DNA MEASUREMENTS OF AN APOMICTIC ISOLATE

OF <u>DIDYMIUM IRIDIS</u>

by

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The author wishes to dedicate this thesis to his wife whose support and encouragement made this study possible.

ABSTRACT

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The nuclear DNA value of a clone derived from an apomictic isolate Panamanian 2-4 w.t. of *Didymium iridis* was measured employing both the Feulgen and absorption microspectrophotometry. This method allowed us to distinguish between homothallic and apomictic isolates. This data demonstrates that there is no alternation of generations in the amoebae and plasmodia stage, indicating that plasmodial development is apomictic. The DNA values also suggest that the Panamanian 2-4 isolate is aneuploid.

ACKNOWLEDGEMENT

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INTRODUCTION

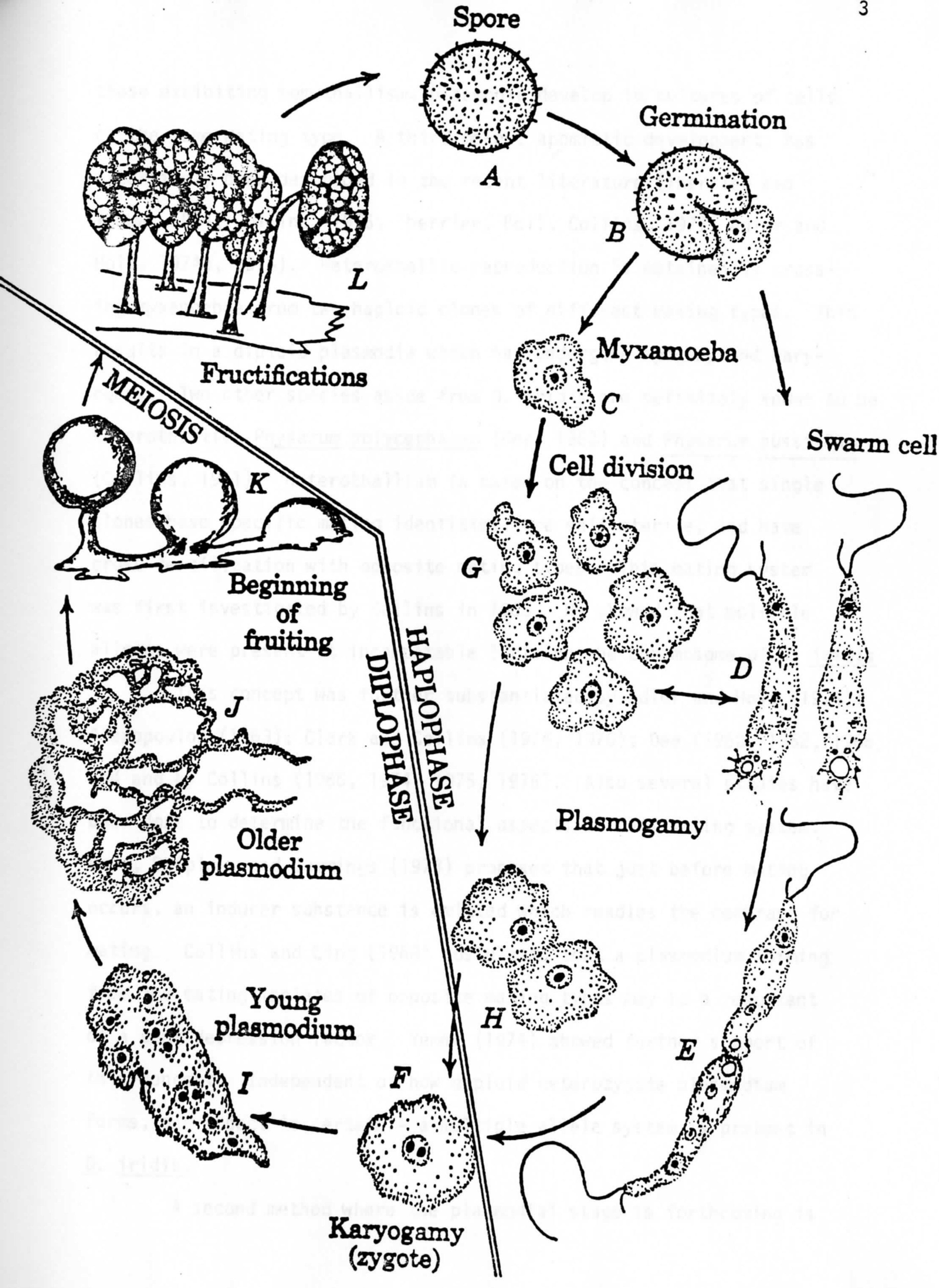
Since DeBarry first investigated the life history of the Mycetozoa in 1860, the Myxomycete have been a great source of scientific information. The unique life cycle of this organism, including both vegetative phases of its life cycle (Plate 1), has provided through many studies pertinent information which includes studies of the nuclear cycle, DNA synthesis, mitotic synchronization, photoreception, genetic analysis, and structure and function of bio-membranes. The two vegetative phases are basically a unicellular myxamoeboid haplophase, a multinucleate plasmodia and sporulation stage. The acellular slime _Didymium iridis_ chosen for this study provides an excellent opportunity for studies regarding the ploidy levels that may occur in these two particular stages.

Depending upon environmental conditions, the myxamoebae can follow one of two pathways. After its germination from a spore a single protoplast (Collins, 1961) emerges in the form of an amoebae. If an aqueous medium is present it transforms into a swarm cell with a biflagetted tail (Aldrich, 1968). These two forms are interconvertable depending upon moisture conditions. Both forms of myxamoebae obtain their food by phagocytosis of bacteria. Once the food source is exhausted the amoebae encyst form a polysaccharide (Gutles et al., 1961) and remains quiescent till environmental conditions favor reentering the amoeboid stage. Cell division occurs in the amoeboid stage.

The plasmodial stage can be obtained by several means. In those strains which exhibit heterothallism, myxamoebae or swarm cells of proper mating types are required (Collins, 1961, 1963, 1973). In


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

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

those exhibiting homothallism, plasmodia develop in cultures of cells of the same mating type. A third means, apomictic development, has extensively been described in the recent literature (Therrien and Yemma, 1974; Collins, 1976; Therrien, Bell, Collins, 1977; Adler and Holt, 1974b, 1975). Heterothallic reproduction is obtained by crossing myxamoebae from two haploid clones of different mating types. This results in a diploid plasmodia which has undergone syngamy and karyogamy. Two other species aside from D. iridis are definitely known to be heterothallic, Physarum polycephalum (Dee, 1960) and Physarum pussillum (Collins, 1961). Heterothallism is based on the concept that single clones have specific mating identities, are self-sterile, and have cross fertilization with opposite mating types. This mating system was first investigated by Collins in 1963. He showed that multiple alleles were present at incompatable locis on the chromosome of D. iridis.

This concept was further substantiated by Adler and Holt (1974); Alexopoulos (1963); Clark and Collins (1974, 1976); Dee (1960, 1962, 1966) and and by Collins (1965, 1974, 1975, 1976). Also several studies have been done to determine the functional aspects of this mating system. Ross, Shipley, and Cummings (1973) proposed that just before mating occurs, an inducer substance is emitted which readies the membrane for mating. Collins and Ling (1968) postulated that a plasmodium forming from the mating isolates of opposite mating types may be a resultant of a gene depression factor. Yemma (1974) showed further support of this concept. Independent of how diploid heterozygote plasmodium forms, one aspect is certain - a multiple allele system is present in D. iridis.

A second method where the plasmodial stage is forthcoming is homothallism: Homothallism is a reproductive method where no mating types are present and plasmogamy and karyogamy occur prior to formation of a plasmodia. Several species have been reported to be homothallic: Didymium difforme (Schuneman, 1930); Physaru oblonga (Ross, 1957); Fuligo cinerea (Collins, 1961); and Didymium nigripes (Kerr, 1961). The third reproductive means, apomictic reproduction, occurs when myxamoebae do not display mating types, but still yield plasmodia. Here karyogamy does precede plasmodial formation (Therrien, 1972). Two other species have been reported apomictic, P. polycephalum (Cooke and Dee, 1974), and Echinostelium minutum (Haskings, 1978). The apomictic isolates do not display an alternation of a 2c amoebae and 4c plasmodial classification. However, there is no difference in the nuclear DNA content of the two vegetative stages.

A fourth method that could be added to this list is "selfing." Such isolates yield plasmodia when properly mated and plasmodia appearing in clonal myxamoebae cultures as well. This type of reproduction was first studied by Collins and Ling (1968), and further researched by Therrien and Yemma (1972, 1975). They found that the ploidy level in the plasmodia did not increase to a diploid value. This indicated that although syngamy (cell fusion) occurred, karyogamy (nuclear fusion) did not. This belief had been purported earlier in 1966 by Ross who stated that the n-2n situation is not necessarily a prerequisite for plasmodial formation. Selfing, however, may just be a form of apomictic development.

A fifth category has been recently determined by Therrien and Collins (1976) where a heterothallic isolate may be called "induced apogamy." Here a polyploid clone is crossed with certain haploid clones which results in a plasmodia that is either haploid or polyploid, depending on which clone is producing the inducing factor. Further research by Collins (1978) retracted the "induction process" or extended to favor chromosome elimination to account for the heteroploid plasmodia.

Once the multinucleated plasmodia is deprived of food or allowed to dry up a resistant structure called a sclerotium is formed. When the nutrient medium is renewed, the sclerotium resumes the active state of a growing plasmodia.

In the final stage of the life cycle, sporulation, spores are released into the environment. The spores can remain dormant for months or even years (Alexopoulos, 1963), depending upon environmental conditions. Once conditions are favorable, myxamoebae are released and the life cycle is resumed.

The purpose of this paper is to present data in the form of histograms demonstrating nuclear DNA measurements by means of microspectrophotometry of two isolates of D. iridis Pan 2-4 and Pan 2-4 w.t. By the use of histograms it can be determined if there is an alternation of generations when plasmodia are formed, i.e. if the amoebae and plasmodia exhibit a change in ploidy levels and therefore give insight regarding the underlying mechanisms of plasmodial formation. The ploidy level was ascertained by employing a Feulgen-cytophotometry method, because myxomycete chromosomes are extremely difficult to count. The two-wavelength method developed by Patau (1957) and Ornstein (1952) was utilized in this experiment. This analytical tool, concurrent with this particular histochemical procedure, is a reliable method of obtaining ploidy levels as proven by Boivin and Venderly

(1948, 1949), Ris and Mirsky (1949), Lessler (1953), and Kasten (1959). The Feulgen-DNA staining is a valid approach to this study as long as the following conditions exist: 1) the microspectrophotometric readings are accurate and 2) the Feulgen reaction is specific for DNA only.

MATERIALS AND METHODS

Organism and Culture Conditions

All myxamoebae Didymium iridis used in this study were obtained from Dale Therrien of Pennsylvania State University. All isolates were obtained in the amoeboid stage and were labeled Panamanian 2-4 (Pan 2-4), Panamanian 2-4 w.t. (Pan 2-4 w.t.), Honduran 1-2 (Hon 1-2). Any new plates made were taken from these stock cultures.

Clones were maintained in an incubator at a temperature of 21°C (Yemma, 1972; Yemma and Therrien, 1974) with an alternating 12 hour light and dark cycle. Using a solid media (Yemma, 1972) amoebae were grown on bacterial lawns of Escherichia coli. Plasmodial growth was maintained on plasmodial media (Gayther, 1972) using an additional food source of sterilized oat flakes.

All strains of clones were grown to log phase upon which they were fixed with 10% buffered formalin (ph 7.0) for a period of 12-18 hours. After fixation, amoebae were washed off utilizing a bent glass rod and subjected to two washings of 70% alcohol. In-between each washing, clones were centrifuged at 10,000 rpms for 10 minutes with the supernatent removed after each washing. The bacteria-clean plug was smeared on previously albuminized slides and placed on a warming tray overnight.

Plasmodial growth was obtained by two methods. The first method utilized a procedure of removing a small amoeboid laden chunk of agar from two different mating types and placing them side by side on previously E. coli lawned agar plates. Plasmodial growth was observed 100% of the time in this method. The second method involved clones which formed plasmodia without the benefit of an opposite mating type. Here, small amoeboid laden chunks of agar were placed on bacterial lawn plates. Plasmodia formed between 50-90% of the incubation period. The plasmodia were harvested from each of the plates and placed on plasmodial media (Gayther, 1972 Unpublished results) containing sterilized oat flakes. Once a large enough amount of plasmodia was obtained, plasmodia was transferred to plain agar for a period of 24 hours to free it of any bacterial content. The plasmodial tissue was then fixed with 10% buffered formalin. Fixation was for a period of 18-24 hours and then washed twice with 70% alcohol. The fixed tissue was then dehydrated through a series of alcohol rinses and embedded in a paraffin block. The plasmodia was then sectioned at 8 microns and placed on previously albuminized slides and dried overnight.

Cytochemical Methods

The amoebae and plasmodia slides were stained simultaneously using the Feulgen nucleal reaction for specific isolation of deoxyribonucleic acid (Feulgen and Rosenback, 1924; as modified by Therrien, 1966, and Bryant and Howard, 1969). Plasmodial slides were cleared in xylene to remove paraffin and then hydrated in a series of alcohol washes before the staining procedure began. The staining followed this sequence:

(1) All slides were placed in 5.0 N hydrochloric acid for a 43 minute period at room temperature.

(2) Stained for one hour in freshly mixed Schiff's reagent. The basic fuchsin used was manufactured by Fisher Scientific Company (C.I. #42500).

(3) Rinsed twice in freshly prepared bisulfite rinse for

5 minutes (Yemma, 1972).

(4) Rinsed in distilled water, then dehydrated in a graded ethanol series.

(5) Cleared in xylene and mounted cover slips with permount.

Cytophotometric Methods

All quantitative DNA measurement readings were made with a Zeis Universal Microspectrophotometer. The objective lens used was a Zeis oil immersion objective x 100 N.A. 1.25, with Zeis immersion oil 518C Din 5884. Before all readings, instrument alignment and phototube linearity response were checked. An absorption curve was used to determine maximum and minimum wavelength. All readings were conducted at these two wavelengths: 560^{λ} and 505^{λ} .

The two-wavelength method as described by Ornstein (1952) and Patau (1952) was employed because it corrects for errors caused by heterogenous distribution. Employing the two wavelengths 505 and 560, four microspectrophotometric readings were required for each nuclear value. Each reading was conducted in a random fashion with the nucleus centered in the appropriate field aperture to insure the most accurate measurements. The four readings I_{o1}, I_{s1}, I_{o2}, I_{s2} were used in the calculating of the amount of chromophore (M), $M = KAL_1C$. The absorptivity constant K_1 was omitted since relative not absolute values were needed. L_1 was determined using the formula $L_1=(1-T_1)$ and $L_2=(1-T_2)$. The constants T_1 and T_2 were determined by the manipulation of the wavelengths 560 and 505 readings, where $T_1= Is/I_o$ and $T_2=Is/I_o$ respectively. The correction factor C, which eliminated any influence of unoccupied portions of the measured area was determined by the calculation $C=(2-Q)^{-1} \ln(Q-1)^{-1}$. Q values are generated by the ratio L_2/L_1, ($Q=L_2/L_1$) which corresponds to the required 2:1 ratio ascertained in the absorption curve. A table formulated by Patau (1952) lists all C values and corresponding Q values.

All relative DNA calculations were conducted on a Mandahl Model 470V5 computer. Statistical methods utilized in this study for comparison made among experimental organisms regarding mean DNA content was the student t-test.

RESULTS

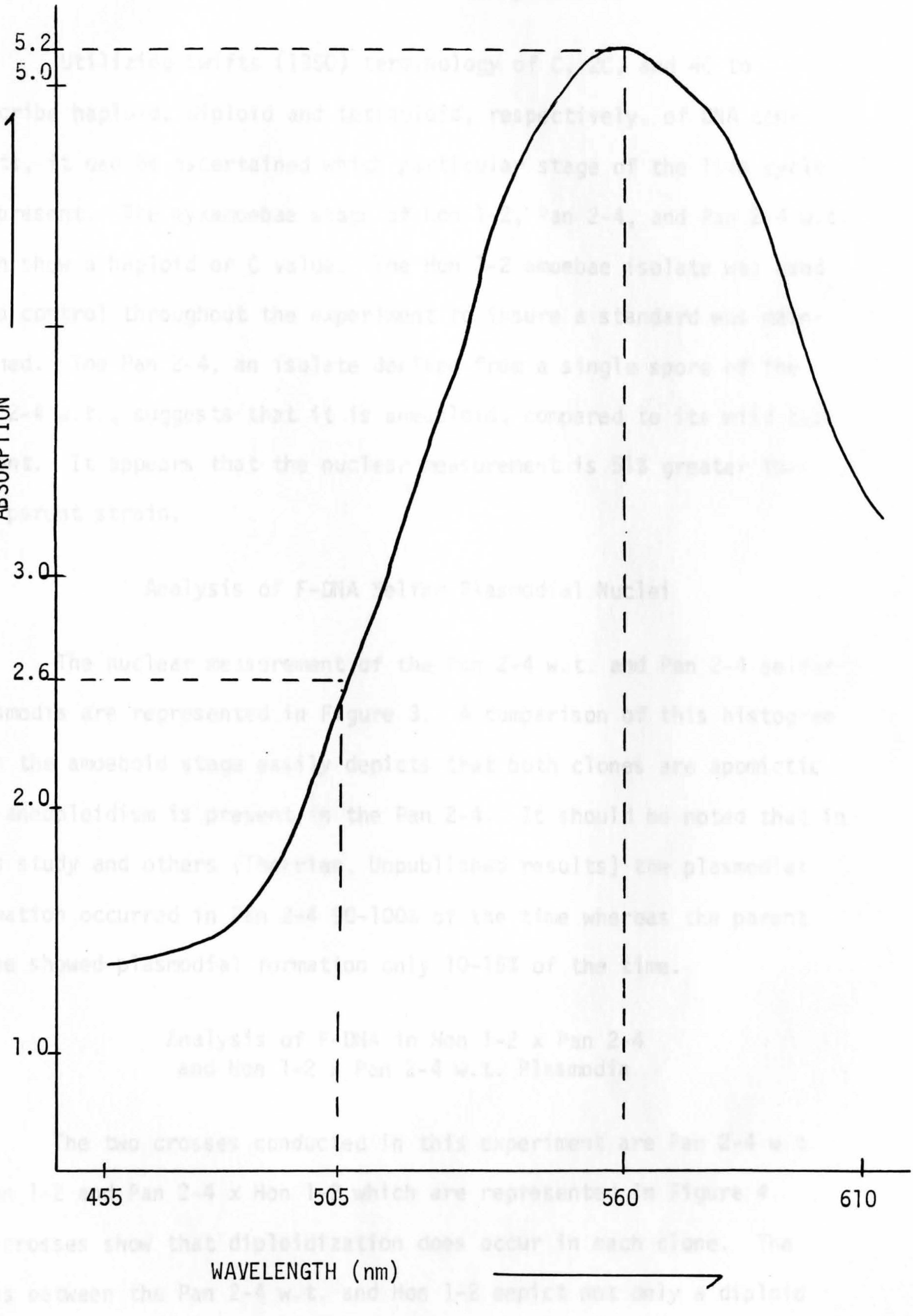
The results of this investigation are presented in the form of histograms, graphs, DNA comparison table, and student's t-test. Histograms are used because they enable the detection of any changes or shifts in ploidy level or mitotic activity in each cell population. The number of nuclei is plotted on the ordinate, and the relative dye concentration is on the abscissa. Since the two-wavelength method for cytophotometry was employed in order to make measurements, an absorption curve was plotted in order to obtain the maximum and half-maximum wavelength. In Figure 1 the wavelengths are easily detected as the maximum absorption at 560 nm and the half-maximum absorption at 505 nm.

Microspectrophotometric Analysis of Nuclear DNA in Didymium iridis

Deoxyribonucleic acid was measured in the myxamoebal stage and plasmodial stage of Pan 2-4, Pan 2-4 w.t. and Hon 1-2 isolates and plasmodial stage of Pan 2-4, Pan 2-4 w.t. and Hon 1-2. The nuclei appear to be haploid and demonstrate a unimodal distribution, which is characteristic of a population of cells lacking a G_1 stage as described by Rusch (1969). For each stage, 100 nuclei were measured to insure that the histograms were not influenced by chance measurements of small populations of nuclei of different ploidy levels. Several repetitions were made of each group and the results are shown on Figures 2-4. Control slides were run with experimental slides during the staining process to insure strict specificity of the stain.

FIGURE I

Absorption Spectrum of Feulgen Stained Plasmodial Nuclei of *Didymium iridis*


5.2
5.0
ABSORPTION
3.0
2.6
2.0
1.0
455
505
560
610
WAVELENGTH (nm)

Analysis of F-DNA in the Myxamoebae

Utilizing Swifts (1950) terminology of C, 2C, and 4C to describe haploid, diploid and tetraploid, respectively, of DNA contents, it can be ascertained which particular stage of the life cycle is present. The myxamoebae stage of Hon 1-2, Pan 2-4, and Pan 2-4 w.t. each show a haploid or C value. The Hon 1-2 amoebae isolate was used as a control throughout the experiment to insure a standard was maintained. The Pan 2-4, an isolate derived from a single spore of the Pan 2-4 w.t., suggests that it is aneuploid, compared to its wild type parent. It appears that the nuclear measurement is 51% greater than the parent strain.

Analysis of F-DNA Selfer Plasmodial Nuclei

The nuclear measurement of the Pan 2-4 w.t. and Pan 2-4 selfer plasmodia are represented in Figure 3. A comparison of this histogram with the amoeboid stage easily depicts that both clones are apomictic and aneuploidism is present in the Pan 2-4. It should be noted that in this study and others (Therrien, Unpublished results) the plasmodial formation occurred in Pan 2-4 90-100% of the time whereas the parent clone showed plasmodial formation only 10-15% of the time.

Analysis of F-DNA in Hon 1-2 x Pan 2-4 and Hon 1-2 x Pan 2-4 w.t. Plasmodia

The two crosses conducted in this experiment are Pan 2-4 w.t. x Hon 1-2 and Pan 2-4 x Hon 1-2 which are represented in Figure 4. The crosses show that diploidization does occur in each clone. The cross between the Pan 2-4 w.t. and Hon 1-2 depict not only a diploid

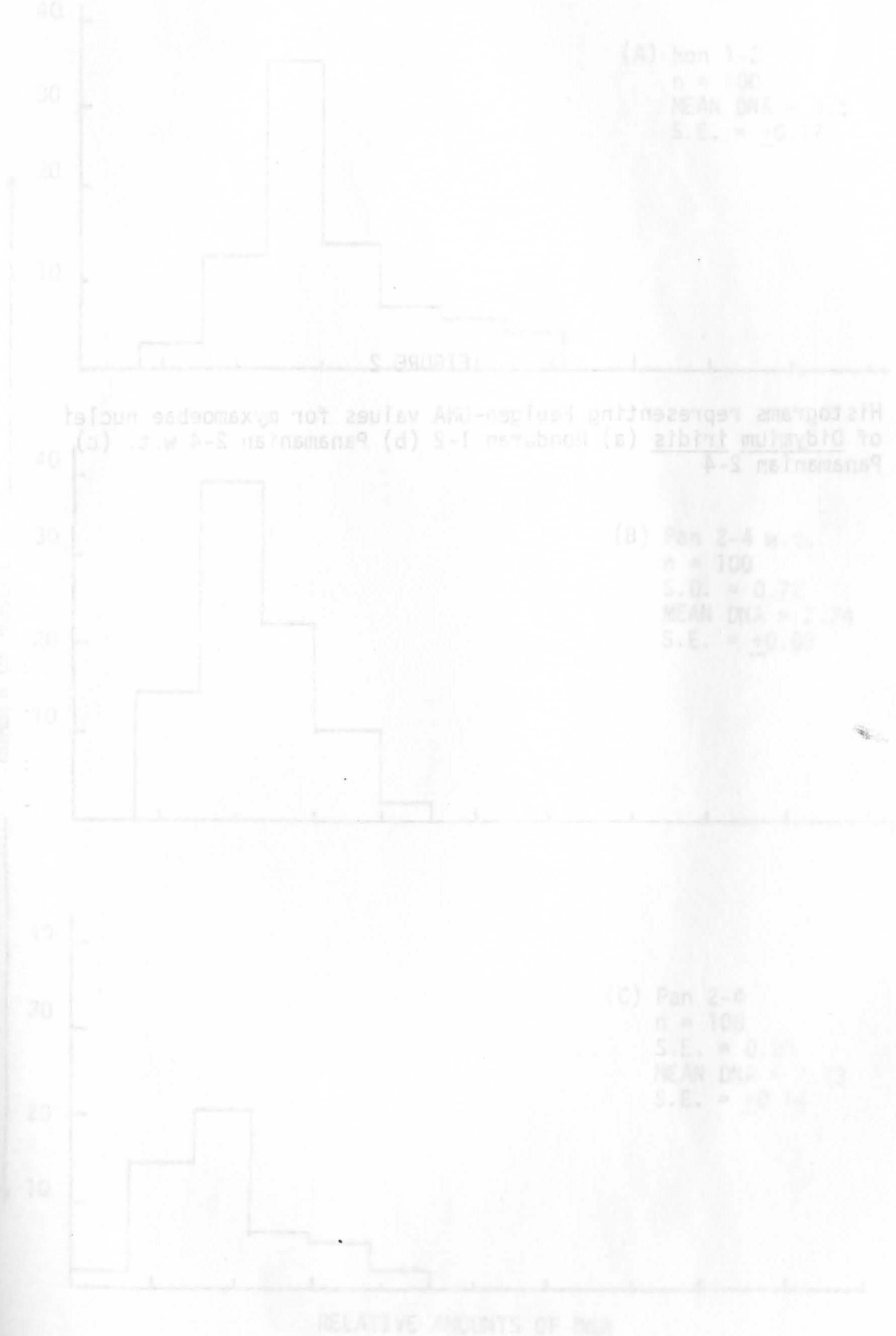

40
30
20
10
(A) Hon 1-2
n = 100
40
30
20
10
(B) Pan 2-4 w.t.
n = 100
30
20
10
(C) Pan 2-4
RELATIVE AMOUNTS OF DNA

FIGURE 2

Histograms representing Feulgen-DNA values for myxamoebae nuclei of Didymium iridis (a) Honduran 1-2 (b) Panamanian 2-4 w.t. (c) Panamanian 2-4

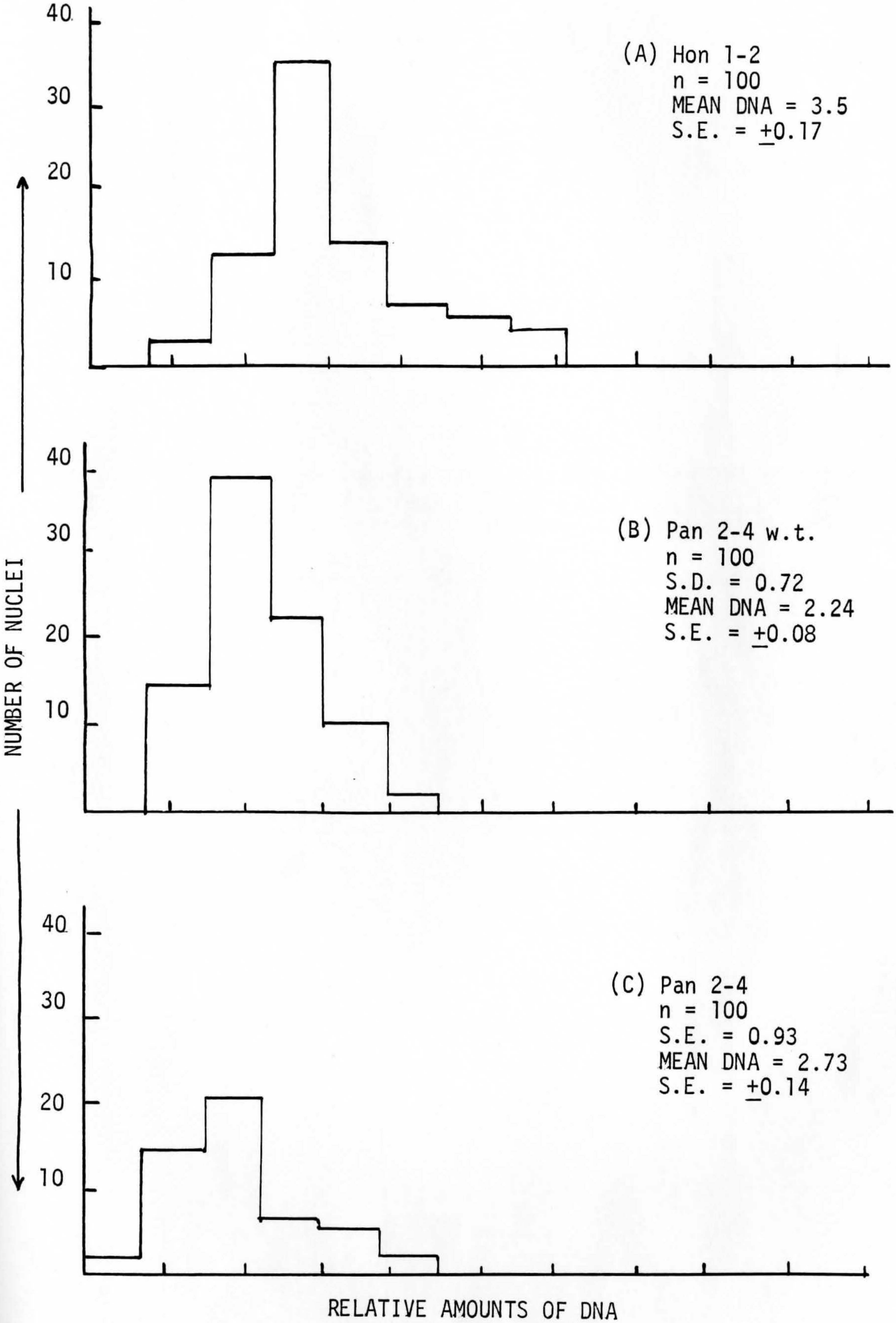
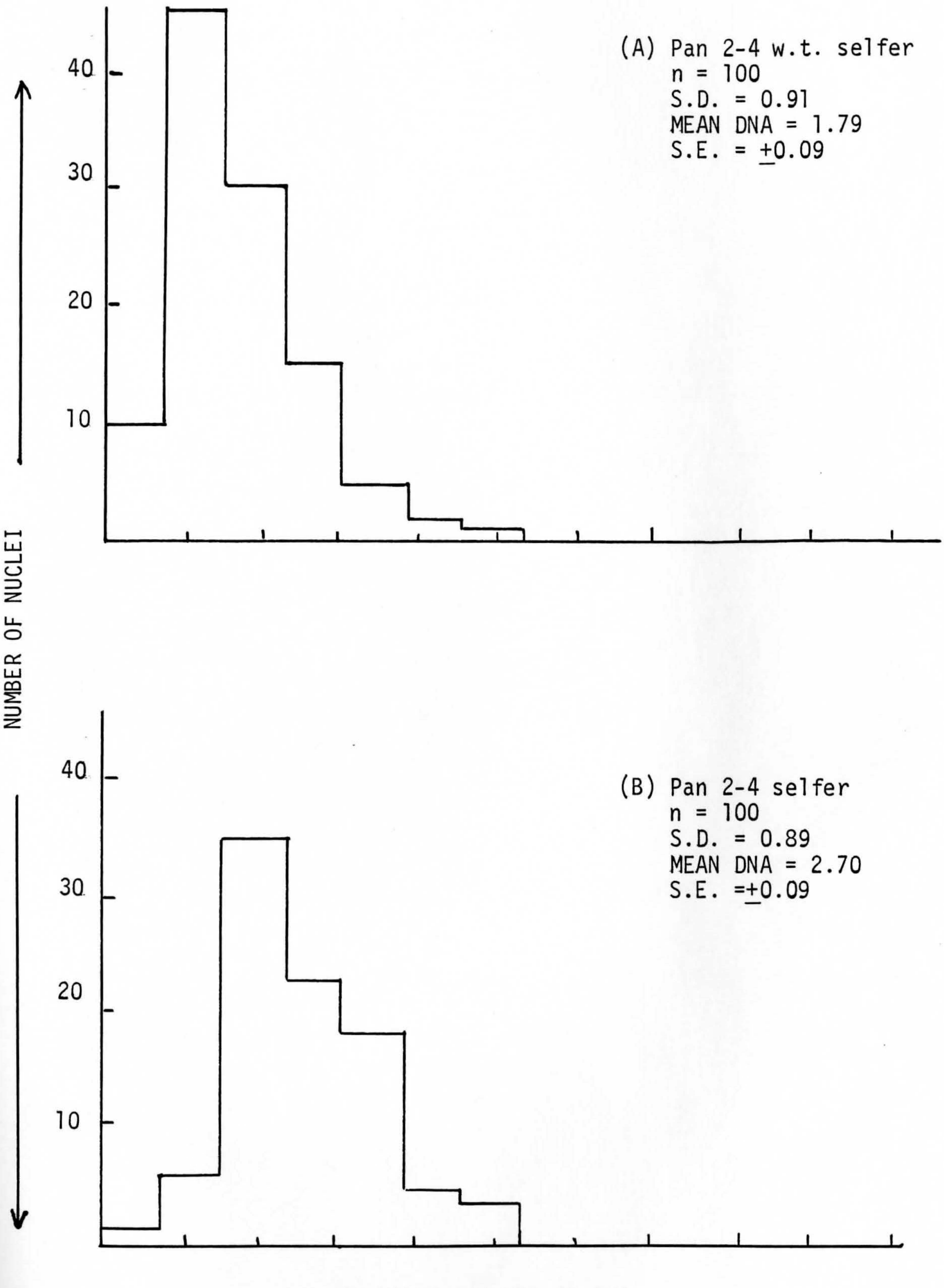

40
30
20
10
(A) Hon 1-2
n = 100
MEAN DNA = 3.5
S.E. = +0.17
40
30
20
10
(B) Pan 2-4 w.t.
n = 100
S.D. = 0.72
MEAN DNA = 2.24
S.E. = +0.08
NUMBER OF NUCLEI
40
30
20
10
(C) Pan 2-4
n = 100
S.E. = 0.93
MEAN DNA = 2.73
S.E. = +0.14
RELATIVE AMOUNTS OF DNA

FIGURE 3

Histograms representing Feulgen-DNA values for selfer plasmodial nuclei of *Didymium iridis*; (a) Panamanian 2-4 w.t. self plasmodia; (b) Panamanian 2-4 self plasmodia


(A) Pan 2-4 w.t. selfer
n = 100
S.D. = 0.91
MEAN DNA = 1.79
S.E. = ±0.09
(B) Pan 2-4 selfer
n = 100
S.D. = 0.89
MEAN DNA = 2.70
S.E. =±0.09
40
30
20
10
NUMBER OF NUCLEI
RELATIVE AMOUNTS OF DNA

Histograms representing relative Feulgen-DNA content: [illegible] (b) Panamanian 2-4 w.t. x Honduran [illegible] plasmodium; (c) Panamanian 2-4 x Honduran 1-2

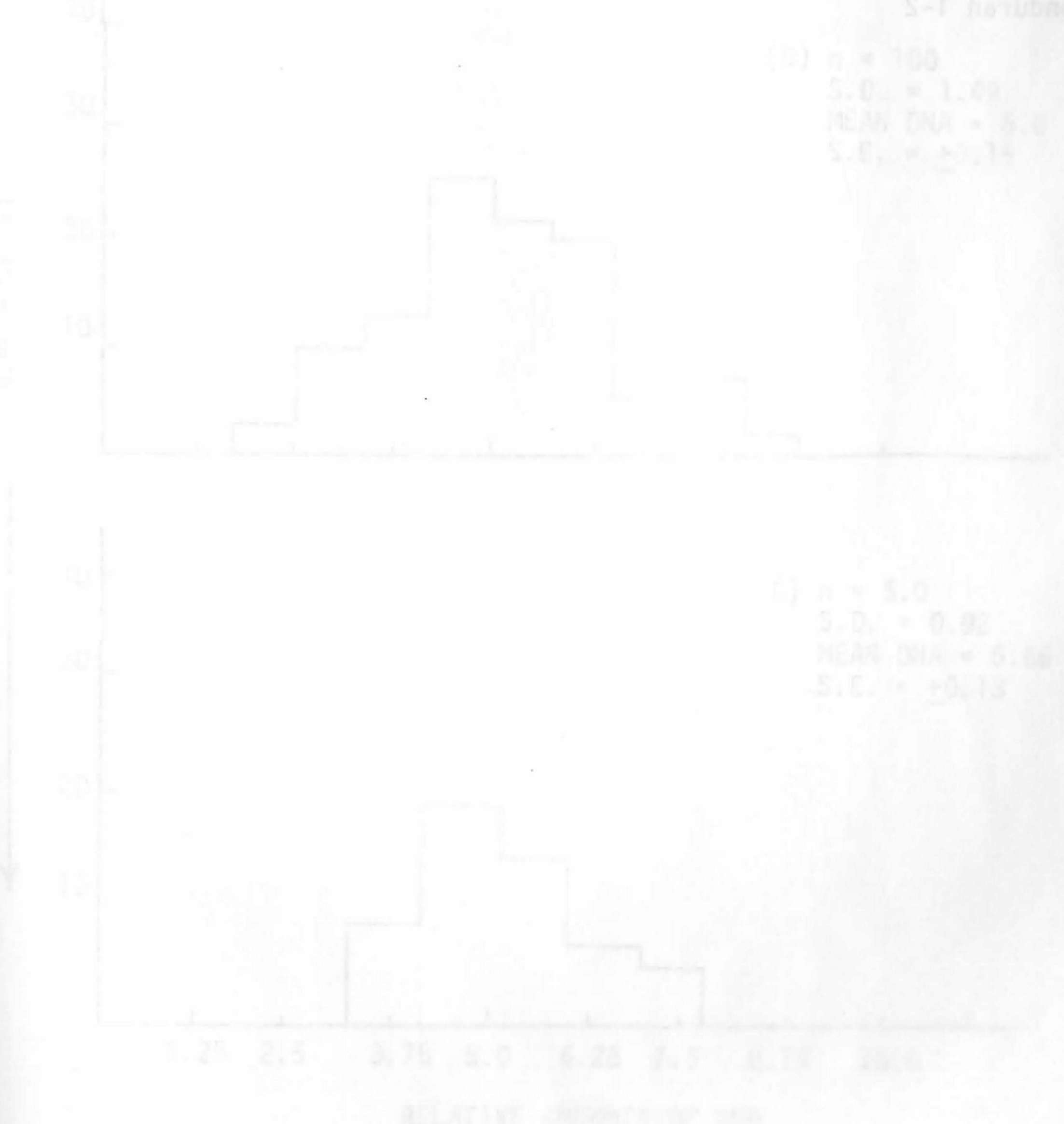
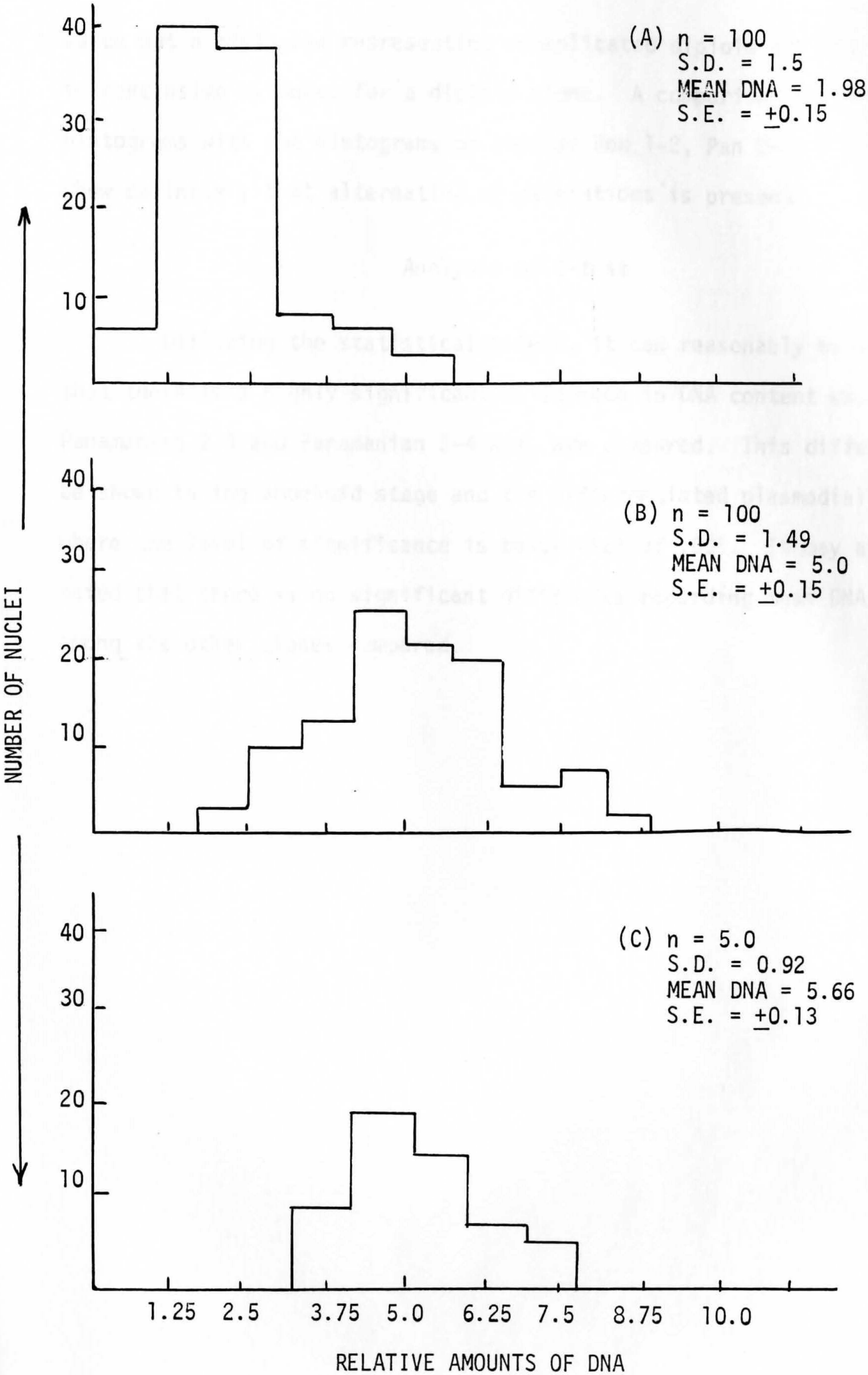


FIGURE 4

Histograms representing relative Feulgen-DNA content. (a,b) Panamanian 2-4 w.t. x Honduran 1-2 plasmodium; (c) Panamanian 2-4 x Honduran 1-2


(A) n = 100
S.D. = 1.5
MEAN DNA = 1.98
S.E. = ±0.15
(B) n = 100
S.D. = 1.49
MEAN DNA = 5.0
S.E. = ±0.15
(C) n = 5.0
S.D. = 0.92
MEAN DNA = 5.66
S.E. = ±0.13
40
30
20
10
NUMBER OF NUCLEI
1.25
2.5
3.75
5.0
6.25
7.5
8.75
10.0
RELATIVE AMOUNTS OF DNA

value but a histogram representing unreplicated diploidization, which is conclusive evidence for a diploid clone. A comparison of these three histograms with the histograms of amoebae Hon 1-2, Pan 2-4, Pan 2-4 w.t. show definitely that alternating of generations is present.

Analysis of t-test

Utilizing the statistical t-test, it can reasonably be ascertained that there is a highly significant difference in DNA content when the Panamanian 2-4 and Panamanian 2-4 w.t. are compared. This difference can be shown in the amoeboid stage and the differentiated plasmodial stage where the level of significance is below that of .001. It may also be noted that there is no significant difference regarding mean DNA levels among the other clones compared.

TABLE I

DNA Comparison of Clone

Clone	$\overline{X}$DNA	S.E.±	S.D.	DNA Diff.	t-test
Hon 1-2 Pan 2-4	3.5	0.17	1.56	.72	* .98
Pan 2-4 w.t. Hon 1-2	2.24	0.08	0.72	1.26	*1.32
Pan 2-4 Pan 2-4 w.t.	2.73	0.14	0.93	.72	**2.80
Plasmodia					
Pan 2-4 x Hon 1-2	5.66	0.13	0.92	.66	*1.1
Pan 2-4 w.t. x Hon 1-2	5.00	0.15	1.49		
Pan 2-4 selfer	2.70	0.09	.89	.91	**45.5
Pan 2-4 w.t. selfer	1.79	0.09	.91		

*sig. at .05
**sig. at .001

DISCUSSION

The results of this experiment clearly indicate that the Panamanian 2-4 clone of the heterothallic myxomycetes of D. iridis is apomictic and is in an aneuploid state, supporting previous investigations of Collins and Ling 1968, Yemma and Therrien 1972, 1974, 1977, Haskins 1977, and Clark and Collins 1976. The results also indicate that plasmodia produced from self-fertile amoebae occur at a greater instance in the Panamanian 2-4 clone than in its parent apomictic clone Panamanian 2-4 w.t. These conclusions can also be supported by the statistical analysis of the student t-test.

The apomictic development is not new in the myxomycetes, it has previously been reported by several other investigations: Gehenio and Luget 1950; von Stosch et al., 1964; Kerr 1967; Anderson, Cooke, Dee, 1976; Therrien and Collins 1975; Therrien and Yemma 1974, 1977; and Haskins, 1977. These studies indicate that the n-2n situation is not an obligatory prerequisite to plasmodial formation, but that meiosis and karyogamy do not occur and that the plasmodia was in an extended G_2 period (Ross, 1967; Adler and Holt, 1975). The differentiation from amoeboid to plasmodial stage has been postulated to result from a series of compatible multiple alleles at a single locus (Collins 1963, 1964). This process is thought to be active at both membrane and gene physiochemical levels (Ross and Shipley, 1972; Olive, 1975). This study supports this hypothesis. It is also possible that the differentian event could also result from a homothallic process. Whereby in homothallism a monospore culture can exhibit plasmodial development where myxamoebae do not contain different mating alleles, "Any two cells from a single clone may fuse to form a zygote which in turn, develop into a plasmodium." Therrien and Yemma, 1974. Some of the species that have been reported to develop this way are Didymium difforme (Schunemann, 1930), Physarella oblonga (Ross, 1957), Fuligo cinerea (Collins, 1961), and Didymium nigripes (Kerr, 1961). Since the plasmodia in this experiment are haploid, homothallism can be excluded as a possibility for their development (Cooke and Dee, 1974) since homothallic plasmodia display a diploid content. Regardless of the genetics of formation there are two possibilities for the development of a multinucleate plasmodia from a uninucleate amoebae: cell fusion with nuclear fusion (coalescence), or repeated nuclear divisions within a single cell (apogamy).

The results presented indicate that the self plasmodia Pan 2-4 is in a haploid state or apomictic. This conclusion is based on the comparison of histograms in both amoeboid and plasmodial stage, where the DNA content is 2.73 and 2.70, respectively (Figures 2 and 3) indicative of apogamy. Since there is no display of alternation of haploid or diploid state, according to the DNA content, it can be concluded that it is apomictic. Further support can be postulated by comparing the known heterothallic isolates which are known; Therrien and Yemma 1974, 1975; Collins and Therrien, 1976.

This apomictic condition can also be supported utilizing Table 1, which shows that there is a significant difference in the Panamanian 2-4 w.t. parent and the Panamanian 2-4 isolate. This difference in relative DNA contents is depicted not only in the amoeboid but also in the plasmodial stage. This significance in DNA contents supports the view that ploidy level may play a role in the amoebae to plasmodial transition. These results also indicate that the Pan 2-4 is aneuploid. It appears that the DNA content of the Pan 2-4 is greater than its parent clone Pan 2-4 w.t., which satisfies the criteria for aneuploidism.

However, while no actual karotype is known for D. iridis it has been repeatedly demonstrated that DNA content can be correlated with ploidy levels (Collins and Therrien, 1976). It should also be noted in reference to the occurance of the self plasmodia in each isolate that the occurance of self plasmodia in the Pan 2-4 w.t. strain was between 10-15%, where the Pan 2-4 showed a 90-100% occurance of self plasmodia (Therrien, Unpublished results). This correlates with a higher mean DNA content in this strain. This 5-10 fold increase suggests some genetic or cytological factor that is instrumental in an increase in the ability to produce plasmodia apomicticly in the Pan 2-4 than in its wild type parent.

The complementation of the aneuploid state of the Pan 2-4 isolate with its increase in occurance of self plasmodial formation suggest that the mating loci is a regulatory locus (Yemma, 1972). A suggested mechanism to account for the increase in plasmodia formation is due to an increased amount of DNA, presumably establishing a heterozygous condition at the mating loci, which could initiate the conversion of amoebae to plasmodia, by passing karyogamy (Collins, Therrien and Betterly, 1978; Adler and Holt, 1975). This heterozygous condition could be the result of chromosomal elimination, for review of chromosomal elimination (Collins, Therrien, and Betterly 1978).

Whether an inducer is released into the media as suggested by Youngman, Adler, Shinnick, and Holt by differentiating amoebae of P. polycephalum signaling neighboring amoebae to participate is not known. A report much earlier than this by Ross, 1966, suggests the same mechanism, "it is not the mere doubling of chromosome number as a result of karyogamy that initiates the change from amoebae to plasmodia, but some other trigger is responsible." Work is presently being conducted using an antigen-antibody complex to isolate this substance. (Personal communication, C.D. Therrien). The possibility of amoebae working in concerted fashion raises many questions. Questions pertaining to membrane changes to facilitate the amoebae-plasmodia transition, the chemical nature of the inducer, and the prospect of isolating and producing this inducer to affect change in indifferentiating cells.

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