EVIDENCE FOR A MATING INDUCER MOLECULE NECESSARY FOR CELL FUSION AND ZYGOTE FORMATION IN **DIDYMIUM IRIDIS**

by

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ABSTRACT

Evidence for a Mating Inducer Molecule
Necessary for Cell Fusion and Zygote
Formation in **Didymium iridis**

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Master of Science
Youngstown State University, 1989

Haploid myxamoeboid mating types, CR5-11A[11] and HON 1-2A[1], of the acellular myxomycete **Didymium iridis**, were isolated and grown in liquid culture. Previously it has been postulated that a molecule, possibly a hormone, is responsible for the induction of mating competency through receptor site synthesis. The goal of this study was to isolate and characterize the molecule and to determine its mode of action. Experiments were designed in order to learn whether it acts only upon the cells which produce it (self induction) or does it have an effect only on opposite mating types (cross induction). In order to do this, separate experiments, one involving a membrane spin culture and the other a separate spin culture, were maintained. The membrane separated culture contained a .45um filter which allowed any diffusable molecule to freely pass through, but not cells, which would allow cross induction to occur while the separate cultures would allow self induction only. Three daily samples were taken, two of which acted as controls of the two clones while the third sample was a

cross of the two clones. The latter contained induced cells which should produce zygotes, or fused cells. These samples were subjected to proper fixation, collection, and staining utilizing the Feulgen reaction. A microspectrophotometric analysis was then performed to quantitatively measure cellular nuclear DNA content, and to provide a means for the detection of cellular fusion. Additionally, experimentally crossed compatible mating types were plated to solid growth media in order to detect any diploid plasmodia formed from fused cells. Plasmodia were similarly prepared and analyzed. Interestingly, it was found that plasmodia can only form on solid media, although zygotes are readily formed in liquid media. Also, competency of cells to fuse, or receptor formation, required that they first undergo a period of maturation.

The use of High Pressure Liquid Chromatography (HPLC) techniques was also employed in order that the inducer molecule be studied. Samples were taken from separate liquid cultures of the two clones, frozen and subsequently analyzed. Also, a control sample containing only the media was run and analyzed.

It was demonstrated that the inducer molecule is similarly produced by both mating types, its production is paralleled by cell numbers, and it is important in cellular maturation and subsequent fusion through receptor site formation. It was also found that mating competency is self induced.

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CHAPTER I

Introduction

The myxomycetes are eukaryotic organisms which demonstrate the most basic of life cycles, which consist of single cells of two types, myxamoebae and swarm cells, which upon fusion of compatible mating types produce a diploid plasmodium. They demonstrate most of the characteristics that are associated with the cell differentiation processes of the higher plants and animals (Ashworth and Dee, 1975). Because of this, they are an ideal research subject.

Didymium iridis belongs to the group of acellular myxomycetes. Its life cycle is illustrated in Figure 1. Each spore of D. iridis is capable of germinating into a haploid form, either a myxamoeba or a biflagellate swarm cell. These two forms are interconvertible, the myxamoeba being prevalent in drier conditions, while the swarm cell is found primarily in an aqueous environment. The cells can increase in number through mitotic divisions until log phase is reached. Once log phase is reached, these cells can then act as gametes in sexual fusions. The species D. iridis exhibits genetically a bipolar multiallelic mating system. There must be two different alleles located at the mating locus, which comprise

compatible mating types, in order that zygote and subsequent plasmodial formation occur.

Once a zygote is formed, a diploid microplasmodium will develop. The resulting microplasmodium will develop into a large multinucleated plasmodium by successive nuclear divisions or by fusing with other zygotes or plasmodia. The plasmodia after a time will give rise to sporangia and spores if certain environmental conditions are present (Ashworth and Dee, 1975).

The fusion of somatic cells is a well known occurrence. For example, it occurs normally in the polykaryocytes of bone and muscle and occurs in all diploid organisms upon zygote formation, and occurs abnormally in cancer cells. However, the mechanism which initiates fusion is a largely unknown phenomenon. Since the slime molds are eukaryotic organisms which possess both a haplo and diplo phase as a normal part of their life cycle (Ross, 1967), they are ideal for studies including cell fusion and differentiation.

In 1958 Wickerham had reported zygote formation upon the mixing of yeast cells of two opposite mating types. He proposed at this time a mechanism similar to agglutination, or clumping. Data presented by Yemma and Perry (1985) has shown however, that rapid agglutination is not the mechanism for zygote formation in **D. iridis**. In 1973 Ross first presented the idea that sexual fusion is the result of chemical induction which can initiate cell surface changes. These changes allow the recognition and subsequent membrane

fusion of compatible mating types (Ross, 1973). Later, in 1977 Crandall postulated that a diffusable molecule, possibly a hormone, caused the initiation of these cell surface changes. Studies using a Colonia isolate of **Physarum polycephalum** indirectly demonstrated the presence of diffusable substance through a .2um Nucleopore filter which enhanced the conversion of amoebae to plasmodia (Youngman et al., 1977). Pallotta et al. (1979) also postulated the existence of an extracellular inducer, produced by growing cells, which was required for cell fusion. Mating is envisioned as a two part process: (1) The formation and release by cells of a diffusable substance. The substance may be produced by cells of one mating type which induce themselves or there may be cross induction of compatible mating types (Shipley and Holt, 1982). (2) The recognition of this substance by compatible mating type cells and the subsequent synthesis of specific membrane receptors prequisite to fusion and zygote formation. Zygote formation in **D. iridis** has been found to be a time dependent process (Yemma and Perry, 1985). This supports the two step mating process envisioned, as a period of time (induction period) would be required for the receptor site synthesis. However, not all cells have the capacity to fuse and form zygotes (Yemma and Perry, 1985). One possible explanation is that the affector molecules become increasingly diluted or that cells must reach a level of maturity before they can

become competent to mate. The purpose of this study was first to determine the mode of action of the inducer molecule, i.e., does it act on the cells which produce it (self-induction), or does it act only on cells of compatible or opposite mating types (cross-induction), and secondly to isolate and characterize the inducer molecule.

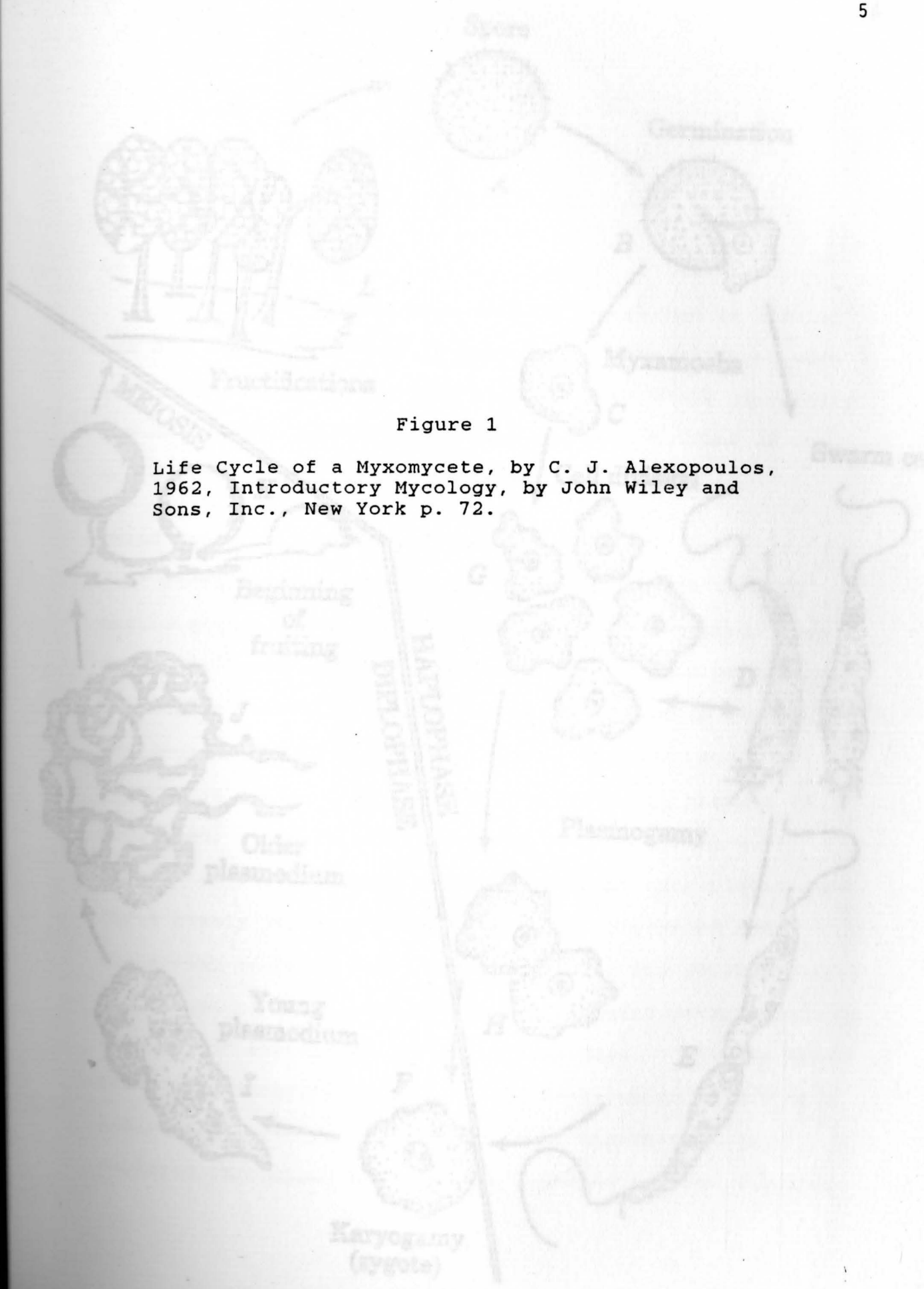
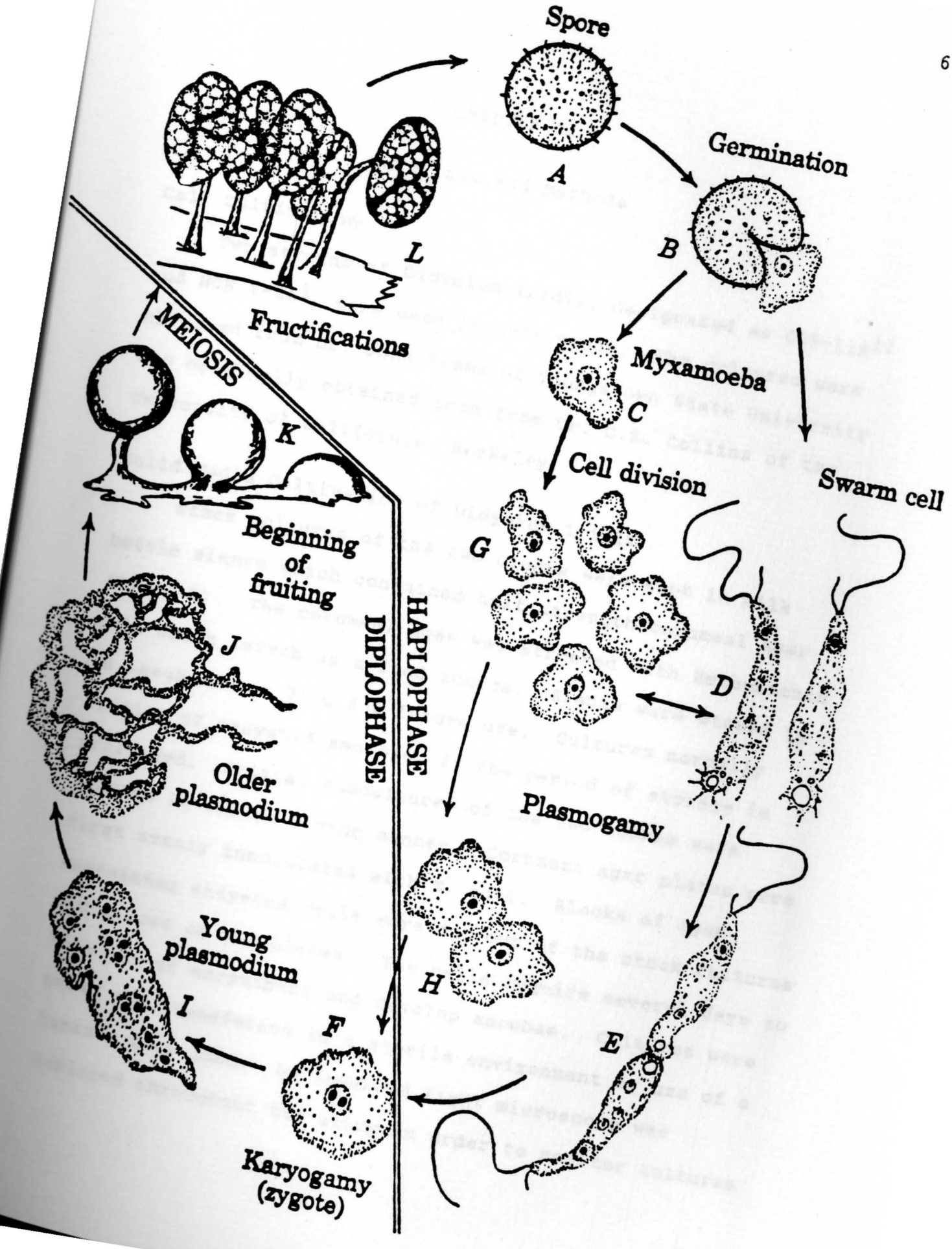


Figure 1

Life Cycle of a Myxomycete, by C. J. Alexopoulos, 1962, Introductory Mycology, by John Wiley and Sons, Inc., New York p. 72.


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

CHAPTER II

Materials and Methods

Cell Cultivation

Two strains of **Didymium iridis**, designated as CR5-11A[11] and HON 1-2A[1], were used in this study. The cultures were obtained from Dr. John Yemma of Youngstown State University who originally obtained them from Dr. O.R. Collins of the University of California, Berkeley.

Solid Media Cultivation of **Didymium iridis**

Stock cultures of the two clones were kept in milk bottle slants which contained half strength cornmeal agar (Table 1). The cornmeal agar was streaked with **Escherichia coli** which serves as a food source. Slants were stored in an incubator at 21 C for future use. Cultures normally consist of encysted amoebae, if the period of storage is prolonged. Initial subcultures of the two clones were started in the following manner. Cornmeal agar plates were first evenly innoculated with **E. coli**. Blocks of agar containing encysted cells were cut out of the stock cultures and placed on the plates. The cells require several days to come out of encystment and develop amoebae. Cultures were grown and transferred in a sterile environment by use of a laminar flow hood. An inverted light microscope was employed throughout the study in order to monitor cultures

TABLE 1

HALF STRENGTH CORNMEAL AGAR

8.5 grams Difco Corn Meal Agar
8.0 grams Difco Bacto-Agar
1.0 liter Distilled Water

The media was mixed and then autoclaved at 121 C, 15 psi, for fifteen minutes. Sterile petri dishes were then filled with this media.

for growth and contamination. Any plates showing signs of contamination were discarded.

Liquid cultures developed from solid agar stocks permit a convenient and useful method for preparing cells for study and reduce contamination to minimal levels. The liquid cultures were maintained in tissue culture flasks which contained cells removed from cornmeal agar slants and maintained in sterile HPLC (High Pressure Liquid Chromatography) grade water. A small amount of **E. coli** was added to the water in order to provide nutrients for the cells. These cultures were also stored in an incubator at 21 C and essentially comprised our working cultures. When clones were to be stored, this was done by adding approximately 1 - 2 drops of liquid culture to cornmeal agar plates which had been previously streaked with **E. coli** and stored at 21 C.

Liquid Cultivation of **Didymium iridis**

In addition to solid media, as already noted, **Didymium iridis** can also be grown in a liquid media. The method requires sterile HPLC water be pipetted into culture flasks so that the bottom is covered. To this a liquid suspension of **E. coli** is added. Next, either 1 - 2 drops of liquid containing cells, or media containing cells on the surface, as the case may be, is placed in the liquid. Extensive use of liquid cultured cells proved very beneficial throughout this study because of enhanced growth in this media, the

ease of observation and as noted, a reduced probability of contamination. Solid media was employed essentially to maintain stock cultures and to grow plasmodium when necessary.

Cultivation of Cells in Spin Culture

When enough cells had been obtained in liquid culture they were transferred to a spin liquid culture apparatus. The spin cultures can be maintained as two distinct and separate units, or as a continuous unit, separated by a .45um membrane filter. The culture also contains a stirrer, to prevent clumping of the cells and to ensure proper aeration. The liquid was maintained at approximately 100 ml using E. coli and sterile HPLC water. This allowed enough liquid for proper maintenance of experimental cells, and permitted daily cell counts to be made. In cases where the spin cultures were to be separated by the membrane, this volume allowed easy mixing of the two sides and equilibration between the two to occur. Cells of two mating types were counted daily using a hemocytometer.

In order to carry out experiments in which cellular fusion was studied three samples were taken from each spin culture daily. These consisted of 4 ml of each separate mating type culture, which acted as controls, and 2 ml from each mating type culture (for a total of 4 ml) which were mixed, resulting in a crossed culture. These were allowed to stand for one hour, and subsequently scored for zygote

formation. At the end of the one hour all three samples were prefixed in 10% buffered formalin for ten minutes. The samples were then centrifuged at 100 G's for fifteen minutes. The supernatant was decanted and the remaining pellets were fixed in 10% buffered formalin for twenty-four hours. Samples were once again centrifuged at 100 G's for fifteen minutes and the supernatant drawn off. The cells were next post-fixed in 70% ethanol for twenty-four hours. At the end of this period they were centrifuged at 100 G's for fifteen minutes and the supernatant decanted. The pellets, or cells, were then pipetted onto albuminized slides and allowed to air-dry. A total of three spin culture experiments were carried out; two were totally separate cultures and a third with cells in two compartments made continuous through a .45um membrane. When plasmodia were required they were obtained by mixing 1 - 2 drops of compatible mating type cells on cornmeal agar plates which were previously streaked with **E. coli**. The plasmodia which developed were first fixed and cleared and then floated off the surface of the agar and mounted on albuminized slides.

Feulgen Reaction For Quantitative Measurement of Nuclear DNA

The Feulgen Reaction (Feulgen and Rossenbeck, 1924) is both a specific and quantitative stain for deoxyribonucleic acid. Hydrolysis of nuclei in 5 N HCl for 45 minutes (Yemma and Penza, 1987) removes the purines and exposes the free aldehyde groups on the adjacent deoxyribose moieties.

The stain then complexes to the free aldehyde groups. Hydrolysis time is crucial; if hydrolysis is allowed to continue for too long the depolymerization of DNA will take place. If the hydrolysis time is too short, the result will be incomplete hydrolysis.

The stain was prepared as follows:

1. 10 grams of Basic Fuschin obtained from Fisher Scientific Company, color index number 42500, and 22 grams of potassium metabisulfite were added to one liter of 1 N HCl.
2. This solution was mechanically stirred for two hours.
3. The stain was next wrapped in aluminum foil to prevent any exposure to light and placed in a dark cabinet overnight.
4. 5-10 grams of activated charcoal from Fisher Scientific were added to the stain and it was suction-filtered until it became a colorless solution.

Feulgen Staining Procedure

Tissures were stained using the following protocol:

1. Slides were placed in 70% ethanol for five minutes.
2. They were next moved to distilled water for one minute.
3. Tissures were hydrolyzed for 45 minutes in 5 N HCl (Yemma and Therrien, 1972).
4. Tissures were stained for two hours in Schiff's Reagent (Lillie, 1951) which has been fortified with freshly prepared 10% potassium metabisulfite in a ratio of 4 parts stain to 1 part potassium metabisulfite.
5. Following staining tissues were treated in two 5 minute washes of 10% potassium metabisulfite.

6. Tissues were subsequently dehydrated in a graded ethanol series of 70%, 95%, and 100%.

7. Tissues were next placed in xylene for ten minutes for clearing; afterwhich they were coverslipped using permount.

The slides used were Special Select purchased from VWR Scientific Inc. while the coverslips were #1.5, 24x50 mm from Corning Glass Company.

Microspectrophotomer Quantitative Measurements

The microspectrophotometer is a valuable instrument which permits the quantification of cellular macromolecules including deoxyribonucleic acid. There are two methods which can be employed in order to make measurements: the two wavelength method and the plug method. The two wavelength method of Patau (1924) was employed to obtain DNA measurements throughout this study. The two wavelength method is applicable when the distribution of the cellular material is irregular or nonhomogeneous in distribution, such as cells demonstrating mitotic figures or numerous chromocenters. The procedure requires that an absorption curve first be plotted in order to select the two measuring wavelengths. In order to do this, a homogeneous area is selected within the object and two measurements, including an off and an on reading for each cell, are taken at different selected wavelengths, begining at 425 nm and continuing to 625 nm. The measurements are designated as I_o, or the background light, and I_s, the light which passes

through the specimen. The percent transmittance can then be calculated as $\%T = I_s/I_o$. The optical density, or extinction, can then be determined since O.D. = log 1/T. The extinction versus the wavelength is plotted and a curve is obtained. The maximum absorbance is determined and the wavelength at which this occurs is designated E_2. This will be the upper, or maximum, wavelength at which measurements are made. The lower wavelength, designated as E_1, is chosen so that

$$2E_1 = E_2$$

where:

E_2 = log 1/T = log I_o/I_s at λ_2 (maximum wavelength)

E_1 = log 1/T = log I_o/I_s at λ_1 (half maximum wavelength)

The maximum and half maximum wavelengths chosen were 565 nm and 515 nm respectively (Figure 2).

Once the two wavelengths have been selected, nonhomogeneous areas of the object may be measured. This is done by first selecting an aperture, located in the photohead of the microspectrophotomer, which just encircles the desired area and then taking the measurements at the two wavelengths.

The amount of dye (DNA) in the quantitatively measured area, or aperture, is given as:

$$M = KAL_1C$$

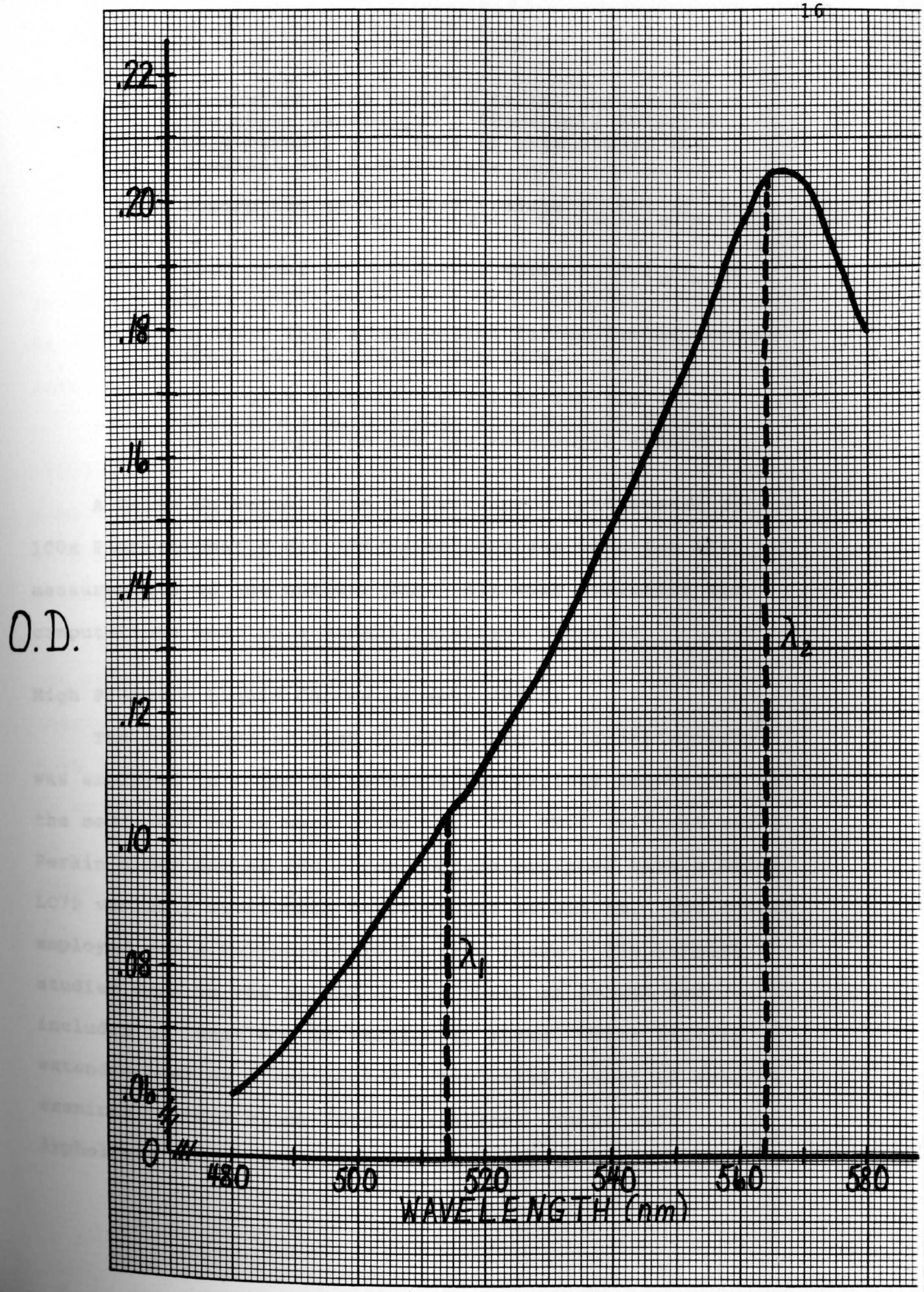
where:

A = area of the aperture (πr^2)

Figure 2

Absorption Curve for Feulgen

Stained DNA of **D. iridis**


O.D.
.22
.20
.18
.16
.14
.12
.10
.08
.06
0
480
500
520
540
560
580
WAVELENGTH (nm)
λ_1
λ_2

K = a constant, 1/E where E is the extinction at the upper wavelength. Since only relative measurements were made in this study, the constant was disregarded.

L_2 = absorption at λ_2 = $(1-T_2)$ where $T_2 = I_s/I_o$ at λ_2

L_1 = absorption at λ_1 = $(1-T_1)$ where $T_1 = I_s/I_o$ at λ_1

Q = L_2/L_1. This a correction factor for unoccupied space in the reading aperture.

C = $1/(2-Q)LN\ 1/(Q-1)$. C values for the corresponding Q values are found in Patau's (1952) paper on the two wavelength method.

A Zeiss Universal Type 01 Microspectrophotometer with a 100x Planachromatic oil immersion lens was used for all measurements of the nuclear DNA. All were evaluated and computations made on a Amdahl mainframe computer.

High Pressure Liquid Chromatography (HPLC)

The technique of High Pressure Liquid Chromatography was employed in order to separate the various components of the media the cells were grown in. The HPLC utilized was a Perkin Elmer Series 400 chromatograph equipped with a Model LC75 variable wavelength detector set at 215 nm. The column employed was a Polypore C-18 Reverse Phase. The samples studied were in the period of log phase of growth and included at least one in stationary phase, which usually extended over an 8 day period. The total number of samples examined in this study totaled sixteen. These were lypholyzed prior to examination in order to concentrate the

solute present in the liquid. They were then reconstituted in 1.0 ml of sterile HPLC water.

The second set of samples consisted of daily samples taken from separate spin cultures which contained only sterile HPLC water and **E. coli**. The samples were immediately frozen and, as noted, were taken for seven days. At the end of this time all samples were lypholyzed. They were also reconstituted in 1.0 ml of sterile HPLC water.

Prior to analysis all samples were filtered through a .45um filter (Gelman Sciences, Ann Arbor, Michigan) into a sample vial. Five microliters of each sample was injected onto a Polypore column using a Perkin Elmer ISS-100 injector with a 200 ul loop.

The mobile phase, which consisted of .035 H_2SO_4, was run at a flow rate of .25 ml/minute for a total run time of thirty minutes. Following each run, the column was flushed with HPLC water. An oven supporting the column was utilized in order to keep the system at a constant temperature of 32 C. All data was collected and analyzed using a Perkin Elmer computer.

CHAPTER III

Results

Data representing quantitative DNA measurements recorded in this study are presented in Figures 3 - 17. These include haploid myxamoebae, results of crosses made between compatible mating types and resulting diploid plasmodium. The analysis of nuclear DNA is illustrated by means of histograms. Data presented in this manner is convenient. They display nuclear DNA frequency and the distribution of cell populations, facilitating the observation of minor shifts in DNA that may be due to its diffuse condition or increased template activity (Yemma and Therrien, 1972), or ploidy level. The number of nuclei measured was plotted on the ordinate and the relative dye concentrations of Feulgen stained DNA were correspondingly plotted on the abscissa. Twenty-five nuclei were usually measured in order to provide an adequate random representation of the cell population. All measurements were limited to interphase nuclei, and are reported in arbitrary units as relative DNA values. The mean DNA values, standard deviation, and standard errors are also given. The results of initial experiments representing the growth of cells and crosses of compatible mating types carried out over an eight day period in liquid culture media

are presented. Cultures were grown in joined separate spin cultures separated by a membrane (.45um) impervious to cell passage but not to molecular diffusion. A following experiment was conducted under essentially the same conditions but instead included separate spin cultures (non-continous). These experiments were designed in order to determine whether induction of cells, that is, competence for mating or generation of mating factors or receptors, (Yemma and Soltis, 1988) were cross induced or self induced. All cell culturing was done in liquid media as described in methods.

In the initial experiment, the membrane spin culture, it should be noted that the histograms representing nuclear DNA measured in the controls essentially presented a unimodal distribution as was expected for both the CR5-11A^{11} and HON 1-2A^{1} mating type clones (Figures 3 - 10). Since growing cells are represented throughout this study, cells having recently completed mitosis (G_1), as well as S and G_2 cells are represented. The average DNA concentration for the CR5-11A^{11} strain for all measured periods was .466 while for that of the HON 1-2A^{1} strain was .519. All measured cells were in the interphase stage of the cell cycle. Normally cells of the myxomycetes show no appreciable G_1 period but rather a transitional one, but do possess a lengthy G_2 period of 12 - 20 hours (Rusch, 1969), which accounts for the unimodal distribution observed for those cells. Note for example, that for Day 1 (Figure 3) the

HON 1-2A^{1} strain demonstrates a maximum relative DNA concentration for G_2 cells of 1.0, and values of .1 minimum arbitrary units for G_1 cells which have recently completed mitosis. The CR5-11A^{11} strain demonstrates many more G_1 cells than the former and of course a smaller number of cells in G_2. Note that in both there are many cells in S phase as expected. Whereas a bimodal distribution of DNA concentrations is demonstrated for the CR5-11A^{11}x HON 1-2A^{1} crosses of compatible mating types; here two cellular populations are represented, namely a haploid and diploid unreplicated (G_1) population of zygotes. These cells, having been previously induced in flask cultures, when transferred to the spin cultures demonstrated fusion competency at this time period. The mean DNA concentration for the measured cellular population for crosses over an eight day period was .542.

It is important to note that the mean of a bimodal population must be observed with care for it obscures the presence of two separate population.

The controls for Day 2 (Figure 4) present a unimodal distribution with cells being in the G_1 and G_2 haploid category. Here more cells are in G_1 than the previous day's sample; cells having just completed mitosis would account for this. The experimental cross for this time period contains cells where the majority have DNA contents similar to the controls. Also observed however, is a bimodal

distribution with a population of cells in the diploid or zygote category.

Presented in Figure 5 are the results of Day 3. Once again controls contain haploid cells in both the G_1 and G_2 phases; it should be noted however, that in this time period there are greater numbers of G_1 cells present than in previous time periods which provides insight as to the rate of mitosis one can expect at this time period. It is evident that a high mitotic rate tends to obscure the expected unimodal distribution. The experimental cross demonstrates a bimodal distribution with diploid zygotes being present. Haploid unfused cells in G_1 and G_2, as in previous time periods, are abundantly present. Note that diploid cells are present at relative units 1.1 to 1.2 and 1.4 to 1.5. Apparently cells can fuse while at the same time being engaged in DNA synthesis.

The CR5-11A^{11} control of Day 4 (Figure 6) is comprised primarily of haploid cells in the G_1 and S phase, having recently completed mitosis. The HON 1-2A^{1} control, a haploid population, is composed of cells in both the G_1 and G_2 stages. Collectively the controls are indicative of a proliferative population of cells. This tends to influence the shape of the histogram and gives the appearance, in a sense, of a bimodal distribution of DNA values. The CR5-11A^{11} control is made up essentially of cells which have recently completed mitosis and some in S phase, while the HON 1-2A^{1} contains G_1, S, and G_2 cells. The experimental cross also

shows haploid unfused cells in both G_1 and G_2 as previously described. Additionally, a bimodal distribution is demonstrated with zygotes present.

For Day 5 (Figure 7) the CR5-11A^{11} control has haploid cells in both G_1 and G_2. Regarding the HON 1-2A^{1} control the majority of cells are in the G_1 stage or early G_2. It is once again indicated that many of these cells have recently completed mitosis. Interestingly enough a number of G cells demonstrate rather low nuclear DNA values. These represent cells in G_1 arrest and more than likely are dying cells. It is important to recall that these cells are grown in spin culture and are subject to a certain degree of trauma. The appearance of G_1 arrested cells leads to a definite population of these cells as well as those in G_2. The experimental cross has haploid cells both in G_1 and G_2. As previously stated a bimodal distribution is demonstrated which includes the presence of zygotes at values similar to other time periods for crosses.

Presented in Figure 8 are the results for Day 6. The two controls both contain haploid cells in both G_1 and G_2. The cross also has cells in the G_1 and G_2 stages. Once again a bimodal distribution of DNA content is exhibited with zygotes being present.

The results of Day 7 are shown in Figure 9. Both controls possess haploid cells in both the G_1 and G_2 phases. In the HON 1-2A^{1} control a number of cells appear to be in G_1

arrest, while most cells appear to be in various stages of the G_2 phase. The appearance of G_1 cells in arrest gives rise to the appearance of a bimodal distribution since progression through the cell cycle does not occur readily regarding these cells. This is more than likely due to the progressive age of the culture and the death of some cells. Note that in the cross haploid cells in G_1 and G_2 are present and a bimodal distribution is once again evident and demonstrates the presence of zygotes. It can be noted that in this time period, as in those previously presented, there appears to be a lack of late haploid G_2 cells.

In Day 8 (Figure 10) the CR5-11A^{11} control contains haploid cells in G_1 and late G_2 as expected. Also note that for the first time degenerating cells are clearly evident. It is quite possible that in this late period, those cells which appear to be in G_1 arrest are now undergoing degenerative changes. The HON 1-2A^{1} control contains cells primarily in the S and G_2 phase. The experimental cross contains some cells having a nuclear DNA concentration which is quite low, which previously had not been observed. These appear to be, as previously stated, degenerating cells in G_1 arrest, while it also appears that for the first time, many late G_2 cells are present which tends to obscure the bimodal distribution of cells. Interestingly, Day 8 presents the stationary phase of cell growth. The aging population demonstrates, as expected, dying cells and cells which appear to be in G_1 arrest.

The separate spin culture also demonstrated essentially the same type of daily cell cycle data as that observed for the membrane spin culture both containing pre-induced populations. The characteristic unimodal distribution of mean DNA concentrations for both controls, CR5-11A^{11} and HON 1-2A^{1} are observed (Figures 11 - 16). The mean nuclear DNA for all the time periods studied for the CR5-11A^{11} is .310 while it is .407 for the HON 1-2A^{1}. The observed mean DNA values compared well with those from the previous experiment. However, it should be noted that induction efficiency may be enhanced by the continuous system separated by a membrane, since the inducer molecule is permitted to achieve equilibrium between both sides of the culture. Because of differing levels of DNA synthetic activity some differences can be expected. The crosses in most cases demonstrated a bimodal distribution due to the appearance of small numbers of zygotes. The measured nuclear mean DNA concentration for represented time periods is .466. Both controls and crosses are presented in Figures 11 - 16.

The controls for Day 1 (Figure 11) demonstrate a unimodal distribution with most haploid cells being in G_1 and G_2. A large number are in G_1, which is indicative of a high level of mitotic activity. The experimental cross exhibits haploid cells in both the G_1 and G_2 stages of the cell cycle. Also presented is a bimodal distribution with

fused haploid cells or zygotes being present.

Presented in Figure 12 are the results of Day 2 separate spin culture data. It should be noted that the CR5-11A[11] control is composed primarily of cells in G_1, indicating a high level of mitotic activity for this period. The HON 1-2A[1] control exhibits cells mainly in G_1 with some cells in G_2. The experimental cross shows a bimodal distribution with zygotes being present and showing DNA synthetic activity. Many cells are haploid and are in the early G_1, S and G_2 stages respectively, denoting rapid growth.

Shown in Figure 13 are the results of Day 4. Both the controls are composed primarily of cells in the G_1 phase of the cell cycle. The cross has both G_1 and G_2 cells present. However, a bimodal distribution is not readily apparent. It appears that late G_2 cells are present, i.e., cells at replicated haploid levels (G_2) and early G_1 diploid levels. This tends to obscure the bimodal distribution. Note that at this time period that many cells of both controls appear to have recently completed mitosis, and thus many immature cells are present. This time period interestingly appears to provide data which implies that the membrane spin culture technique is more efficient than the separate cultures, regarding zygote formation. This may be due to the probable distribution of inducer molecules being in equilibrium with both sides of the cultured cells in the former and not being so in the later or separate cultures. This data along with

HPLC data presented later indicates that the inducer molecule, although produced by separate compatible mating types, is nevertheless similar.

In Day 5 (Figure 14) the CR5-11A^{11} control has a majority of cells in G_1, the result more than likely, of recent mitosis, and the presence of degenerating cells. The HON 1-2A^{1} control shows cells present in both G_1 and G_2. The experimental cross also has cells both in G_1 and G_2. Also in the experimental cross, note the bimodal distribution which demonstrates the presence of zygotes. The zygotes appear to be in varying stages of DNA synthetic activity.

The controls for Day 7 (Figure 15) show a unimodal distribution with many cells being in G_1, having recently completed mitosis and some in G_1 arrest. The HON 1-2A^{1} control also exhibits many cells with haploid G_1 nuclear DNA values. The experimental cross demonstrates a bimodal distribution with zygotes in the 1.20 to 1.35 relative DNA units. Since here .90 units would represent diploid unreplicated cells (2C, G_1), these cells appear to be in the synthetic phase, and are approaching the 4C DNA level.

Presented in Figure 16 are the results for Day 8. Both the CR5-11A^{11} and the HON 1-2A^{1} controls contain cells in the G_1, S, and G_2 phases. The experimental also posesses cells in both the G_1 and G_2 stages of the cell cycle. Additionally, note the bimodal distribution and the presence

of zygotes. At this later time period it is evident that many cells are in late G_1 arrest. It is interesting to note this appears to occur only in aged cultures.

A follow up replicate experiment from which microspectrophotometric data was obtained in order to determine the reproducibility of the separate spin data was run. Once again a unimodal distribution of DNA concentrations was consistently obtained in most cases for the controls. Their respective mean DNA concentrations for all time periods are .446 (CR5-11A^{11}) and .425 (HON 1-2A^{1}) and demonstrated good correlation. Also, the CR5-11A^{11} x HON 1-2A^{1} cross demonstrated a bimodal distribution where zygotes were present in similar time periods, confirming the previously presented data. The mean DNA concentration is .716. It is important to point out that any slight variability can be attributed to a difference in DNA synthetic activity or a difference in the number of zygotes formed at a given time. A summary of the data is presented in Table 2.

Measurements of plasmodial nuclei (Figure 17) show a unimodal distribution of DNA concentration with the mean being 2.63. This is consistent with cells in the 4C category of nuclear DNA. Also noted is the absence of a G_1 phase. Zygotes are approximately one half the plasmodial values indicating that cellular fusion occurs essentially between G_1 cells and that those in G_2 are then committed to division. In these experiments, to ensure that these

which microspectrophotometrically demonstrated zygote formation for designated time periods were in fact present, the aliquots of crosses were always plated to solid media and scored for plasmodial formation. In all cases plasmodia developed as expected. It is important to recall that in our experiments plasmodia were never observed to form in liquid culture, but readily did so on solid media.

Crosses of both uninduced and induced mating types were also performed on solid media. This was done in order to determine the amount of time required for plasmodial formation, under these conditions of induced and non-induced cells. Uninduced would require that cells be grown in required numbers ($5.0x10^5$) in order to allow induction (Shipley and Holt, 1982), (Yemma and Perry, 1985), (Alberts and Therrien, 1985) to occur; the other would be a cross between previously induced cells crossed in much smaller numbers. For zygote formation to occur in already induced cells, it was ensured that induction could not occur otherwise because of the short amount of time allowed for mating (one hour) as well as low cell counts. Uninduced mating types required an average of seven days for plasmodial formation to take place while pre-induced mating types required only three to four days (Table 3). It is important to note that if the cells are allowed to encyst the result is a loss of induction.

In a separate experiment, in which uninduced encysted cells were treated with liquid media in which previously induced cells were grown plasmodia quickly appeared in three days in 8 of 10 cases on solid media. Normally, as explained later, crosses between uninduced encysted cells normally takes 7 to 10 days. As previously stated, it was also noted that while zygote formation occurred in liquid media, plasmodia formation never did. Plasmodia formation required a solid media. Additionally, plasmodium will not form when only common mating types are present (Yemma and Therrien, 1972); compatible and opposite mating types are required as previously stated. It has also been consistently noted in our experiments that the zygotes produced in a compatible cross were always low in number which appeared to possibly relate to the degree of maturation of the cells regardless of numbers, that is, even when the numbers of cells undergoing induction were large, the number of zygotes produced was consistently small in comparision (Tables 4 - 6).

Experiments were carried out in order to test the hypothesis of the maturation requirement and plasmodia formation. Compatible mating types were crossed and observed for plasmodia formation. When the plasmodial development was observed, cells in the vicinity of the plasmodium which had not yet formed plasmodium were replated and scored for plasmodial formation. In 60% of these

replatings plasmodium formation occurred within 3 - 4 days (Table 7).

The results of experiments done in order to study and characterize the inducer molecule are presented in Figures 18 - 25. These studies were performed by the use of High Pressure Liquid Chromatography techniques, giving rise to data which is the result of molecular separation. Data is presented as graphs where retention time is plotted against voltage. Where comparision of peak size is necessary, these are given in relative units of area (Tables 5 - 6). In Figure 18 the results of the **E. coli** and water are presented as controls in order to eliminate any peaks caused by the media and **E. coli** in which the cells were grown. Note there are two large peaks consistently and characteristically present at approximately five and thirteen minutes. Figures 20 - 25 are those of the experimental strains on days 1, 5 and 7. The area of interest is between approximately eight and eleven minutes. There are three peaks within this area but it is the first that is considered most significant. This peak relates well with approximate cell numbers present in culture but does not appear to be accumulated to increasingly high levels. Rather it increases and decreases as cell numbers do so. The other two peaks are thought to be altered forms of the inducer molecule. It is also well to note that the number of zygotes per day remains at approximately the same level,

and notably peaks Days 5 and 6. Our own experience suggests this molecule is highly labile, as when it was left out at room temperature for a day and then rerun on the HPLC radically differing results were obtained.

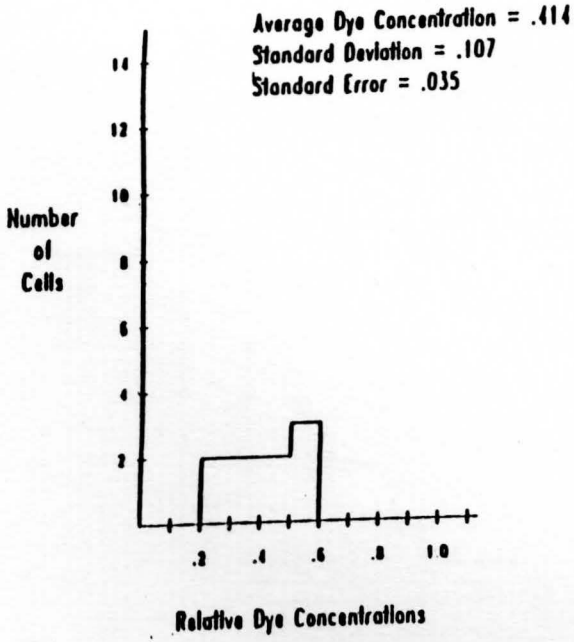
Figure 3

Feulgen DNA Values for Day 1

of Membrane Spin Culture

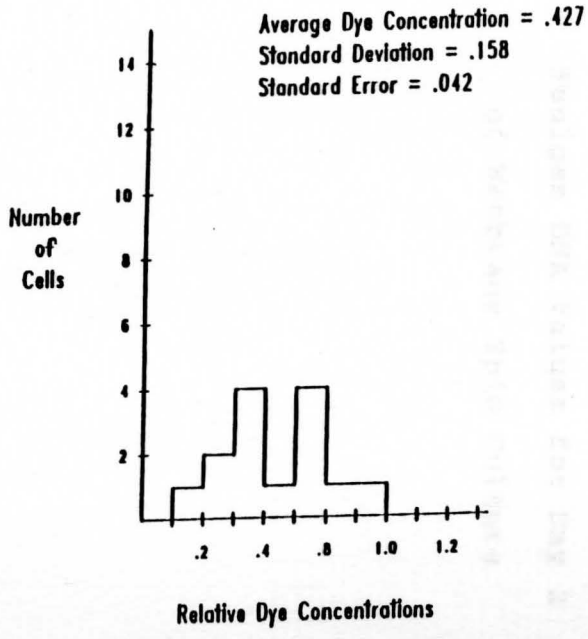
Membrane Spin Culture

Day 1 CR5-11A''



Membrane Spin Culture

DAY 1 HON 1-2A'



Membrane Spin Culture

Day 1 CR5-11A'' x HON 1-2A'

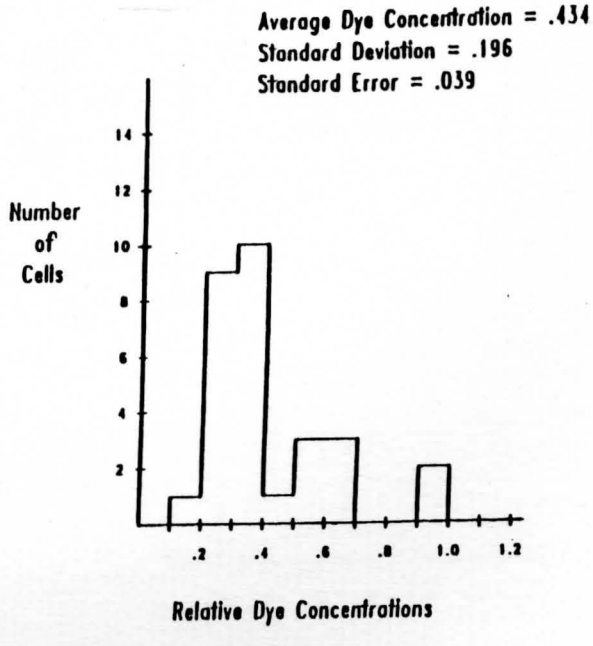


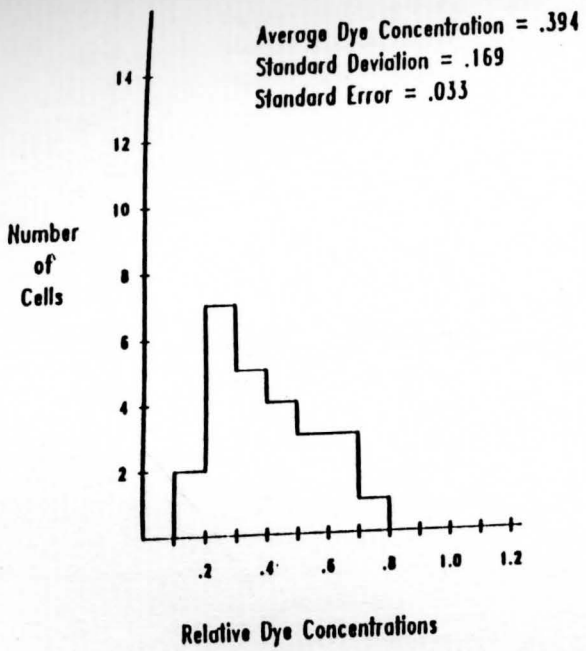
Figure 4

Feulgen DNA Values for Day 2

of Membrane Spin Culture

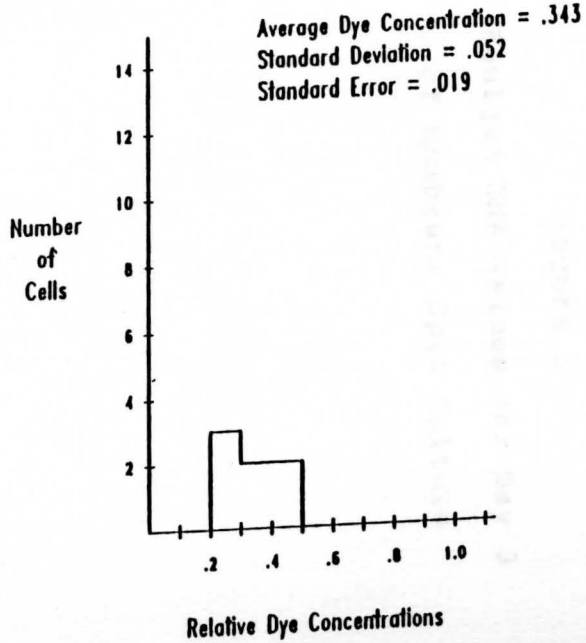
Membrane Spin Culture

Day 2 CR5-11A''



Membrane Spin Culture

DAY 2 HON 1-2A'



Membrane Spin Culture

Day 2 CR5-11A'' x HON 1-2A'

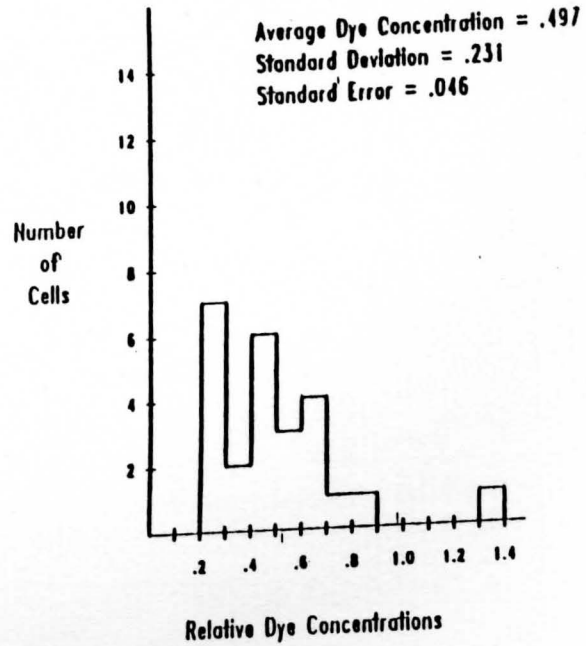


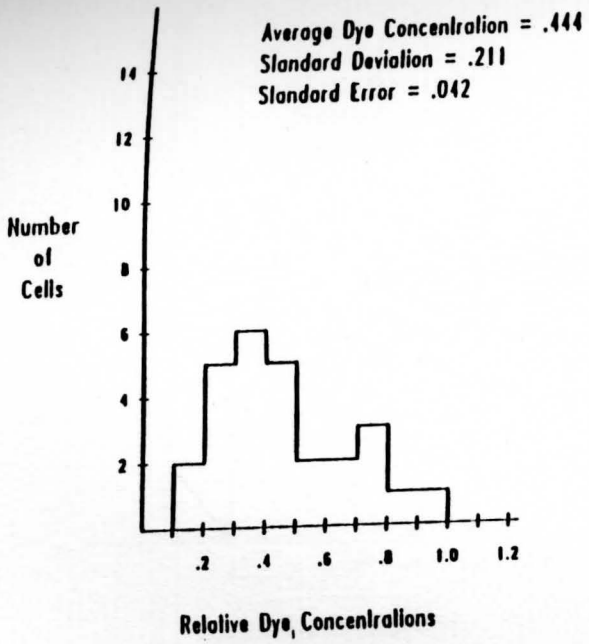
Figure 5

Feulgen DNA Values for Day 3

of Membrane Spin Culture

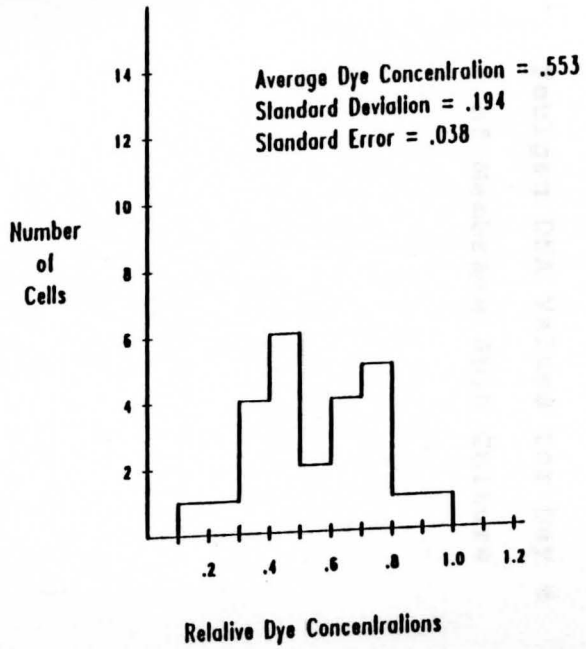
Membrane Spin Cullure

Day 3 CR5-11A''



Membrane Spin Cullure

Day 3 HON 1-2A'



Membrane Spin Cullure

Day 3 CR5-11A'' x HON 1-2A'

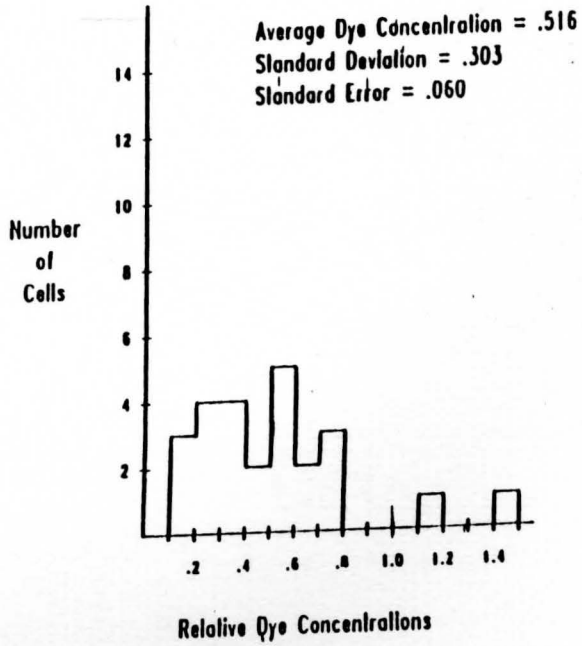


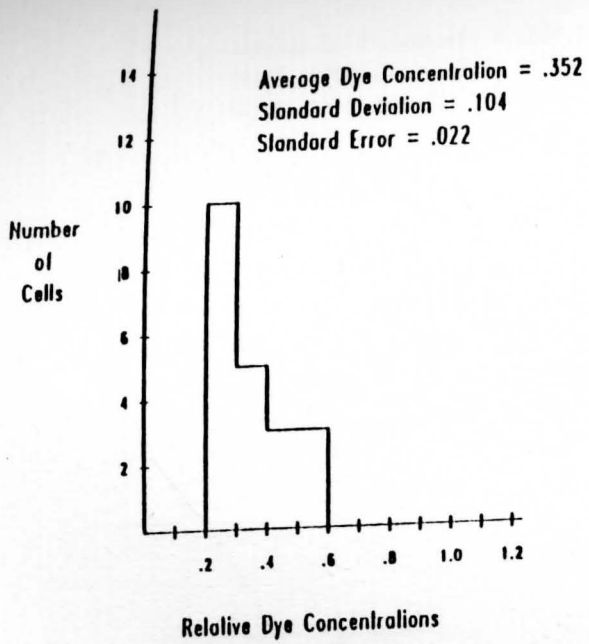
Figure 6

Feulgen DNA Values for Day 4

of Membrane Spin Culture

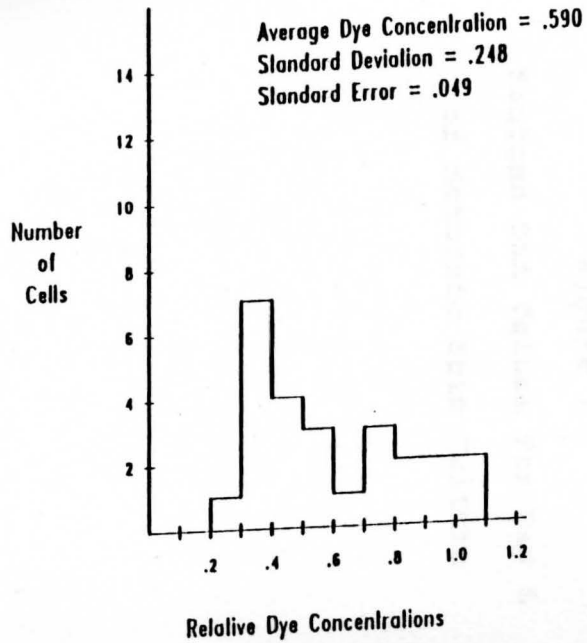
Membrane Spin Cullure

Day 4 CR5-11A''



Membrane Spin Cullure

Day 4 HON 1-2A'



Membrane Spin Cullure

Day 4 CR5-11A'' x HON 1-2A'

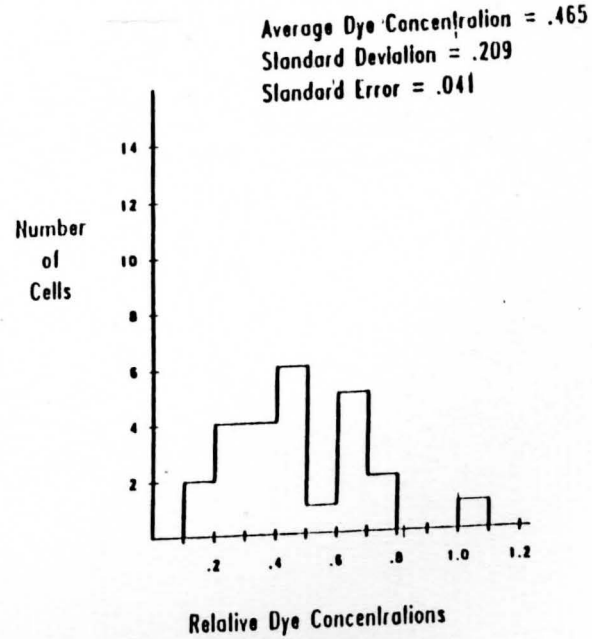


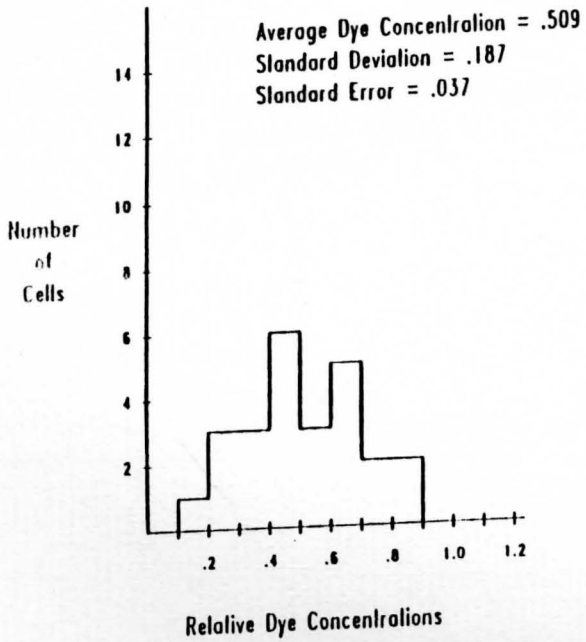
Figure 7

Feulgen DNA Values for Day 5

of Membrane Spin Culture

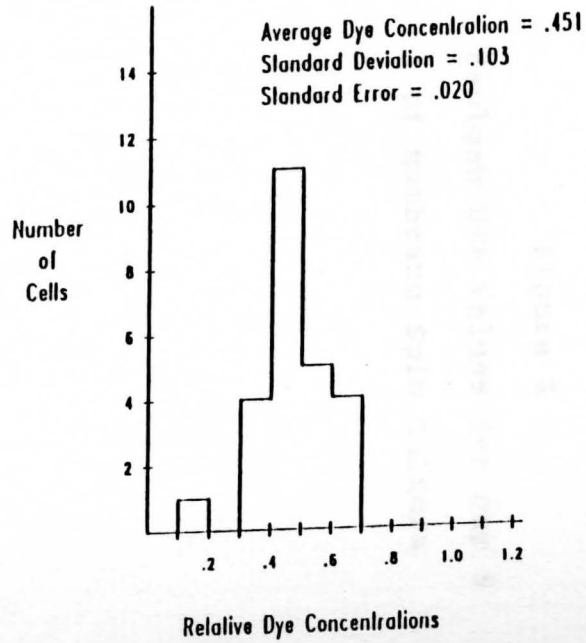
Membrane Spin Cullure

Day 5 CR5-11A''



Membrane Spin Cullure

Day 5 HON 1-2A'



Membrane Spin Çullure

Day 5 CR5-11A'' x HON 1-2A'

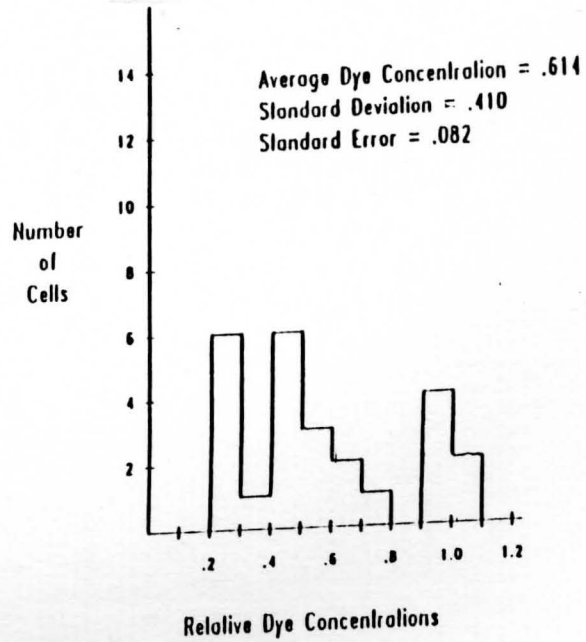


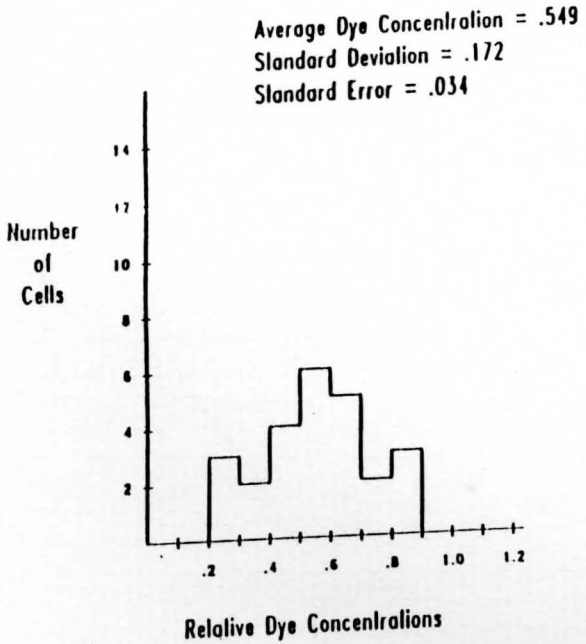
Figure 8

Feulgen DNA Values for Day 6

of Membrane Spin Culture

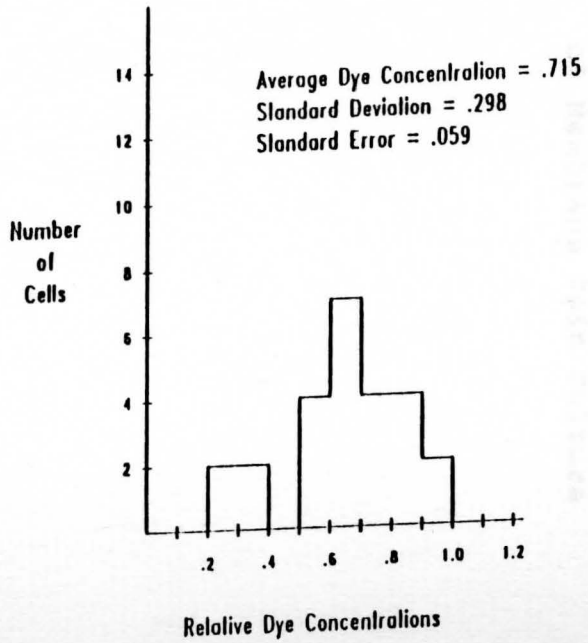
Membrane Spin Cullure

Day 6 CR5-11A''



Membrane Spin Cullure

Day 6 HON 1-2A'



Membrane Spin Cullure

Day 6 CR5-11A'' x HON 1-2A'

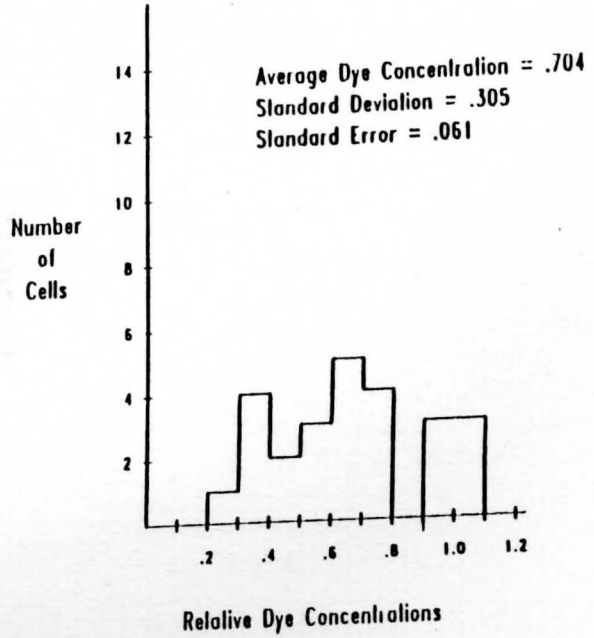


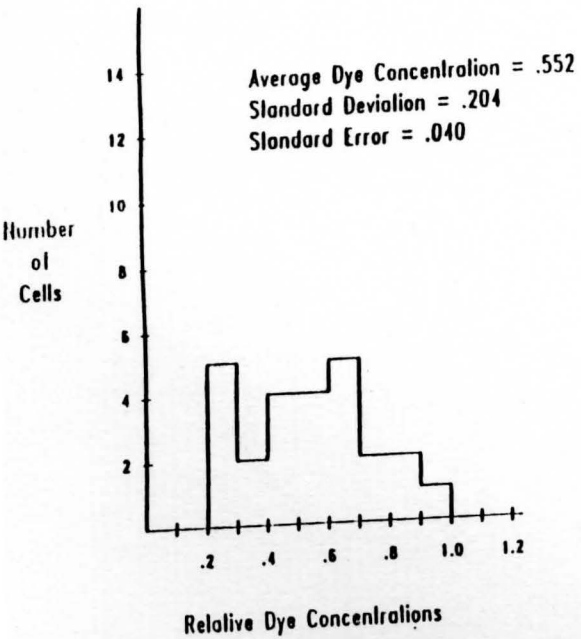
Figure 9

Feulgen DNA Values for Day 7

of Membrane Spin Culture

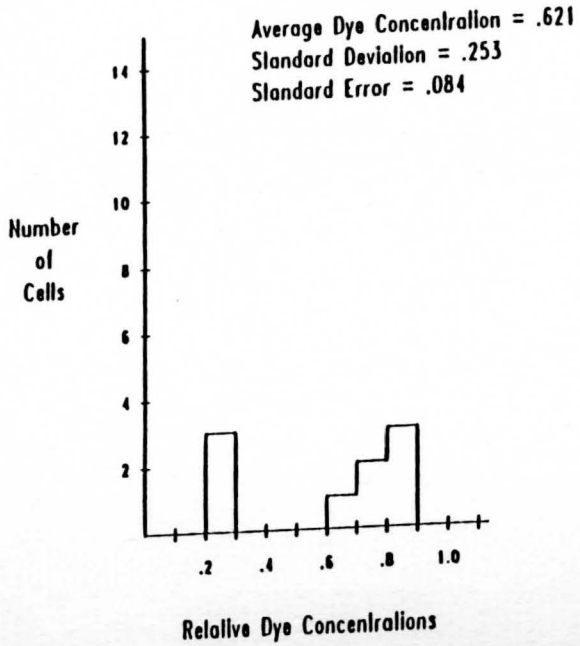
Membrane Spin Cullure

Day 7 CR5-11A''



Membrane Spin Cullure

Day 7 HON 1-2A'



Membrane Spin Cullure

Day 7 CR5-11A'' x HON 1-2A'

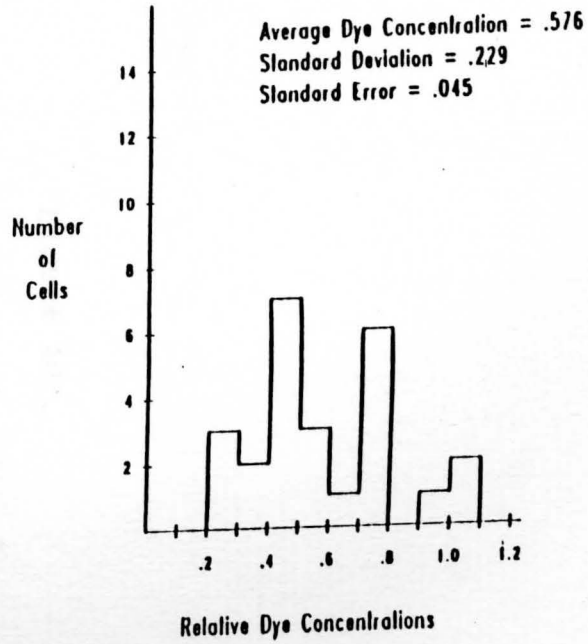


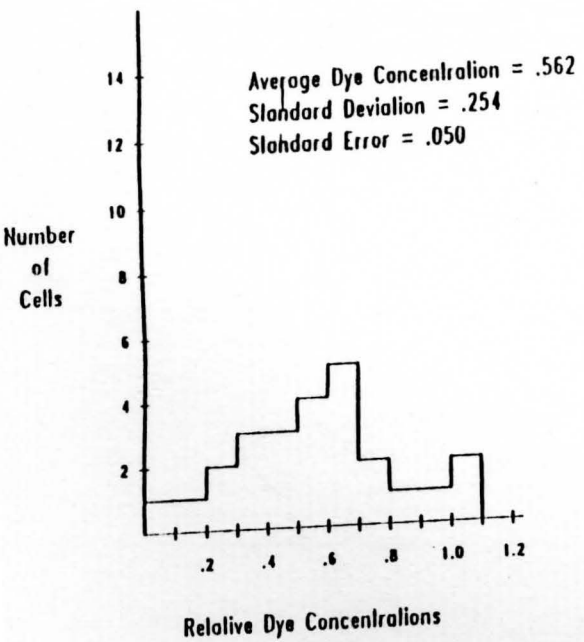
Figure 10

Feulgen DNA Values for Day 8

of Membrane Spin Culture

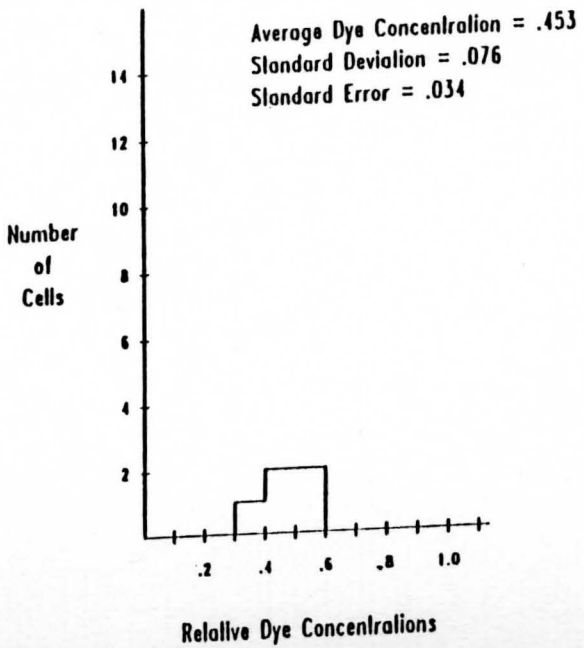
Membrane Spin Cullure

Day 8 CR5-11A''



Membrane Spin Cullure

Day 8 HON 1-2A'



Membrane Spin Cullure

Day 8 CR5-11A'' x HON 1-2A'

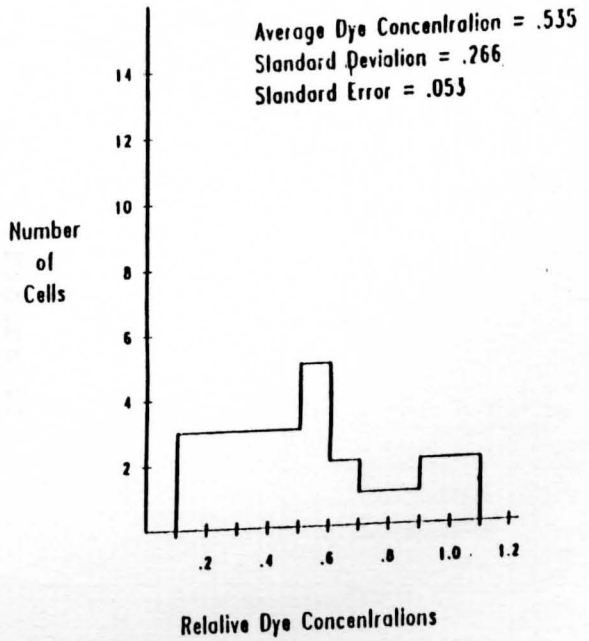


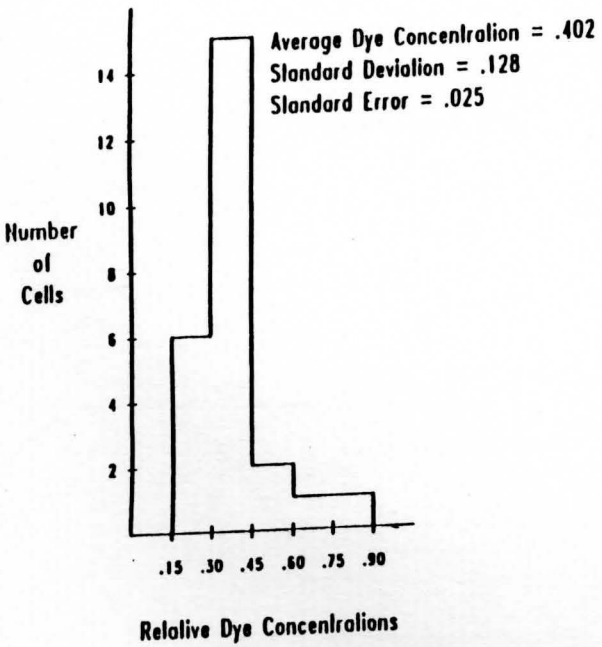
Figure 11

Feulgen DNA Values for Day 1

of Separate Spin Culture

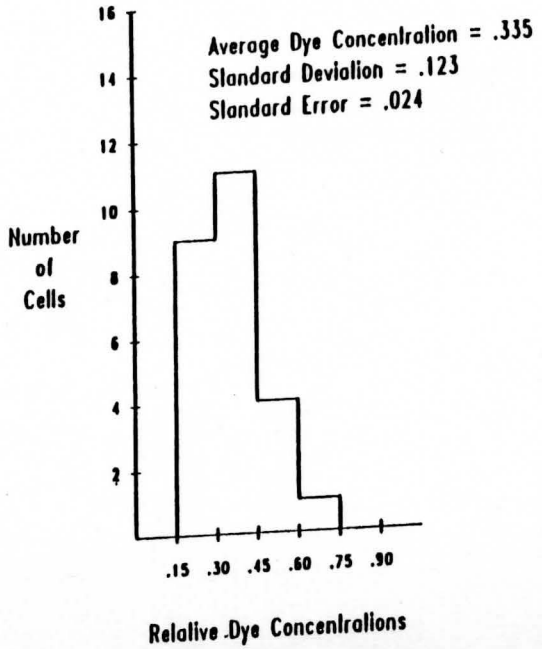
Separale Spin Cullure I

Day 1 CR5-11A''



Separale Spin Cullure I

Day 1 HON 1-2A''



Separale Spin Cullure I

Day 1 CR5-11A'' x HON 1-2A'

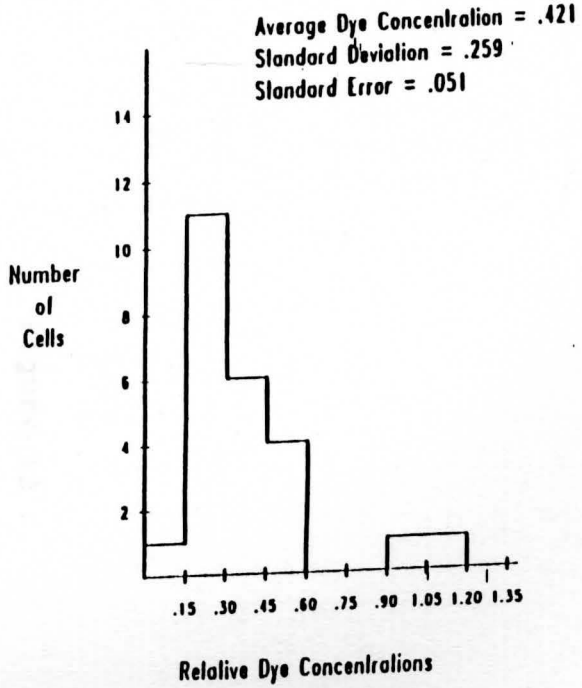


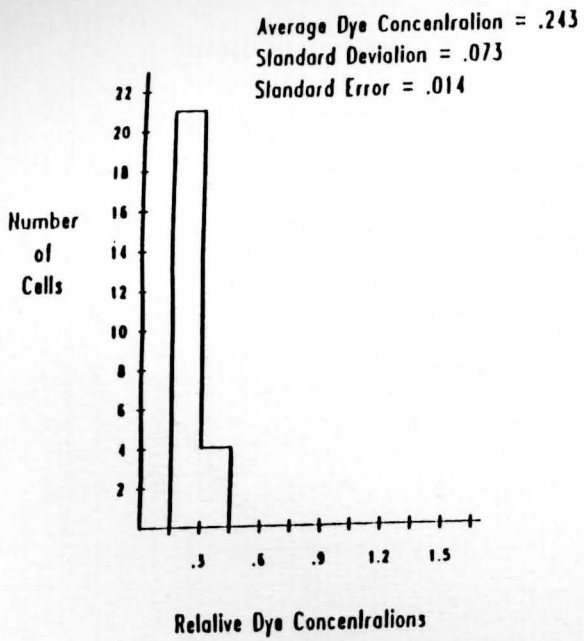
Figure 12

Feulgen DNA Values for Day 2

of Separate Spin Culture

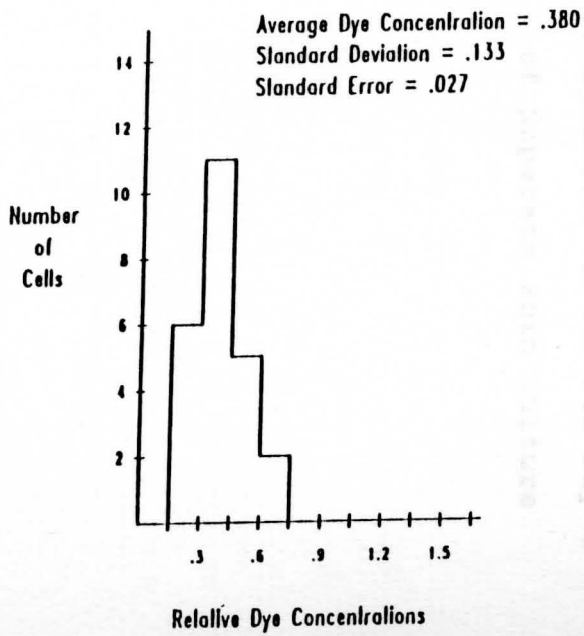
Separale Spin Cullure I

Day 2 CR5-11A''



Separale Spin Cullure I

Day 2 HON 1-2A'



Separale Spin Cullure I

Day 2 CR5-11A'' x HON 1-2A'

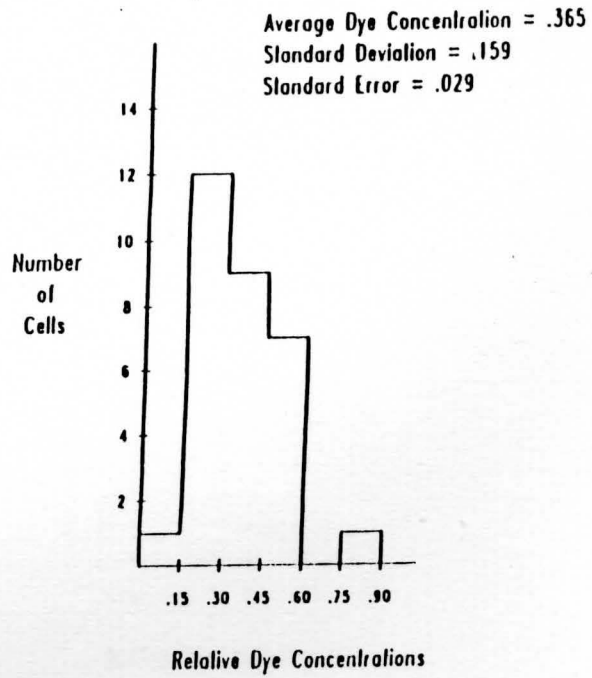


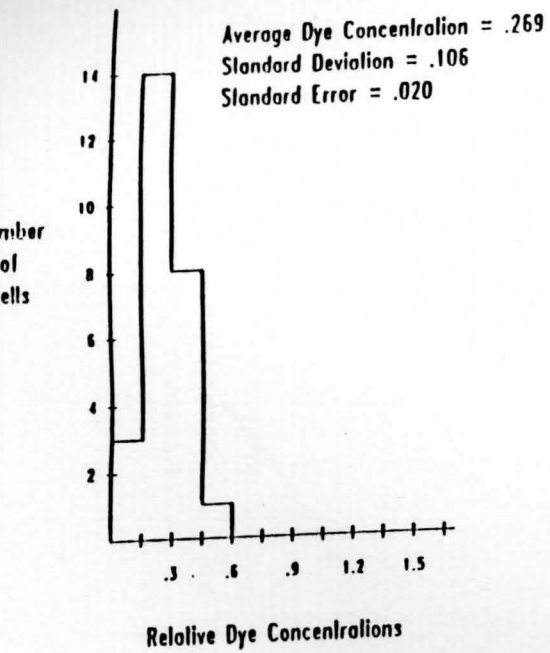
Figure 13

Feulgen DNA Values for Day 4

of Separate Spin Culture

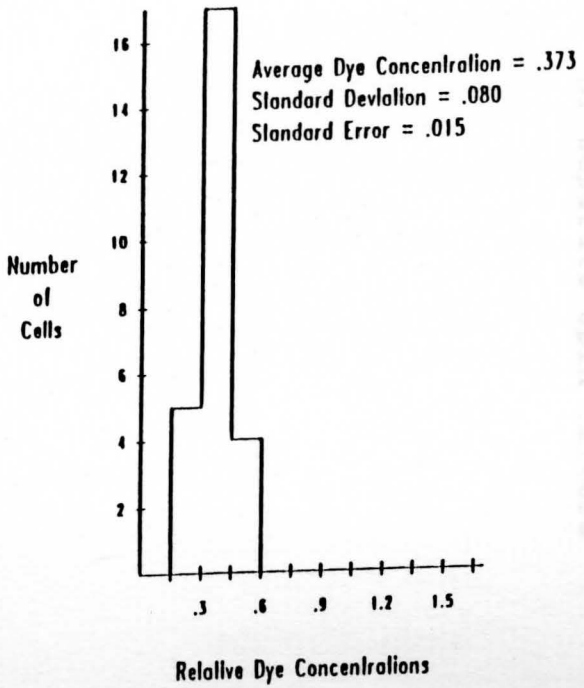
Separale Spin Cullure I

Day 4 CR5-11A''



Separale Spin Cullure I

Day 4 HON 1-2A'



Separale Spin Cullure I

Day 4 CR5-11A'' x HON 1-2A'

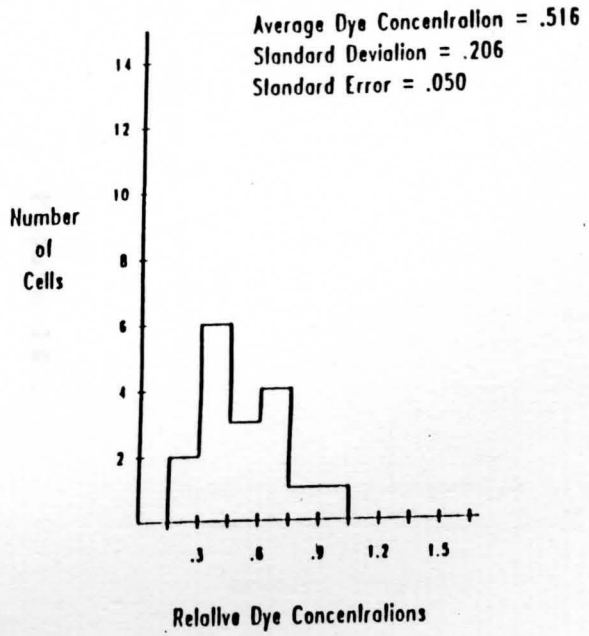


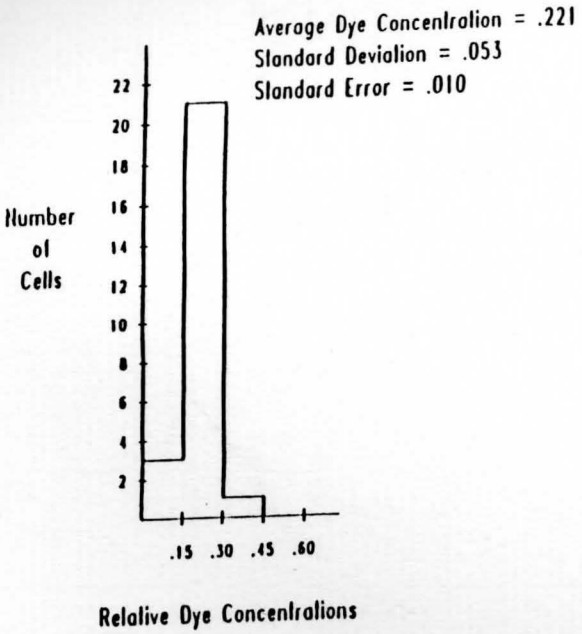
Figure 14

Feulgen DNA Values for Day 5

of Separate Spin Culture

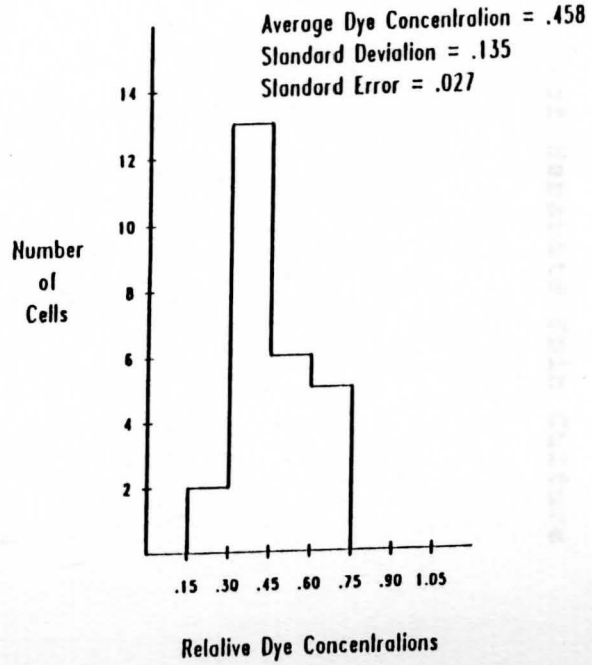
Separate Spin Culture I

Day 5 CR5-11A''



Separate Spin Culture I

Day 5 HON 1-2A'



Separate Spin Culture I

Day 5 CR5-11A'' x HON 1-2A'

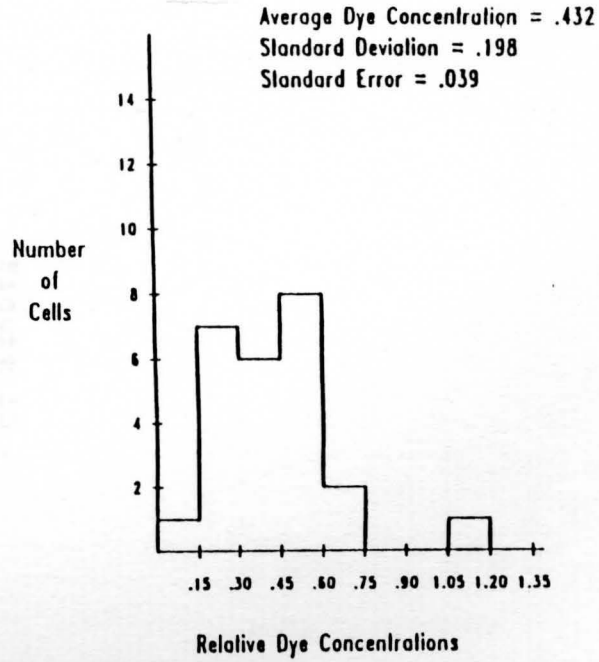


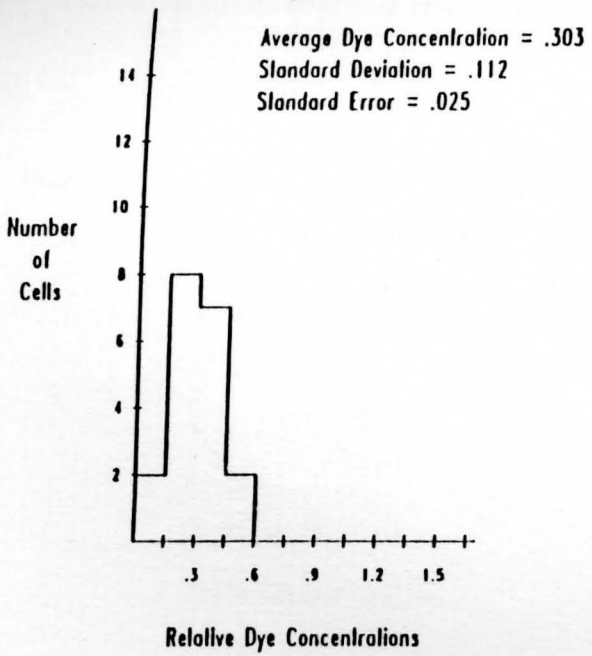
Figure 15

Feulgen DNA Values for Day 7

of Separate Spin Culture

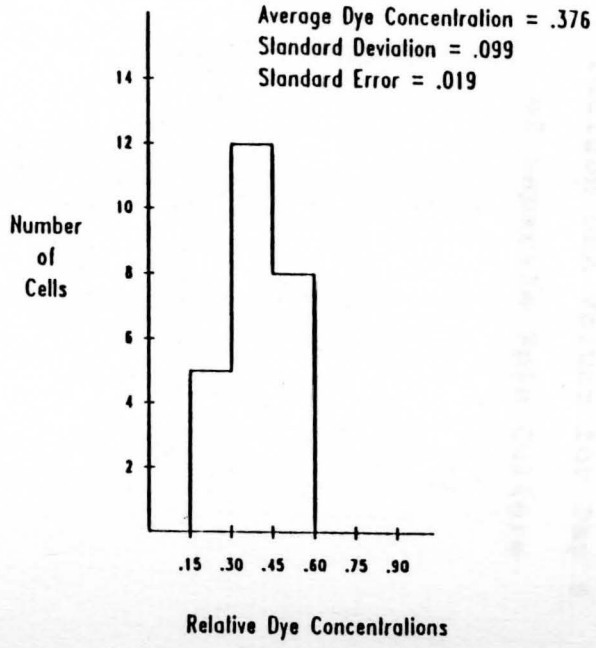
Separate Spin Culture I

Day 7 CR5-11A''



Separate Spin Culture I

Day 7 HON 1-2A'



Separate Spin Culture I

Day 7 CR5-11A'' x HON 1-2A'

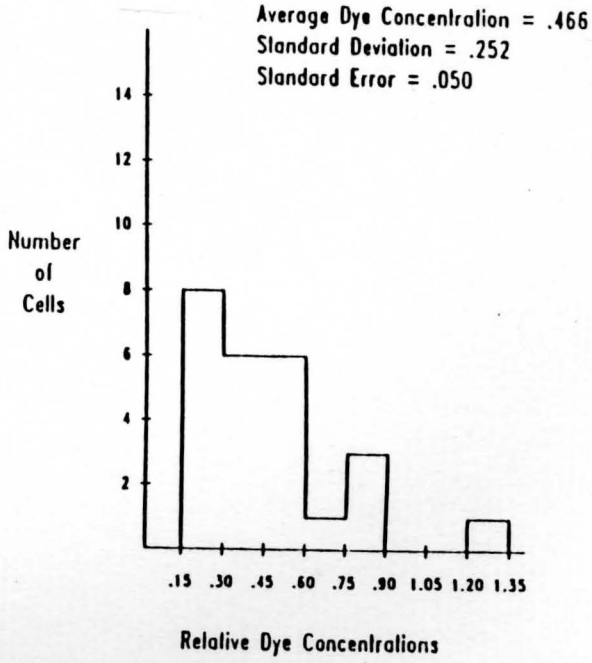


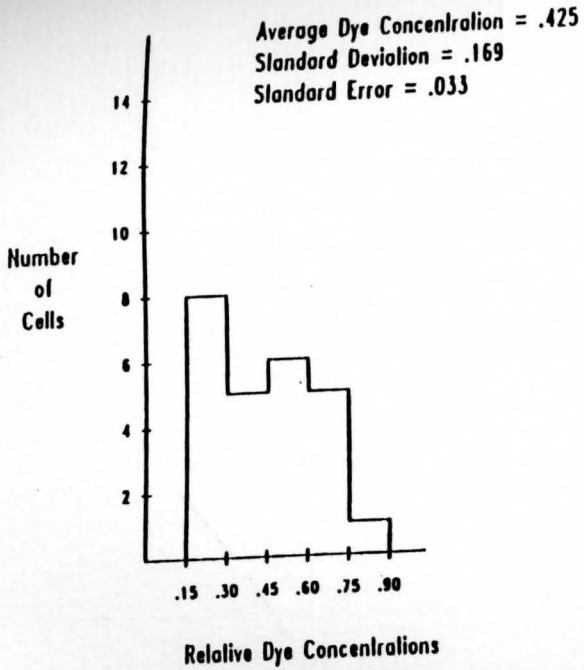
Figure 16

Feulgen DNA Values for Day 8

of Separate Spin Culture

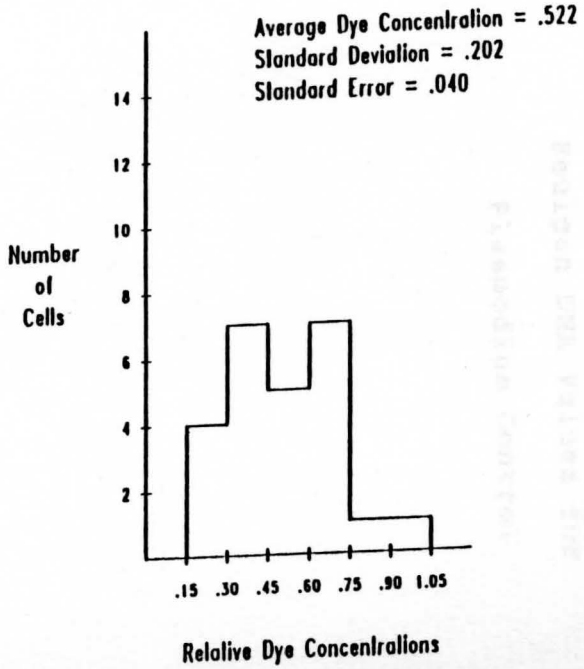
Separale Spin Cullure I

Day 8 CR5-11A''



Separale Spin Cullure I

Day 8 HON 1-2A'



Separale Spin Cullure I

Day 8 CR5-11A'' x HON 1-2A'

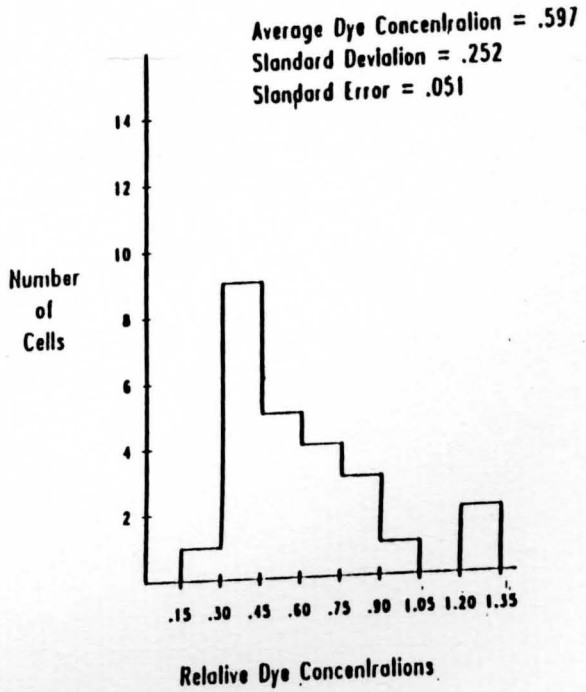


Figure 17

Feulgen DNA Values for

Plasmodium Control

Plasmodium

Average Dye Concentration = 2.636
Standard Deviation = .444
Standard Error = .062

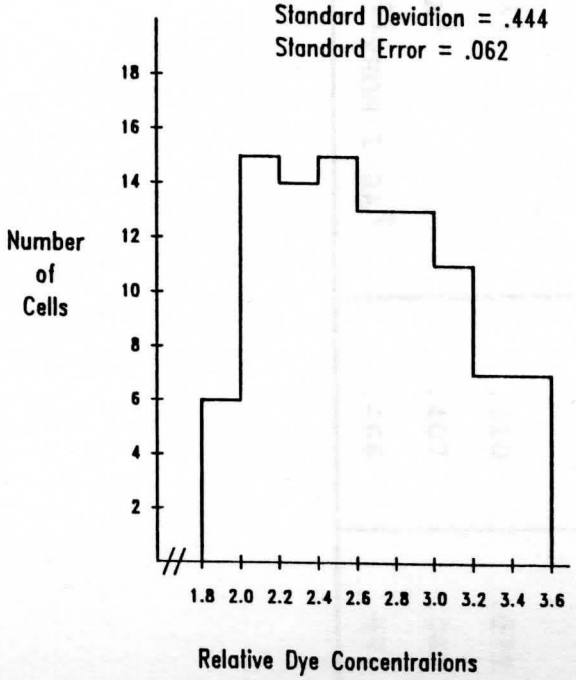


TABLE 2

SUMMARY OF MICROSPECTROPHOTOMETER DATA
(average DNA values)

Study	Mean DNA	S.D.	S.E.
Membrane			
CR5-11A^{11}	.466	.072	.005
HON 1-2A^{1}	.519	.113	.012
CR5-11A^{11}xHON 1-2A^{1}	.542	.081	.006
Separate			
CR5-11A^{11}	.310	.077	.005
HON 1-2A^{1}	.407	.063	.003
CR5-11A^{11}xHON 1-2A^{1}	.466	.074	.005

TABLE 3

AMOUNT OF TIME NEEDED FOR PLASMODIUM FORMATION
(days)

	Time
Encysted Cells	6 - 7
Non-encysted Cells	3 - 4

TABLE 4

MEMBRANE SPIN CULTURE CELL COUNTS
(# cells/ml)

	CR5-11A[11]	HON 1-2A[1]	Total # Zygotes
Day 1	9.20×10^4	2.80×10^4	1
Day 2	1.58×10^5	3.40×10^4	1
Day 3	1.98×10^5	5.80×10^4	2
Day 4	1.86×10^5	5.40×10^4	1
Day 5	2.16×10^5	4.80×10^4	6
Day 6	2.28×10^5	5.00×10^4	6
Day 7	2.04×10^5	5.80×10^4	3
Day 8	1.82×10^5	4.20×10^4	1

TABLE 5

SUMMARY OF DATA FOR CR5-11A[11]

	Cell* Counts (#/ml)	# Zygotes	Plasmodium Formation	Inducer˜ Molecule	Altered˜ Inducer Molecules
Day 1	1.95×10^4	2	+	43236	48272
Day 2	1.68×10^4	2	+	30416	48504
Day 3	2.35×10^4	1	+	33972	22264
Day 4	2.40×10^4	2	+	58696	99372
Day 5	2.55×10^4	4	+	15024	10860
Day 6	2.67×10^4	1	+	10316	37128
Day 7	3.07×10^4	2	+	45324	50832
Day 8	4.80×10^4	2	+		

*Data is an average of the duplicated separate spin culture studies

˜Area under the peak in relative units

TABLE 6

SUMMARY OF DATA FOR HON 1-2A[1]

	Cell* Counts (#/ml)	# Zygotes	Plasmodium Formation	Inducer˜ Molecule	Altered˜ Inducer Molecules
Day 1	1.30×10^4	2	+		
Day 2	1.20×10^4	2	+		
Day 3	1.80×10^4	1	+	67496	60348
Day 4	1.78×10^4	2	+	11608	5728
Day 5	2.18×10^4	4	+	72180	67176
Day 6	1.95×10^4	1	+	7868	2328
Day 7	2.15×10^4	2	+	19004	N.D.
Day 8	2.70×10^4	2	+		

*Data is an average of the duplicated separate spin culture studies

˜Area under peak in relative units

N.D.:Area not detectable

TABLE 7

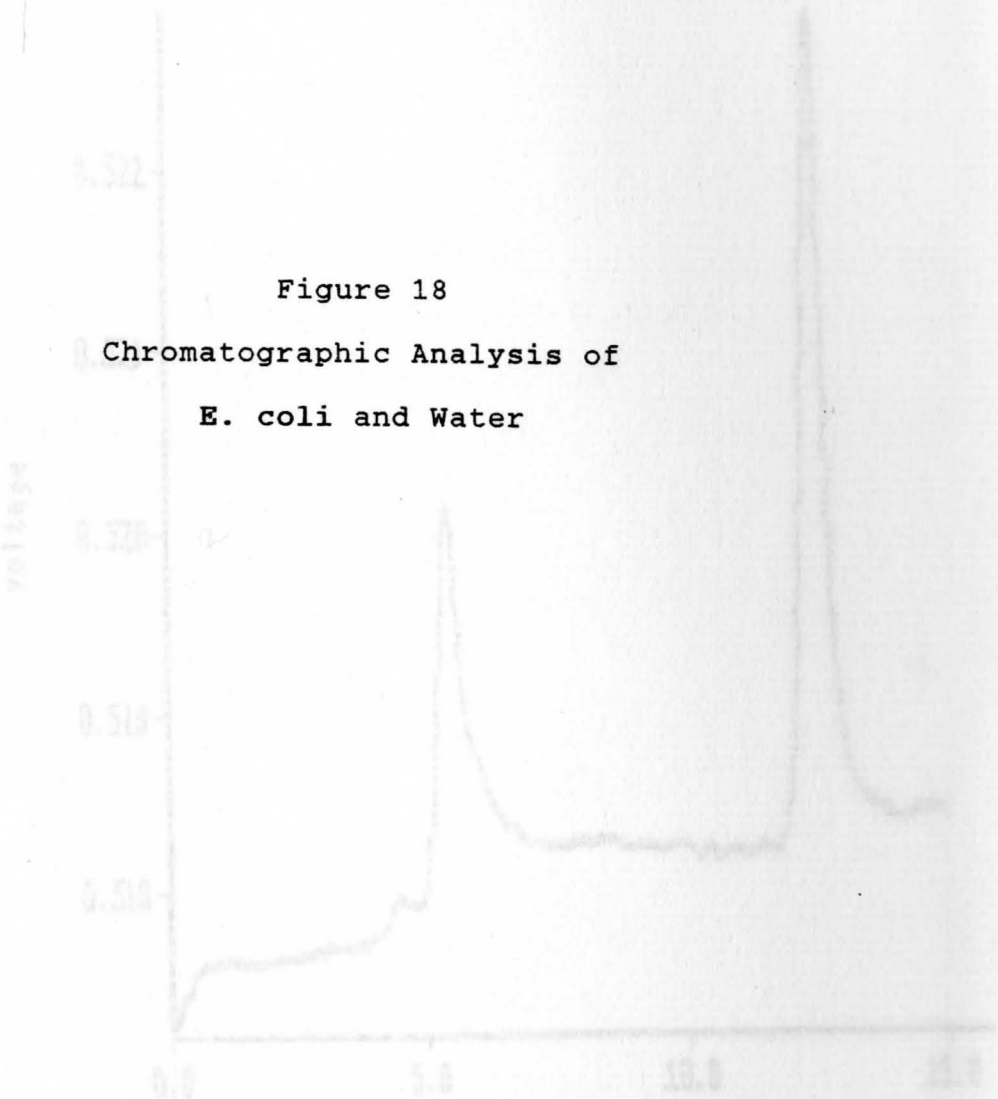
Plasmodial Formation in Replated Cells

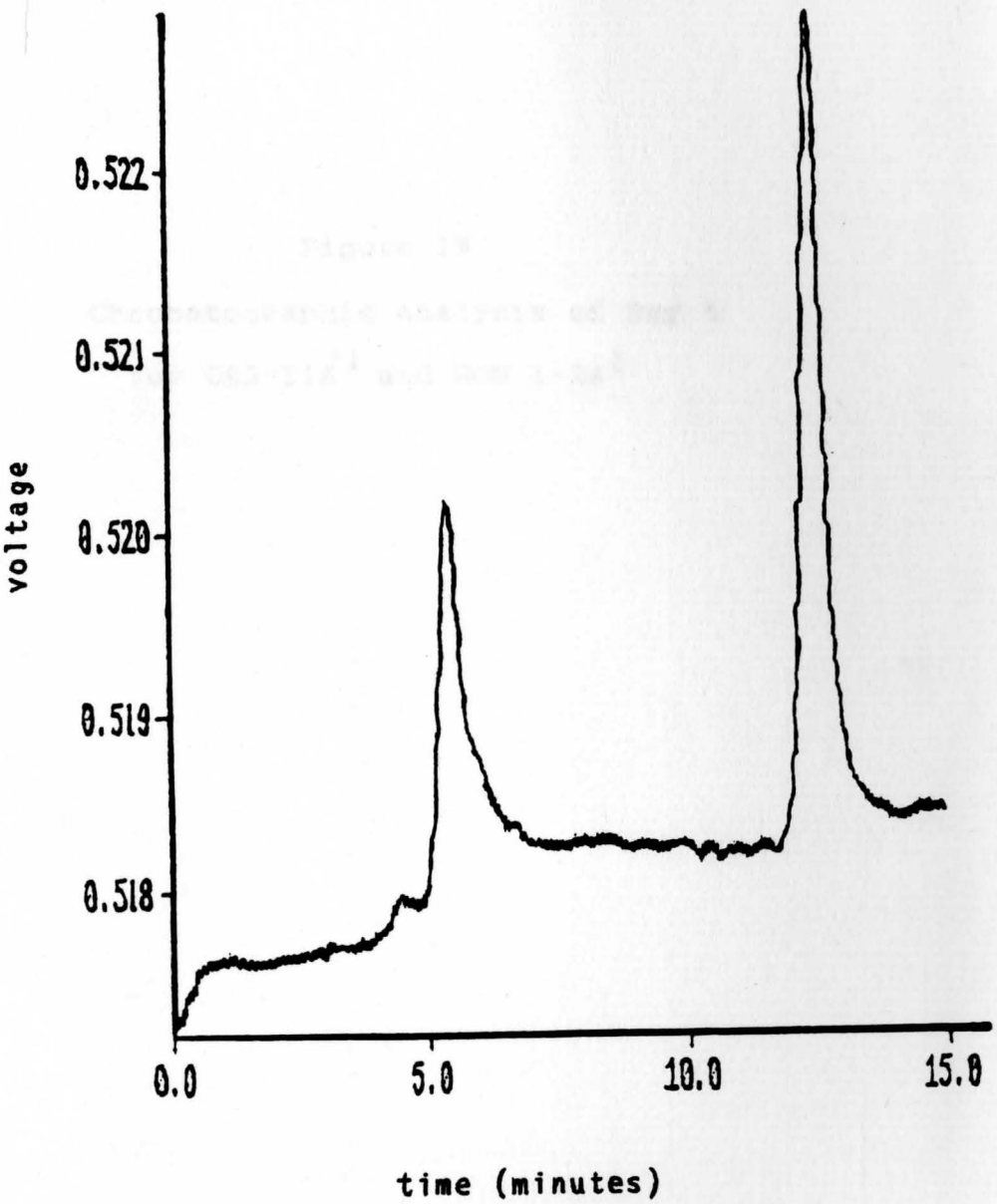
Number of Platings	Time for Plasmodial Development (days)	Percentage
10	3 - 4	60% positive

Figure 18

Chromatographic Analysis of

E. coli and Water




0.522
0.521
0.520
0.519
0.518
voltage
0.0
5.0
10.0
15.0
time (minutes)

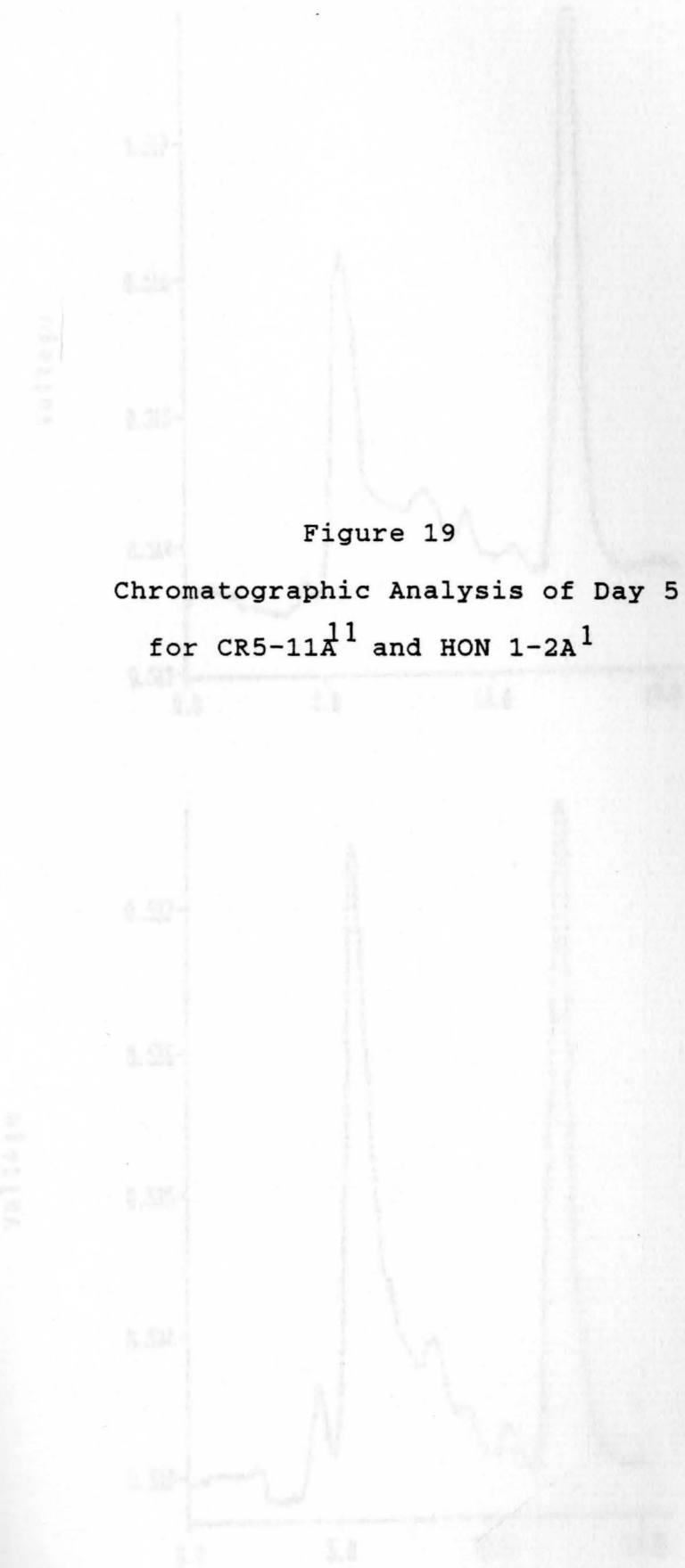
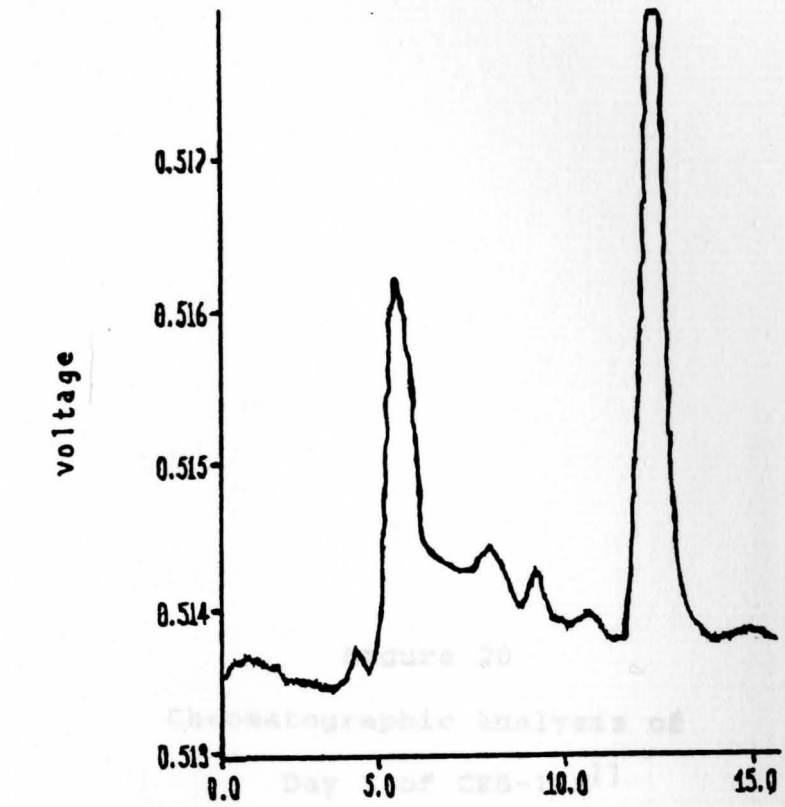


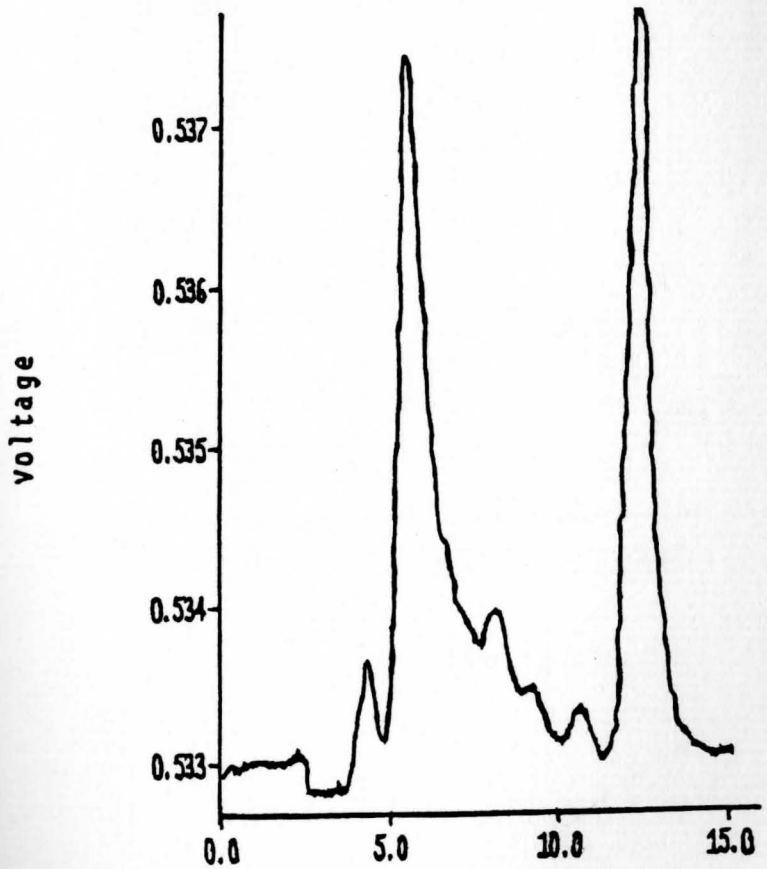
Figure 19

Chromatographic Analysis of Day 5

for CR5-11A[11] and HON 1-2A[1]



HON 1-2A[1]

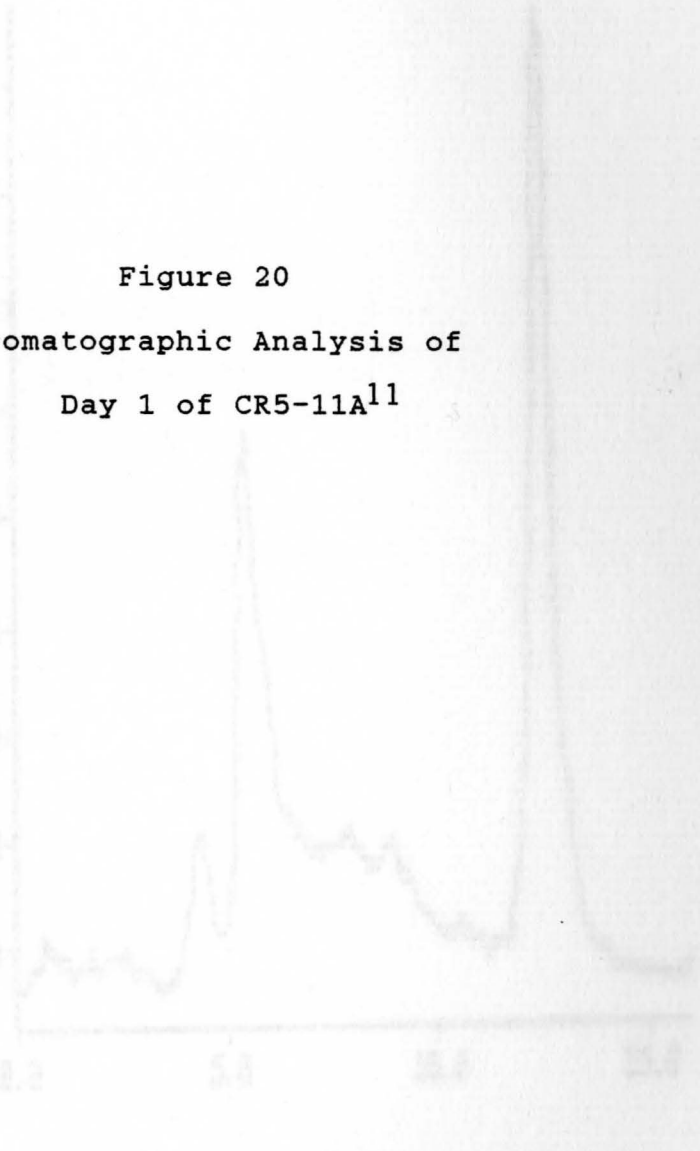


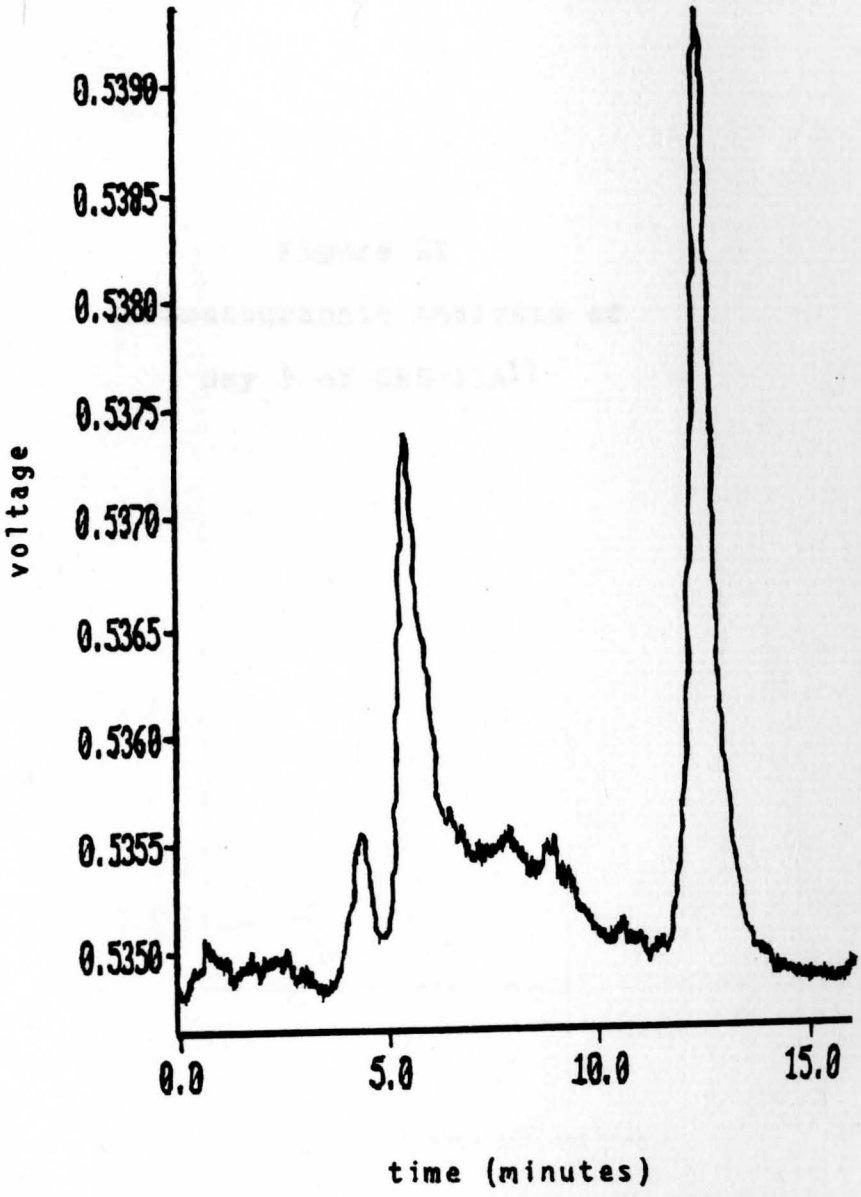
CR5-11A[11]

Figure 20

Chromatographic Analysis of

Day 1 of CR5-11A[11]




0.5390
0.5385
0.5380
0.5375
0.5370
0.5365
0.5360
0.5355
0.5350
voltage
0.0
5.0
10.0
15.0
time (minutes)

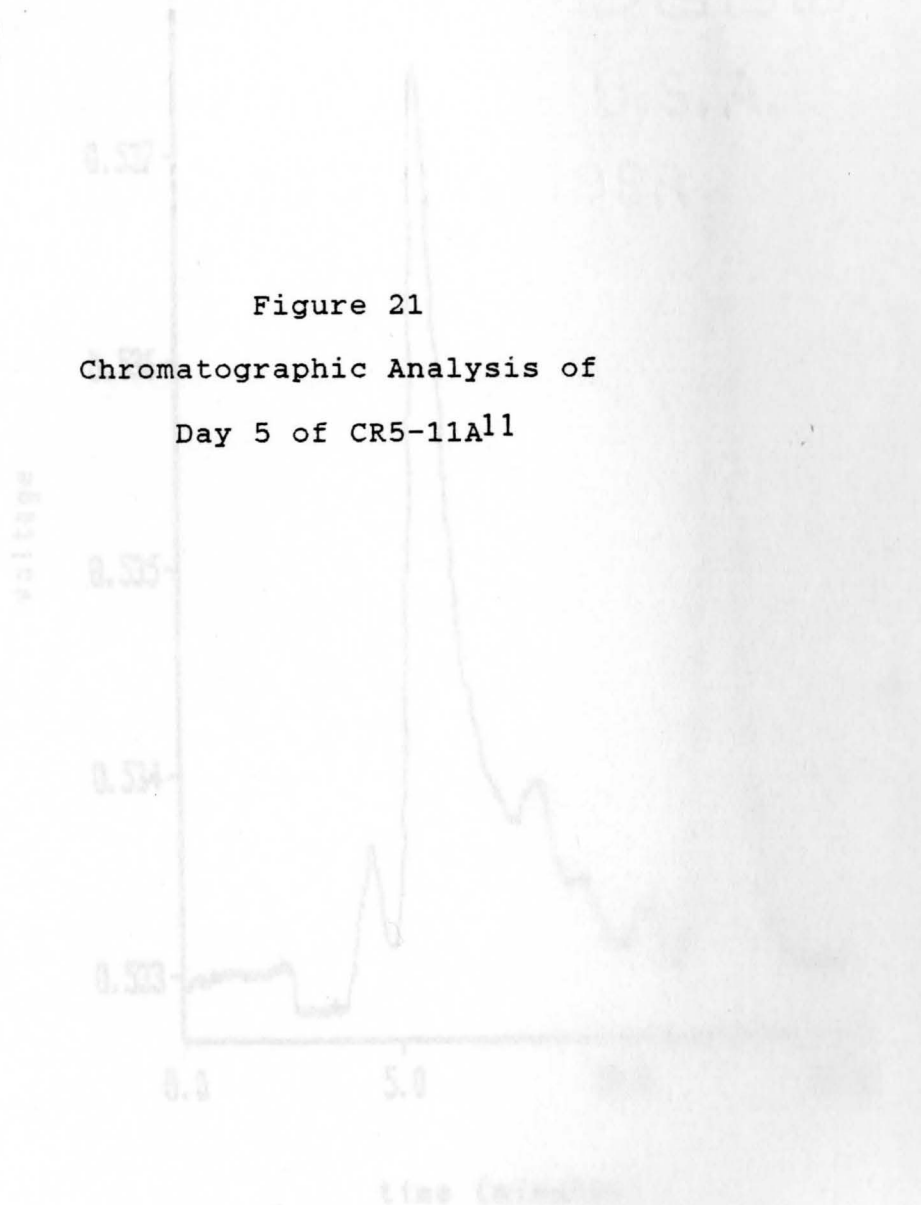


Figure 21

Chromatographic Analysis of

Day 5 of CR5-11A[11]

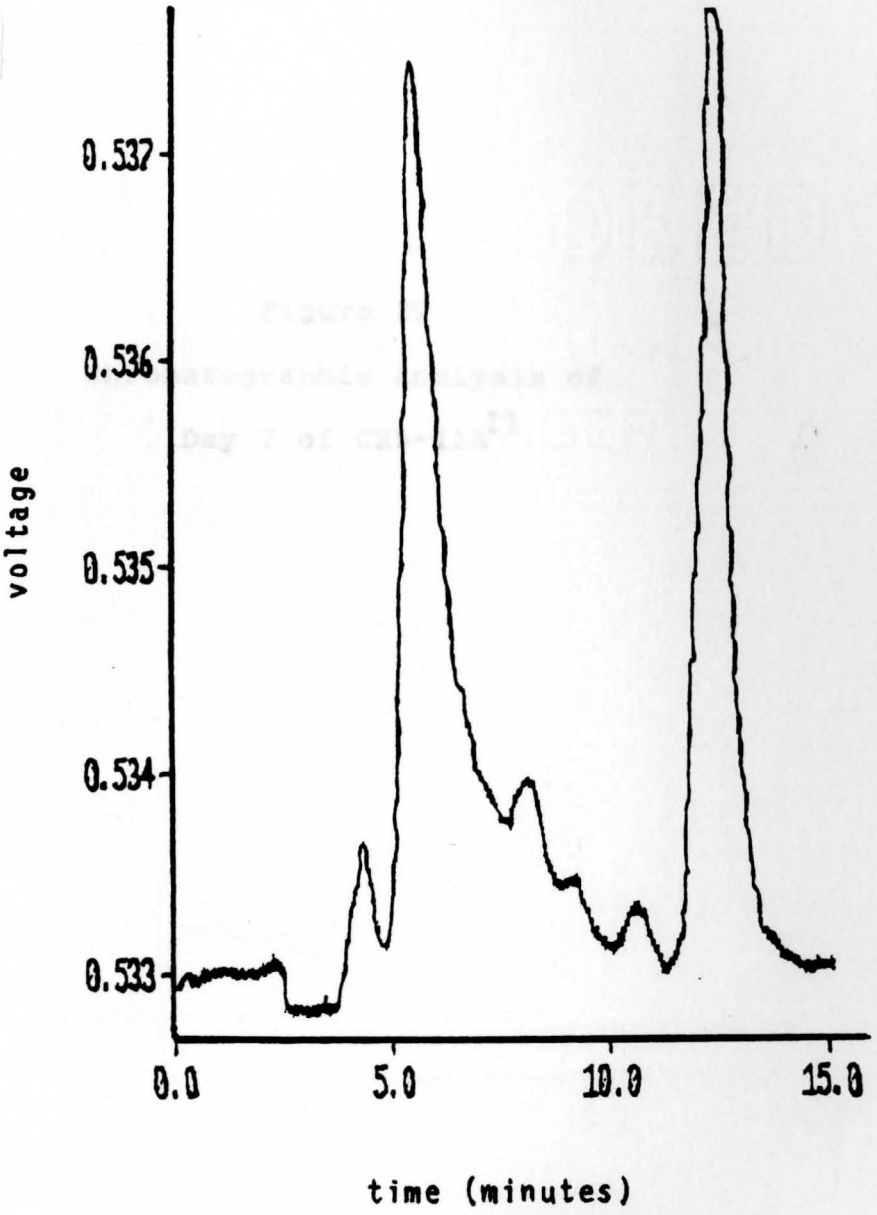
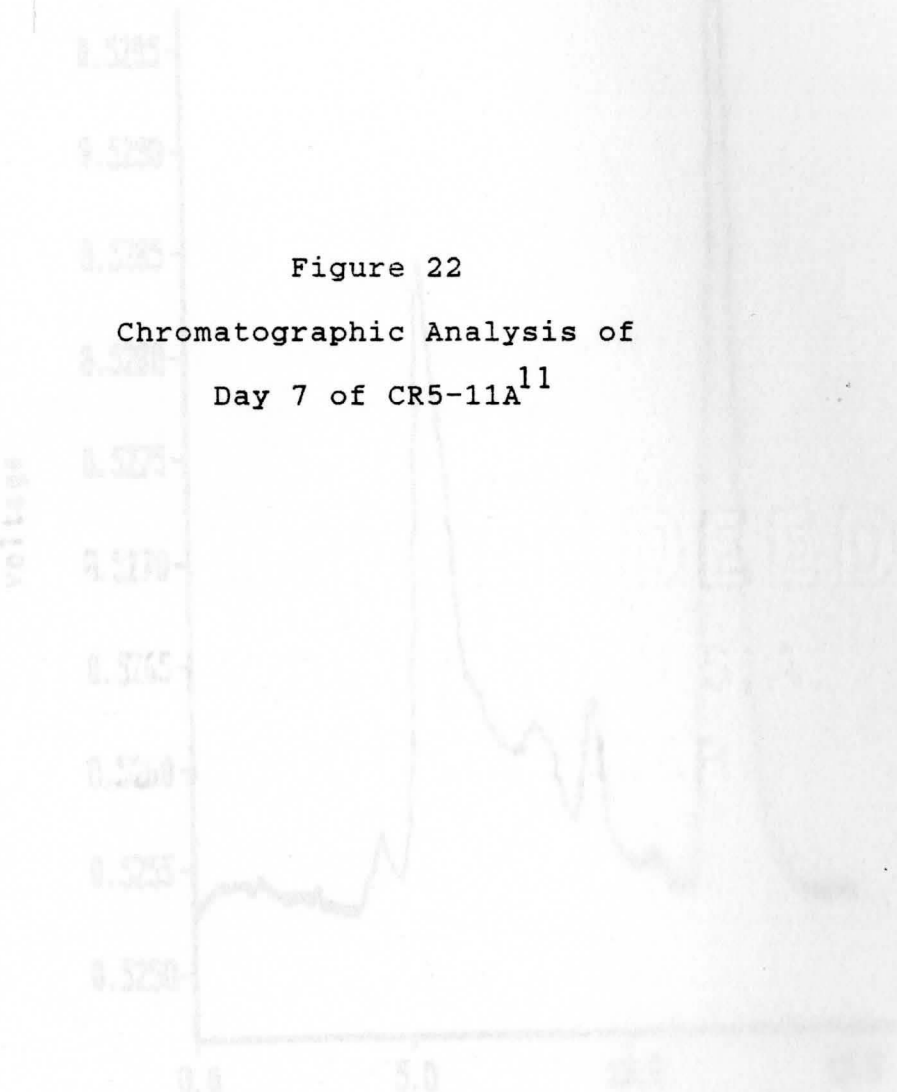
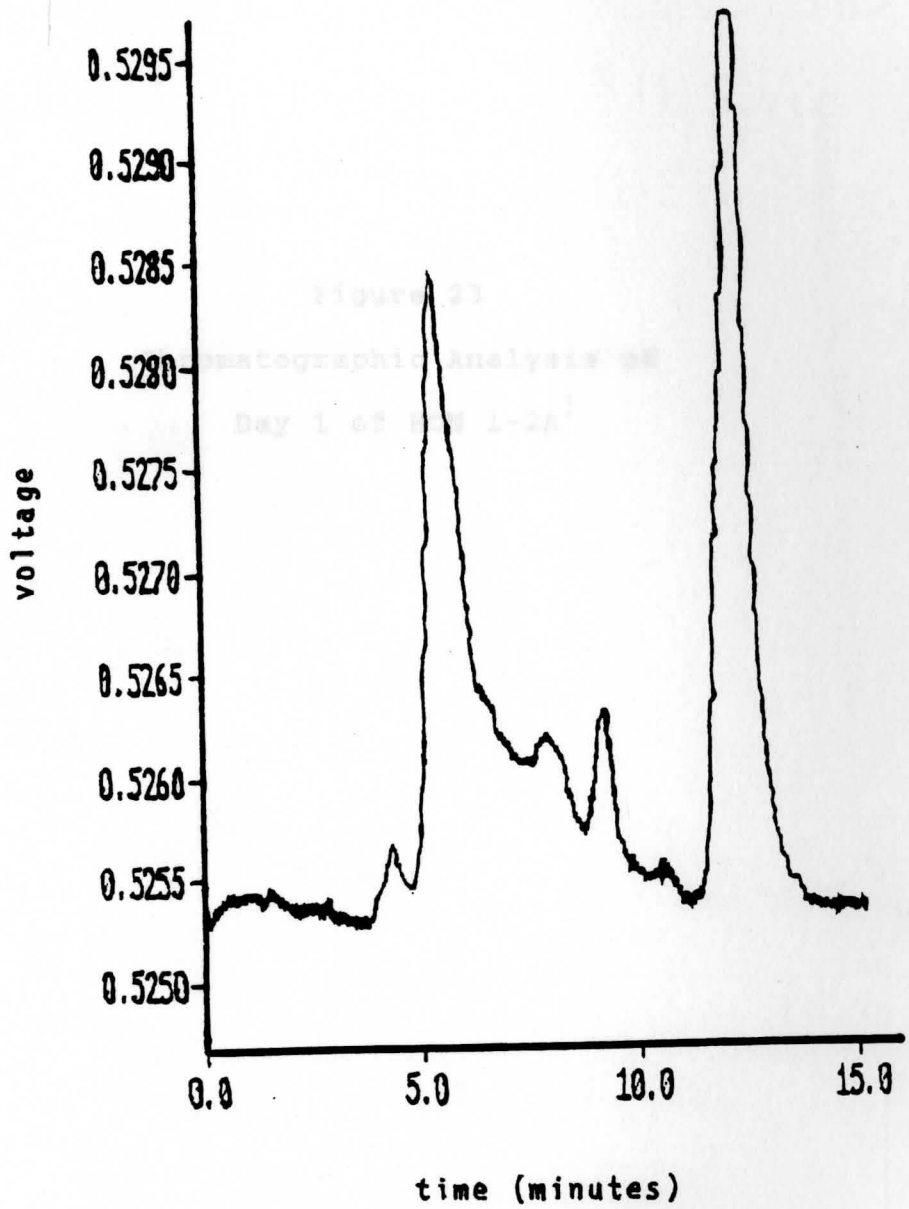

0.537
0.536
0.535
0.534
0.533
voltage
0.0
5.0
10.0
15.0
time (minutes)

Figure 22

Chromatographic Analysis of

Day 7 of CR5-11A[11]

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0.5290
0.5285
0.5280
0.5275
0.5270
0.5265
0.5260
0.5255
0.5250
voltage
0.0
5.0
10.0
15.0
time (minutes)

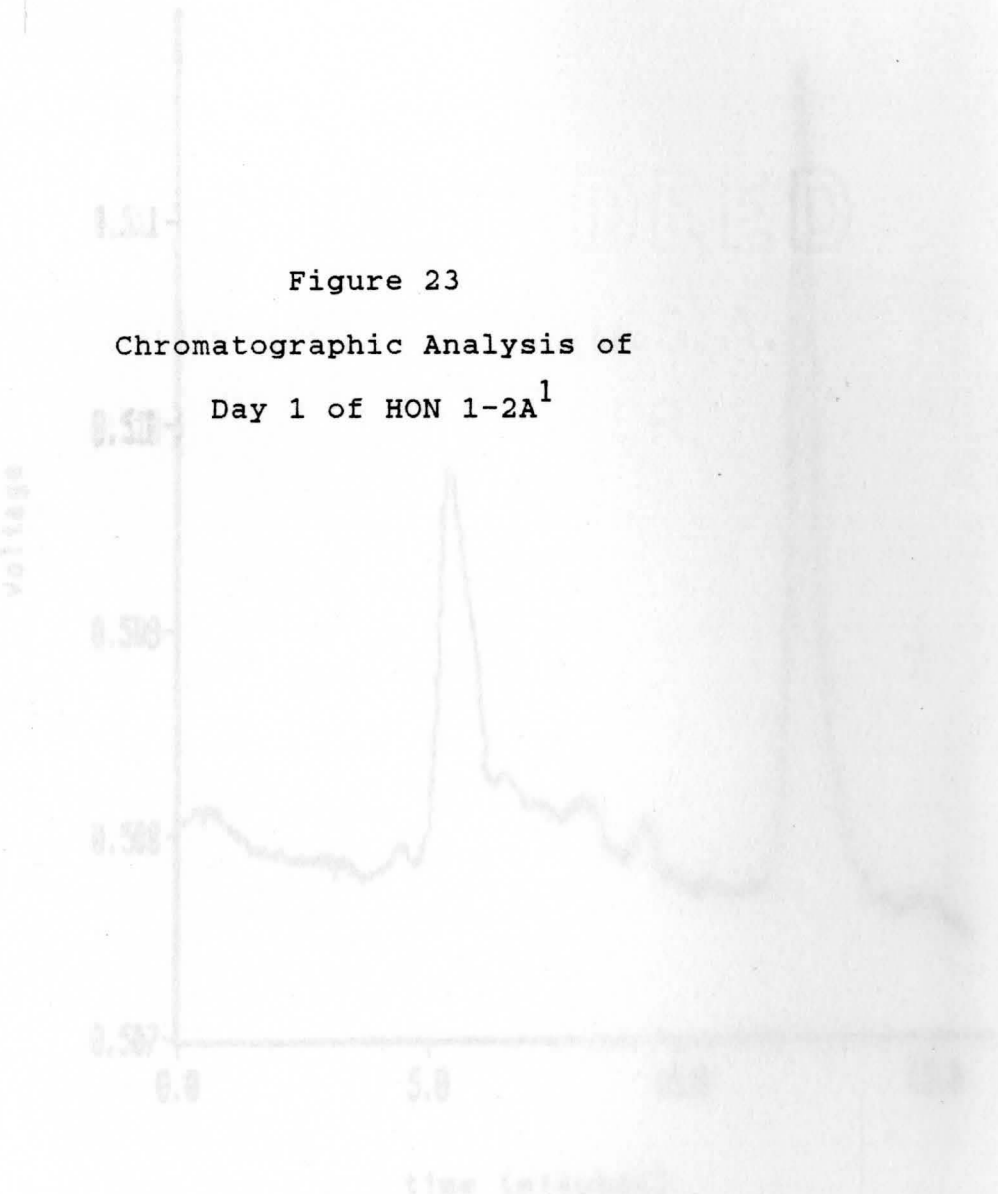
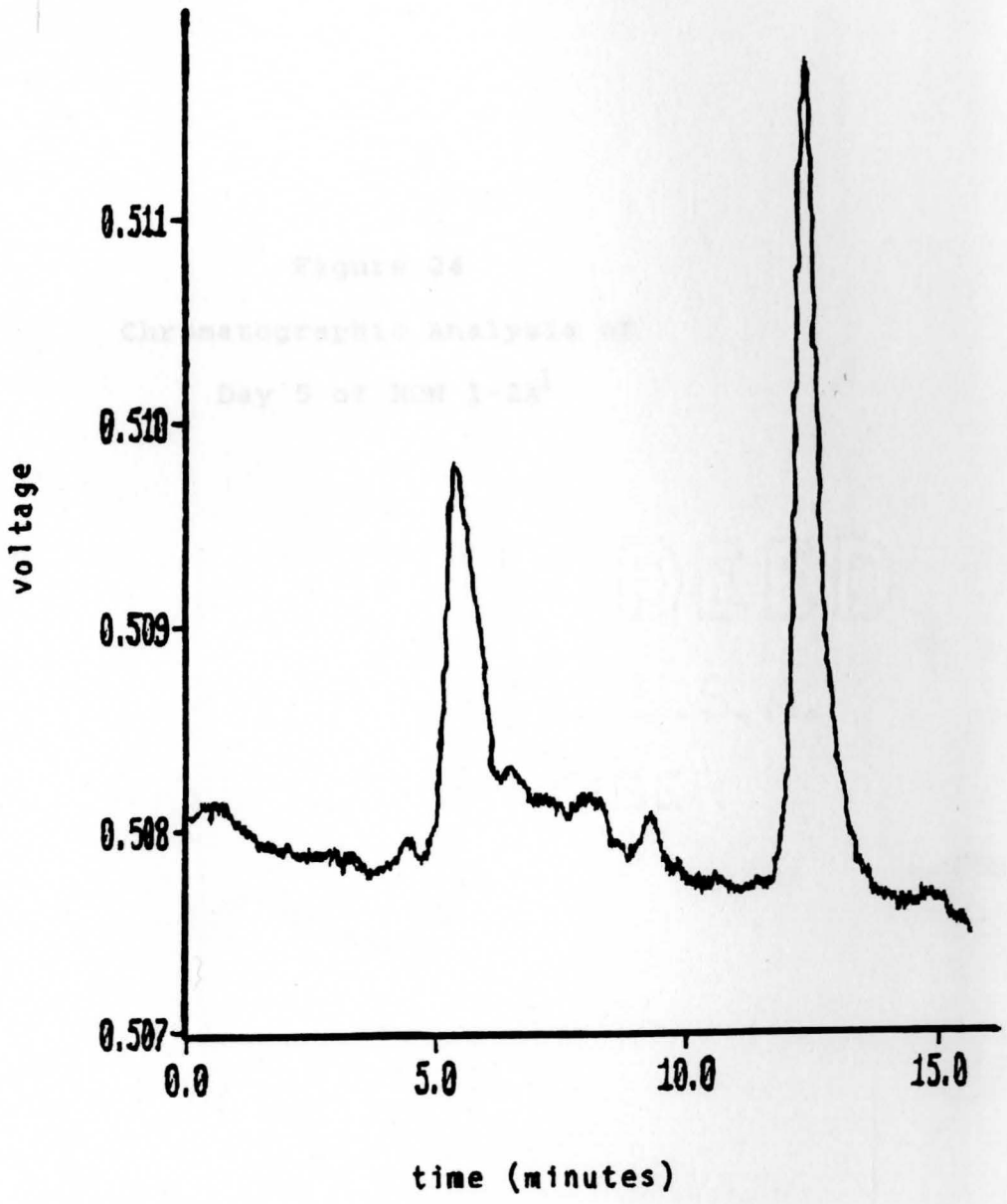


Figure 23

Chromatographic Analysis of

Day 1 of HON 1-2A[1]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0.509
0.508
0.507
voltage
0.0
5.0
10.0
15.0
time (minutes)

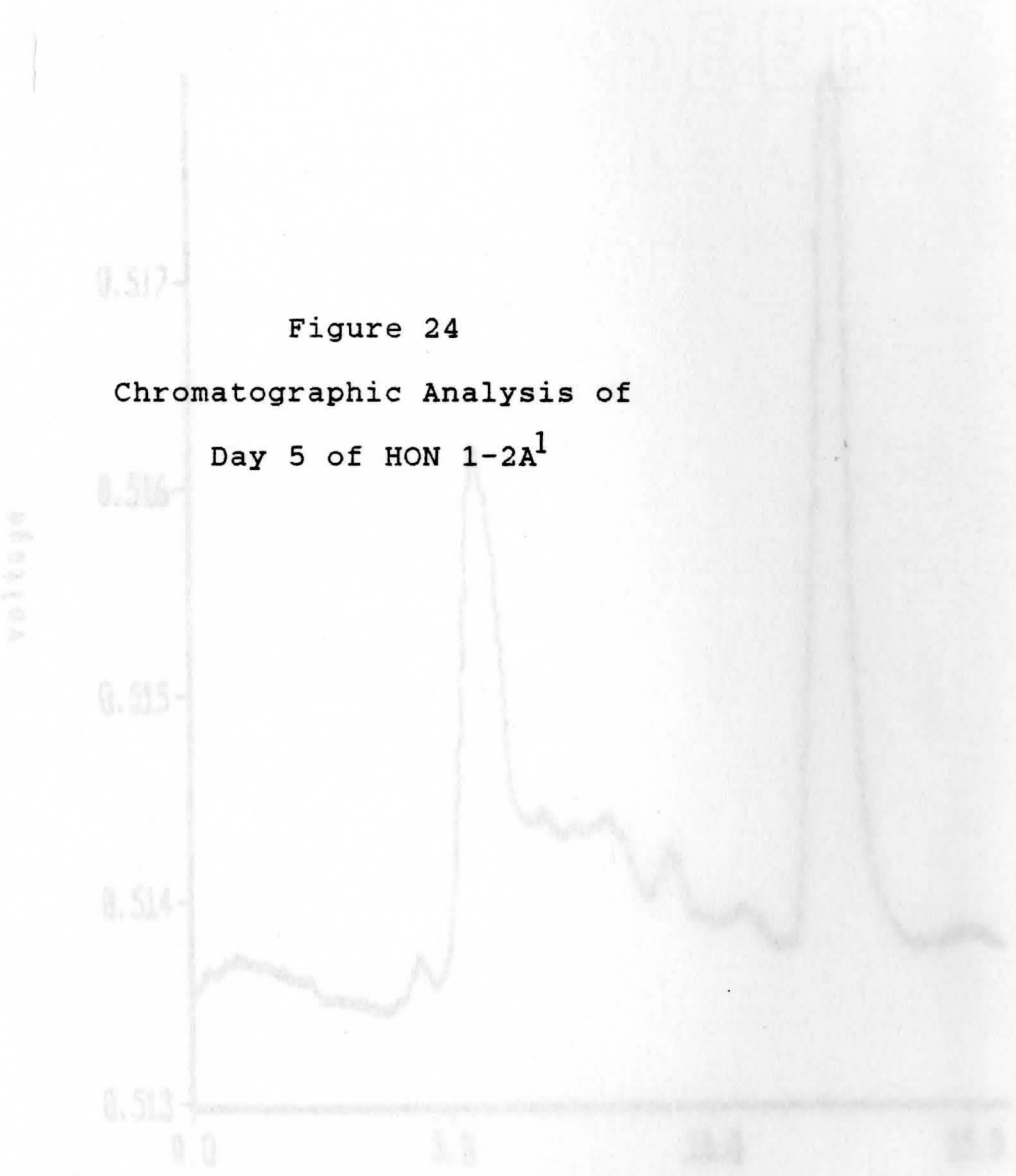
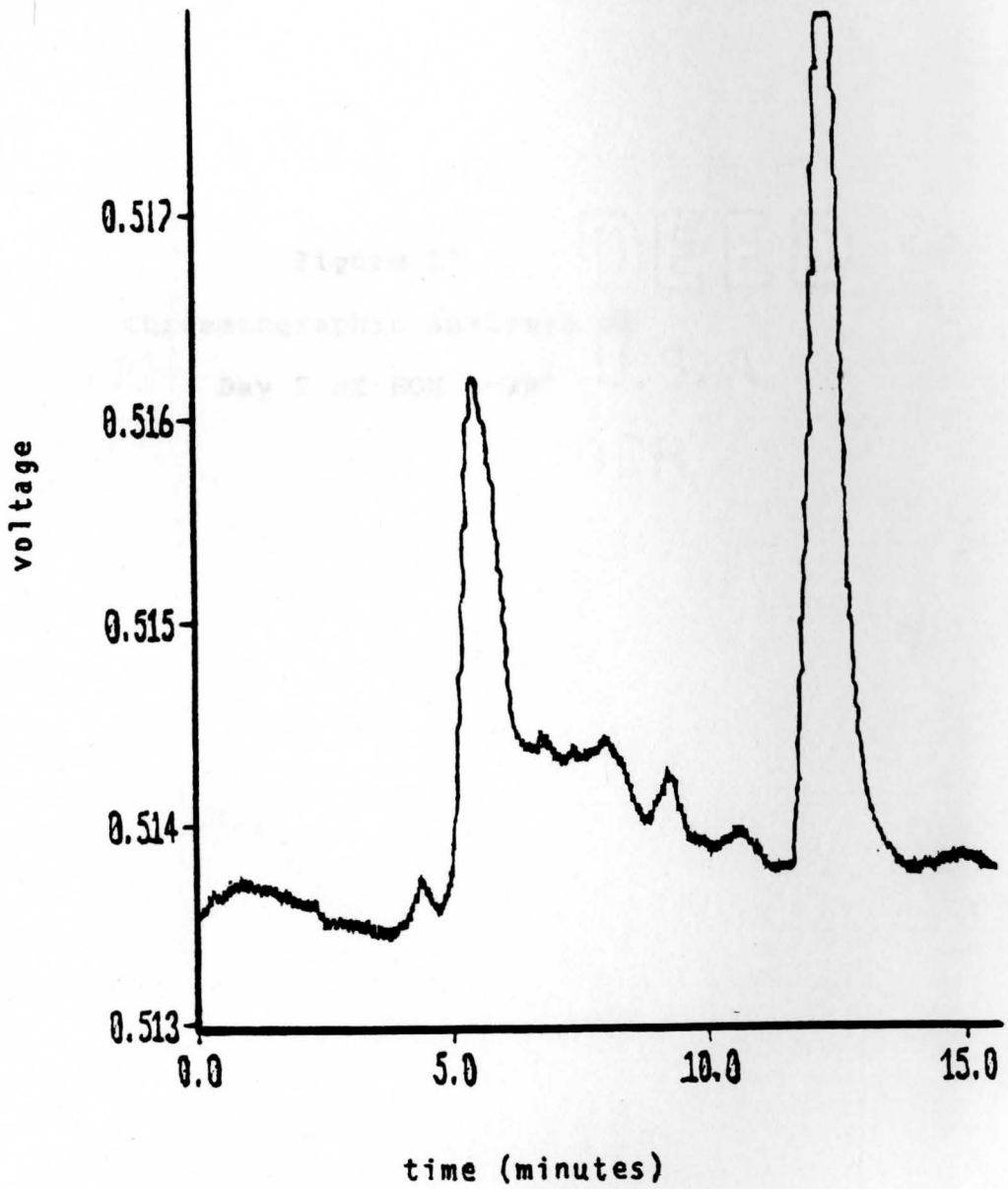


Figure 24

Chromatographic Analysis of

Day 5 of HON 1-2A[1]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0.515
0.514
0.513
voltage
0.0
5.0
10.0
15.0
time (minutes)

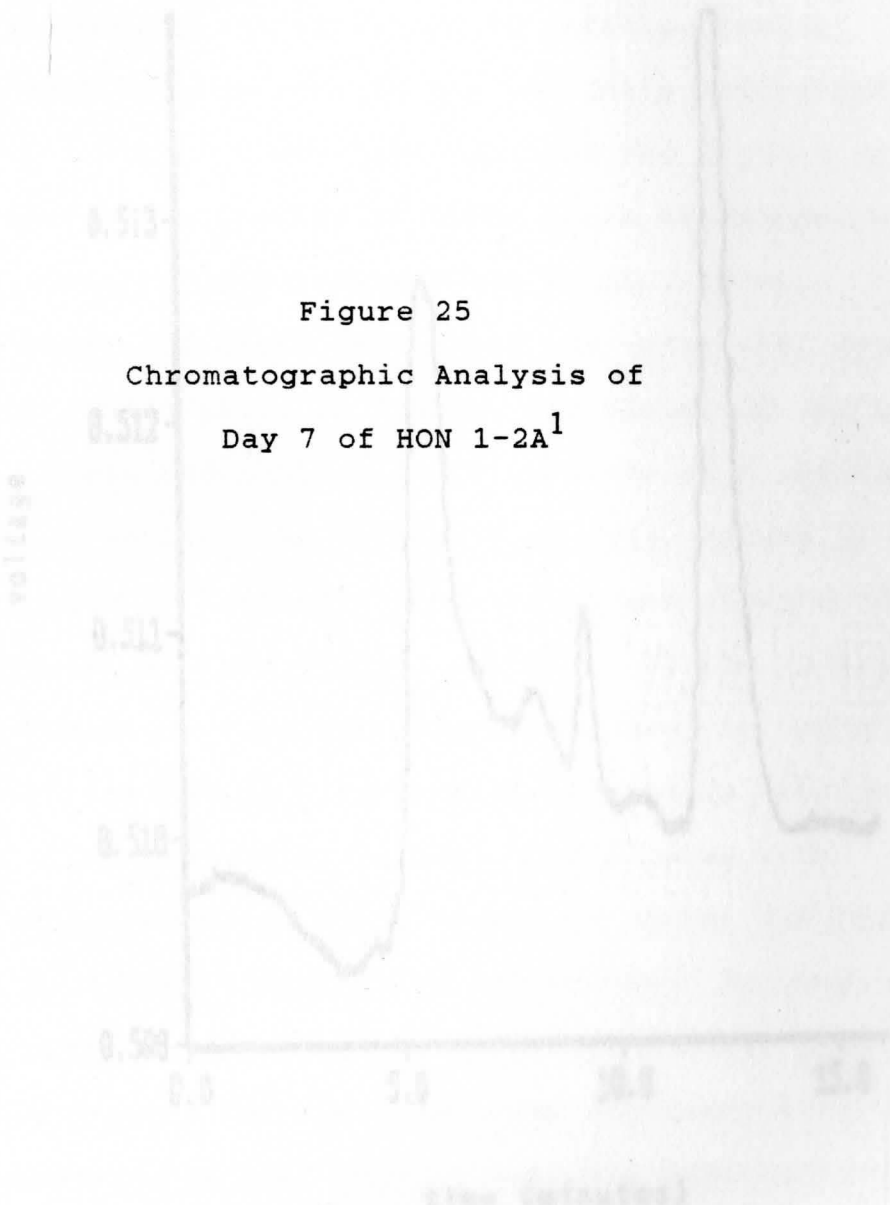
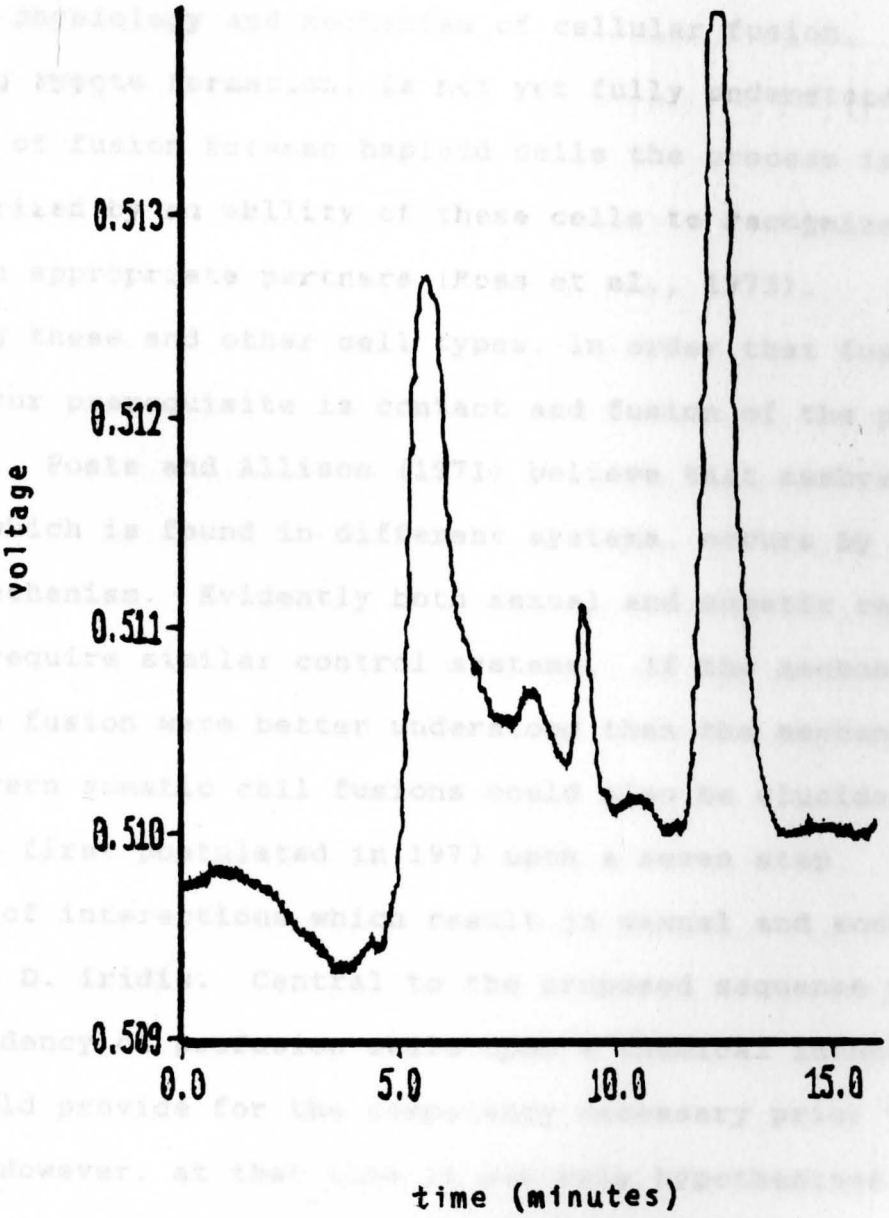


Figure 25

Chromatographic Analysis of

Day 7 of HON 1-2A[1]


0.513
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0.511
0.510
0.509
voltage
0.0
5.0
10.0
15.0
time (minutes)

CHAPTER IV

Discussion

The physiology and mechanism of cellular fusion, including zygote formation, is not yet fully understood. In the case of fusion between haploid cells the process is characterized by an ability of these cells to recognize and fuse with appropriate partners (Ross et al., 1973). Regarding these and other cell types, in order that fusion might occur prerequisite is contact and fusion of the plasma membrane. Poste and Allison (1971) believe that membrane fusion, which is found in different systems, occurs by a common mechanism. Evidently both sexual and somatic cell fusions require similar control systems. If the mechanisms of gamete fusion were better understood then the mechanisms which govern somatic cell fusions could also be elucidated.

Ross first postulated in 1973 upon a seven step sequence of interactions which result in sexual and somatic fusion in **D. iridis**. Central to the proposed sequence was the dependency of prefusion cells upon a chemical inducer, which would provide for the competency necessary prior to fusion. However, at that time it was only hypothesized that the inducing molecule was actually produced; no evidence for such a molecule had ever been presented. Later, Youngman et al. (1977), using the Colonia isolate of **P. polycephalum**

hypothesized that a molecule capable of diffusing through a .2um Nucleopore filter could enhance the conversion of amoebae to plasmodia. Also, in 1979 the possible existence of an inducing molecule released by growing cells and required for cell fusion was indirectly demonstrated (Pallota et al., 1979). However, the mechanism of action by which such a compound acts has never been elucidated, nor has the actual molecule ever been isolated. Furthermore, it was not made clear whether this compound acts on the cells themselves which produce it (self induction) or if once produced by one mating type, or comparable cell type, is only effective on another compatible mating type cell (cross induction). Induction of mating competency or fusion necessarily leads to zygote formation and subsequent diploid plasmodia. The objective of this study was (1) To determine if induction of fusing competency does in fact take place, and also (2) To determine if induction was self induced or, on the other hand, was cross induced in **D. iridis** and (3) To determine if such a molecule exists by isolating and characterizing it. Evidence for the presence of mating receptors in **D. iridis** was demonstrated previously (Yemma and Soltis, 1988).

In order to answer these questions experiments were designed to test these hypotheses. In the initial experiment cross induction of compatible mating types as a prerequisite for mating competency and zygote formation was

studied. This experiment utilized a spin culture technique previously described, where mating types were separated by a diffusable membrane which would allow a molecular substance to pass through, but not whole cells. HPLC data also presented indicates that although cells in spin cultures had been previously induced, that induction must be an ongoing process, for the inducer molecule appears to be very labile, time dependent, and is produced in small amounts. Also, daughter cells produced as a result of mitosis must be induced as they mature for zygotes are minimally produced in crossed cultures which may contain a large volume of cells. Data regarding maturation of cells is also presented.

Three daily aliquots of each culture were taken over an eight day period, totalling 4 ml each. These experiments ensured that because of a low volume of crossed mating types that zygote production could only occur if cells were pre-induced for mating competency. Two aliquots, each of compatible mating types, acted as controls, with a cross of the two strains representing the experimentals. The data is presented in the form of histograms (Figures 3 - 10) representing measured nuclear DNA in the compatible mating types, CR5-11A^{11} and HON 1-2A^{1} , prior to crossing (controls) and post crossing (experimentals). The crosses were allowed to stand for one hour prior to fixing and processing and represented $2.8x10^{4}$ - $2.28x10^{5}$ cells/ml over the time period indicated. Yemma and Perry (1985) demonstrated that significant zygote formation occurred in

20 - 30 minutes and achieved a maximum in pre-induced cells in approximately 60 minutes, once competency for mating was achieved. In that study it was required that $5.0x10^5$ cells be in culture in order that induction occur, representing a rather high cellular density. Shipley and Holt (1982), Yemma and Perry (1985), and Alberts and Therrien (1985) had previously reported the requirement of a similar cellular density in order that mating competency might occur; this is in agreement with our data. Data presented in this study also has demonstrated that induction of cells is negated upon encystment and requires replating, or culturing. These cells must be grown for approximately 7 days to achieve a large enough number of cells in order to reinduce the population whereas crossed induced cells normally demonstrate plasmodial formation, or the diploid state, in 3 - 4 days. It should also be noted that the results of experiments in which crossed uninduced cells were plated on solid media, then treated with liquid media removed from cultures in which induced cells were previously grown, 8 out of 10 cultures demonstrated plasmodial formation in 3 - 4 days. These cultures did not at that time contain a cellular population of $5.0x10^5$ cells.

Cytophotometric data regarding controls consistently demonstrated a unimodal distribution as expected at most time periods where nuclear DNA was measured, except those in which appreciable arrested G_1 cells were present. At these

time periods age of the culture and cellular death contributed to these findings. Cells possessing a DNA value of approximately .5 for example, are considered haploid G_1 cells (1C), or unreplicated. Those cells possessing DNA values of approximately 1.0 when compared to the controls demonstrate measured DNA values equivalent to replicated haploid cells (2C) or G_2. It has been previously demonstrated that growing cells of **D. iridis** do not possess an appreciable G_1 phase of the cell cycle, for these cells are produced as a result of recent mitosis and quickly enter the S phase of the cell cycle, but do have a G_2 period of considerable length (approximately 12 hours) (Rusch, 1969). Many haploid cells demonstrate a progression from S phase to the G_2 stage of the cell cycle, resulting in haploid replicated DNA values.

The histograms representing DNA values of the crossed mating types, however, in almost all cases show a bimodal distribution of the DNA values representing the cellular population, indicating that there are two distinct cell populations present containing both haploid and diploid cells. It is important to recall that plasmodia are diploid multinucleated organisms whose nuclei also possess an extended G_2 phase, and because of this their DNA content is considered to be essentially 4C. A 2C cell, whether the result of immediate zygote formation, and therefore diploid, or a haploid replicated G_2 cell, would have DNA values approximately half that of the G_2 plasmodia, while an

unreplicated G_1 haploid cell (1C) would possess DNA values approximately half those of the unreplicated G_1 diploid cells. The two cellular populations present in the crosses are made up of unfused haploid cells and diploid zygotes (2C), which are the result of sexual fusion between haploid cells. These cells have DNA values of 1.0 and above, approximately double the mean values of the controls. A break is clearly present in the histograms when zygotes appear in a predominantly haploid population. In these crosses the zygote population was always plated to solid media in order to demonstrate the presence of zygotes. Their presence could then be confirmed by the development of diploid plasmodia (Figure 17). In all cases where zygotes were detected in crosses between compatible mating types by cytophotometric methods, plasmodia did indeed develop within 3 to 4 days. Sexual fusion evidently occurs primarily between non-replicated haploid cells or those in early S phase, as this second population (zygotes) possesses mean DNA values twice that of the mean 1C controls. These cells also demonstrated DNA values which were approximately one half that of the 4C plasmodium control (Figure 17). The mean DNA value for all the experimental crosses was higher than that of the controls, but not appreciably so as expected. This is due to the fact that the number of zygotes produced are small when compared to the represented cellular population essentially made up of unfused haploid

cells in varying degrees of maturation ranging from G_1 cells which have recently completed mitosis to G_2 cells which have replicated their DNA. Yemma and Therrien (1974) were able to demonstrate microspectrophotometrically that stable zygote formation occurred rather quickly in a non-random process upon mixing myxamoebae of compatible mating types. This implied at that time that specific membrane activity was required. It is equally important to note that a bimodal distribution is not accurately reflected by a total mean value which tends to obscure the presence of two cellular populations. It is important to recall that zygotes represented here were produced in small aliquots of cells and had to be pre-induced in order that zygote formation occurs. The literature states that 5.0×10^5 cells/ml are required to induce mating competency in these cells as reported by Shipley and Holt (1982), Yemma and Perry (1985) and Alberts and Therrien (1985). The initial data supported the hypothesis that induction does in fact occur for mating competency and that the the inducer molecule is filterable.

In a subsequent experiment cells were grown in separated spin cultures. The results (Figures 11 - 16) of controls representing measured nuclear DNA of CR5-11A[11] and HON 1-2A[1] myxamoebae for each day collected generally exhibited unimodal distributions as observed previously except in those cases where G_2 cells are appreciably present as previously noted. Those cells having a DNA value of .45

represent non-replicated haploid cells (1C) while those with values of approximately .90 are haploid DNA replicated (2C) or G_2 cells. Those of intermediate values are in various states of DNA synthesis. The values presented represent the average of controls measured over an 8 day period. Provided sexual fusion and subsequent zygote formation occurred within the crosses, zygotes would be expected to show DNA values approximately twice that of the 1C haploid cells or .90 or above depending on whether they had initially fused as haploid G_1 cells or were in early G_2, and whether the zygotes had begun to replicate their DNA. Most of the cells measured appear to have recently completed mitosis (haploid G_1) while those cells in the .90 to 1.20 range represent diploid 2C cells in G_2. As can be seen from the histograms, there is a bimodal distribution of the cellular population with some cells demonstrating DNA values of .90, which are considered zygotes. Additionally, these cells possess DNA values one half that of the plasmodium (4C) control as would be expected (Figure 17), since they are diploid G_1 (2C) cells.

In a subsequent repeat of the previous separate spin culture experiment the previous values demonstrated excellent reproducibility. The controls demonstrated a unimodal distribution with the haploid (1C) cells having DNA values of .6. The crossed mating types once again demonstrated a bimodal distribution with the zygotes (2C)

having DNA values twice that of the haploid (1C) controls and one half that of the plasmodium (4C) control. Additionally, some zygotes possessed DNA values approaching the 4C value. Apparently cells can fuse in the 1C unreplicated or while in the S phase condition as evidenced by the presented data but normally do not in the G_2 or replicated haploid condition. It appears that G_2 cells are inevitably programmed to divide, for diploid G_2 zygotes do not normally appear, and histograms representing crosses do not exhibit late G_2 populations.

At the designated time periods three types of crosses were made from aliquots of cells previously described onto solid agar. Two involved plating the two common mating type strains separately with a common mating type, while the third involved plating the previously crossed compatible mating types. The separate plating of the CR5-11A^{11} and HON 1-2A^{1} should not demonstrate plasmodium formation for they are haploid and uncrossed while the cross of the two should have demonstrated plasmodial formation since microspectrophotometric data presented demonstrated that zygotes were present. This occurred in all cases. The separate platings of a common mating type acted to ensure that each designated mating type culture remained pure. Interestingly, it has been consistently evidenced throughout this study that although zygotes readily form in liquid culture plasmodia never do; however plasmodia readily develop from zygotes on solid media.

These experiments yielded important results. It was demonstrated that cells of compatible mating types undergo self induction regarding the production of receptors in order to become competent to undergo sexual fusion and that subsequent zygote formation occur. Although it is important to add that when growing cell cultures are made continuous by a selective membrane, the efficiency of the inducer appears to be enhanced due to an equilibrium produced between the two separate sides. Presented culture data and HPLC data indicate that the inducer molecule is similar for both strains. The lack of significant numbers of zygotes present in the small aliquots can be explained as previously stated by the relatively short time allowed for zygote formation (one hour) and the low pre-induced cell counts in the collected aliquots. Also, it is important to note that results obtained in this study indicate that maturation of mating cell types is necessary in order that induction of cells takes place and zygote formation occurs. For sexual fusion to occur contact must occur between cells of compatible mating types and the chances of this happening in one hour given the low cell counts reported is low. The presented data also demonstrates that these cells were pre-induced; it is highly unlikely that the synthesis of the inducing molecule, its recognition by the cells and the formation of the appropriate receptor could occur within one hour for all cells present and at the cellular number/ml

indicated (Tables 4 - 6).

When encysted amoebae of compatible mating strains were crossed on cornmeal agar, plasmodium formation occurred within six to seven days. Apparently large cell numbers per milliliter are required. This was previously demonstrated by Holt and Shipley (1982), Yemma and Perry (1985), and Alberts and Therrien (1985), who agreed that competency for fusion or mating in D. iridis required $5.0x10^5$ cells/ml. Yet, when these same strains in the amoeboid form were grown in large numbers and subcultured in crosses containing significantly lower numbers (Tables 4 - 6) than $5.0x10^5$, plasmodium formation was seen within three to four days (Table 3). Evidently, the cells must produce an inducer molecule in order to become competent to mate with other compatible mating types. When these mating types were allowed to encyst and then were subsequently crossed plasmodium formation did not occur until the sixth or seventh day. However, as previously noted if uninduced cells are plated and quickly treated with media removed from growing induced cells, plasmodia develop in 3 - 4 days, regardless of the low cell numbers. It is apparent that encystment of the cells causes a loss of induction. It also appears that receptor formation through an inducer molecule is under genetic control and is under rather precise regulation by the inducer. This is logical as cells only enter encystment because of harsh environmental conditions in order to survive. Mating would not be a top priority at

this time.

The data presented in order to answer the question "why" when zygotes do appear many haploid cells are still present and do not at that time form large numbers of zygotes points to the necessity for cell maturation in order to be induced. Thus there is a constant necessity for the production and presence of inducer molecule. Also it is important to note that the inducer molecule is labile and the lability is time dependent. This is supported by presented HPLC data. It also appears that an increase in inducer concentration does not incite greater zygote formation which supports the need for maturation to take place among cells before they are capable of forming zygotes (Tables 5 - 6). In order for the cells to produce the inducer molecule, they must first reach a developmental level of maturity. This was demonstrated by replating cells, which had not yet formed plasmodium, but were removed from crossed cultures which had already demonstrated plasmodial formation. Sixty percent of the replated cells eventually gave rise to plasmodium (Table 7); the only difference between them before and after their replating appears to be their degree of maturation. One explanation for this percentage not being 100% is the occurrence of transfers containing a common mating type. This is quite probable as there is no way to microscopically distinguish between different mating types. This experiment also helped

to further explain the low number of zygotes formed in the crosses, as previously mentioned. A large number of those cells were still developmentally immature, and were not producing the inducer molecule or were incapable of being induced at that time since mitosis is occurring at a rather high frequency in many of these cells. Total maturity of a culture, it appears, is achieved by the eighth day of growth. Following this time period the cells then exhibit a stationary phase regarding additional growth and therefore mitosis. Since the number of cells competent and available for mating is then low, cellular death (G_1 arrest) and large numbers of G_2 cells are observed (Figure 9) in this late time period. It is important to note that although the maturity of cells is necessary in order to achieve mating competency and to produce the proper receptors, it is equally important to note that cells which enter the G_2 phase of the cell cycle are committed to division in cultures approaching log phase of growth. In those which are in stationary phase many dying and G_1, as well as G_2, cells remain in an arrested state (Figure 9), as previously mentioned. Shipley and Holt (1982) stated that a critical density of cells, 5.0×10^5/ml, is needed for cell induction and subsequent fusion to occur and this data was confirmed by Yemma and Perry (1985) and Alberts and Therrien (1985). However, viewing the daily cell counts for the spin culture experiments (Tables 4 - 6), it can be noted that the maximum number of cells was 2.28×10^5/ml, which is approximately half

that number, while the lowest cell count fell to 1.20×10^4 /ml. Yet in all these studies zygote formation occurred. This data suggests that in liquid culture which contains pre-induced cells the mechanism of receptor formation and mating is probably much more efficient.

HPLC data is presented in Figures 18 - 25. These experiments were carried out in order to isolate and characterize the inducer molecule. A control of only **E. coli** and water (Figure 18) was run in order to discover the presence of diffusable molecules normally produced by these two components since the cell culture media was made up only of these two prior to cellular innoculation. This allowed for the selection of any molecules produced and secreted by the cultured cells only. Three new peaks representing isolated molecules were consistently found between eight and eleven minutes for the time periods studied. It is quite important to note that the analysis demonstrated identical retention patterns for both mating types (Figure 19), indicating that the inducer for both have the same or very similar molecular composition. It is believed that all three of these are important regarding induction. However, the first of these represents that of most interest for it especially parallels the rise and fall of cellular numbers and especially reflects the peaking of cell and zygote numbers at day 5 for both mating types. Although Day 4 in the CR5-11A[11] shows a peak for the inducer this appears to

be most affective residually in Day 5. In addition, the membrane spin culture as well as the separate spin culture (Tables 4 - 6) showed a peaking of zygote formation at day 5. That this peak may represent metabolic breakdown products was ruled out because a gradual cumulative increase in the peak would then be expected and this was not seen. In addition Day 1 for each clone shows these peaks present in small amounts. If they are normally produced metabolic products they should have been present in greater concentrations at this time period. It is reasonable to consider the two remaining peaks to be other forms of the inducer. We have often observed in our laboratory that the inducer compound is quite labile, thus the other two observed peaks may represent other molecular forms of the inducer or the inducer in various degrees of decomposition. When the compound is examined by HPLC techniques a day or so after its isolation decomposition products are consistently seen. Freezing of the compound does not appear to alter these results. A similar molecular substance studied in P. **polycephalum** was also found to be extremely labile (Youngman 1985). Also, it has been demonstrated that the other two remaining peaks greatly parallel the concentration of the first peak or the inducer molecule. It is interesting to note however, that very low levels for the second and third peaks are found in Day 7 of the HON 1-2A[1]. This indicates that the first peak does in fact represent the inducer, and the other two peaks an altered form of the inducer, possibly

a decomposition product of the inducer terminating in a final product not detectable at 215 nm. Attempts were made to characterize this substance; for example, its peak absorbance is at 215 nm. Normally ubiquitous metabolic products cannot be isolated at this wavelength. The work of White et al. (1985), in their investigations of maturation hormones in Mucoraceous fungi, has discovered that this molecule was a trisporic acid which absorbed in the same area of the UV spectrum as our molecule. They also discovered other related compounds, possibly decomposition products of trisporic acid.

Because of its life cycle, **D. iridis** has shown itself to be an ideal research subject. This study has helped us gain a more thorough understanding of the mechanism of sexual fusion and the factors which initiate it in this organism. But further studies need to be done in order to further characterize the inducing factor and to isolate the actual mating receptor it induces.

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