QUANTITATIVE CYTOPHOTOMETRIC AND AUTORADIOGRAPHIC ANALYSIS OF THE NUCLEAR CHANGES IN THE MYXOMYCETE *DIDYMIUM IRIDIS* DURING GROWTH AND DEVELOPMENT OF THE PLASMODIUM

by

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ABSTRACT

QUANTITATIVE CYTOPHOTOMETRIC AND AUTORADIOGRAPHIC ANALYSIS OF THE NUCLEAR CHANGES IN THE MYXOMYCETE DIDYMIUM IRIDIS DURING GROWTH AND DEVELOPMENT OF THE PLASMODIUM

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Microspectrophotometric and cytochemical examinations were made of the nuclear changes in basic protein, RNA, and DNA in the developing plasmodium of Didymium iridis, at the fourth, eighth, and twelfth day of its progression through the growth cycle prior to sporulation. Data was collected from cultures which had no indications of differentiation and there was no renewal of growth medium made during the course of the investigation. Relative determinations of basic protein and RNA were obtained using the double staining technique of Napthol Yellow-S/Azure B in conjunction with absorption microspectrophotometry. Cytophotometric measurements of the Feulgen-DNA nucleal reaction provided quantification of DNA. Autoradiography using tritiated thymidine and liquid-emulsion techniques provided information on periods of DNA synthesis.

The results of the investigation include a net decrease in protein and RNA to a point of stabilization which correlates well with increased age of the organism and decreased nutrient supply in the cultures. No loss of DNA was observed under either of these conditions. These findings are in complete accord with macroscopic observations of the initially rapid then decreasing growth rates

until a point was reached where actual decrease in plasmodial mass was detected. Feulgen-autoradiography results showed the cultures of four and twelve day plasmodia in the replicated G_2 condition. The 4C DNA content of the four day organisms indicates rapid growth and high metabolic activity but it has been determined that the replicated DNA of the twelve day organisms would not have been succeeded by a mitotic division until some later time, presumably following the initial sporulation processes. Microspectrophotometric determinations of DNA in the eight day cultures showed a 2C DNA content, indicating no synthesis occurs during this period. This implies that a G_1 period of some duration exists in the plasmodial slime mold which may extend up to the point of the final replication of DNA (G_2 condition) prior to sporulation. The observed morphological growth patterns also support these conclusions.

ACKNOWLEDGMENTS

I would like to express my deep gratitude for the guidance and assistance given by Dr. John J. Yemma in this study.

I am grateful to Justina Wilcox and Leonard Perry, members of the department staff, for their continual cooperation in helping me to secure chemicals and equipment necessary to pursue this investigation.

I would also like to thank Dr. O.R. Collins for providing the clones of *Didymium iridis* used throughout this study.

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INTRODUCTION

Didymium iridis, a myxomycete, or true acellular slime mold, has proven to be an invaluable tool for use in all types of investigations involving the cell cycle, including the processes of growth and differentiation. The naturally occuring synchrony of DNA synthesis and mitosis, which is of normal incidence in some parts of the life cycle, particularly in the plasmodial stage, in organisms of this type (Nygaard, Guttes, and Rusch, 1960; Guttes, Guttes, and Rusch, 1961; Braun, Mittermayer, and Rusch, 1965) permit analyses based on what could be considered a single macroscopic cell. The stages in the life cycle of this group have been well defined and are described in detail by Gray and Alexopoulos (1968). The simple culturing techniques required to maintain *D. iridis* in this study, have been described by Collins (1963).

A general review of the complete life cycle of this organism would serve to clarify the mode of development and ploidy level of the vegetative or plasmodial stage used in this study (see Plate 1).

Each germinating spore of *D. iridis* produces one haploid myxamoeba or swarm cell (biflagellate), these two forms being interconvertible, the latter being dependent on aqueous environmental conditions. The population of cells then increases in size by successive mitotic divisions until achieving the log phase of growth. These cells can then serve as gametes in sexual fusions. If plasmodial production requires that fusing cells to be of separate but compatible mating types, it is homothallic.

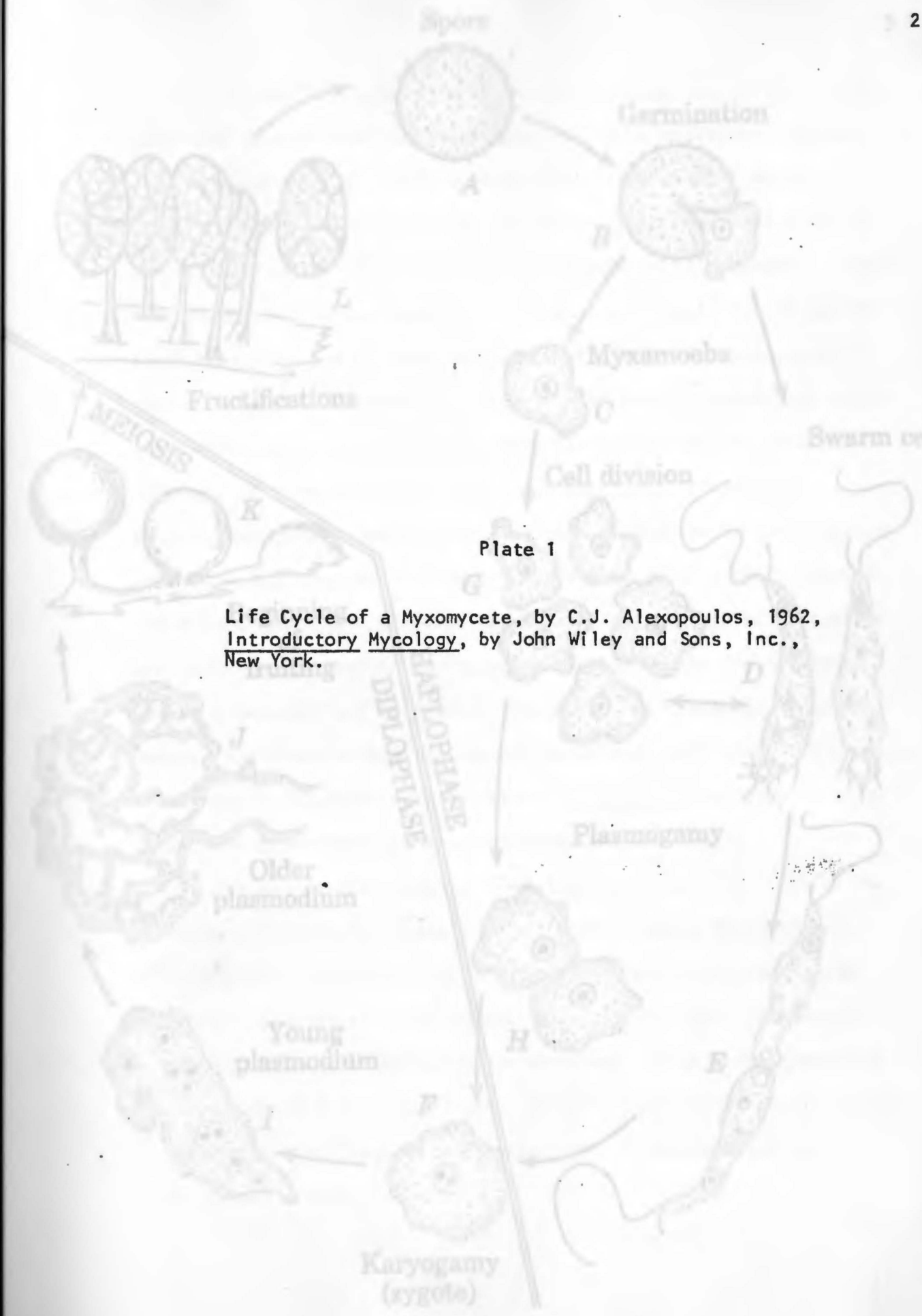
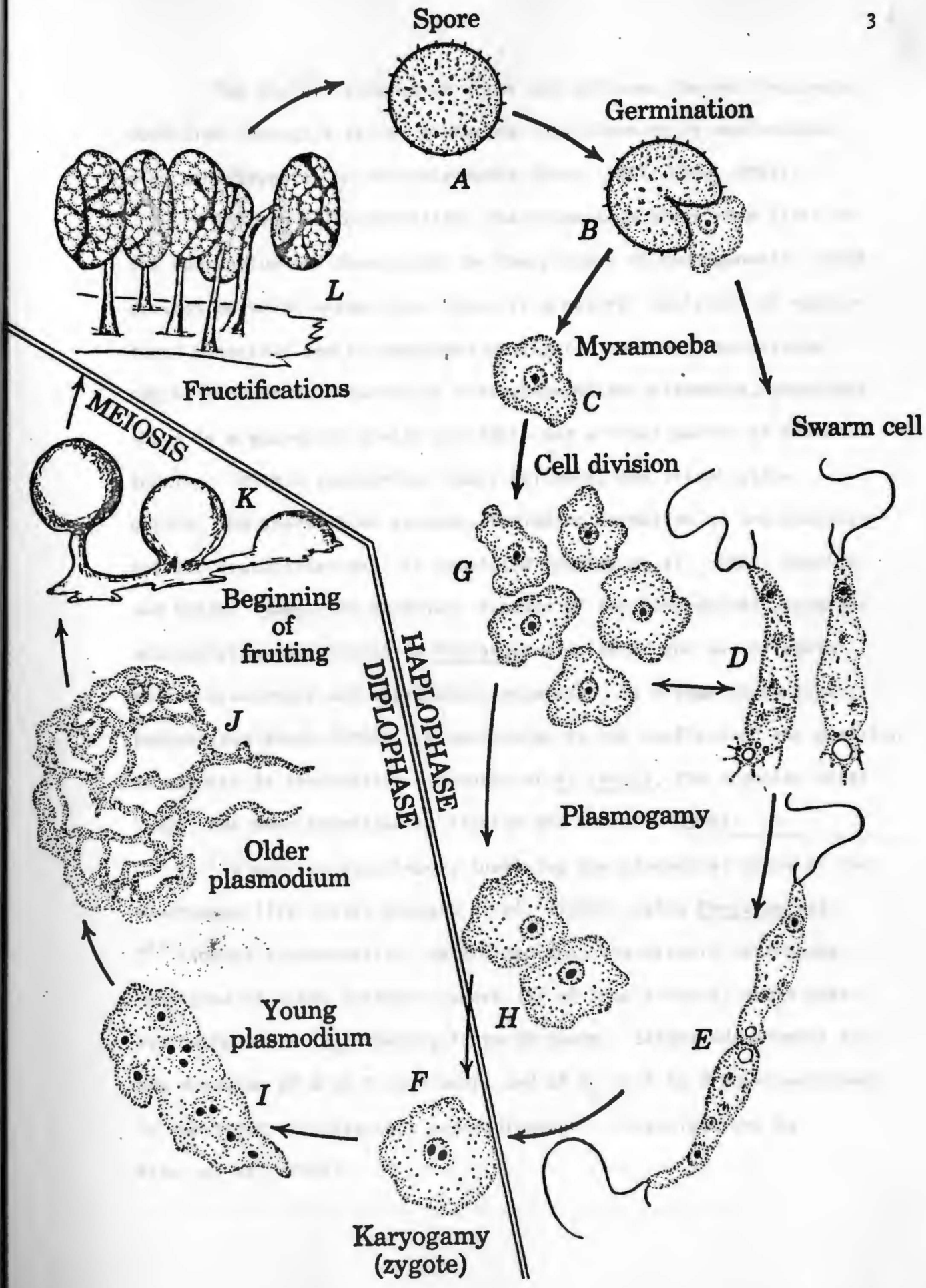


Plate 1

Life Cycle of a Myxomycete, by C.J. Alexopoulos, 1962, Introductory Mycology, by John Wiley and Sons, Inc., New York.


Spore
A
Germination
B
L
Fructifications
Myxamoeba
C
Swarm cell
Cell division
MEIOSIS
K
G
Beginning
of
fruiting
D
HAPLOPHASE
DIPLOPHASE
J
Plasmogamy
Older
plasmodium
H
E
Young
plasmodium
F
I
Karyogamy
(zygote)

The diploid plasmodium grows and achieves the multinucleate condition through a series of nuclear divisions or by coalescence with other zygotes or microplasmodia (Kerr, 1961; Ross, 1967).

Through differentiation, the plasmodium gives rise first to the sporangium and then spores by the process of sporogenesis. This process normally ensues when there is a natural depletion of nutritional materials and is dependent on light. However, sporulation can be induced by a period of starvation of the plasmodia, providing there is a source of niacin available and a brief period of illumination. Within twenty-four hours following the illumination period, the sporulation process, including formation of the characteristic fructifications, is completed (Guttes et al., 1961; Cummins and Rusch, 1968). An excellent account of the biochemical processes accompanying sporulation in Physarum, elucidated the use of radioactive precursors and extraction procedures, is presented by Sauer, Babcock and Rusch (1969). A resolution to the conflict of the position of meiosis in sporulating plasmodia of D. iridis, the organism under study, has been submitted by Aldrich and Carroll (1970).

In earlier experiments involving the plasmodial phase of the myxomycete life cycle, Nygaard et al. (1960), using Physarum and C^{14} isotope incorporation, determined that the mitotic interphase consisted of a DNA synthetic phase (S) of 1 to 2 hours, and a post-synthetic phase (G_2) lasting 12 to 20 hours. Slight adjustments in the duration of S to 3 to 4 hours and of G_2 to 6 to 8 hours were made in the light of subsequent autoradiographic investigations by Braun et al. (1965).

Further studies concerning the chemical analysis of D. iridis plasmodia and closely related myxomycetes, include microspectrophotometric determinations of the DNA content in plasmodia, myxamoebae, and sporangia of D. iridis by Therrien (1966), Yemma and Therrien (1972), and Therrien and Yemma (1974). Rusch (1969) performed extensive investigations of Physarum plasmodia from growth in the vegetative stage through the changes accompanying the sporulation process. The reported alterations observed in metabolic activity were determined by the use of extraction techniques and autoradiography. These studies demonstrated protein, RNA and associated nuclear DNA changes in that organism, which seem to compare quite well with those taking place in D. iridis as shown by the present study. Also of interest are recent studies carried out by Mohberg and Rusch (1971) analyzing the RNA, DNA and protein content of nuclei extracted at various stages of the life cycle of Physarum, in both growing and starving plasmodia. They reported high protein and RNA values in growing organisms and a decrease of RNA in starvation.

The purpose of this investigation is to elucidate the growth cycle of the heterothallic strain of Didymium iridis with special attention given to those events occuring during the development of the plasmodial stage. This has been accomplished by recording the changes in nuclear protein, RNA and DNA composition which occur as the organism ages. Also an attempt has been made to answer questions such as, "Do these changes agree with those known to occur in closely related organisms?"

To expedite the answering of this question, quantitative and qualitative determinations of DNA activity were obtained using

Feulgen-microspectrophotometry coupled with autoradiography. The validity of the quantitative aspect and specificity of the Feulgen reaction for DNA analysis has been well documented by Swift (1950), Lessler (1953) and others. The fact that both Azure B and Napthol Yellow-S bind stoichiometrically to RNA (Flax and Himes, 1952) and basic protein (Deitch, 1955), respectively, has provided investigators with a powerful tool for the quantitative determination of these cellular constituents. The reliability of microspectrophotometric measurements has been established by Ris and Mirsky (1949) and Mayall and Mendelsohn (1970), as well as by many others, using comparisons of results obtained by this method with those from extraction techniques. Relative determinations of basic protein and RNA were made cytophotometrically using the staining combination of Napthol Yellow-S/Azure B.

An analogous experiment using mammalian Chinese Hamster Cells (Kimball, Perdue, Chu, and Ortiz, 1971) has been reported. However, their results necessarily relate to an asynchronous population of cells that experience a pronounced G_1 period.

MATERIALS AND METHODS

Tissue Preparation

The cultures of Didymium iridis used in this study were originally obtained from Dr. O.R. Collins, Department of Botany, University of California, Berkeley. Isolates of compatible mating types, designated Honduran 1-2A[1] and Panamanian 2-7A[8] were investigated.

Cultivation of the Organism.

Clones of myxamoebae of Didymium iridis, developed from single spore isolates, were sustained on slants of half strength corn meal agar with Escherichia coli serving as a food source. The two isolates were transferred to separate plates of the sterile medium which had been previously inoculated with 2 ml. of E. coli suspension.

The medium was prepared by dissolving 8.5 grams of Difco Corn Meal Agar and 8.0 grams Difco Agar in 1.0 liter of distilled water and then autoclaved (Collins, 1963; Yemma et al., 1974). Subcultures from each clone to fresh media and bacterium suspension were made once the log phase of growth had been achieved, and transfers were made each week. All cultures of plasmodia and amoebae were maintained in an incubator at 21 degrees C (Yemma and Therrien, 1972; Yemma et al., 1974).

To obtain a plasmodium, one can mix (cross) amoebae of two compatible mating types (Honduran 1-2A[1] and Panamanian 2-7A[8]) on a plate of the medium to which E. coli suspension has been added (Collins, 1963; Therrien, 1966). The plasmodial phase is initiated by the sexual fusion of two haploid amoebae with different mating alleles (Yemma et al., 1974; Yemma, unpublished data) and was generally observed within two to three days after crossing. The resulting diploid plasmodium can increase in size by the growth of a single zygote or by the fusion of other zygotes or developing plasmodia (Ross, 1967). One hundred plates of the cross between the two clones were made and were examined every four hours under a low power (10X) microscope objective to record plasmodia formation.

Cultures of four, eight, and twelve day old plasmodia were used in this investigation. To secure plasmodia at each age, it was necessary to transfer any newly formed plasmodia to a separate plate of the medium supplied with the E. coli suspension. These transferred organisms were then allowed to grow for the required period of days without replenishing the food supply. This procedure insured that normal developmental changes from the plasmodial stage to sporulation would occur within a reasonable amount of time. Once the proper age was reached, the plasmodia were either fixed with 10% buffered formalin (pH 7.0) or were labeled with tritiated thymidine (H^3-thymidine), washed to remove the tag not incorporated, and then fixed.

Preparation of Material for Cytochemical Study

Plasmodia Used in Basic Protein/RNA Analysis

Plasmodia of each age group were transferred from the half strength corn meal agar to plates of 2% agar. The proceeding steps for preparation of the plasmodia for staining are those described by Yemma (1971). The cultures were flooded with 10% buffered formalin and fixed for 12 to 14 hours. Following fixation, plasmodia of each of the three age groups were pooled and washed twice in 70% ethanol. Post fixation in 70% ethanol for 12 hours, dehydration in a graded ethanol series, then clearing in two changes of xylene preceeded the embedding in paraffin blocks. Sections of each block were cut at 4 microns and were mounted on slides cleaned in an acid-alcohol solution then stained for quantitative protein/RNA analysis.

Plasmodia Used in Feulgen-DNA and Autoradiography Analysis

Preparation of Labeled Bacteria.

The labeled bacteria and the medium in which they were grown were prepared in the following manner:
The basic growth medium is made by dissolving 2.0 g. NH_4Cl, 6.0 g. Na_2HPO_4, 3.0 g. NaCl, 0.115 g. Na_2SO_4, 0.085 g. $MgCl_2 \cdot 6H_2O$, and 4.0 g. nutrient broth in 1.0 liter of distilled water. The solution is then sterilized for twenty minutes at 15 p.s.i. pressure in an autoclave. Exactly 0.004 g. of H^3-thymidine (specific activity 16.7 ci/mmole; from Schwartz Bioresearch Inc.) was dissolved in 10.0 ml. of sterile distilled water. The solution was drawn into a 10 ml.

syringe and a sterile Millipore filter (0.45 micron) was fitted to the end. The H^3-thymidine solution was dispensed into one liter of growth media. The bacteria were grown in 250 ml. sterile flasks of this medium in a 37 degree C shaker water bath. Before use, the labeled E. coli were centrifuged (3200 rpm for 15 minutes) and washed twice with sterile phosphate buffered saline (0.75 g. KCl, 0.58 g. NaCl, 2.26 g. Na_2HPO_4, 4.6 g. KH_2PO_4 and 1.0 liter of distilled water). Each final pellet of bacteria was diluted to 10.0 ml. with the buffer giving approximately 10^{10} labeled bacteria per milliliter.

Radioactive Labeling of Plasmodia.

Plasmodia of each age group were pooled on petri dishes of 2% agar and were allowed to migrate for two hours to insure viability. The plates were then flooded with 2 ml. of the above suspension of tritium labeled thymidine negative E. coli (10^{10}/ml.) and were incubated for 3 hours and 15 minutes. This incubation time was necessary to allow for a sufficient pulse for breakdown of the bacteria and assimilation of the tritiated precursor into the DNA, and also to insure that the tagged nuclei were in the S or G_2 stage of interphase of the cell cycle, but not beyond that. The plasmodia were then washed with phosphate buffered saline (pH 7.2) to rid the cultures of labeled bacteria not phagocytized, and were fixed in 10% neutral buffered formalin for 12 hours. A thymidine negative strain of E. coli (thymidine requiring due to an inability of the organism to produce thymidylate synthetase) was used since the wild type strains of the bacterium are able to convert thymidine.

to uracil (Simon and Tessman, 1963).

The fixed labeled plasmodia were washed and post fixed for 12 hours in 70% ethanol, dehydrated, cleared, and embedded in paraffin just as described for the plasmodia of the protein/RNA analysis. Blocks were sectioned at 4 microns and the tissue was mounted on slides which had been previously washed in chrome-sulfuric acid and then albuminized.

Cytochemical Methods

Protein/RNA Analysis

The combined stain of Napthol Yellow-S (NYS)/Azure B, as developed by Therrien et al. (1975), to quantitatively localize basic proteins and RNA on the same specimen was employed. The two stains have absorption maxima sufficiently different enough to allow separate quantitative determinations at two wavelengths microspectrophotometrically. The paraffin embedded sections of plasmodia were cleared in xylene and rehydrated in a series of descending concentrations of ethanol to distilled water. All staining of slides for each age of plasmodium was done simultaneously for uniform and comparable results. Prior to staining, treatment of the slides with Deoxyribonuclease (DNase; Worthington Biochemical Co.) was necessary to remove the DNA which would also stain metachromatically with Azure B (Jenson, 1962). Control slides were treated with DNase and later Feulgen stained. The DNase and subsequent staining procedure used, is as follows:

(1) Hydrated slides are dipped in boiling water for 5 seconds to remove formalin.

(2) Sections are incubated in a solution of 15 mg. $MgSO_4 \cdot 7H_2O$, in 100 ml. distilled water, adjusted to pH 6.8) for 4 hours at 38 degrees C. (Brachet, 1953; Das, Patau, and Skoog, 1958).

(3) Slides are rinsed in distilled water.

(4) Stain for 15 minutes in 1% Napthol Yellow-S (C.I. # 10316) in 1% acetic acid.

(5) Differentiate 15 to 24 hours in 1% acetic acid.

(6) Dehydrate in 3 - thirty minute rinses of tertiary butanol (TBA).

(7) Hydrate in descending ethanol series to distilled water.

(8) Stain for 2 hours at 50 degrees C in Azure B (pH 4.0). (C.I. # 50210)

(9) Dip in water

(10) Make 3 - thirty minute changes in TBA.

(11) Differentiate over night (12 hours) in TBA.

(12) Transfer to xylene and apply coverslips with Preservaslide mounting medium (Matheson, Coleman, and Bell).

Napthol Yellow-S will bind stoichiometrically to basic residues of lysine, arginine and histidine (Deitch, 1955). To test the NYS staining reaction, control slides were acetylated in 1% glacial acetic acid in absolute acetic anhydride for one hour at 60 degrees C then rinsed in 100% ethanol (Monne and Slauterback, 1951; Deitch, 1955). This reaction blocks the epsilon amino group of lysine and terminal amino groups.

The representative acetylation reaction is

$$R\text{-}NH_2 + (CH_3CO)_2 \text{ ----- } R\text{-}NHCOCH_3 + CH_3COOH$$

(Monne and Slauterback, 1951).

Slide preparation for autoradiography and DNA analysis.

Sections of the H^3-thymidine labeled plasmodia were cleared in xylene to remove the paraffin and hydrated to distilled water through a descending ethanol series. Slides were then prepared for liquid-emulsion autoradiography according to the protocol used at the Los Alamos Scientific Laboratory, Los Alamos, New Mexico. The technique is described in detail by Prescott (1964) and proceeds as follows:

(1) Kodak NTB-3 liquid emulsion is warmed in total darkness in a water bath at 43 degrees C for 45 minutes. Using a Wratten series number 1 safe light filter over a 15 watt bulb, add 10 ml. of emulsion to 30 ml. of deionized water in a coplin jar, stir gently and return jar to water bath.

(2) Dip a clean slide into the emulsion and allow to dry for 3 hours in the dark. Develop and check for background grains. If the emulsion is fresh, background should be very low.

(3) Dip slides, one at a time, into the emulsion for 5 seconds.

(4) Wipe emulsion from the back of the slides and place in a light tight box in a rack, to dry (3 to 4 hours).

(5) Store slides in boxes at 4 degrees C, developing a slide periodically to determine exposure time. Six weeks was found to be a suitable duration for exposure in this investigation.

(6) Slides are developed for 2 to 4 minutes in complete darkness in Kodak D-19 developer cooled to 18 degrees C before use. Rinse in water for 10 seconds, then place in Kodak acid-fixer (at 18 degrees C) for 5 minutes.

(7) Slides are then washed in running water for 30 minutes and rinsed in distilled water, then stained.

During the period of exposure, the labeled specimen is covered with nuclear emulsion and they remain in contact for a period of time. During this time the radioactive atoms within the specimen (H^3) decay. The beta particles from the tritium strike the silver bromide within the emulsion forming a latent image of metallic silver. Later the silver bromide with latent images is then further reduced by the developer and the subsequently formed silver grains can be used to localize structures within the cell or organism (Perry, 1964). Due to the low energy of the beta particles of tritium, thin sectioning, and close contact between emulsion and specimen, the latent image produced will be just above the radiation source, which in this case, is the nucleus (Cleaver, 1967).

That tritiated thymidine is a specific label for DNA and therefore can be useful in studying growth and maturation of cell populations has been well documented (Reichard and Estborn, 1951; Lu and Winnuck, 1954; Cleaver, 1967). However, there are some assumptions that must be made about the labeled compound when used in these studies. They are as follows (Cronkite et al., 1959):

(1) Labeled thymidine does not exchange with the unlabeled compound after it has been incorporated into DNA, and is stable.

(2) DNA turnover is due solely to mitosis and death.

(3) Thymidine is not stored for any significant period of time within the cell before it is used in DNA synthesis.

(4) DNA synthesis destines a cell to divide.

(5) There is no significant radiation injury to tissues caused by the H^3 beta particles.

(6) In vivo labeling makes it possible to elucidate cell kinetics.

From experimental work previously done using tritiated thymidine labeling of DNA, there is no evidence to indicate a significant loss of label in the subsequent procedures of dehydration and paraffin embedding (Rogers, 1967). However, hydrolysis of tissue during the Feulgen reaction is known to cause substantial loss of label if hydrolysis time exceeds that necessary for maximum staining intensity. At this point, pyrimidine bases of DNA are disrupted and H^3-thymidine is lost (Cleaver, 1967). For this reason, hydrolysis of a slightly shorter duration was used.

The autoradiographs obtained by the method described were not used for quantitative grain density determinations, but for qualitative purposes. However, control steps as suggested by Rogers (1967) were taken to increase the accuracy of the results. Control slides of unlabeled plasmodia were treated concurrently with the experimental slides to determine background (silver grains developed from sources other than the radioactivity of the specimen). Also, experimental slides were exposed to light and then returned to the light tight boxes with the other autoradiographs to undergo identical conditions of exposure and development. This precaution

serves to detect instances where the emulsion may not be recording radioactivity as developed silver grains, as in instances when there is a chemical interaction of the emulsion and specimen or when there is a fading of the latent image during exposure. This latter possibility is not likely, though, since tritium labeled liquid-emulsion autoradiographs have been reported to have undergone exposures of up to eight months with little detectable loss of latent images (Prescott, 1964). To insure uniformity of grain development, all sections compared were cut at four microns and were exposed and developed simultaneously (Fitzgerald and Carol, 1970).

Background was determined using similar areas on control and experimental slides. Grain counts over a measured area of the slide, away from radioactive regions, were made and the mean number of background grains per unit area was subtracted when determinations of whether or not a nucleus was positively labeled were made (Rogers, 1967).

Slides were stained at this point, following development, utilizing the Feulgen reaction for quantitative DNA determinations. This dual technique provides for localization of radioactive precursor (H^3-thymidine) and subsequent DNA measurement on the same nuclei.

The slides were examined under oil immersion for H^3-tagging of nuclei. Entire pieces of plasmodium were randomly chosen and coordinates of the microscope stage were recorded for each piece. This procedure would allow for DNA determinations to be made on the same nuclei as were included in the frequency determinations, once

the silver grains were removed for DNA analysis. The frequencies of labeled nuclei in each piece of plasmodium and the size of the nuclei, as determined using an ocular micrometer, were then noted for organisms of all three ages.

The silver grains were removed from the slides by placing them in a solution of equal parts of 5% sodium chloride and 5% cupric sulfate for thirty minutes at room temperature (Bloch, et al., 1967). Coverslips were mounted using Cargille's index of refraction oil (R.I. = 1.564) to minimize light loss due to scatter from refractive index boundaries. DNA determinations were then made on these same tissues.

Feulgen-DNA Analysis

To localize and quantify deoxyribonucleic acid (DNA), the Feulgen reaction was used (Feulgen and Rossenbeck, 1924; as modified by Bryant and Howard, 1969). Hydrolysis was carried out at room temperature for 35 minutes in 5 N hydrochloric acid rather than in 1 N hydrochloric acid at 60 degrees C for 8 minutes (DeCosse and Aiello, 1966) since the former method was found to produce optimum staining results with the organism used here (Yemma and Therrien, 1972).

Feulgen Staining Procedure

The following steps for the Feulgen stain were employed:

(1) Hydrolysis in 5 N HCl for 35 minutes at room temperature.

(2) Stain for 1 hour in Schiff's reagent (Lillie, 1951) fortified with freshly mixed 10% potassium metabisulfite in a ratio

of 1 to 4 with the reagent (10 ml. to 40 ml. Schiff's reagent). The basic fuchsin used to prepare the dye was from Fisher Scientific Company (C.I. #42500).

(3) Rinse three times for 5 minutes each in bisulfite rinse prepared from 5 ml. of 1.0 N HCl, 5 ml. 10% potassium metabisulfite and 100 ml. of distilled water.

(4) Rinse in distilled water.

(5) Dehydrate in ascending ethanol series.

(6) Clear in xylene and mount in Cargille's index of refraction oil (R.I. = 1.564).

Cytophotometric Methods

Protein/RNA Measurements

A Zeiss Type 01 microspectrophotometer was used for all cytophotometric determinations and all measurements were made with a Planachromat oil immersion objective, N.A. 1.30, X 100. The alignment of the instrument and linearity of the phototube were checked each time the instrument was used. A Zeiss continuous interference-filter monochromator was used to isolate chosen wavelengths of light.

The slides of plasmodia used in the determination of basic protein and RNA were double stained with Napthol Yellow S and Azure B as described previously. A representative spectral absorption curve for the combined stain was used to determine the proper wavelengths at which to make measurements (see Figure 1).

Mean relative basic protein/RNA values were determined microspectrophotometrically using the method for spheres as described

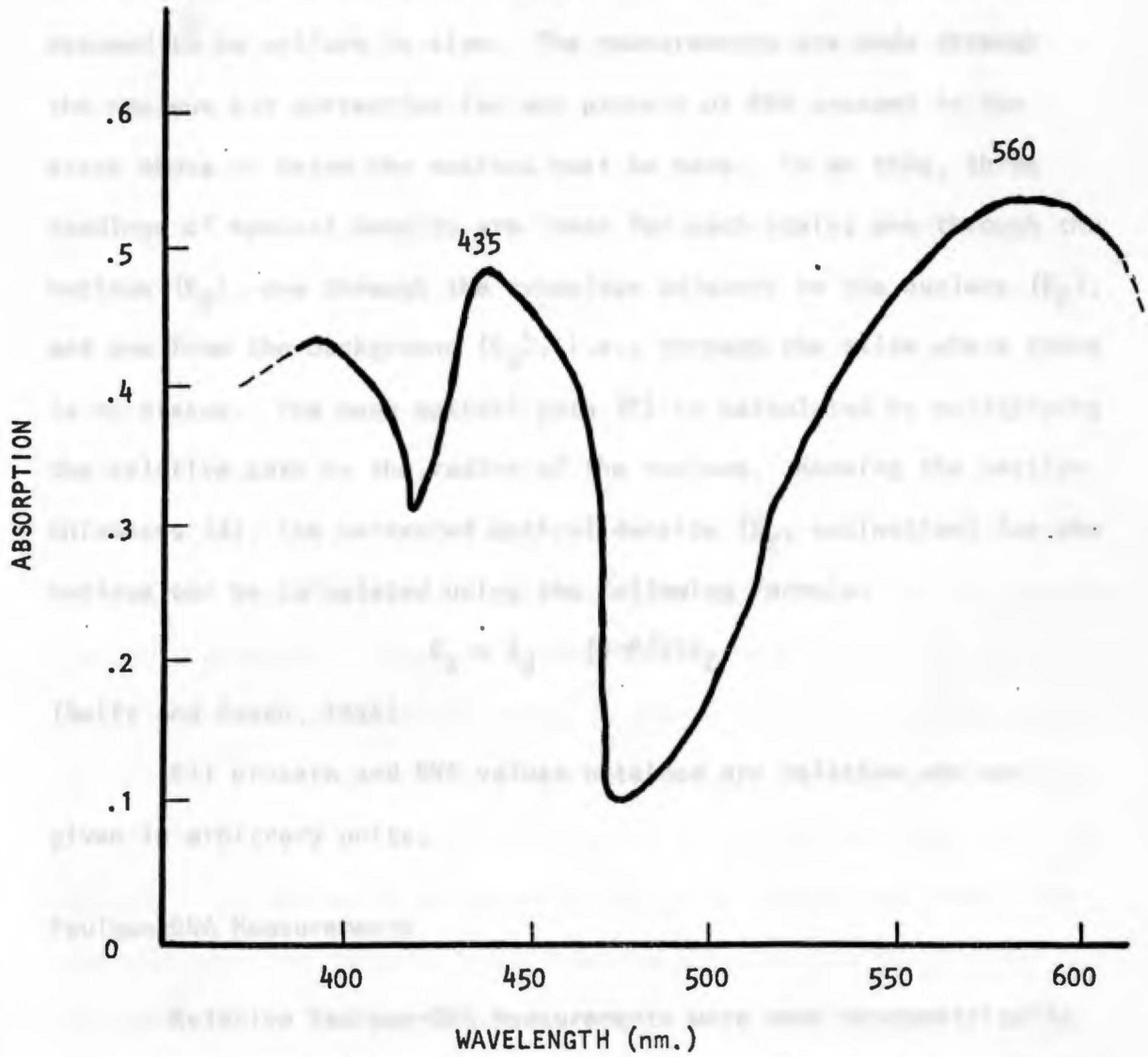


Fig. 1.--Absorption spectrum of Napthol Yellow S/ Azure B stained plasmodia of Didymium iridis.

by Swift and Rasch (1956). An ocular micrometer was used to determine average nuclear sizes based on 100 randomly chosen nuclei from each experimental group. Nuclei of each age group of plasmodia were assumed to be uniform in size. The measurements are made through the nucleus but correction for any protein or RNA present in the areas above or below the nucleus must be made. To do this, three readings of optical density are taken for each stain; one through the nucleus (E_N), one through the cytoplasm adjacent to the nucleus (E_C), and one from the background (E_0), i.e., through the slide where there is no tissue. The mean optical path (P) is calculated by multiplying the relative path by the radius of the nucleus. Knowing the section thickness (d), the corrected optical density (E_X, extinction) for the nucleus can be calculated using the following formula:

$$E_X = E_N - (1-P/d)E_C$$

(Swift and Rasch, 1956).

All protein and RNA values obtained are relative and are given in arbitrary units.

Feulgen-DNA Measurements

Relative Feulgen-DNA measurements were made photometrically (Ris and Mirsky, 1949) once the silver grains had been removed from the slides. Nuclei in those sections of plasmodia previously mapped to determine labeling frequency, were examined. Instrumentation was checked for proper alignment prior to use, as formerly stated. In this study, the two-wavelength method for quantitative Feulgen-DNA measurement (Patau, 1952; Ornstein, 1952; Mendelsohn, 1961) was utilized. Optical density measurements made by this method are desirable

since it minimizes the distributional error (caused by heterogeneity of staining within the specimen) and eliminates the need for direct measurement of nuclear area (Mayall and Mendelsohn, 1970). The selection of the two wavelengths is critical for reliable estimation of the absorbing material and they should give specific absorptivities of two to one for the chromophore when uniformly stained material is in the field. Once the wavelengths are chosen, areas with heterogeneous dye distributions can be measured. Hydrolysis and staining of all material should be done at one time to produce comparable results and absorption curves for the chromophore-molecular complex that are identical (Swift and Rasch, 1956).

Several absorption curves for the Feulgen stained specimens were made. A characteristic spectral absorption curve for the Feulgen stained plasmodia is shown in Figure 2. The two wavelengths suitable for the dye were chosen conforming to the extinction requirements of the method. The photometric field was adjusted to completely circumscribe the nucleus with a minimal amount of unoccupied space past its borders. The amount of chromophore (M) to be determined within the measured area (A) is calculated from the equation $M = KAL_1Q$. The constant, K, was eliminated in this study since only relative values and not absolute values, were necessary. Transmissions (T_1 and T_2) were taken at wavelengths 1 and 2 for each nucleus and from these values, L_1 and L_2 were computed so that $L_1 = (1-T_1)$ and $L_2 = (1-T_2)$. The ratio, Q, corresponds to L_2/L_1 and can be used to determine the correction factor (C) for the distributional error (Swift and Rasch, 1956; Leuchtenberger, 1958). The C value that corresponds to a particular Q ratio can be found in a table formulated by Patau (1952).

All calculations of the data compiled for the relative values of basic protein, RNA, and DNA have been done with an IBM 370 Model 145 computer for the sake of accuracy.

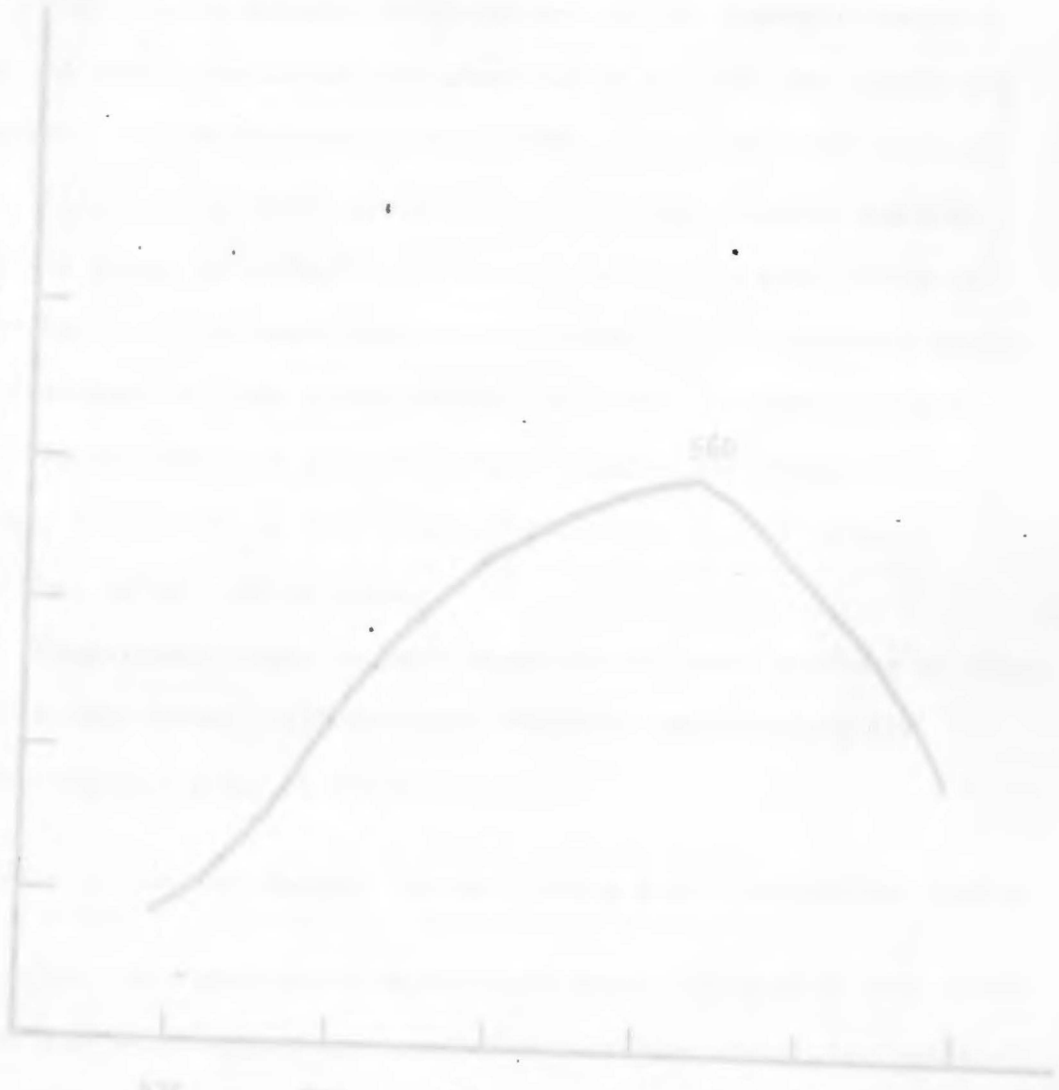


Fig. 2.--Absorption spectrum of Feulgen-stained plasmodial nuclei of Didymium iridis.

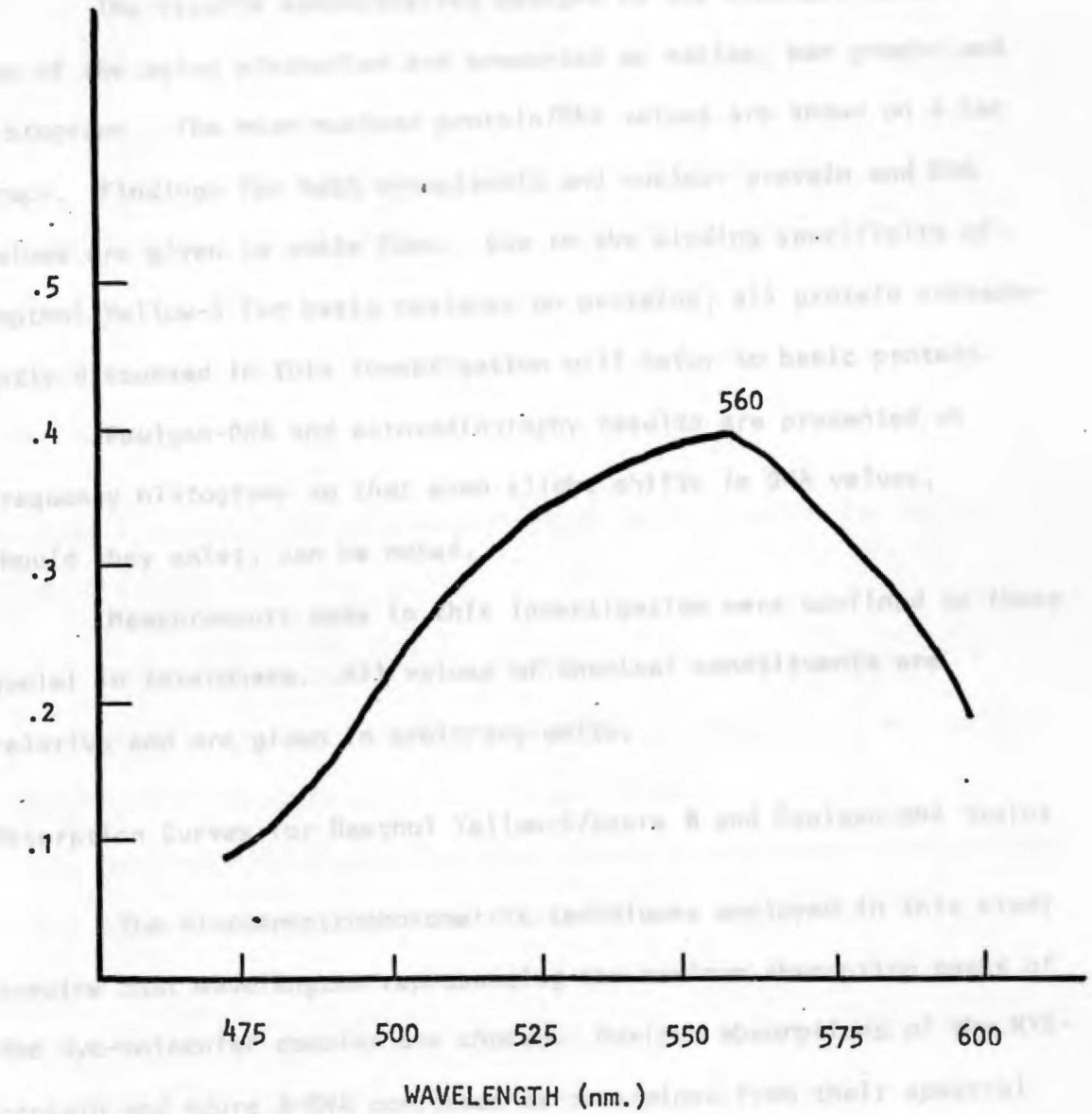


Fig. 2.--Absorption spectrum of Feulgen stained plasmodial nuclei of Didymium iridis.

RESULTS

The results demonstrating changes in the chemical composition of the aging plasmodium are presented as tables, bar graphs and histograms. The mean nuclear protein/RNA values are shown on a bar graph. Findings for both cytoplasmic and nuclear protein and RNA values are given in table form. Due to the binding specificity of Napthol Yellow-S for basic residues on proteins, all protein subsequently discussed in this investigation will refer to basic protein.

Feulgen-DNA and autoradiography results are presented on frequency histograms so that even slight shifts in DNA values, should they exist, can be noted.

Measurements made in this investigation were confined to those nuclei in interphase. All values of chemical constituents are relative and are given in arbitrary units.

Absorption Curves for Napthol Yellow-S/Azure B and Feulgen-DNA Stains

The microspectrophotometric techniques employed in this study require that wavelengths representing the maximum absorption peaks of the dye-molecular complex are chosen. Maximum absorptions of the NYS-protein and Azure B-RNA complexes as determined from their spectral absorption curves, were found to be 435 nm and 590 nm, respectively. Slides of NYS/Azure B stained plasmodia were used for determination of absorption maxima. Due to the instability of measurements of Azure B at 590 nm, caused by excessive refractivity at this wavelength, an alternate wavelength of 560 nm was used. This change was necessary

to insure the stability of absorbance readings and therefore ultimately, the accuracy of the results. The extinctions at this later wavelength were still at the peak of the absorption curve of the dye. The chosen wavelength does not overlap the absorbance range of the Napthol Yellow-S chromophore, thus permitting the two dyes to be used as "coupled stains".

A plot of the spectral absorption curve of Feulgen-stained plasmodial nuclei demonstrated the maximum absorption of the Feulgen-DNA complex at 560 nm and the half maximum at 500 nm, which is similar to that recorded by Yemma (1971) and many others who have employed this technique.

Microspectrophotometric Determinations of Basic Protein and RNA in Didymium iridis

Slides of plasmodia were stained with Napthol Yellow-S/ Azure B combination and relative quantitative measurements of basic protein and ribonucleic acid content were made.

Control slides were run simultaneously with experimental slides in the above staining procedures to ensure the strict specificity of each stain. Those controls treated with DNase and later Feulgen stained did not produce a staining reaction, indicating that DNA had been effectively removed and that Azure B had specifically stained only RNA. The controls acetylated with acetic anhydride, as described in materials and methods, and ran in NYS produced negligible staining and absorbance at 435 nm. This implies only minimal attachment and is probably due to some binding of the dye to the hydroxyl group of tyrosine (Therrien, 1967). The specificity of the dye for basic

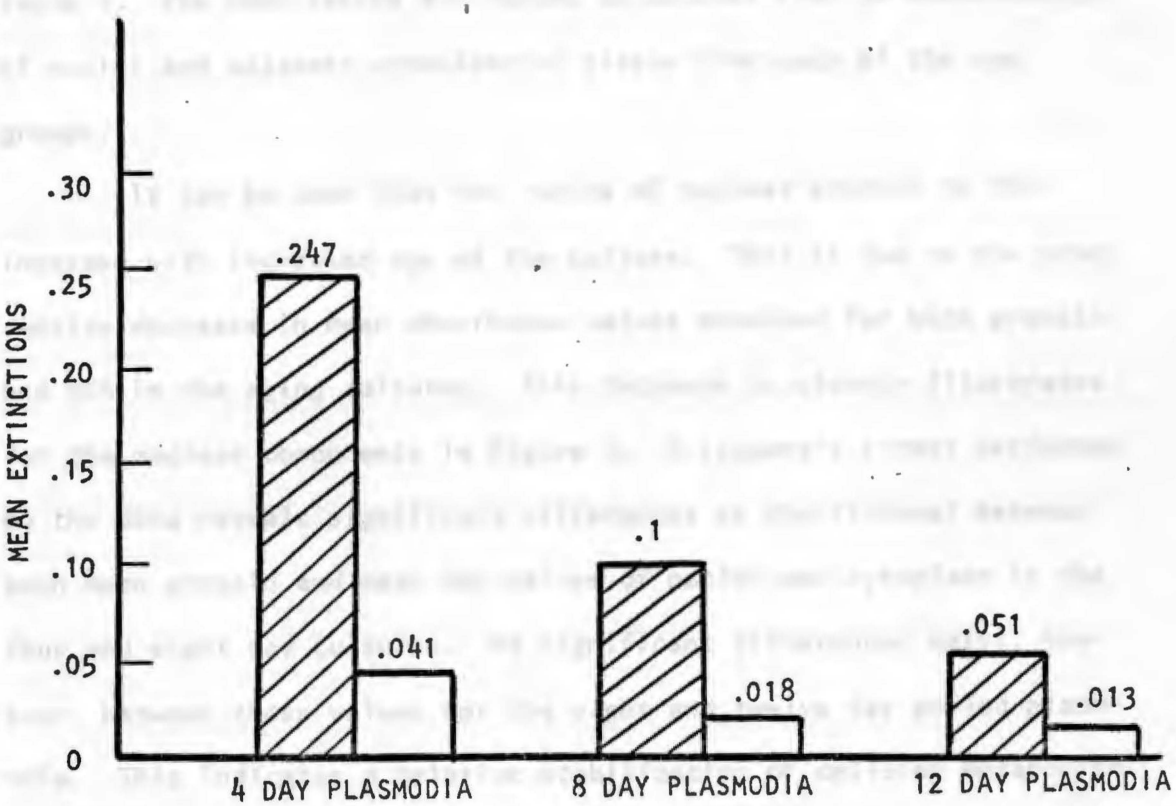


Fig. 3.--Bar graph representing mean relative RNA and basic protein values of plasmodial nuclei of Didymium iridis. Cross hatching on the graph designates the RNA values.

protein residues is thus demonstrated since these groups are blocked in the acetylation reaction.

The nuclear and cytoplasmic protein and RNA values were measured cytophotometrically in four, eight, and twelve day old cultures of plasmodia. Results of the mean protein/RNA content of nuclei and cytoplasm are given with standard errors and mean nuclear size in Table 1. The mean ratios are values calculated from 50 measurements of nuclei and adjacent cytoplasm of tissue from each of the age groups.

It can be seen that the ratios of nuclear protein to RNA increase with increased age of the culture. This is due to the progressive decrease in mean absorbance values obtained for both protein and RNA in the aging cultures. This decrease is clearly illustrated for the nuclear components in Figure 3. A student's t-test performed on the data reveals significant differences at the 1% level between both mean protein and mean RNA values of nuclei and cytoplasm in the four and eight day cultures. No significant differences exist, however, between those values for the eight and twelve day pooled plasmodia. This indicates a relative stabilization of cellular metabolism at approximately eight days of growth. Macroscopic observations of growing plasmodia found little increase, if any, in plasmodial size after one week of growth under the culturing conditions described, and an actual decrease in size of some twelve day organisms. This may be due to starvation, as depletion of nutrients was inevitable after several days of rapid growth. Mean nuclear diameters (Table 1) were also found to progressively decrease with the corresponding increased ages of the cultures. This may be significant in view of

TABLE 1.

MEAN VALUES AND STANDARD ERRORS OF BASIC PROTEIN AND RNA CONTENT DETERMINED MICROSPECTROPHOTOMETRICALLY

	Age of Plasmodium (days)		
	4	8	12
Nuclear protein/ RNA ratio*	0.174 ± .000	0.222 ± .006	0.391 ± .006
Nuclear protein·	0.041	0.018	0.013
Nuclear RNA·	0.247	0.100	0.051
Cytoplasmic protein·	0.039	0.020	0.019
Cytoplasmic RNA·	0.267	0.087	0.078
Total nuclear protein	0.040	0.009	0.004
Total nuclear RNA	0.240	0.047	0.014
Mean nuclear diameter (microns)	1.97	1.37	1.05

* Mean of protein/RNA ratios

· Mean optical densities

the findings that some true slime molds maintain a constant nuclear size distribution presumably for optimum metabolic activity (Kerr, 1970).

Autoradiographic and Microspectrophotometric Analysis of Nuclear Deoxyribonucleic Acid in Didymium iridis

The relative determinations of nuclear DNA content using the Feulgen reaction, for plasmodia of four, eight, and twelve days of age, and the corresponding frequencies of nuclei that incorporated tritiated thymidine, are presented in the frequency histograms of Figure 4. The abscissa represents the dye concentration in arbitrary units and the ordinate, the number of nuclei. To accurately represent ploidy levels, 100 nuclei from plasmodia of each experimental unit were analyzed. This procedure insured that the DNA content of a representative sample of each stage was measured, i.e., the histograms are not influenced by the chance measurement, for example, of a small population of nuclei of a different ploidy level. These same nuclei were used to determine frequencies of tritium labeling, though DNA content and presence or lack of label were not directly recorded for each individual nucleus, but were for the population of nuclei represented.

To describe the DNA content of haploid, diploid, or tetraploid nuclei, the respective designations of C, 2C and 4C of Swift (1950) will be used. To indicate the synthetic activity of the interphase nucleus, the designations of Howard and Pelc (1953) of G_1 (presynthetic gap), S (DNA synthesis), and G_2 (post-synthetic gap) will be referred to.

The mean DNA values of the four and twelve day plasmodia (Fig. 4) are nearly equal and indicate they both belong to the replicated diploid or 4C class and therefore must be in the S or G_2 stage of mitotic interphase. That the crossed plasmodium is diploid and that these values do correspond to 4C DNA content has been determined for this organism (Therrien, 1966; Yemma and Therrien, 1972). The mean DNA of the eight day cultures is somewhat less than half the value found in cultures of the other two age groups. This implies a 2C (unreplicated diploid) DNA content of the nuclei which would in turn imply the presence of a G_1 stage in this plasmodium. It is currently held, however, that a G_1 stage is absent or of very short duration in this organism (Rusch, 1969). Thus, should a profile of DNA values be constructed for any of the cultures of plasmodia, a unimodal distribution pattern typical of the myxomycetes, would be observed (Therrien, 1966). The mean DNA value of 6.58 representing that of the eight day cultures correlates with the 2C value found in replicated myxamoebae of Didymium by Therrien and Yemma (1974). The histogram of their results can be compared with those obtained here for the eight day cultures in Figure 5. Note that due to the 2C values of the eight day plasmodia, the scale on the abcissa of its histogram was adjusted for differences between it and the scale of the other two age groups (Fig. 4).

Some polyploid DNA values (greater than 4C) are present on the histograms. The occurence of polyploidy is common in this organism (Therrien and Yemma, 1975) and tends to increase with age of the plasmodium (Kerr, 1968). Thus, the skewness slightly to the right of the twelve day histogram is explained.

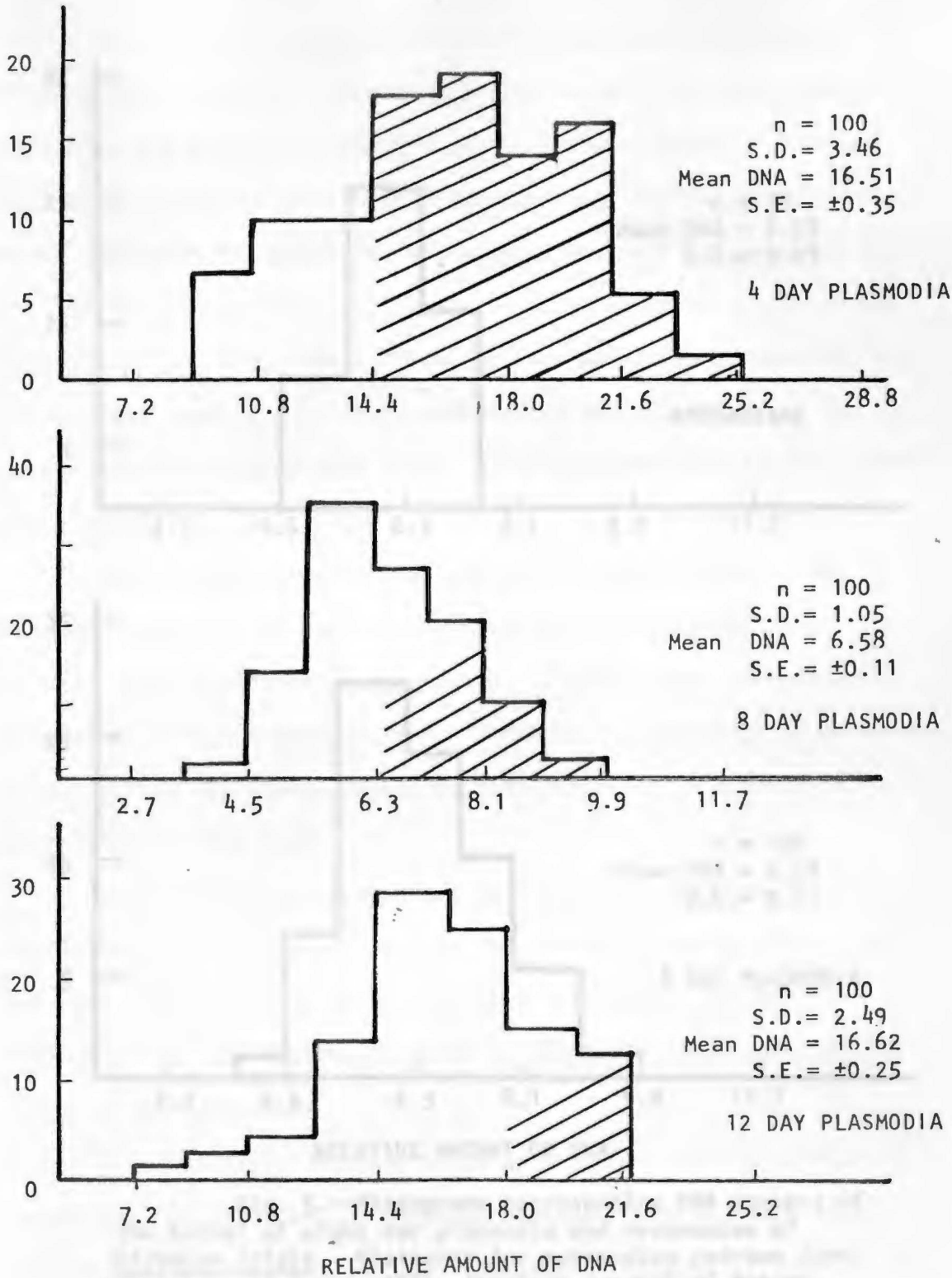


Fig. 4.--Histograms representing Feulgen-DNA values and frequency of tritiated thymidine labeling of plasmodial nuclei of Didymium iridis. Cross hatching on the histograms designates labeled nuclei.

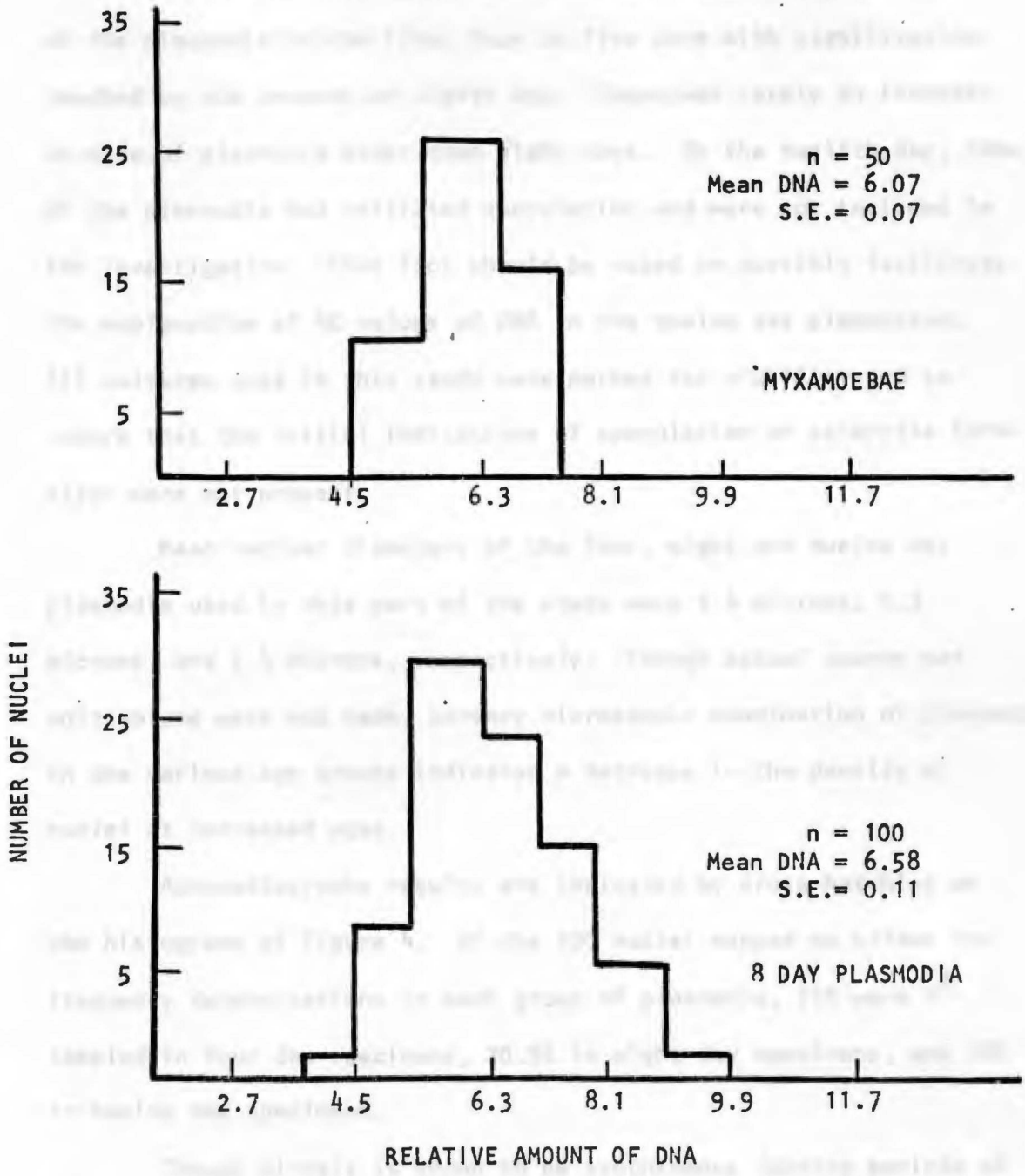


Fig. 5.--Histograms representing DNA content of the nuclei of eight day plasmodia and myxamoebae of Didymium iridis. Histogram for myxamoebae redrawn from Therrien and Yemma, 1974, American Journal of Botany, 61:400-404.

Visual observations of these cultures showed rapid growth of the plasmodia in the first four to five days with stabilization reached by the seventh or eighth day. There was rarely an increase in size of plasmodia older than eight days. By the twelfth day, some of the plasmodia had initiated sporulation and were not included in the investigation. This fact should be noted to possibly facilitate the explanation of 4C values of DNA in the twelve day plasmodium. All cultures used in this study were marked for viability and to insure that the initial indications of sporulation or sclerotia formation were not present.

Mean nuclear diameters of the four, eight and twelve day plasmodia used in this part of the study were 3.4 microns, 2.3 microns, and 2.4 microns, respectively. Though actual counts per unit volume were not made, cursory microscopic examination of plasmodia in the various age groups indicated a decrease in the density of nuclei at increased ages.

Autoradiography results are indicated by cross-hatching on the histograms of Figure 4. Of the 100 nuclei mapped on slides for frequency determinations in each group of plasmodia, 71% were H^3-labeled in four day specimens, 20.5% in eight day specimens, and 10% in twelve day specimens.

Though mitosis is known to be synchronous (during periods of growth) in this organism, the results obtained here indicate some variation in the synthesis of DNA. The high frequency of labeling in the four day cultures is highly compatible with the 4C DNA content and observed growth patterns at that age. The 2C category and low labeling frequency of the eight day organisms implies the reduced growth and

stabilization that was observed. The 10% labeling on the twelve day cultures with the 4C DNA content implies that the organism is about to undergo a nuclear division, though an increase in size of the plasmodia at that age was not observed.

DISCUSSION

The data presented here describes the plasmodial growth cycle and developmental changes in Didymium iridis in terms of cytochemical evaluations made at predetermined time intervals. Of special significance were those changes occuring during the nuclear cycle leading up to sporulation.

Results of the protein/RNA analysis shows a progressive decrease in the amount of both of these constituents with increasing plasmodial age. The data correlates well with the observed growth patterns. This can be explained in view of the fact that nutrients were not replenished in the cultures and starvation conditions eventually developed. The relatively high values for nuclear RNA and protein found in the four day cultures corresponds to the observations of rapid growth, which implies considerable template activity (Yemma, 1971; Stevens and Pachler, 1973). The protein/RNA values of the cytoplasm are also indicative of the rapid growth in the four day organisms. With the decrease of available nutrients and cessation of growth of the cultures by the eighth day, a diminished value for total RNA and the subsequent loss of protein (Stevens and Pachler, 1973) was observed in this study. It has been reported in the past, using various biochemical extraction procedures that starved or aging plasmodia of many species other than D. iridis, decrease in protein and nucleic acid content, though mitosis continues to occur at a slower rate with an increase in the duration of G_2 and loss of synchrony

(Daniel and Baldwin, 1964; Daniel, 1966; Cummins and Rusch, 1968; Sauer et al., 1969; Rusch, 1970). Results of a study by Mohberg and Rusch (1971) on nuclei extracted from Physarum plasmodia showed that RNA and protein values for growing organisms were high, just as the present data indicates. They also reported a drop in RNA upon starvation, no change in DNA content, but an increase in nuclear protein content under these conditions. The increase in protein was explained by the authors as unavoidable contamination of the nuclear fraction with cytoplasm in their extraction procedure. Studies of O'Brig and Gottlieb (1970) have noted increased levels of ribonucleases and degenerative enzymes in conjunction with decreased dry weight of protein, RNA and DNA in aging fungal populations.

The decrease in size of an aging or starving plasmodium has been described by Guttes et al. (1961) in studies with Physarum polycephalum, a closely related organism. This may be the result of inherent metabolic changes occuring during the developmental process and would explain the low protein and RNA content of the twelve day plasmodium. Endogenous oxidations of protein and nucleic acid could occur and would provide energy for movement (to search for food) and maintenance of essential cell substances (Cummins and Rusch, 1968). Though there is a net loss of protein and RNA in both the nuclei and cytoplasm at low growth rates and diminished nutrient supply, a steady state is reached between the eighth and twelfth days when no detectable plasmodial growth occurs, could be considered to constitute a G_1 stage in this organism. In Physarum, it has been noted that by the seventh day, a drop of 56% of the original protein and 72% of the initial RNA content had occured (Daniel, 1966). The

results of the study just cited, however, were based on the extraction of those cellular constituents from a plasmodium. Specific protein/RNA ratios for the represented nuclei within a plasmodium were not recorded.

The present study also demonstrates that there is a definite correlation between nuclear size and age of the plasmodium. This can be explained by the observations of Kerr (1970) that nuclear size distributions of plasmodia seem to relate to their metabolic activity.

The results of starvation (which appear to have occured here in older cultures) and the exposure to light, which could not be avoided in the preparation of the organisms, are prerequisites for the onset of sporulation (Daniel, 1966; Rusch, 1969; Sauer et al., 1969). That sporulation did occur in the twelve cultures insures that these conditions were met.

The values in Table 1 may erroneously imply that in some cases there is a higher concentration of RNA in the nucleus than in the cytoplasm. It has been shown that only 3% or less of the total plasmodial RNA is located in the nucleus, but the results of previous studies using pulse chase labeled RNA and sedimentation profiles have determined that completed ribosomal RNA is stored for an extended period of time in the nucleus (Braun, Mittermayer, and Rusch, 1966). The RNA values obtained in this study reflect this phenomenon.

Since plasmodial differentiation and growth are distinct processes (Daniel and Rusch, 1962) the metabolic changes associated with the transition of the cell state are of significance. In terms of energy requirements, it would seem that the growth limiting conditions, necessarily preceeding sporulation, would induce the

organism to shift its metabolic processes in such a way as to satisfy its energy requirements with a minimum amount of adverse effects on the organism. This could be accomplished by increasing metabolic efficiency in the turnover of metabolites and in energy yielding processes or by specialization (Daniel and Baldwin, 1964). The resultant state (spores) of the organism, by undergoing specialization, represents one of minimum entropy and high resistance to environmental conditions with the quest for survival accomplished (Alexopoulos, 1962).

The outcome of the Feulgen-DNA and autoradiography investigation is in agreement with, and will be discussed in the light of, the results of the protein/RNA analysis. However, since only identical age groups and not identical specimens were used for the studies, no attempt was made to directly correlate a particular DNA value with a particular protein/RNA content for a nucleus. Only trends in biochemical alterations occuring in a given developing plasmodium over a period of time, were under investigation.

Since synchrony of mitotic divisions (though not 100% synchronous) exists within a single growing plasmodium, it logically follows that all nuclei within that organism are in the same stage of the cell cycle (Nygaard et al., 1960). Values for mean DNA content are known to be equal throughout somatic cells of a particular species and therefore provide a parameter from which to observe change (Bloch and Godman, 1955). Thus, pooled plasmodia of the same age, collected over a time interval, adequately represent any overall chemical changes occuring within a population of nuclei during that designated period of time.

To serve as a foundation for the data presented regarding the DNA analysis, a few statements will be made about what is known to occur in other closely related organisms. It has been previously determined using isotope incorporation experiments with P. polycephalum, that the DNA synthetic period (S) of interphase lasts for 3 to 4 hours immediately following mitosis in actively growing plasmodia that exhibit a total intermitotic period of from 8 to 10 hours (Nygaard et al., 1960; Braun et al., 1965). A prolonged G_2 or post-synthetic gap and lack of a G_1 has been noted in Physarum, indicating that in the plasmodium, DNA is in the replicated state about 90% of the time (Therrien, 1966; Kessler, 1967; Rusch, 1969). The absence of a presynthetic gap period is common in rapidly dividing cell cultures since all substances produced are used for growth (Swift, 1950; Cummins, 1969).

If the growth and mitotic patterns established for Physarum hold true for Didymium iridis, the nuclear DNA profile for Didymium should exhibit a unimodal distribution, and does so, as is demonstrated here (Fig. 4) and previously (Yemma and Therrien, 1972; Therrien and Yemma, 1974).

The 4C DNA content of the plasmodial nuclei in the four day cultures, and the large majority of nuclei that incorporated the tritium label, demonstrates a high level of mitotic activity which is indicative of the rapid growth at this age. The same conclusion was reached from the high protein/RNA values observed for this same time period previously discussed. The 4C DNA content of the nuclei of this young plasmodium also reasserts that the development of this heterothallic organism is initiated through sexual zygote formation

(Ross, 1967; Yemma, 1975 - unpublished data) as contrasted to the clonally produced plasmodia of the same species which is not preceeded by karyogamy and is therefore haploid (Therrien and Yemma, 1975). Synthesis of DNA in 99% of the nuclei in a single growing plasmodium is initiated simultaneously, but the rate and duration of synthesis varies in the individual nuclei. This was determined in autoradiography studies by Braun et al., (1965). That only 71% of the nuclei in the 4 day organisms were labeled and not the expected 99%, is probably a result of 30% of the nuclei having had completed their DNA synthesis when incubation in the tritium-labeled bacteria was begun.

The eight day plasmodia demonstrated a 2C DNA content which compares with the mean DNA value of D. iridis myxamoebae (see Figure 5) in the replicated state, found by Therrien and Yemma, (1974). These findings are not consistent with previous observations reported in the literature, that the organism is in an extended G_2 period and lacks a G_1 in the plasmodial and myxamoebae forms (Therrien and Yemma, 1974). The observed metabolic stabilization of the eight day plasmodia, with cessation of growth and decreased nuclear size, correlates with the nuclear DNA changes and implies that this organism may be experiencing a G_1, typical of cells that specialize to some degree or differentiate. The possibility that developing plasmodia might experience a G_1 under starvation conditions has been suggested by Rusch (1969). He speculates that the purpose of this would be to initiate the production of substances used in the developmental changes accompanying the sporulation process which will ensue. It is expected, then, that a G_1 period does exist

at some point in the life span of the plasmodium prior to sporulation. This G_1 seems to last for an extended period of time. In fact, the data presented supports the conclusion that it persists up to the time of presporulation, and at this point, a round of DNA synthesis occurs giving rise to the G_2 condition. The presence of a G_1 in the cell cycle of this organism once again would produce a unimodal distribution on the DNA histogram (though shifted to the left) since the organism would still maintain relative mitotic synchrony and is homogenous with respect to DNA condition.

In recent studies on selfed plasmodia (clonally-produced) of this species by Therrien and Yemma (1975), a G_1 was observed at one point in the thirty-six hour investigation period. However, these studies were done using a haploid, abnormal plasmodium, and not a normal diploid, as was employed in this study. The existence of a G_1 of some duration in this organism, therefore seems plausible since no growth, which is indicative of G_1 arrest, was reported in that study as well as in the present one.

The 20.5% labeling frequency of the eight day plasmodia was not entirely expected to occur considering the 2C DNA content observed and in view of the generally synchronous initiation of DNA synthesis. The reason for this was attributed to the loss of mitotic synchrony that occurs in starvation and aging as previously mentioned. This would mean that a fraction of the nuclei had commenced replication of their DNA at some time during the incubation period with the labeled bacteria. An alternative interpretation might be that when the eight day cultures of plasmodia were pooled and subsequently labeled for a 3½ hour duration, fusion of plasmodia predominantly in

the G_1 period with those not yet having at that time completed the last division prior to G_1 arrest, occured. If fusion did take place between organisms in the conditions just described, some nuclei would then be able to incorporate the tritiated thymidine in the prophase preceeding G_1 arrest (Guttes et al., 1967). The time required for a synchronous mitosis to occur after coalescence, if it is to occur, is estimated to be six to seven hours (Guttes and Guttes, 1964). Therefore, if a plasmodium in S fused with one in G_1, the observed labeling is accounted for. Since none of the DNA values of the eight day plasmodia could be considered 4C, a few are high enough to indicate that at least a partial DNA synthesis may have occured in one of the coalescing plasmodia.

It is also possible that selfing could have occured in the cultures, (since the Honduran $1\text{-}2A^1$ clone used in the original cross is also known to produce plasmodia without prior nuclear fusion). In this case, the 2C DNA content would simply indicate that the normally haploid organism (Therrien and Yemma, 1975) is in a replicated state. The occurence of the 20.5% labeled nuclei would therefore imply that approximately two hours of S had elapsed prior to the incorporation of tritiated thymidine into the organism and only a few nuclei were synthesizing DNA at that time (Braun et al., 1965). That pooled plasmodia were employed in this study, however, would tend to negate this possibility of a selfed plasmodium since the 2C value for DNA would have been determined at other time periods also. In addition, selfing is a rare event unless high densities of amoebae are maintained in culture and viability of selfed plasmodia is low (Therrien and Yemma, 1975). Though highly

unlikely, this possibility had to be mentioned and ruled out.

By the twelfth day, the aging plasmodia have decreased in mass and in number of nuclei per unit area. This latter observation does not agree with those investigations of Guttes et al., (1961) in which no net decrease in nuclear density was found in starving plasmodia.

The Feulgen-DNA analysis shows that the DNA content of the twelve day plasmodia is 4C. Ten percent of those nuclei measured had incorporated the tritium label. This low rate of incorporation was probably due to tagging late in the S period when the majority of nuclei had already replicated their DNA. In fact, the data indicate that tagging occured only during the last 30 to 45 minutes of the synthetic phase since a 4C DNA content was observed for this time period. Also, loss of mitotic synchrony due to nutrient depletion and subsequent starvation could explain the partial labeling observed. Further discussion of the 4C value obtained is warranted by its implications to the sporulation process. It was noted previously that some of the cultures of twelve day plasmodia had begun the sporulation process by the time they were to be treated. Since the ages of all the pooled plasmodia were within hours of one another, it is conceivable that most of the organisms were close to initiating that process at that age.

The requirements for sporulation to occur in a starved plasmodium, as were determined using Physarum (Daniel and Rusch, 1962; Sauer et al., 1969) include the synthesis of DNA at some point late in the starvation period, which must persist for a minimum of 4 days. The twelve day plasmodia would appear to have

fulfilled this DNA synthesis requirement in preparation for sporulation.

The intermitotic period of starving plasmodia has been estimated to be from 24 to 36 hours (Guttes et al., 1961). If this is the case in Didymium, it is not likely that a mitosis would succeed the observed DNA replication before the onset of the processes of biochemical differentiation and morphogenesis in sporulation. The mitosis would have to occur at some later period during sporogenesis. The feasibility of this scheme of events is supported by the findings of Mohberg and Rusch (1971). They have determined a 4C ploidy level in sporulating nuclei of Physarum prior to the precleavage mitosis. The same results were observed for Didymium by Yemma (1971).

The possibility that a particular plasmodium would not go on to sporulate within the following 24 to 36 hours cannot be excluded, since even though the requirements for sporulation are met for any one plasmodium, the differentiation process does not inevitably take place (Rusch, 1969). If this were the case, the observed DNA replication would be eventually followed by a division.

On the other hand, it is possible to assume that the 4C nuclei would proceed through the sporulation process, migrate up the papillae, and finally experience a precleavage mitosis within the sporangia. A doubling of the DNA prior to sporangium formation has been observed in the selfed plasmodium of this species by Therrien and Yemma (1975). Also, Aldrich and Carroll (1970) have observed a precleavage mitosis in D. iridis in the sporangium

about 15 hours after the illumination period.

The possible advantage of 4C nuclei entering the developing sporangia would be to decrease the energy expenditure of the organism already adversely effected by the limiting environmental conditions and to provide for a larger number of spores produced per nucleus in the subsequent meiotic divisions within the sporangium. These meiotic divisions have been positioned from 9 to 15 hours after the cleavage of the sporangia in D. iridis (Aldrich and Carroll, 1970).

Further investigation into the differentiation processes of this particular organism appears to be necessary in order to state with more certainty the significance of the metabolic state of the eight and twelve day plasmodia. Also, for a more comprehensive view of the changes that take place within the aging organism throughout its growth cycle, more frequent time intervals during its growth period could be investigated. Intervals of 2 or 3 days would seem to be a good choice for this type of study.

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