination in Dictyostelium discoideum. The same chromatographic profiles which were used to determine adenine phosphate levels were used in this analysis. Procedures are detailed in the Materials and Methods.

Symbols:

- (●) GTP levels
- (○) GDP levels
the same time as the ATP drop and thus the involvement of GTP in energy requiring processes (i.e. protein synthesis) is implicated. While the changes in nucleoside triphosphate levels appear to raise more questions than they answer, they do provide support to a view of the dormant spore as a metabolically competent entity.
DISCUSSION

Fungal spore germination is a unique system in which to study cell differentiation. Upon treatment with an activating agent or placement in a suitable environment, the metabolically quiescent spore suddenly comes to life and begins taking up oxygen, swelling, and synthesizing various biological macromolecules. In most fungi this process results in the emergence of a germ tube which differentiates into an actively growing mycelium. An understanding of fungal spore germination and dormancy may provide methods by which to control plant and human mycoses which are disseminated by spores and in addition provide a suitable paradigm for cell differentiation.

One of the simplest and best understood systems for studying fungal spore germination can be found in the cellular slime mold Dictyostelium discoideum. Germination can be induced by a number of methods (see Introduction) and synchronous germination is completed within five hours. The four recognized stages are: activation, post-activation lag, swelling, and emergence. Unlike most fungal spore systems, germination results in the emergence of a single myxamoeba, which appears to be more closely related to higher eucaryotic cells.
than to fungi. A number of physio-chemical methods are available for delaying or irreversibly blocking each of the four developmental stages.

In fungal spores it is difficult to address the subject of germination without prior consideration of spore dormancy. Early work on dormancy by developmental biologists involved analyzing the component parts of the macromolecular machinery in the dormant spore. It was hypothesized that since there was a lag before the onset of RNA or protein synthesis, the dormant spore might lack some essential factor (i.e., synthetase enzymes, functional tRNA etc.) that would block germination. However, in all the fungal spores examined to date, most of the protein synthetic machinery appears to be present and functional (for review see, Van Etten et al., 1976). Our preliminary results suggest that *Dictyostelium discoideum* spores conform to this general pattern.

The mature dormant spore contains preformed 25s and 17s rRNAs contained in abundant ribosomes, visible in the electron micrographs of Gregg and Badmann (1970). These ribosomes form polyribosomal complexes before the onset of new rRNA synthesis (Giri and Ennis, 1977), suggesting that these ribosomes are functional. Additionally, the dormant spore has preformed 5.8s and 5.8s ribosomal associated RNAs (Fig. 4), which also
appear in a number of other fungal spores in approximately a 1:1 ratio (Van Etten et al., 1976). Though not tested here, ribosomes have been isolated from a number of fungal spore types including the pycnidiospores of the fungus Botryodiplodia theobromae (Van Etten, 1971), the zoospores of Blastocladiella emersonii (Schmoye and Lovett, 1969) and the conidia of Aspergillus oryzae (Kimura et al., 1965). In all three cases these ribosomes have been found to be biologically active. Thus the notion of inactive ribosomes contributing to spore dormancy is largely unsupported.

The dormant spores also contain approximately 3.0 ug of polyadenylate containing RNA per milligram of total spore RNA (see Table 1). This 'mRNA' component has been translated and appears to code for a large number of proteins including actin (Giri and Ennis, 1978; Doybenko and Ennis, 1980; MacLeod et al., 1980). While a few of these proteins appear to be unique, no function has been assigned to these proteins. Presumably this mRNA is transcribed late in sporulation and becomes stored in the dormant spore. Conceivably, this mRNA may have at least two functions: (1) it may merely be residual mRNA left over during sporulation and may have absolutely no function for germination per se; (2) it may also code for a critical number of proteins which serve to initiate later germination events. While it
is immediately obvious that both possibilities can exist simultaneously, a clear answer will not be obtainable until the translation products of this mRNA population can be identified and assigned a function.

Studies in other fungi have not proved to be any better. In the fungus *Rhizopus stolonifer* (Freer and Van Etten, 1978), and *B. emersonii* (Lovett and Sikkema; 1981) preformed mRNA has been isolated and translated in a cell free system. The translation products have been compared to other stages of germination and no unique proteins were coded for by the preformed mRNA. One exception appears to be the fungus *Botryodiplodia theobromae*. The mature conidia appear to lack detectable levels of cytochrome oxidase and oligomycin sensitive ATPase activity. The oligomycin sensitive ATPase has 12 peptides — 11 of which appear to be synthesized de novo from stored mRNA in the cytoplasm. These peptides assemble into a functioning enzyme system within 45 to 50 minutes of germination (for review see, Brambl, 1981).

Cloned hybridization probes have recently been used to study the coding complexity of the stored mRNA in at least two fungi, *Dictyostelium discoideum* and *Aspergillus nidulans*. Camonis et al., (1981) found that a large number of the mRNA species in the spore are the same as those found in other stages of the life cycle in the cellular slime mold. However 20 to 30%
of the mRNA mass appears specific to the spore cell. While the abundance of these spore specific mRNAs was not given by the authors, it is likely that they are moderately abundant (≤ 50 copies per cell) or even rare (≤ 1 copy per cell) since two dimensional gels failed to detect them (Dowbenko and Ennis, 1980).

Timberlake and Barnard (1981) have isolated a gene cluster expressed specifically in the asexual spores of A. nidulans. In Aspergillus approximately 3000 genes (approx. 13% of the haploid genome) is specifically expressed during sporulation, and 600 of these genes appear to be expressed in the mature conidia in the form of 'stored mRNA'. A gene cluster was isolated from a lambda-DNA 'library' of the Aspergillus genome probed with spore specific mRNA. This clone carried six genes which were coordinately transcribed during conidiophore development. While their techniques appear complex, their results lack conclusion. The function of these genes is unknown, and the authors can only speculate on their involvement with germination.

One question continues to elude these developmental mycologists! If spores contain functional, preformed mRNA and ribosomes, what forces (factors) block or prevent polysome formation? In some fungi this question can be easily answered. When the spores of R. stolonifer are dry harvested no polysomes are detected. However
when they are wet harvested, polysomes appear immediately; thus specific hydration may play a role (Nickerson et al., 1981). In Blastocladiella initial results suggest that the preformed mRNA is bound in some 'ribonucleoprotein-like' complex (Jaworski and Stumhofer, 1981). In Discoidium this question has not been addressed. Studies on polysome initiation in SCI spores and heat activated spores may provide some interesting clues unobtainable in other systems.

The dormant spores of D. discoidium also possess transfer RNAs which are capable of being charged. These tRNA do not appear to be undermethylated, and can be isolated by conventional means from whole cell RNA (see Table 2 and Figure 6). This supports a general hypothesis; that dormant spores of all the fungi tested to date, have functional tRNA (Van Etten et al., 1976). The charge ratio of the dormant spore tRNA was also investigated, and found to be relatively low when compared to other spore systems (Table 3). In Blastocladiella, the dormant zoospore have a markedly different pattern of germination. Upon release, these aquatic spores go through a period of high motility before encysting and producing a germ tube. Therefore their relatedness to the cellular slime mold is at best minimal.

The charge ratios for the three cognate tRNAs of tryptophan, methionine, and phenylalanine were also
determined for the dormant spore and compared to the
vegetatively growing amoebae. It was assumed that the
vegetative cell has charge ratios suitable for protein
synthesis, and has competent amino acid levels. The
data suggest that all three tRNAs have charge ratios
similar to that found in the vegetative cell, suggesting
these levels are sufficient for protein synthesis. These
data are limited in their scope since these charge ratios
were not examined following activation and throughout
germination. A further detailed study would include
studies on the isoaccepting species, of the various aminoacyl-
tRNAs and their changes with germination. Studies in
B. theobromae (Van Etten et al., 1969) and Aspergillus
oryzae (Horikoshi et al., 1969) suggest that tRNA do
not appear to play a major role in the regulation of
spore dormancy.

Several lines of evidence suggest that tryptophan,
phenylalanine and methionine are not metabolically
necessary for germination. A mixture of all three
amino acids are required to promote germination, however
only tryptophan appears to have an undetectable pool
level (Ernis, 1981b). The cognate tRNA for this amino
acid is present and a significant portion appears to be
charged (Table 4). Cotter and Raper (1966) demonstrated
that D- as well as L- amino acid mixtures would activate
spores. The incorporation kinetics of tryptophan,
phenylalanine and methionine into TCA precipitable material during germination is the same as any other amino acid (Tisa, 1979). These findings do not support the hypothesis that these amino acids are metabolically required for germination. Additionally, respiratory quotient determinations suggest that carbohydrates are the main energy source during early germination (Jackson et al., in press). Cotter (1976) has proposed that the hydrophobic side chains of these three amino acids may induce protein conformational shifts in a trigger protein for germination.

A dogma among many developmental biologists is that development and differentiation are mediated by differential gene action. RNA and protein synthesis are predominant events during fungal spore germination, thus this area has been intensively studied and many reviews are available (Lovett, 1976; Lovett and Sikkema, 1981; Van Etten et al., 1976; 1977; 1981). Studies on RNA synthesis during spore germination in Dictyostelium discoideum have had several inherent problems: (1) sucrose density gradients have been used which do not adequately separate the low molecular weight RNA's, (2) a mutant strain (strain B) has been used which does not respond to the heat shock process as does wildtype spores (see Introduction), (3) labelling periods have been continuous and do not correspond to the four
developmentally defined stages of germination. By pulse-labelling spores during discreet stage specific intervals, and analysing the RNA using a combination of 1.7% and 10% polyacrylamide gels, several conclusions can be drawn.

First, there is little or no RNA synthesis during post-activation lag. This result was not evident in previous studies (Giri and Ennis, 1978). Secondly, tRNA synthesis appears first and the amount of label in the 25s, 17s, 5.8s and 5s RNAs increases with each pulse period, suggesting a generalized increase in the rate of synthesis with germination. This may result from an increase in the amount of polymerase activity transcribing these species, or from a relaxation of the chromatin structure in the region of these genes increasing their availability to transcription.

Third, transfer RNA synthesis did not initiate until after the second hour of germination. The largest amount of tRNA synthesis occurs during myxamoebae emergence. Giri and Ennis (1978) suggested that tRNA is synthesized first. However their sucrose density gradients could not resolve 4s from 5.0s or 5.8s RNA. Fourth, poly(A)+ RNA appeared to peak around the middle of swelling and dropped slightly with emergence. This peak in poly(A)+ RNA synthesis is consistent with other results showing
that a large number of proteins are being synthesized at this time which include trehalase, (Cotter and Raper, 1970), beta-glucosidase (Tisa and Cotter, 1980), alkaline phosphatase (Tisa and Cotter, 1979), and actin (MacLeod et al., 1980). The data of Giri and Ennis (1978) suggest that approximately 50% of all the new RNA synthesized at any time during germination is poly(A) containing. I have attempted to repeat their results using DMSO activation and oligo-dT cellulose chromatography, but with no success. At no time did I find the poly(A) content of the labelled RNA to be above 3% during the first two hours of germination (data not shown).

The requisite for RNA synthesis during fungal spore germination is uncertain when a number of fungi are examined. Many of the studies directed in this area are based on the use of RNA synthesis inhibitors. Germination has been found to occur in Blastocladiella emersonii (Lovett, 1968) and Alomyces arbuscula (Burke et al., 1972) in the presence of actinomycin D. The pycnidiospores of E. theobromae can germinate in the presence of daunorubicin (Brambl et al., 1979). The absence of RNA synthesis also does not affect the germination of Alternaria soloni (Hollomon, 1970) and Pernospora rubacina (Hollomon, 1979) or the mitospores of Alomyces macrogynus (Smith and Burke, 1980). Fungal spores that appear to require RNA synthesis include
Aspergillus nidulans (Emerson and Humphrey, 1970) and Neurospora crassa (Inoue and Ishikawa, 1970).

The resistant nature of the fungal spore makes finding a reliable inhibitor difficult. In our examination, only the inhibitor 4-nitroquinoline-1-oxide appeared effective enough to block RNA synthesis completely without affecting spore germination kinetics or O₂ uptake. Irradiation with ultraviolet light demonstrated the same stage specific phenomena. RNA synthesis is inhibited and the spores remain irreversibly blocked at the swelling stage of germination. Both these inhibitors exploit the inability of Dictyostelium discoideum spores to carry out excision repair (Demsar and Cotter, 1981).

It has been proposed that the excision repair enzymes may appear later during vegetative growth (Hashimoto and Wada, 1980). Additionally, I have found that UV and 4-NQO can block emergence and RNA synthesis in the microcysts of Polysphondylium pallidum (Hamer, unpublished results). It is conceivable in the light of other similarities (Tisa and Cotter, 1980b) that all three resting stages of the Dictyosteliales may possess this excision repair deficiency.

4-Nitroquinoline-1-oxide is a potent mutagen and carcinogen, termed a UV 'mimic'. It has been demonstrated that 4-NQO damaged DNA is subject to the same repair system that is induced following irradiation, namely nucleotide excision repair (Setlow, 1968). Support for
this work comes from the fact that 4-NQO purine adducts can be excised from the DNA of wildtype E. coli (Ikenaga et al., 1975) as well as from normal human cells but not from E. coli uvr A- and xeroderma pigmentosum cells (Cleaver, 1968) which are unable to excise pyrimidine dimers. Preliminary evidence suggests that dormant spores are sensitive to low quantities of 4NQO for the reasons stated above. There also appears to be a time requirement for 4-NQO to block emergence (see Table 7). This delay could be explained by the time required for the chemical activation of 4NQO to its active form (see results section F). As expected 4NQO is a potent inhibitor of RNA synthesis during germination, when added early. However, later addition of the drug appears to be less inhibitory (Fig. 19). This may be due to the time requirement involved in the drug's activation. Currently experiments are underway to determine if the vegetative amoebae of Dictyostelium discoideum are capable of repairing 4-NQO induced damage.

Ennis (1981a) has demonstrated that the anthracycline antibiotic, nogalamycin, can inhibit RNA synthesis by 88% in germinating spores, while only blocking emergence. This conclusion may be premature, since the residual 12% of the label in the nogalamycin treated spores occurs in complete transcripts. We also found that there were three nogalamycins available from Upjohn Co.: U12,241,
drug he used. All three require solubilizing in 95% ethanol before use and only U12,241 and U15,167 block RNA synthesis to any extent and inhibit myxamoebae emergence. Finally, the presence of 0.95% ethanol in the control cultures drastically altered heat-induced germination kinetics. It may be possible that low concentrations of ethanol do not affect germination in DMSO-activated, strain B, spores. However the application of other methods of blocking RNA synthesis proved more useful.

Irradiation of dormant spores with ultraviolet light specifically blocks the emergence stage of germination without significantly affecting the earlier three stages. It has long been known that spores of the cellular slime mold are more sensitive to UV light than their vegetative counterparts (Gilles et al., 1976; Liverant and Pereira de Silva, 1976; Ford and Deering, 1979); however, the physiological phenomena associated with this sensitivity have only recently been investigated (Demsar and Cotter, 1981).

The primary target of UV has been found to be the formation of pyrimidine dimers on DNA templates (Squerbier, 1976). Lesions of this sort have been found to cause depressed rates of RNA synthesis due to UV induced chain termination sites (Fukuyama et al., 1967;
Sauerbier et al., 1970). In the irradiated spores of *D. discoideum* these same events seem to occur. RNA synthesis in UV treated spores is inhibited by over 90% when measured by the incorporation of $^{3}$H-uracil. Furthermore, the level of labelled precursor accumulation (in spores irradiated prior to emergence) suggests the presence of UV induced chain-termination sites which abruptly stop the production of complete transcripts. Electrophoretic analysis of RNA extracted from pulse-labelled irradiated spores supports this finding (Fig. 25). Thus the residual 10% of the label appears to accumulate in prematurely terminated transcripts. This data also demonstrated that UV has no effect on uracil uptake.

Irradiation with ultraviolet light clearly demonstrates that RNA synthesis is not required for the swelling stage of germination. An immediate objection is that ultraviolet light may affect other metabolic processes besides the production of thymine dimers in DNA. However this criticism should be carefully weighed. The mode of action of ultraviolet light on eucaryotic cells has been extensively researched, much more so than the mode of action of any chemical inhibitor; thus this criticism holds much more for studies involving inhibitors than ultraviolet light. Studies on eucaryotic cells involving radiation mapping of transcriptional units are
founded on the mode of action of ultraviolet light, being the formation of thymine dimer on the DNA template (Miskin et al., 1981).

The spore swelling stage of germination in D. discoideum is extremely sensitive to minute environmental changes (Cotter et al., 1979). Small changes in the dissolved oxygen concentration, low concentrations of various solvents, small temperature changes, the age of the spore itself, will all alter the kinetics of spore swelling. Despite this rigid sensitivity, irradiated spores take up oxygen and swell with normal kinetics. Therefore, in the light of these findings and others stated elsewhere, we find the above criticism unsupported.

Using ultraviolet irradiation as a biological probe we have been able to determine which RNA species (if any) are limiting germination in the UV blocked spore. Polyacrylamide gel electrophoresis reveals that spores have a total complement of cellular RNAs before the onset of germination. Dormant spores are also known to contain a population of preformed mRNAs capable of being translated (Giri and Ennis, 1978). The tRNA of dormant spores is also capable of being charged. Thus no particular class of RNA appears to be limiting at the onset of germination. When spores are irradiated the 25s, 17s, 5s, 5s and 4s RNAs remain intact. Therefore, the normal synthesis of these species during germination
may represent preparation for vegetative growth rather than a requirement for spore germination. It has been proposed that the cellular slime mold may lack control mechanisms for regulating rRNA synthesis during development (Loomis, 1975) and thus germination may contain both germination specific and growth specific synthesis.

Poly(U) hybridization is a convenient method for determining the distribution of poly(A) containing RNA in a cell (Bishop et al., 1974). Nonirradiated germinating spores undergo an approximate twofold increase in their poly(A) content during germination. Irradiated spores however show a dramatic decrease in their poly(A) containing RNA during germination. These data are consistent with the idea that poly(A)+ RNA synthesis is inhibited by damaged DNA templates, and that poly(A)+ RNA may be one of the limiting components required for complete germination leading to the release of free myxamoebae.

Since ultraviolet irradiation appears to be a convenient means of terminating RNA synthesis, an attempt was made to measure the half-life of the poly(A)++ RNA during germination. As of yet there has not been a comparable study in any other fungal spore. To date there have been three extensive studies on mRNA decay during growth and development of D. discoideum; Palatnik.
et al. (1980), Margolskee and Lodish (1980), and recently Ennis (1981). In all of these studies inhibitors have been used to terminate RNA synthesis and the decay of the poly(A)+ RNA has been assessed by a number of techniques. During growth, the half-life of the poly(A)+ RNA is approximately 240 minutes, which shortens slightly with the onset of multicellular development.

Using a filter hybridization technique the poly(A)+ RNA half-life during germination was found to be approximately 15 minutes. Several findings support this result; (1) the rate of cellular protein synthesis following irradiation drops after fifteen minutes (Fig. 23); (2) Ennis (1981a) has speculated that mRNAs may decay rapidly during germination since the duration of synthesis of certain stage specific proteins is extremely short; (3) Densar et al., (submitted) has shown that the accumulation of certain stage specific enzymes is terminated within 20 minutes after irradiation. Recently, the laboratory of Dr. Lodish has demonstrated that the bulk of the developmentally programmed transcripts that appear following aggregation, decay with a half-life of about 15-20 minutes (Chung et al., 1981). Additional half-life studies on the developmentally programmed discoidin I mRNA indicate that this transcript decays with a half-life of about 20 minutes (Adrien Tsang, personal communication). Thus a precedent for a rapidly
decaying mRNA population in *D. discoideum* has been established.

It should be stated that this measurement of mRNA half-life is of bulk mRNA and therefore an approximate average. This fact has been well documented in yeast cells. The half-life of bulk mRNA in yeast (measured by studying polysome decay, etc.) is about 20 minutes (Kuo et al., 1973; Hartwell et al., 1970; Hartshison et al., 1967). However Chia and McCaughlin (1979) studied the disappearance in synthetic capacity of mRNA by using a temperature sensitive strain, ts136, which was defective in cytoplasmic RNA production at 37°C. Cell cultures were pulse labelled with $^35$S-methionine and then shifted to the nonpermissive temperature. The radio-labelled proteins were displayed on 2-dimensional gels at various time intervals. Thus, the disappearance of individual mRNA species could be monitored.

Messenger RNA half lives ranged from 4.5 to 41 minutes with an average value of 22 minutes for 80 individual mRNAs. The authors point out that the kinetics of exponential decay and their sum closely parallels the decay of the overall rate of protein synthesis. It is likely that a similar diversity in the mRNA half-lives exists in the cellular slime molds. While our estimate represents an average of mRNA half-lives, a short mRNA decay is consistent with the idea that spore germination...
is largely under transcriptional control (Dowbenko and Ennis, 1980).

Prior work on the effects of ultraviolet light on germinating spores elucidated a control period for myxamoebae emergence, termed 'competency' (Demsar and Cotter, 1981). 'Competency' during heat-induced spore germination in *D. discoideum* has been defined as a period 30 minutes prior to the release of myxamoebae when germinating spores become resistant to ultraviolet irradiation (Demsar and Cotter, 1981). Irradiation as little as fifteen minutes after this point allows for increase in the final myxamoebae population. Irradiation any time prior to this thirty minute period prevents emergence. From the above findings on the effects of UV irradiation on spore germination we can postulate that competency may be a period when the essential gene product(s) for emergence are synthesized. These products may assist or regulate the degradation of the inner spore wall (Cotter, 1975). Metabolic events prior to the competency point may be required for germination; however, alone they are not sufficient for complete germination. Further work is under way to characterize this transcriptional regulatory period.
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Protein synthesis during fungal spore germination.


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