QUANTITATIVE CYTOPHOTOMETRIC ANALYSIS OF NUCLEAR DNA CONCENTRATIONS BY USE OF CRESYL VIOLET ACETATE VERSUS BASIC FUCHSIN

by

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ABSTRACT

QUANTITATIVE CYTOPHOTOMETRIC ANALYSIS OF NUCLEAR DNA CONCENTRATIONS BY USE OF CRESYL VIOLET ACETATE VERSUS BASIC FUCHSIN

Andrew C. Bidinotto

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Cresyl violet, a basic, oxazine dye, was used in conjunction with the Feulgen reaction to study its staining properties and possible stoichiometric properties in comparison to basic fuchsin, which is a stoichiometric stain. The two-wavelength method of microspectrophotometry was employed to measure nuclear DNA concentrations in liver tissue, sperm, plasmodium, and myxamoeba cells.

It was determined that cresyl violet is specific and stoichiometric for nuclear DNA and complexes with hydrolytic formed aldehyde groups on the DNA molecule. Furthermore, it was also demonstrated that the use of cresyl violet acetate can readily be used to study subtle nuclear DNA changes which occur during the cell cycle.

ACKNOWLEDGEMENT

The author would like to sincerely thank Dr. John J. Yemma, because without his patience, dedication, encouragement, and guidance, this accomplishment would not have been possible. Also, a special thanks is reserved for the author's parents, John and Mary Bidinotto. Their continuous support and love made this rocky road smoother. Finally, this thesis is dedicated to Julianne Urban, the author's fiance. Her considerable understanding, support, and constant love helped fuel the desire to attain this goal.

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SYMBOL	DEFINITION	UNITS OF REFERENCE
A	Area	U
C	Correction factor for unoccupied space: $C=(2-Q)^{-1}\ \ln(Q-1)^{-1}$	
E	Extinction	
I_s	Flux of photons on chromophore	
I_o	Transmitted flux	
K	Specific absorptivity constant of the chromophore at a defined wavelength	
L	Parameter equivalent to one minus the transmission (1-T) at a defined wavelength	
M	Chromophore mass in the measured field	
Q	Ratio of L_2/L_1	
T	Transmission of the field	

Introduction

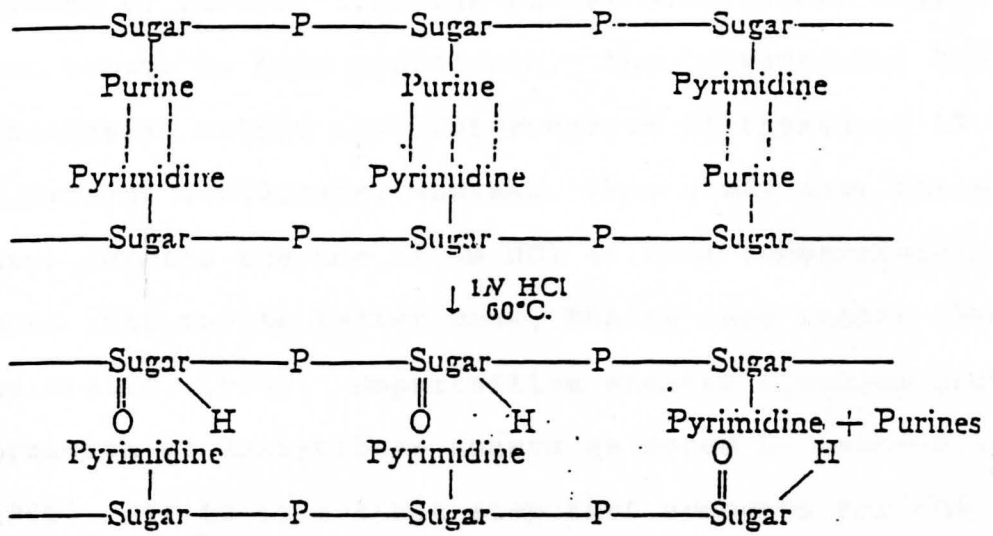
The quantitative measurement of nuclear deoxyribonucleic acid (DNA) by use of stoichiometric staining found its roots in 1865 with the work of Hugo Schiff, a German organic chemist. His research involved the reaction between aromatic amines and aldehydes, known as the Schiff-base reaction (Kasten, 1960). His work included an extensive study of triphenylmethane basic dye (rosaniline), also known as fuchsin due to its fuchsia color. In 1867, Schiff noted that fuchsin solution could be decolorized upon addition of sulphur dioxide, only to regain its color upon compounding an aldehyde to the solution. Although his reasoning for the mechanism of changes was found to be incorrect, he paved the way for future experimentation.

Upon further investigation, basic fuchsin, a mixture of pararosaniline, rosaniline, and magenta-II dyes, was found to react like that of the schiff-base reaction dye. With the addition of SO_2, basic fuchsin produces a colorless derivative, leukofuchsin (Schiff's reagent), which can react with aldehydes to form a colored dye complex (Kasten, 1959). In 1924, Feulgen and Rossenbeck developed a histochemical application for Schiff's reagent which permitted the localization of DNA in nuclei. This method became known as the Feulgen reaction. Although this reaction is complex and not totally defined (Figure 1), an accepted view of the mechanism of action as described by (DiStefano, 1948; Lessler, 1953; Kasten, 1960) is that the Feulgen reaction

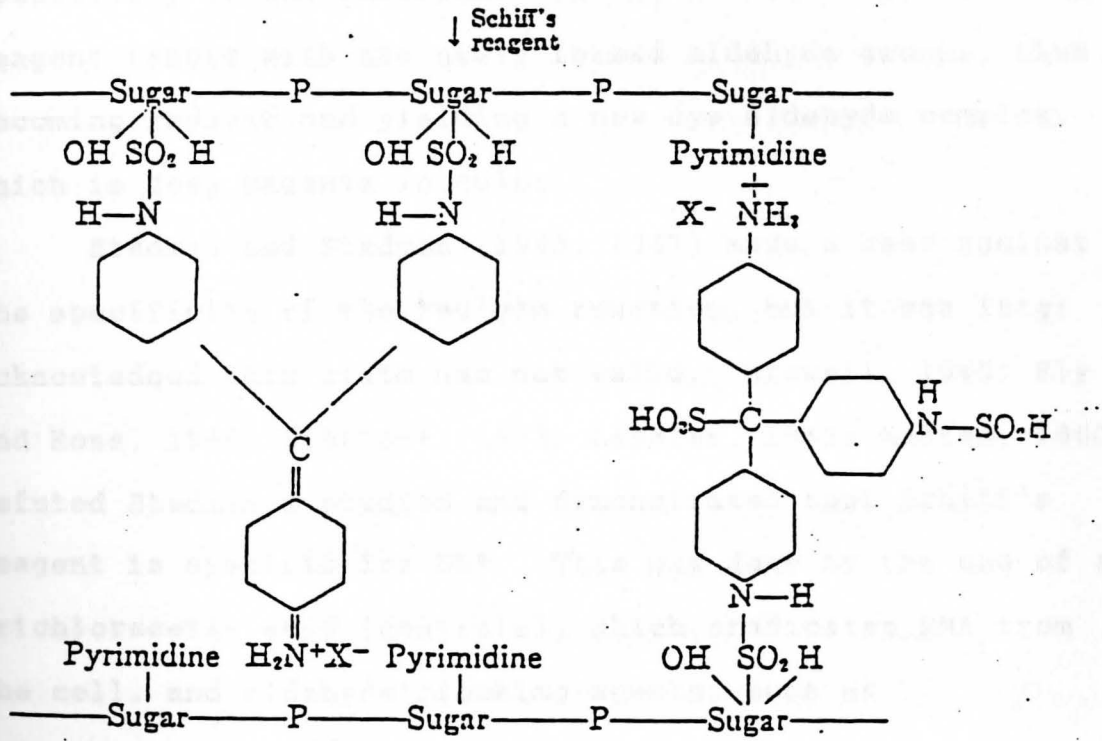
Figure 1

Mechanism of the Feulgen Reaction

THE CHEMISTRY OF SCHIFF'S REAGENT



Representation of hydrolyzed DNA



Aldehyde dye product

Mechanism of Feulgen reaction.

(Kasten, 1960)

takes place in two separate steps. First, depurination, the removal of purines from the purine-deoxyribose complex of DNA, occurs by mild hydrolysis. The conventional "hot hydrolysis" method involves emersion of tissue in 1N HCl at 60 degrees centigrade; whereas, this study uses the method which permits the use of 5N HCl at room temperature. It was found that in the latter case, DNA is more stable (DeCosse and Aiello, 1966). Depurination enables aldehyde group formation of deoxyribose sugars as noted by Osborne and Heyl (1908). It is this first step that accounts for the specificity of the reaction. In the second step, Schiff's reagent reacts with the newly formed aldehyde groups; thus, becoming reduced and yielding a new dye aldehyde complex, which is deep magenta in color.

Stedman and Stedman (1943, 1947) made a case against the specificity of the Feulgen reaction, but it was later acknowledged this claim was not valid. Stowell, 1945; Ely and Ross, 1949; Sibatani, 1953; Lessler, 1953; Kasten, 1960 refuted Stedman's studies and demonstrated that Schiff's reagent is specific for DNA. This was done by the use of 4% trichloracetic acid (controls), which eradicates DNA from the cell, and aldehyde blocking agents, such as hydroxylamine and thioseimcarbazide. In both cases the cell did not stain, which showed that Schiff's reagent is not only specific for staining DNA, but also the aldehyde groups of the DNA. Currently, there is general agreement that under proper controls and conditions that Schiff's reagent,

when used in the Feulgen method, is stoichiometric and can therefore be used in the quantitative measurement of DNA concentrations (Yemma, 1985, 1991).

Refuting skepticism by Ely and Ross (1949), Ris and Mirsky (1949) demonstrated that relative DNA concentrations in several different species of animals could be determined by microspectrophotometric analysis. Also, Swift (1956) concurred that post-hydrolytic DNA can be accurately measured. Ornstein (1952) and Mendelsohn (1961) developed the microspectrophotometric two-wavelength method for the reliable quantitative measurement of DNA. This method reduces error by correcting heterogeneous distribution of stained DNA. Therefore, almost any conceivable distribution of DNA can be measured. It is agreed that with careful procedures and controls quantitation of relative DNA concentrations are valid and extremely reproducable.

Although basic fuchsin, when implemented in the Feulgen reaction, has successfully been shown to be useful in both qualitative and quantitative measurements, many factors have been shown to affect its specificity, intensity, and stability (Orozs, 1992). For example, temperature, dye impurities, pH, and light have all been shown to affect the results of the Feulgen reaction. Fixation, also being among the list of factors as determined in previous studies done in our laboratory, is a critical process in the preparation of tissues for the Feulgen reaction (Kelley, 1984). Pearse (1968) noticed that nucleic

acids may vary in physical state depending on the type of fixative used. The goal of fixation is to maximally stabilize nuclear DNA, stop catabolic degradation, and to make insoluble protein components of the cell. The literature on this topic indicates (Kurnich, 1955; Ruch, 1966; Swift, 1966; Yemma and Therrien, 1972) that formalin buffered at pH 7.0 is most suitable for quantitative photometry. Kelley (1984) also carried out studies on different fixatives and their affect on the intensity of the Feulgen reaction. It was concluded that 10% buffered formalin gave the best results.

In addition to fixation, the concentration and source of SO_2 plays a key role in the effectiveness of the reaction, for it is the SO_2 that changes basic fuchsin to its leuco state enabling it to react with the aldehyde groups on the DNA. Schibsted, 1932; Rumpf, 1932; Tobie, 1942 showed that an excess of SO_2 decreases the sensitivity of the reagent *in vitro*, and noted levels that are too deficient hindered color development. A standard ratio of sulphur dioxide:basic fuchsin does not seem to be agreed upon due to various sources of SO_2 used and the complexity of the reaction. DeTomasi (1936) found that overtime sulphur dioxide levels decrease in Schiff's reagent which in-turn raises the pH of the solution. Adding to this, Bedi and Horobin (1980) found that increasing the pH leads to a decrease in stain intensity. This particular problem led scientists to search for reliable sources of sulphur dioxide

for preparation of Schiff's reagent.

At first, sulphur dioxide gas was bubbled-in aqueous basic fuchsin solution, but this method was hard to standardize. Introductions of sodium bisulfite ($NaHSO_3$) and sodium hydrosulfite ($Na_2S_2O_4$) offered more variety of sulphur dioxide producing chemicals. Of all the modifications found, potassium metabisulfite ($K_2S_2O_5$) is most commonly implemented. It was first introduced by DeTomasi (1936); he showed it to be very useful when preparing Schiff's reagent, as well as having increased stability over its counterparts. For a more thorough list of the history of sulphur dioxide sources (Kasten, 1959) refer to Table-1.

The shelf-life of Schiff's reagent has also been studied in some detail (Orozs, 1992). The life-span of the reagent, which affects the intensity and specificity of the Feulgen reaction, depends greatly upon the ingredients, the process of compounding, and the method of storage. Various times of expiration have been reported as 2 days (Chace, 1906), 5 months (Tobie, 1938), 13 months (Tobie, 1942), 6 months (Kasten, 1960), 1-2 months (Dutt, 1979), and 2-4 weeks (Orozs, 1992). In view of this, the details of which specific entities cause deterioration of the reagent were sought. Elftman (1959a) demonstrated the depletion of SO_2, as well as the oxidation of sulfite to sulphate, is a major component. In addition, Bedi and Horobin (1980) found the intensity of the stain fell steadily after an eradict first

TABLE 1

Sources of SO_2, References, and Schiff's Reagent Composition

SO_2 source and references	SO_2 (gm)	Basic fuchsin (gm)	Acid (ml)
SO_2 gas			
Schiff, 1866		?	
Chautard, 1886		0.05	
Francois, 1897		0.012	1.2ml H_2SO_4
Bitto, 1897		0.25	
Tolman and Trescott 1906	0.5	0.05	
Chace, 1906	1.6	0.05	
Feulgen and Rossenbeck, 1924		0.5	
Crocker, 1925		0.06	
Widstrom, 1928		0.35	
Schibsted, 1932	0.085	1.0	
Tobie, 1938	0.5	0.05	
Tobie, 1942	0.2	0.1	
Wild, 1953	11.3	0.05	
Vogel, 1956		0.1	
Staple, 1957		0.5	
$Na_2S_2O_4$			
Prud'homme, 1904	0.87	0.013	1ml H_2SO_4
Wertheim, 1922	2.0	0.002	
Alexander et al, 1950	0.5	0.5	
Na_2SO_3			
Fincke, 1914	2.5	0.1	1.5ml conc HCl
Elvove, 1917	1.0	0.1	1ml conc. HCl
Scott, 1945	1.0	0.1	2ml conc. HCl
Mowry, 1958	2.5	1.0	4ml conc. HCl

Table 1 (continued)

SO_2 source and references	SO_2 (gm)	Basic fuchsin (gm)	Acid (ml)
$SOCl_2$			
Baarger and DeLamater, 1948	0.25 ml	0.1	
$K_2S_2O_5$			
DeTomasi, 1936	0.45	0.45	10ml 1N HCl
Coleman, 1938	0.95	0.48	10ml 1N HCl
Stowell, 1945	0.91	0.45	10ml 1N HCl
Hotchkiss, 1948	0.98	0.49	10ml 2N HCL
Lillie, 1951a	1.9	1.0	100ml .15N HCL
Hormann et al., 1958	0.3	0.3	10ml 1N H_2SO_4
$NaHSO_3$			
Mohler, 1890	8.6	0.013	1.5ml H_2SO_4
Margolena, 1932	0.5	0.45	20ml 5N HCl
Benseley, 1939	0.83	0.83	20ml 1N HCl
Wild, 1953	0.5	0.125	1ml con.H_2SO_4
Vogel, 1956	1.0	0.1	1ml HCl

few days, which was due to changes in the organic Oxy-sulphur system. Many ideas evolved from this work in order to resolve these problems. For example, Ely and Ross (1949) suggested storing Schiff's reagent in a light-resistant container in the refrigerator; thereby, reducing the loss of SO_2 by evaporation. This was agreed to be the most effective method for retaining stability. Also, Middleton and Hymas (1931) suggested using antioxidants. Even with these modifications, the general view of the stability of Schiff's reagent is that it is of relative short duration.

The success basic fuchsin exhibited with the localization and quantitation of DNA concentrations led scientists to investigate other possible stains that could be employed in the same manner. These dyes became known as Schiff-Type dyes. Ostergren (1948) followed up on several other dyes that could possibly be used in place of basic fuchsin. The importance of discovering another suitable dye stems from the fact that many investigators agree that under certain conditions basic fuchsin can be carcinogenic (Tatken and Lewis, 1983; Sigma, 1990). The majority of the research done was by Kasten (1957, 1958, 1959, 1960) in which 400 dyes were tested. From this work, many dyes, all containing at least one free amine, were found to demonstrate adequate staining ability. Table 2 is a more comprehensive list of the important Schiff-Type reagents Kasten found. Building on Kasten's work, Floyd (1990) developed the Blue Feulgen

TABLE 2

Important Schiff-Type Reagents and Their Characteristics

Dye	Dye Class	Primary amine	Color stained	Visible peak	U.V. peak
Acridine brown	Acridine	1,2	Brown	458	267
Acridine Yellow	Acridine	2	Yellow	442	264
Acriflavine	Acridine	2	Yellow	445	261
Auramine O	Diphenyl-methane	1	Yellow	430	249
Azure A	Thiazine	1	Blue	625	284
Bizmarck brown R	Disazo	2	Brown	447	283
Bizmarck brown Y	Disazo	2	Brown	435-455	276
Brilliant cresyl blue	Oxazine	2	Blue	615	236
Chrysoidine R	Monoazo	1	Orange	450	278
Chrysoidine Y	Monoazo	1	Orange	440	276
Chrysophos-phine 2G	Acridine	2	Yellow	432	265
Coriphos-phine O	Acridine	1	Yellow	453	265
Cresyl violet	*Oxazine*	*1*	*Violet*	*580*	*268*
Flavophos-phine N	Acridine	2	Yellow	443	266
Neutral red	Azine	1	Brown/red	515	275
Neutral violet	Azine	1	Red	558	276
Phenosafra-nin	Azine	2	Red	515	275

procedure. This method incorporates cresyl violet dye in a modified Feulgen procedure for DNA localization. Floyd noted that this procedure is both faster and easier, and the dye solution has a shelf-life of years compared to the shelf-life of basic fuchsin in the standard Feulgen reaction.

Cresyl violet, belonging to the oxazine dye class, has one free amine. It has been found that the free amine on the dye molecule in the presence of sulphur dioxide allows the dye to attach to DNA aldehyde groups (Kasten, 1958, 1959, 1960). The exact mechanism of attachment is still not completely understood, and more study is necessary. Cresyl violet also was noted to exhibit metachromatic properties, thus staining cartilage a violet color (Kasten, 1959). This may create problems when staining certain tissues.

Although cresyl violet has been shown to be useful in localization of nuclear DNA concentrations, it has not been determined up to this point whether it is a stoichiometric stain. Kasten (1960) mentions studying some of the Schiff-Type stains for stoichiometric value, but these studies were nonconclusive. Dutt (1971b) also looked at Schiff-Type stains, specifically cresyl violet, for quantitative purposes. His research demonstrated some abnormalities; thus, he concluded that more study was necessary. It is the purpose of this study to closely examine the specificity of cresyl violet, its localization of DNA concentrations, and its possible quantitative

properties in comparison to basic fuchsin in more than one organism.

The slime mold is an ideal organism for this study. The relative short life cycle contains both distinct haplophase as well as a distinct diplophase; hence, distinct morphological forms and ease of culturing contribute to its value for use in developmental studies and cell biology. The life cycle of Didymium iridis revolves between a unicellular haploid form and diploid multinucleated stage, which has been documented in publications by Guttes, Guttes, and Rusch (1961), Alexopoulos and Koevenig (1962), and Gray and Alexopoulos (1968). Figure 2 is a detailed representation of the life cycle of Didymium iridis.

The germination of spores into a single protoplast indicates the myxamoeba stage (Collins, 1961). This stage can differentiate into a swarmer, a biflagellate form, depending on enviromental liquidity. It is at this point that the myxamoeba feed on various bacterial strains, such as Eschericha coli, via psuedopodia. If the nutrients are unavailable or depleted, myxamoeba can differentiate into a cyst until more favorable conditions arise. From this point, heterothallic compatible myxamoebal clones can be sexually mated to yield a plasmodial form. It is essential that the myxamoeba be in a log phase of growth, and that each mating strain be in concentrations of 10^5 cells for mating to occur (Yemma and Perry, 1985). It is following syngamy, cellular fusion, that karyogamy occurs in which the

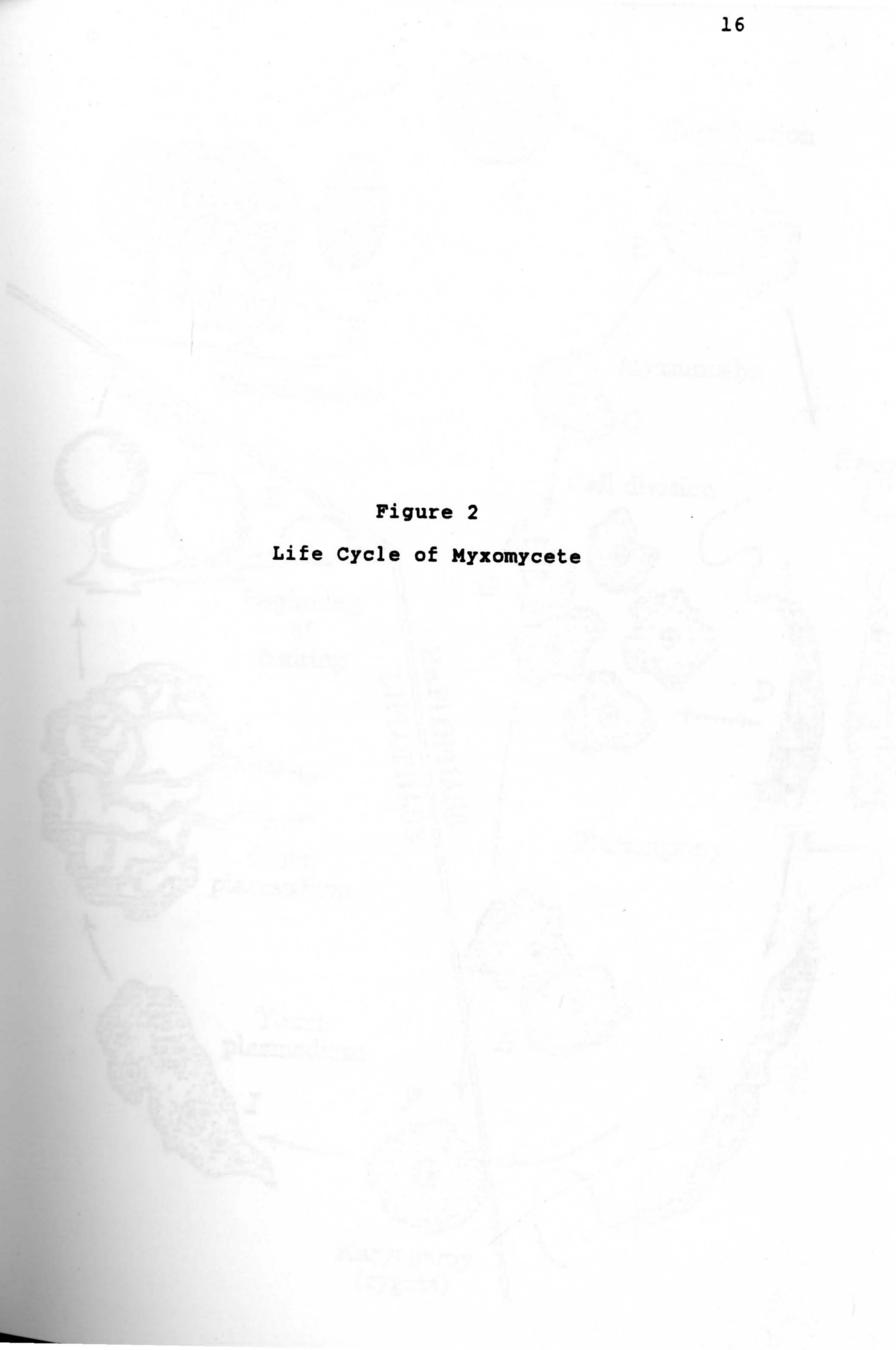
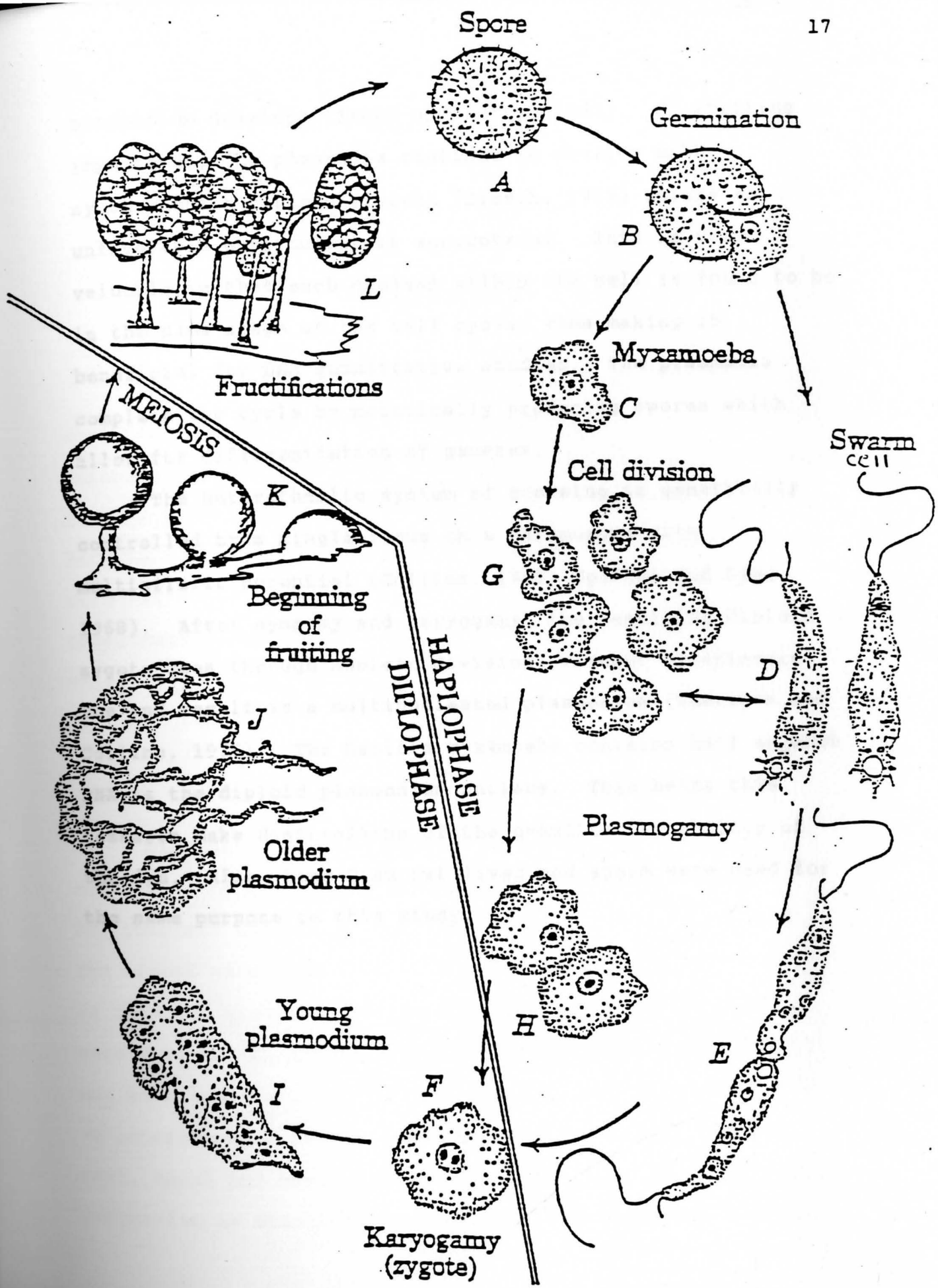


Figure 2

Life Cycle of Myxomycete


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

plasmodium develops (Yemma and Perry, 1985). Of striking importance, the plasmodia continue to develop with synchronous nuclear divisions (Rausch, 1969) unless an unfavorable environment is encountered. This is very valuable in that each nucleus within the cell is found to be in the same stage of the cell cycle, thus making it beneficial for DNA quantitative studies. The plasmodia complete the cycle by meiotically producing spores which allow for differentiation of gametes.

The heterothallic system of crossing is genetically controlled by a single locus on a chromosome with multiallelic potential (Collins, 1963; Collins and Ling, 1968). After syngamy and karyogamy, the resulting diploid zygote goes through nuclear divisions without cytokinesis. The end result is a multinucleated plasmodium (Therrien and Collins, 1976). The haploid myxamoeba contains half as much DNA as the diploid plasmodium nucleus. This helps this research make distinctions of the quantitative ability of the dye. Also, Long-Evan rat liver and sperm were used for the same purpose in this study.

Chapter 2

Materials and Methods

Tissue Source

All isolates of *Didymium iridis* used in this study were supplied by Dr. John J. Yemma, Department of Biological Sciences, Youngstown State University. The two strains used in this study were designated Honduran $7A^2$ (Hon $7A^2$) and Panamanian $2\text{-}21A^7{}_b$ (Pan $2\text{-}21A^7{}_b$). Mammalian liver and sperm were extracted from Long-Evans rats provided by the Department of Biological Sciences, Youngstown State University.

Cultivation of Myxamoeba

Original stocks of myxamoeba clones, *D. iridis*, were maintained on Difco half strength corn meal agar slants. For complete details of agar ingredients refer to Table 3. The clones were nourished with *Escherichia coli* bacteria. In this two organism system, the *E. coli* metabolizes the corn meal and *D. iridis* feeds on the bacteria. At time of use both isolates, Hon $7A^2$ and Pan $2\text{-}21A^7{}_b$, were subcultured on separate agar plates containing half-strength 2% corn meal, which had been previously inoculated with an *E. coli* suspension as described by Yemma and Therrien (1972). The

Table 3

Half-Strength 2% Cornmeal Agar Growth Media

Media

8.0 g Difco Agar

8.5 g Difco Cornmeal Agar

1.0 L Distilled Water

All media was autoclaved at 121°C at 15 psi for 15 min. Approximately 25 ml portions were dispensed in sterile petri plates, and then refrigerated until time of use.

E. coli suspension is approximately equal to a McFarland #2 in turbidity. Two drops of this suspension were placed on each agar plate and spread around by a curved glass rod, otherwise known as a "hockey stick". The plates were then incubated at room temperature in an inverted position for 24 hours. Once assured of an even lawn of growth, each strain of myxamoeba was subcultured on cornmeal agar plates. The plates were then inverted and incubated 4-6 days. The plates were periodically examined for contaminants. When the myxamoeba cultures reach log phase of growth, they were subcultured once again following the steps above, or the two different strains were crossed to yield diploid plasmodium.

Cultivation of Plasmodium

Plasmodial cultures were obtained by taking agar plugs (1 cm x 1 cm) containing log phase myxamoeba from both types of subcultured plates previously prepared. The agar plugs were placed in contact on cornmeal media that had been previously inoculated with an E. coli suspension. The plates were then inverted and incubated at room temperature for 5-7 days. When plasmodial growth is obtained, subcultures of the plasmodium are taken by removing a 1 cm square piece of agar and placing these agar plugs on specialized agar which enhances the growth of the plasmodium. Table 4 describes the ingredients of the specialized plasmodium agar. In 5-8 days the plasmodium

Table 4

Plasmodium Agar

Plasmodium Media

5.0 g Difco Agar

1.0 g Difco Peptone Agar

1.0 g Lactose

1.0 L Distilled Water

All media was autoclaved at 121°C at 15 psi for 15 min. Approximately 25 ml portions were dispensed in sterile petri plates, and then refrigerated until time of use.

grows to suitable size. This is repeated until adequate amounts of plasmodium can be harvested for cytological preparation.

Cytological Preparation of Myxamoeba

The procedure for fixation, harvesting, and slide preparation was carried out in a similar manner for both strains of myxamoeba. Slides of Hon $7A^2$ strain were used for DNA studies. The procedure used in this preparation is as follows:

1) Agar plates containing the myxamoeba were fixed with 10% buffered formalin (pH 7.0) for 24 hours.
2) The cells were "lifted" off the agar by using a rubber policeman and agitation with a pasteur pipet.
3) The cells were transferred to a 50 ml conical centrifuge tube and spun in an Adams Dynac swinging bucket centrifuge at 1500 rev/min for 10 min.
4) The supernatant, which contained bacteria, was discarded. The pellet was washed with 70% ethanol twice to completely remove any remaining bacteria.
5) The cells were then resuspended in 70% ethanol for additional 24 hours.
6) The cells were then centrifuged at 1500 rev/min for 10 min, and the supernate removed.
7) Finally, enough 70% ethanol was added to the pellet to

provide a concentrated suspension of cells.

8) Three drops of the suspension were added to albuminized slides and placed on a slide warmer set at 40°C.

Cytological Preparation of Plasmodia

1) The plasmodial cultures were fixed with 10% buffered formalin (pH 7.0) by flooding the plate for 24 hours.
2) The plasmodia were then lifted from the agar by use of a rubber policeman and pipet.
3) The plasmodial cells were then transferred to a 50 ml conical centrifuge tube and spun at 1500 rev/min for 10 min.
4) The supernate was discarded, and the plasmodia were washed in 70% ethanol 2 times for the removal of bacteria.
5) Plasmodia were then resuspended in 70% ethanol for 24 hours.
6) They were then spun down at 1500 rev/min for 10 min and the supernate was removed.
7) The plug was resuspended in 90% ethanol for 10 min and spun down as before.
8) After removing the supernate, the plasmodia were resuspended in 100% ethanol for 10 min and spun down again.
9) The plug was resuspended in xylene for 10 min and spun down once again. Most of the supernate was discarded

leaving enough to make a very concentrated suspension (approximately 2 ml).

10) The suspension was then paraffin blocked.
11) Five micron sections were cut on an American Optical microtome and placed in a warm water bath.
12) The sections were then placed on albuminized slides and put on a slide warmer set at 40°C.

Cytological Preparation of Rat Tissue

Two Long-Evan male rats were sacrificed by use of a carbon dioxide chamber in this study. Liver and testes tissue were removed from the animals. The fixation, dehydration, and paraffin imbedding procedure is as follows:

1) The liver tissue was cut into approximately 1 cm^3 sections with a scalpel and placed into 10% buffered formalin for 24 hours.
2) The blocks were transferred to 70% ethanol and allowed to sit another 24 hours.
3) The blocks were placed in 90% ethanol for 2 hours.
4) They were transferred into 100% ethanol for 2 hours.
5) The final stage of the dehydration process was to submerge the liver tissue in xylene 2 times for 1 hour each.
6) The tissue was covered with paraffin for 1 hour and left on a warmer such that the paraffin remained in a

liquid state.

7) The paraffin was removed and reapplied for a second time for an hour
8) The paraffin was removed once again, and the tissue was finally paraffin blocked.
9) Five micron sections were cut from the block on an American Optical Microtome and were placed in a warm water bath.
10) The liver sections were then placed on albuminized slides and dried on a slide warmer at 40°C.

Cytological Preparation of Sperm

Long-Evan rat testicles were excised and cut longitudinally while in 10% buffered formalin. The procedure for fixation and slide preparation is as follows:

1) The teste in the 10% buffered formalin was briefly sonicated and a suspension was formed.
2) The suspension was filtered through guaze and spun at 1000 rev/min for 10 min in a 50 ml conical centrifuge tube, such that unwanted tissue was removed.
3) The supernate was transferred to another 50 ml centrifuge tube and spun at 1500 rev/min for 15 min.
4) The pellet containing the sperm cells was resuspended in 10% buffered formalin for 24 hours. The supernate was checked for residual sperm cells and respun if a

numerous amount of sperm were evident.

5) After fixation, the sperm were centrifuged at 1500 rev/min for 10 min and the formalin supernate discarded.
6) The pellet was resuspended in 70% ethanol for 24 hours.
7) The sperm solution again was spun down at 1500 rev/min for 10 min and the supernate removed.
8) Enough 70% ethanol was added to be 1 cm above the pellet, resulting in a concentrated sperm suspension.
9) Two-Three drops of suspension were placed on each albuminized slide, and the slides were placed on a slide warmer set at 40°C.

Table 5 has a complete list of the number of slides made for each tissue and cell sample.

Nuclear Deoxyribonucleic Acid Assay

The Feulgen reaction was carried out according to Feulgen and Rossenbeck (1924), as modified by Therrien (1966) and Bryant and Howard (1969), to localize DNA. The method of Floyd (1990), Blue Feulgen procedure, was also used to localize and quantify DNA. Both basic fuchsin and cresyl violet were used to localize and quantitate nuclear DNA concentrations in all tissue and cell samples, in order to make quantitative comparisons. Tables 6 and 7 are a detailed listing of the compounding of basic fuchsin and

Table 5

Tissue and Cell Slides, Ploidy level, and Groupings

Test Slides

Tissue/Cells	Ploidy	Cresyl	Schiff	Extra
Liver	2N	10	10	20*
Sperm	1N	10	10	20
Plasmodium	2N	10	10	20
Myxamoeba	1N	10	10	20

* This group of slides were used for both types of dye binding property experiments. Experiment-1 10 slides were treated with 4% trichloracetic acid. Experiment-2 10 slides were treated with 2% thiosemicarbazide.

Table 6

Compounding of Schiff's Reagent

Schiff's Reagent Protocol

1.0 g basic fuchsin

2.2 g potassium metabisulfite

100.0 ml 1N HCL

The solution was stirred for 2 hours and left overnight in a cool dark place. After which, 200 mg decolorizing charcoal/500 ml Schiff's reagent was added, and the solution was filtered. This step was repeated until a clear solution appeared. The freshly prepared Schiff's reagent was stored in a light sensitive container at 5°C. At time of use, the solution was fortified with 20 ml 10% potassium metabisulfite for every 80 ml Schiff's reagent.

Table 7

Compounding of Cresyl Violet Solution

(Blue Schiff's Reagent)

Blue Schiff Protocol

0.02 g cresyl violet acetate

1000 ml distilled water

Store violet solution in refrigerator in a light sensitive container. At time of use, add 3 g of sodium dithionite for every 150 ml blue schiff used. The violet color disappears resulting in a clear solution.

cresyl violet for use in the Feulgen reaction. A key requirement for the Feulgen reaction to be accurate in its quantitative measurement is proper hydrolysis of all samples. During the hydrolytic process, purine bases are removed from the DNA resulting in aldehyde group formation. It is at this point the dye can bind as an alkyl sulfonic acid (Nauman et al., 1960) which allows for stoichiometric ability. The stability of the bond enables the quantitation of relative DNA concentrations in individual nuclei (Ris and Mirsky, 1949; Kasten, 1964). This study employed 5N HCL at room temperature for 45 min for hydrolysis, for DeCosse and Aiello (1966) found it to be superior to the conventional 1N HCL method.

The Feulgen reaction used in this study is as follows:

1) Hydration

Slides were emersed in xylene, followed by an ethanol series (100%-70%), and finally distilled water (H_2O^D), 10 min in each solution.

2) Hydrolysis

The slides were placed in 5N HCL at room temperature for 45 min. They were rinsed in distilled water briefly.

3) Staining

Half the slides were placed in Schiff's reagent for 1 hour, and the other half were immersed in cresyl violet

for 20 min.

4) Sulfite Bleach

Slides were then placed in 2 changes of sulfite bleach (10 ml 10% potassium metabisulfite, 10 ml 1N HCL, and 200 ml distilled water) for 10 min each. This removes any erroneous staining and helps the stability of the dye/aldehyde complex.

5) Dehydration

The slides were then dehydrated by first immersing them in distilled water, then an ethanol series (70%-100%), and finally xylene. Immersion was for 10 min. in each solution.

6) Mounting

Finally, each slide was removed from the xylene, and a coverslip was mounted on the slide with permount.

This method was used for all test slides in order to compare the quantitative properties of cresyl violet to that of basic fuchsin. Also, two types of tests were performed to study the attachment specificity of cresyl violet. Experiment-1 (10 liver slides) were treated with 4% TCA (Trichloracetic acid) at 90°C for 15 min (Schneider, 1945) prior to the hydrolysis step in the Feulgen procedure. The TCA eradicates the DNA within the cell. If no staining

takes place upon completion of the Feulgen reaction, the stain is specific for DNA. Experiment-2 (10 liver slides) were treated with 2% thiosemicarbazide solution at 60°C for 3 hours (Kasten, 1959) post-hydrolysis. Thiosemicarbazide is a potent aldehyde blocking agent that adheres to the aldehydes formed post-hydrolysis; thus, interfering with aldehyde specific stains. If no staining is evident in control group-2 following the completion of the Feulgen reaction, then cresyl violet attaches specifically to the aldehyde groups formed on the post-hydrolytic DNA.

Microspectrophotometric Analysis

This study made use of the two-wavelength method of microspectrophotometry (Patau, 1952; Ornstein, 1952; Mendelsohn, 1961) on a Zeiss type 01 microspectrophotometer, using a planachromat oil immersion objective, N.A. 1.30 x 100. The two-wavelength method requires an absorption curve to be run for each chromophore being studied. Since all tissues were fixed and treated under the same conditions, one absorption curve for each dye was sufficient to represent all tissues used. Through this method, errors from heterogeneous distributions of DNA are corrected. Also, Mayall and Mendelsohn (1970) noted it is not necessary to measure the nuclear area directly. These two advantages make this method ideal for this study. Each time before any readings were taken the alignment and linearity of the

phototube were checked.

The selection of the two wavelengths is critical for the correct relative amount of absorbing material, which should give specific absorptivities in a ratio of 2:1 for the chromophore when uniform amounts of stained material is in the field of view. The difference between the transmissions at the two wavelengths are responsible for the estimation of the relative amounts of absorbing material. Wavelength 2 (L_2) is the wavelength at maximum extinction (E_2) of the absorption curve. Wavelength 1 (L_1) is equal to half the maximum extinction (E_1), where $E_2 = 2E_1$. The extinction is equal to the log I_o/I_s; where I_o is the light intensity from the backround and I_s is the intensity of light that passed through the specimen. Therefore E_2 = log I_o/I_s at L_2 and E_1 = log I_o/I_s at L_1. From the absorption curve the two wavelengths can be selected. In this study Schiff's reagent had L_1 = 500 nm and L_2 = 565 nm. Cresyl violet had L_1 = 550 and L_2 = 630.

After the two wavelengths were found, heterogeneous dye concentrations were measured. An aperture, which adjusts the photometric field, was selected to circumscribe the nucleus minimizing the amount of backround light in its borders. One hundred individual nuclei, from each group of tissue being studied, were measured by taking readings on and off the nucleus at the two wavelengths set for that particular dye. The amount of chromophore (M) to be determined within the measured area (A) was calculated by

the equation $M = KAL_1Q$. The constant, K, was dropped due to the fact that this study only used relative values rather than absolute values for DNA determination. For each nucleus, the transmissions (T_1 and T_2) were taken at L_1 and L_2, such that $T_1 = I_s/I_o$ and $T_2 = I_s/I_o$. From this information $L_1 = (1-T_1)$ and $L_2 = (1-T_2)$. The ratio L_2/L_1 was used to determine Q, which was used to determine the correction factor, C, where $C = (2-Q)^{-1} \ln (Q-1)^{-1}$. Q and C values can be found in tabulated form by Patau (1952).

Calculations of all relative DNA values and statistical analysis were performed using the IBM 370 model 145 mainframe computer at Youngstown State University. Dr. John J. Yemma provided the program from which the data was produced.

Chapter 3

Results

Spectral Absorption Curves For Both Basic Fuchsin and Cresyl Violet

The two wavelength method of microspectrophotometry, used in this study, requires the establishment of an absorption curve for each stain being investigated. Absorption curves for both basic fuchsin and cresyl violet were performed using liver tissue (Figures 3 and 4). Two wavelengths (L_1 and L_2) were calculated for each chromophore from the curves. The results were as follows:

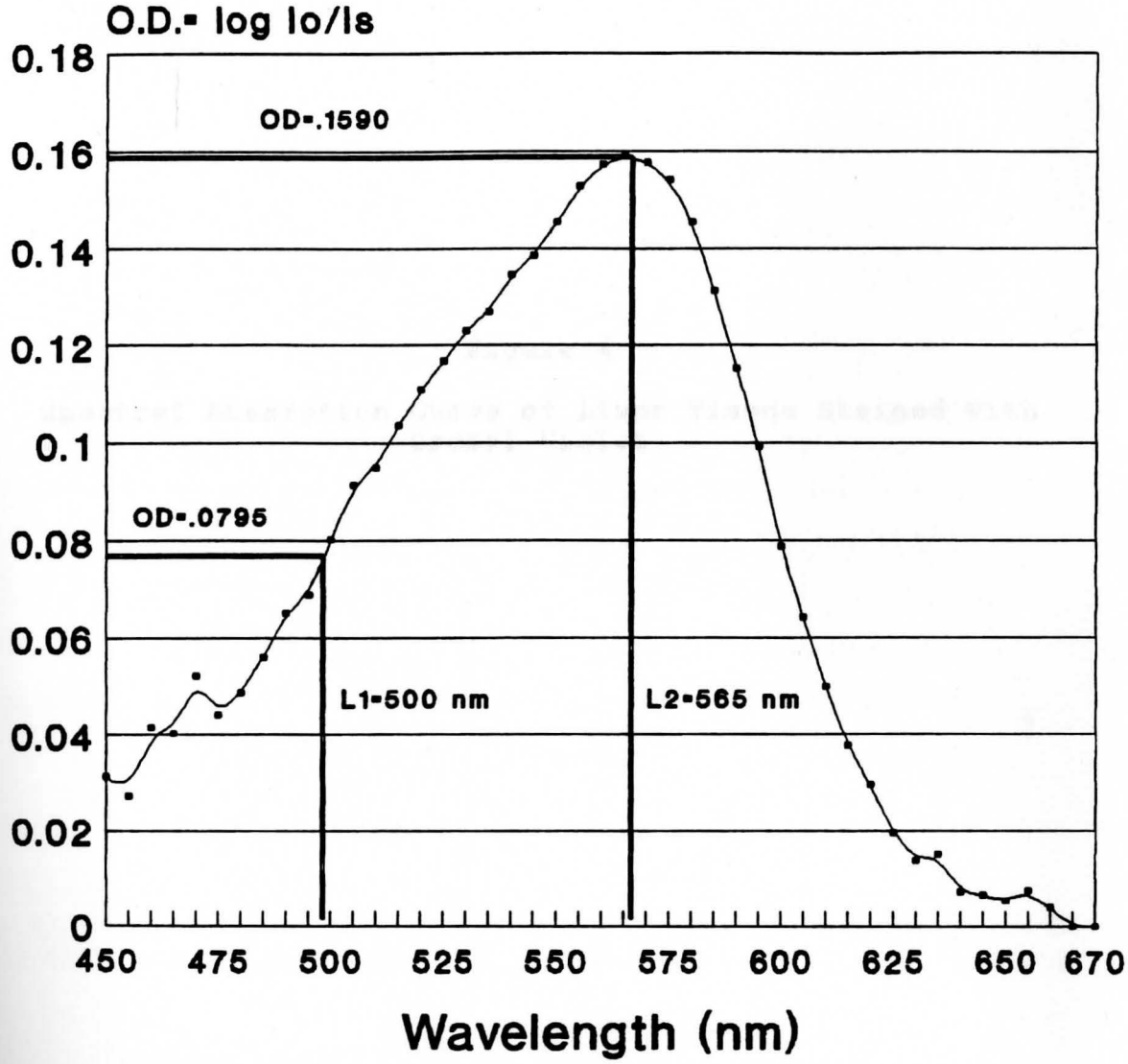
	L_2 (nm)	L_1 (nm)
Stain	Max Absorption	1/2 max absorption
Basic fuchsin	565	500
Cresyl violet	630	550

The results of this study are presented in both graphical form as histograms or in tabular form as the case may be. The histograms represent nuclear DNA frequency distributions of cellular populations. This is a convenient means for monitoring shifts in DNA content within a cellular population. The measurements among the different cell types were limited to interphase nuclei (no mitotic

Figure 3

Spectral Absorption Curve of Liver Tissue Stained With Basic Fuchsin

Absorption Curve
Schiff's Reagent

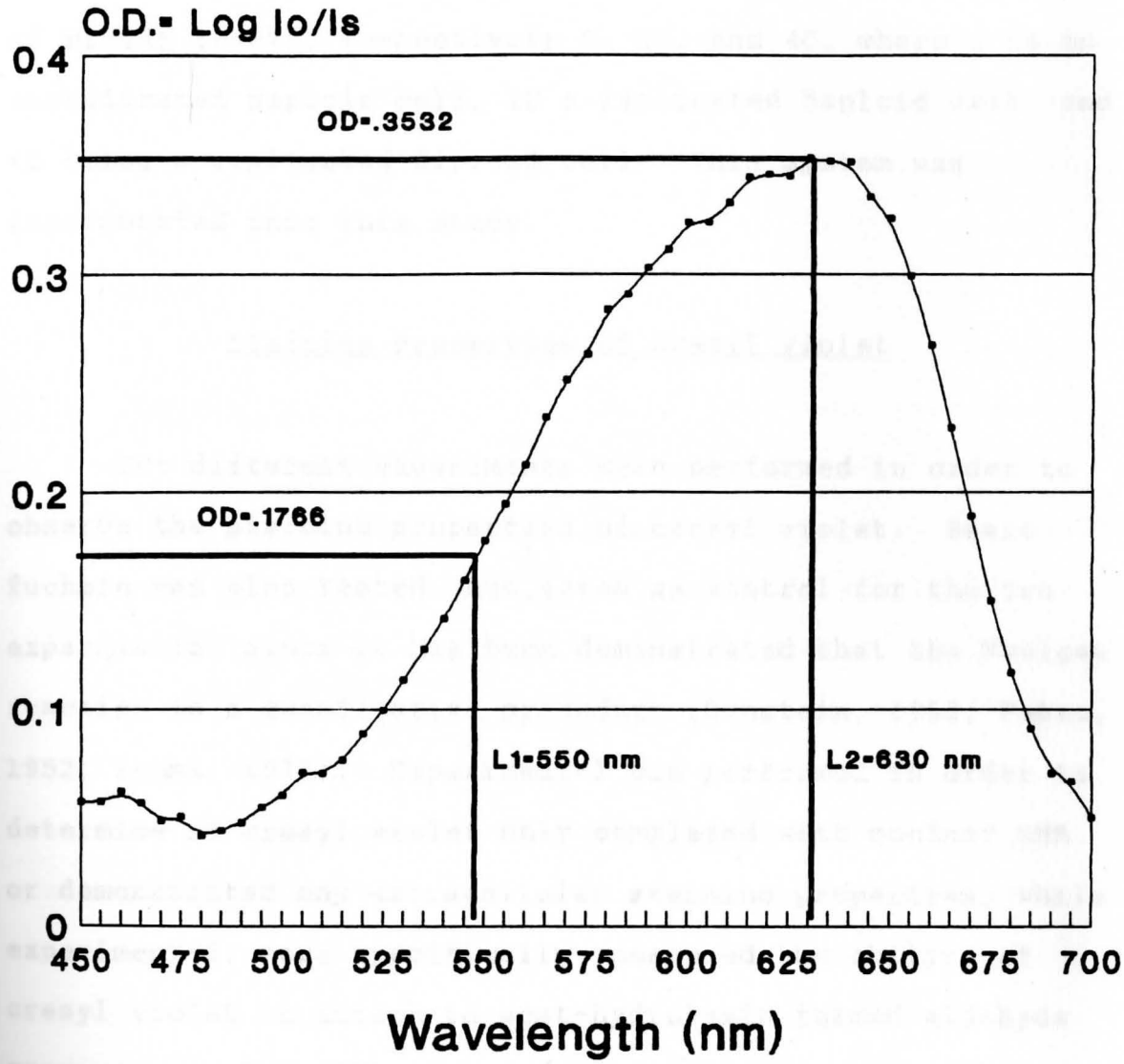


Liver Tissue

Figure 4

Spectral Absorption Curve of Liver Tissue Stained With Cresyl Violet

Absorption Curve
Cresyl Violet



Liver Tissue

figures were observed), and were reported as relative dye concentrations, as is conventional (Yemma, 1972, 1985, 1991).

Swift (1950) designed a system for the interpretation of ploidy levels, respectively C, 2C, and 4C, where C is an unreplicated haploid cell, 2C a replicated haploid cell, and 4C being a replicated diploid cell. This system was incorporated into this study.

Staining Properties of Cresyl Violet

Two different experiments were performed in order to observe the staining properties of cresyl violet. Basic fuchsin was also tested, but acted as control for the two experiments, since it has been demonstrated that the Feulgen reaction is a quantitative procedure (Ornstein, 1952; Patau, 1952; Yemma, 1972). Experiment-1 was performed in order to determine if cresyl violet only complexed with nuclear DNA or demonstrated any extracellular staining properties, while experiment-2, more specifically, measured the ability of cresyl violet to attach to post-hydrolysis formed aldehyde groups on nuclear DNA, in a stoichiometric manner. The results of the two tests were as follows:

Experiment-1

Ten liver slides were treated with 4% trichloracetic

acid (TCA). Five of them were then stained with basic fuchsin, while the other five were stained with cresyl violet. All ten slides treated with TCA did not show any coloration, which was expected due to the fact that TCA removes nuclear DNA. Also, no extracellular staining was evident. This supported the contention that cresyl violet is specific for nuclear DNA as Kasten (1960) demonstrated.

Experiment-2

In this experiment, ten liver slides were treated with an aldehyde blocking agent, 2% thiosemicarbazide (Kasten, 1959), post-hydrolysis. After which, five were stained with basic fuchsin and the remaining five with cresyl violet. All ten treated slides lacked coloration. Since basic fuchsin in the Feulgen reaction, as explained, has been shown to bind aldehyde groups on hydrolyzed DNA stoichiometrically, indicates that cresyl violet also attaches specifically to aldehyde groups on the nuclear DNA that were formed post-hydrolysis as noted by Kasten (1960), although other possible sites of attachment have been suggested (Dutt, 1971b). It is presently agreed that Kasten's hypothesis is correct.

Microspectrophotometric Analysis of Rat Liver and Sperm Nuclei

Analysis of nuclear DNA in liver and sperm tissue of Long evans rats is presented in the form of histograms for both basic fuchsin and cresyl violet stains (Figures 5-8). Histograms are very useful for the presentation of data, for they are sensitive to even minor shifts in nuclear DNA content that may be due to synthetic activity, changes in ploidy level, and template activity, as well as enable one to make reliable quantitative comparisons between dyes used in this study. The relative dye concentrations, being proportional to DNA content, were plotted on the abscissa in arbitrary units, while the corresponding number of nuclei were plotted on the ordinate. One hundred nuclei were randomly measured in order to accurately represent the cell population being measured. Figures 5-8 depict the analysis of both liver and sperm tissue. The mean dye concentration, standard deviation, and standard error were also recorded.

Analysis of Basic Fuchsin Dye

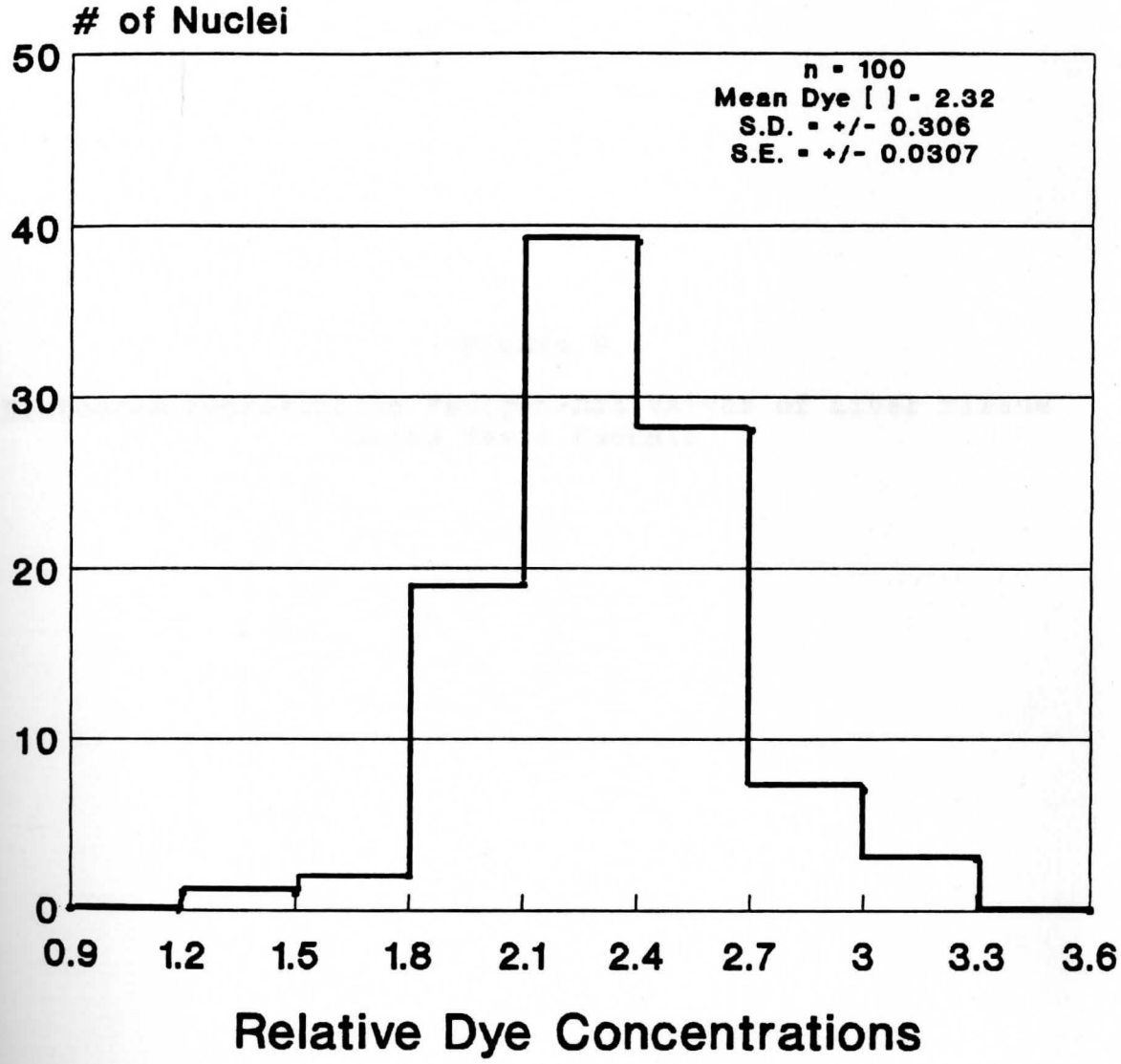
(Liver and Sperm Tissue)

Histograms representing sperm and liver tissue (Figure 5 and 6) demonstrate that the liver tissue has a DNA range (2.7-7.8) in arbitrary units, which is approximately double that of the sperm tissue (0.9-3.6), as expected. Liver tissue also demonstrates variability as expected, due to the diffuse nature of DNA and high metabolic activity, which is greater than that of sperm (Yemma and Perry, 1985). The

Figure 5

Histogram Representing Feulgen-DNA values of Sperm Tissue Using Basic Fuchsin

Histogram
Basic Fuchsin



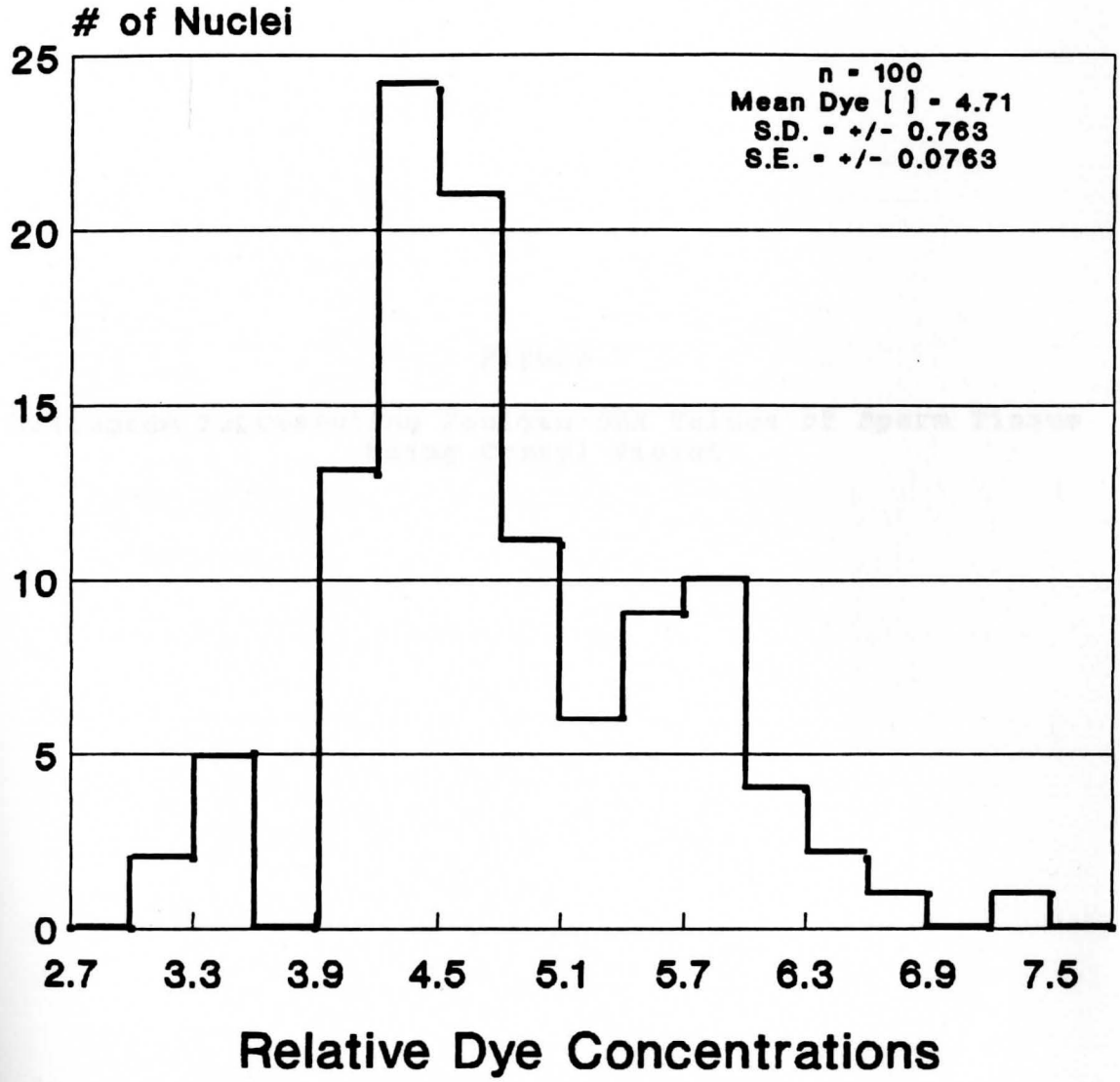
• 0.3 Arb. Units/Div.

Sperm (1N)

Figure 6

Histogram representing Feulgen-DNA Values of Liver Tissue Using Basic Fuchsin

Histogram
Basic Fuchsin



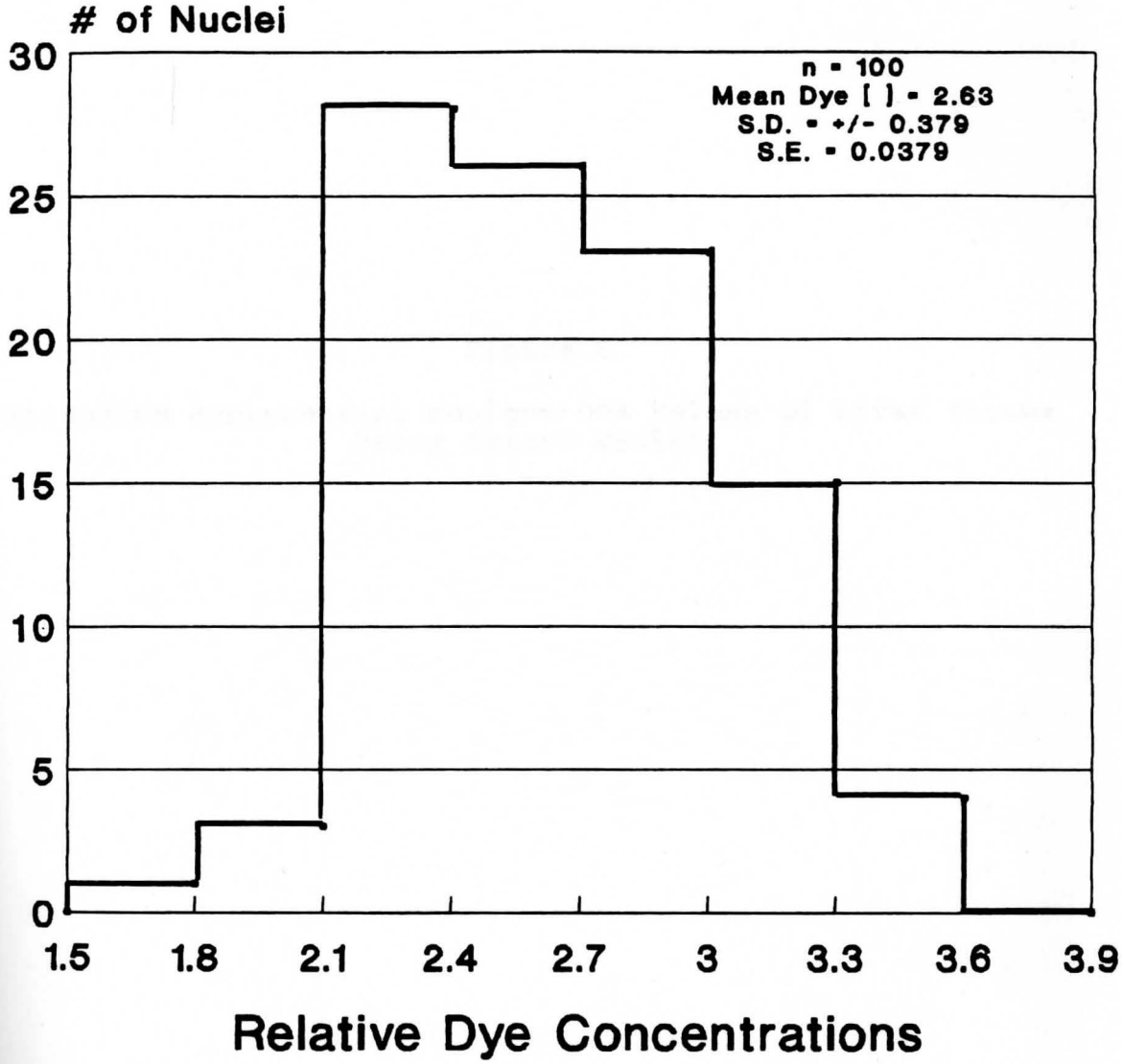
• 0.3 Arb. Units/Div.

Liver (2N)

Figure 7

Histogram Representing Feulgen-DNA Values of Sperm Tissue Using Cresyl Violet

Histogram
Cresyl Violet



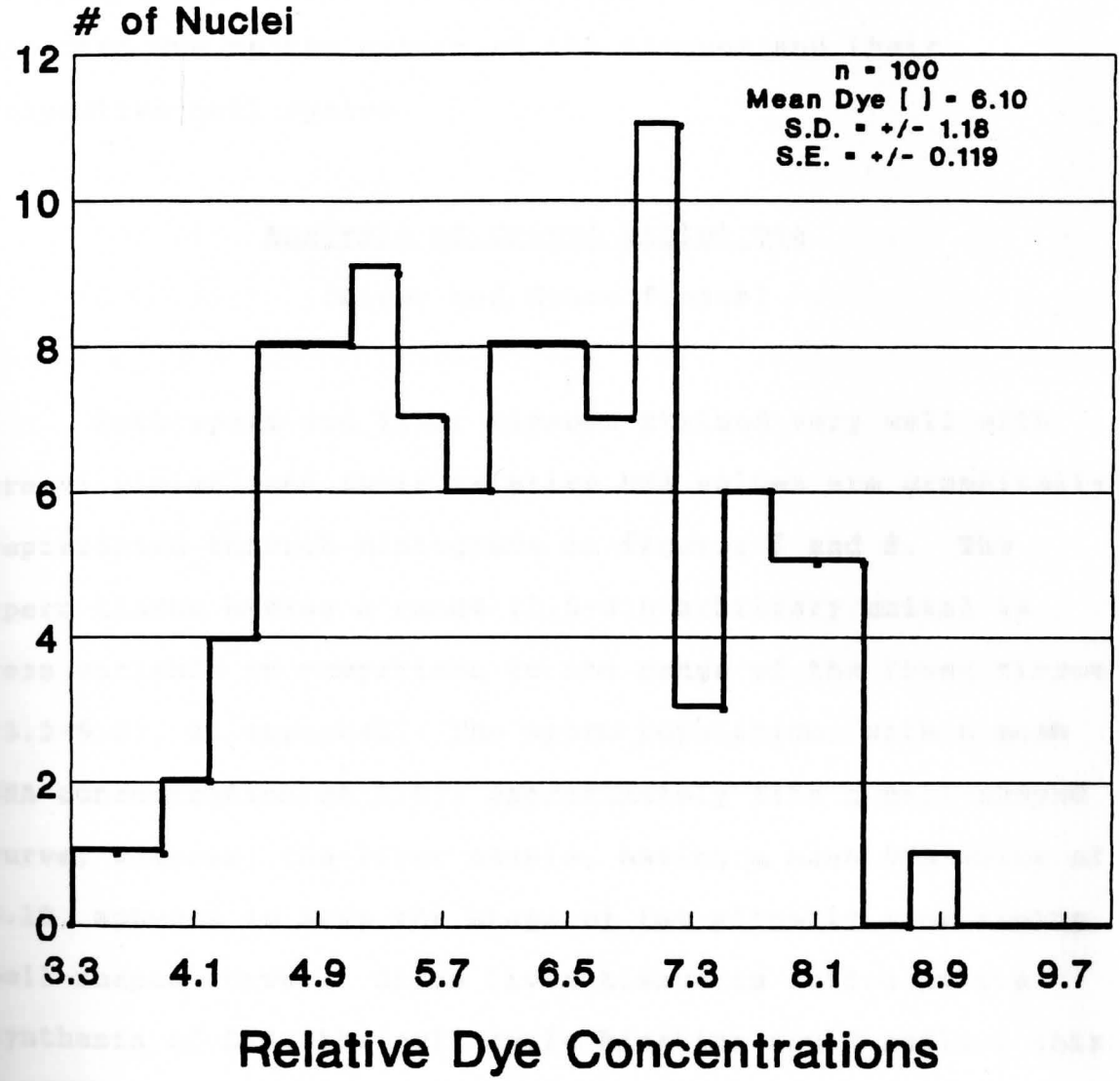
• 0.3 Arb. Units/Div.

Sperm (1N)

Figure 8

Histogram Representing Feulgen-DNA Values of Liver Tissue Using Cresyl Violet

Histogram
Cresyl Violet



• 0.3 Arb. Units/Div.

Liver (2N)

liver tissue having a mean DNA concentration of 4.71 is approximately twice that of the sperm tissue population, which is 2.32. Both means indicate that the liver and sperm tissues are of two different ploidy levels, which is expected due to the nature of the tissues and their respective cell cycles.

Analysis of Cresyl Violet Dye

(Liver and Sperm Tissue)

Both sperm and liver tissues stained very well with cresyl violet, and their relative DNA values are graphically represented through histograms in figures 7 and 8. The sperm tissue having a range (1.5-3.9 arbitrary units) is less variable in comparison to the range of the liver tissue (3.3-9.0), as expected. The sperm population, with a mean DNA concentration of 2.63, approximately fits a bell-shaped curve; whereas, the liver sample, having a mean DNA value of 6.10, appears to have the shape of two slightly overlapping bell-shaped curves. Since liver tissue is active in the synthesis of DNA, the cell cycle kinetics would reflect this variability. In contrast, sperm tissue does not show this activity. The two tissues exhibit differing ploidy levels, with the sperm tissue being haploid, and the liver being mostly diploid with many cells in G_2, and some cells approaching the poly-ploid levels (7.5-9.0 arbitrary units). These results can account for the variability found in the

liver sample, due to the diffuse nature of DNA due to template activity as well as the nature of the tissue.

Comparison Between Basic Fuchsin and Cresyl Violet Dyes

(Liver and Sperm Tissues)

Liver

Both stains effectively demonstrate (Figure 6 and 8) individual cellular DNA variation within a random population of cells as previously explained. This is expected and has been demonstrated many times in previous studies (Yemma, 1985, 1987, 1991) regarding the Feulgen reaction and the use of this procedure for the determination of quantitative DNA measurements. The liver sample stained with cresyl violet appears to be more sensitive when compared with values obtained for basic fuchsin. Also, the mean DNA content of the liver sample stained with cresyl violet (6.10 arbitrary units) is higher than that of the sample stained with basic fuchsin (4.71). It is interesting to note that both stains showed peaks of DNA values at 4.5, but the cresyl violet sample, having more variability and demonstrating a pronounced G_2 phase as well as a large number of cells in S phase, showed another peak at 7.8, almost twice that of the value of the first peak. It is important to note that since these are active tissues, this reflects the cell cycle stage sensitivity that is desirable, which accounts for the notable variability.

Sperm

Both samples (Figures 5 and 7) of sperm populations showed unimodal distributions as expected since cell division does not normally occur in mature sperm cells. The range of activity between the two are nearly equal, their mean DNA concentrations also reflect this similarity, which demonstrates that both populations demonstrate a similar ploidy level. Both show a peak of their population at the DNA value of 2.2.

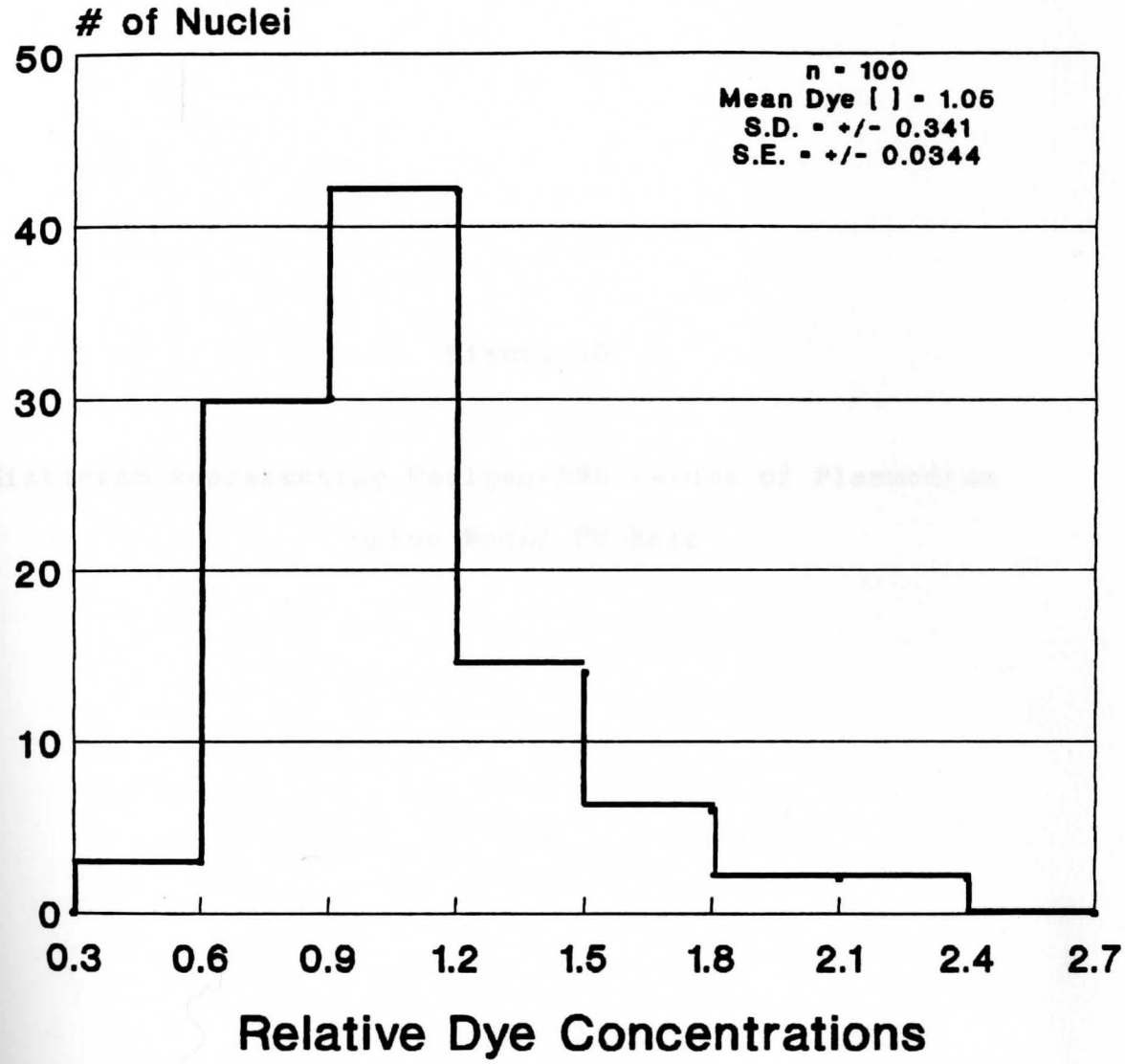
Microspectrophotometric Analysis of Nuclear DNA in Didymium iridis

This study involved the analysis of nuclear DNA of plasmodium and myxamoeba of D. iridis. The intent is to examine the dyes with respect to any effects due to tissue differences of species. The data collected is represented as histograms for each individual dye (Figures 9-12). One hundred nuclei were read as a representative sample of the cellular population and plotted on the ordinate. Their corresponding relative dye concentrations, which are proportional to their relative DNA content, were plotted on the abscissa in arbitrary units. The mean dye concentrations, standard deviation, and standard error were also calculated for all histograms. From this information it is possible to monitor variability due to template activity and ploidy level, as well as compare the results of the stains.

Figure 9

Histogram Representing Feulgen-DNA Values of Myxamoeba Using Basic Fuchsin

Histogram
Basic Fuchsin



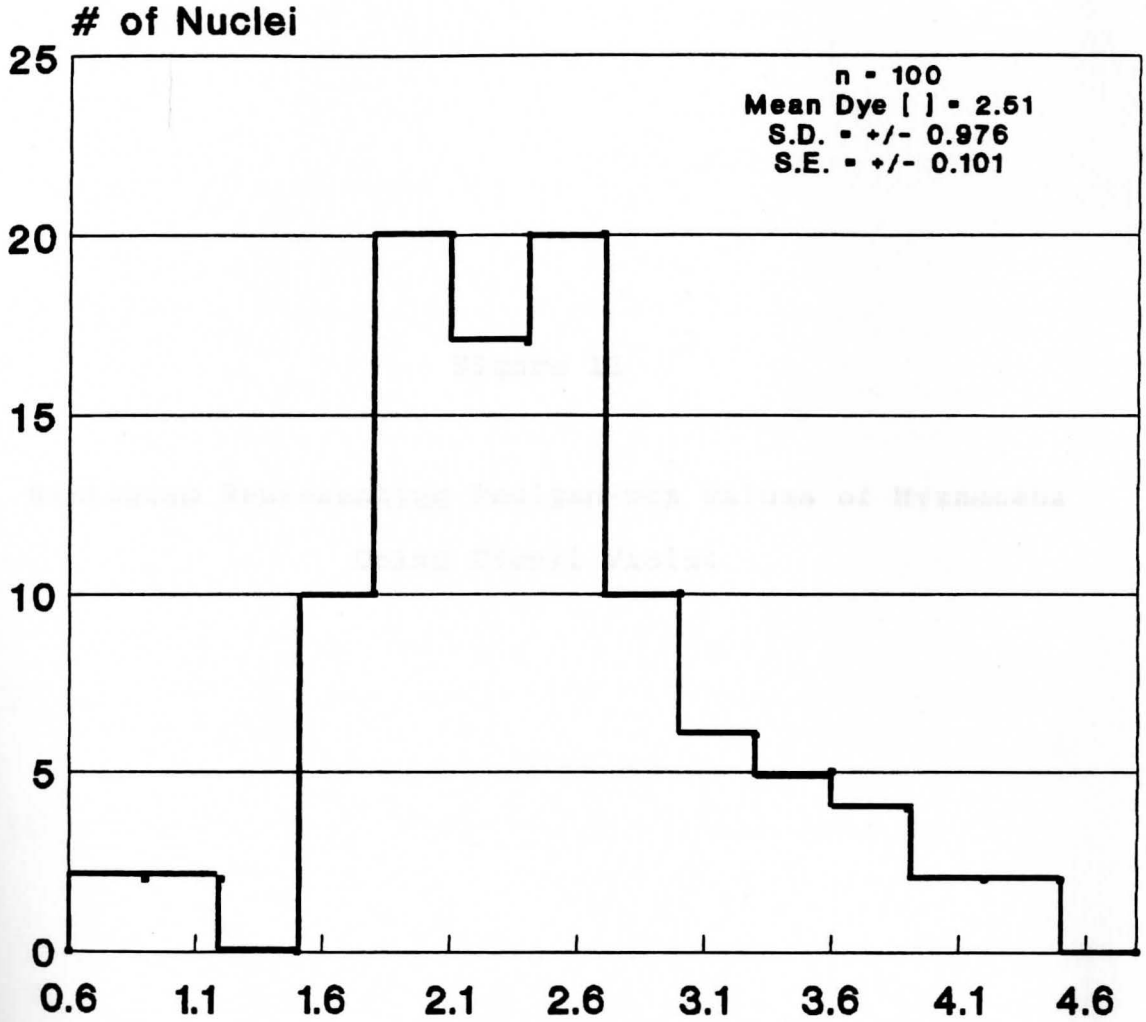
• 0.3 Arb. Units/Div.

Myxamoeba (1N)

Figure 10

Histogram Representing Feulgen-DNA Values of Plasmodium using Basic Fuchsin

Histogram
Basic Fuchsin



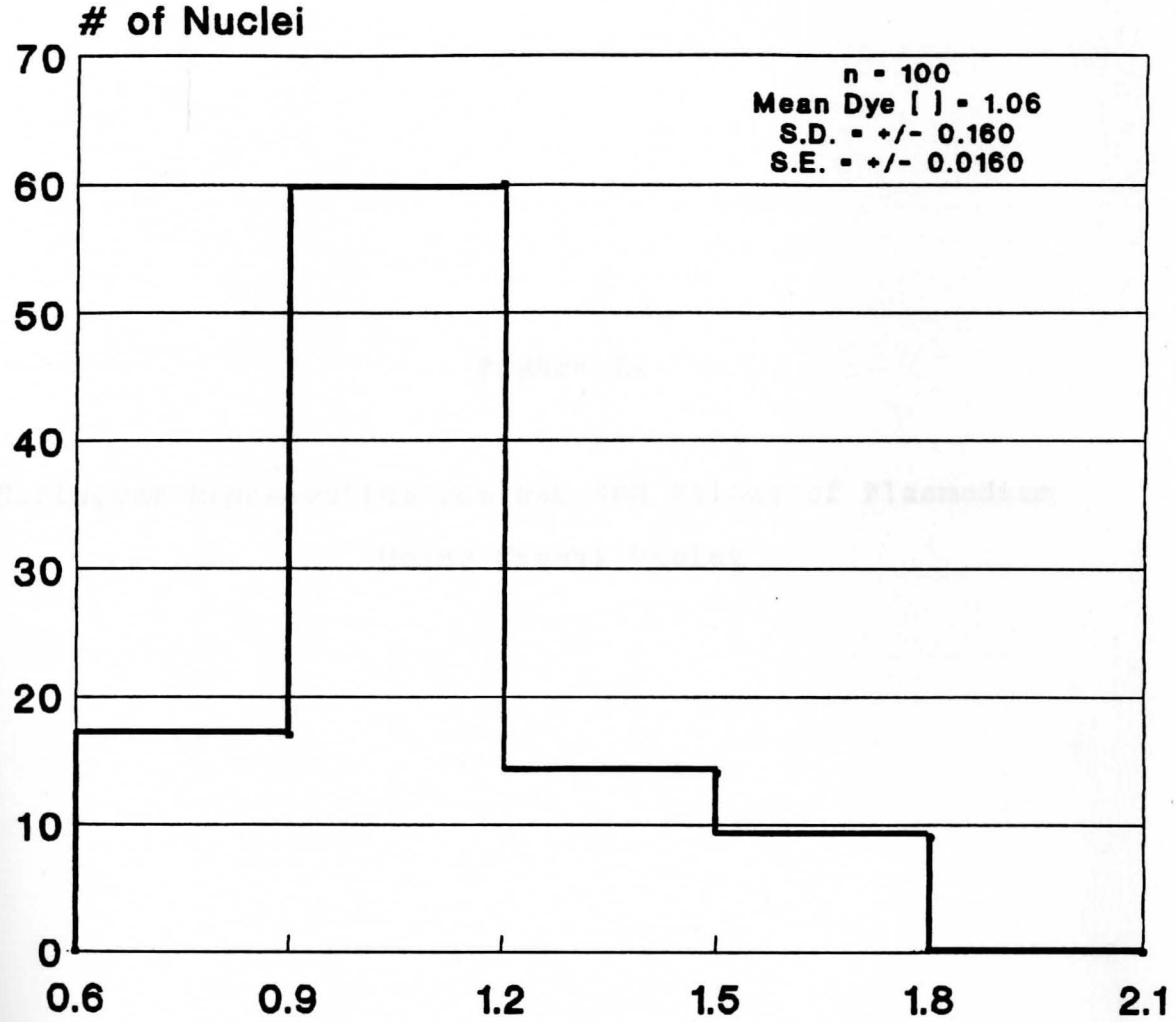
• 0.3 Arb. Units/Div.

Plasmodium (2N)

Figure 11

Histogram Representing Feulgen-DNA Values of Myxamoeba Using Cresyl Violet

Histogram
Cresyl Violet



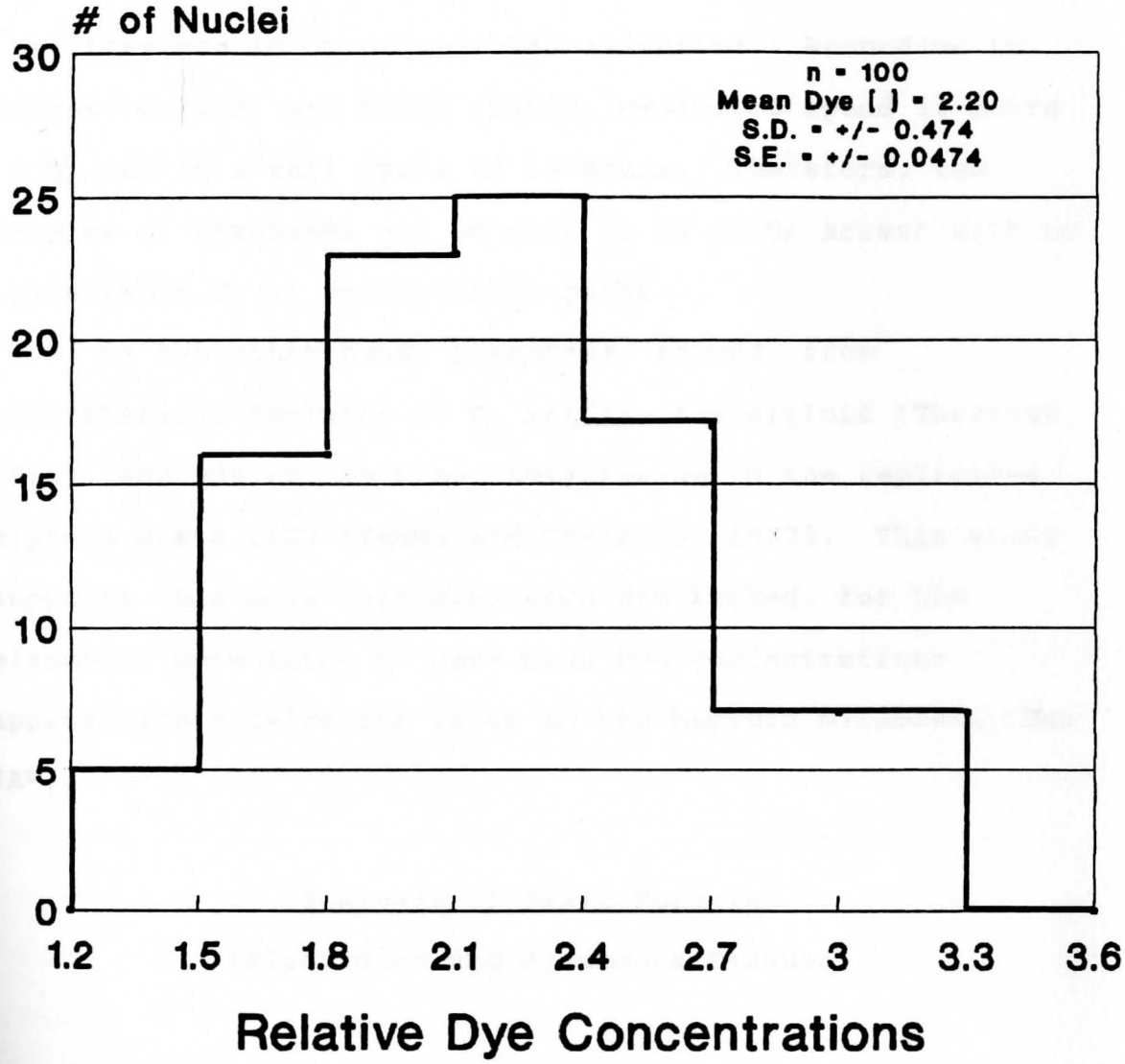
• 0.3 Arb. Units/Div.

Myxamoeba (1N)

Figure 12

Histogram Representing Feulgen-DNA Values of Plasmodium Using Cresyl Violet

Histogram
Cresyl Violet



• 0.3 Arb. Units/Div.

Plasmodium (2N)

The mean DNA concentrations of myxamoeba with both stains indicate the replicated haploid (2C) value for D. iridis. In addition, unimodal distribution of values among the myxamoeba, which is the case in this study, demonstrate that they are in G_2 or post-DNA synthesis. According to Guttes, Guttes, and Rusch (1961), myxamoeba spend 12 hours in G_2 out of a cell cycle of 13 hours. Therefore, the samples of myxamoeba can be said to be in G_2 arrest with no appreciable G_1 or presynthetic phase.

On the other hand, plasmodia, formed from heterothallic isolates of D. iridis, are diploid (Therrien, 1966), and 99% of the time, they reside in the replicated diploid state (4C) (Yemma and Therrien, 1972). This study supports this data regarding each dye tested, for the plasmodia were found to have mean DNA concentrations approximately twice the value of the haploid myxamoeba (Hon $7A^2$).

Analysis of Basic Fuchsin

(Plasmodium and Myxamoeba Tissues)

In the Hon $7A^2$ and plasmodium histograms (Figures 9 and 10), both demonstrate unimodal distributions of the cellular samples and conform to a bell-shaped curve. Also, the ranges of the distributions are nearly equal. Both the Hon $7A^2$ and the plasmodium samples have low variations from the mean DNA content, which is indicative of their cell

cycle behavior. The plasmodium mean DNA concentration (2.51 arbitrary units) is slightly over twice that of the Hon 7A² mean DNA concentration (1.05). This corresponds to the expected difference in ploidy level between the diploid plasmodial tissue and the haploid myxamoeba tissue and supports the numerous studies of Yemma et al. 1972, 1980, 1985, 1987, 1991.

Analysis of Cresyl Violet

(Plasmodium and Myxamoeba Tissues)

Similar to samples stained with basic fuchsin, both Hon 7A² and plasmodium distributions (Figures 11 and 12) stained with cresyl violet are unimodal and represent bell-shaped curves. Their variations are also considerably low, due to their arrest in G_2. The mean DNA concentration of the Plasmodium (2.20) is nearly equal to twice the mean DNA concentration of Hon 7A² sample (1.06). This is consistent with the nature of the cell cycle, but also indicates that the plasmodium and Hon 7A² occupy two different ploidy levels.

Comparison Between Cresyl Violet and Basic Fuchsin Dyes

(Plasmodium and Hon 7A²)

Plasmodium

Comparing both histograms (Figures 10 and 12), it is

apparent that the ranges of both plasmodial samples are nearly equal. Basic fuchsin shows slightly more variation among the distribution, but the mean DNA concentrations of both samples are very close in value. Both dyes show peaks at the DNA value of 2.1, and have fairly close distributions among the rest of the population. It seems evident that both samples are of the same ploidy level from the data which is consistent with the nature of their cell cycle. Statistical analysis of the data further verifies this observation.

Hon 7A[2]

Both myxamoeba samples (Figure 9 and 11) have unimodal distributions which is expected due to their, as previously pointed out, being in G_2 arrest. Also, both samples fit a near perfect bell-shaped curve. Basic fuchsin shows slightly more variation among the sample, which can be attributed to a differential in the S phase as would be expected. However, the mean DNA concentrations of both samples are nearly equal. Furthermore, both models have peaks at the DNA value of 1.1, which is very close to the mean DNA value for both samples. Statistical analysis along with the above data confirm both samples reside at the same ploidy level.

Table 8 is a summary of the data on the histograms, which includes: type of tissue, expected ploidy level, stains used, mean DNA value, and standard deviation.

Table 8

Summary of Histogram Data, Ploidy, and Tissue Types

Table 8

Tissue	Stain	Mean DNA	Standard Deviation
Liver (2N)	Basic Fuchsin	4.71	0.763
Liver (2N)	Cresyl Violet	6.10	1.180
Sperm (1N)	Basic Fuchsin	2.32	0.306
Sperm (1N)	Cresyl Violet	2.63	0.379
Plasmodium (2N)	Basic Fuchsin	2.51	0.976
Plasmodium (2N)	Cresyl Violet	2.20	0.474
Myxamoeba (1N)	Basic Fuchsin	1.05	0.341
Myxamoeba (1N)	Cresyl Violet	1.06	0.160

Statistical Analysis: One-Way Analysis of Variance

One-way analysis of variance is a program provided by Dr. John Yemma, Ph.D. to compare the means of 2 or more samples in order to determine if the samples are significantly similar, or different as the case may be. In this study, it was necessary to compare the mean DNA values of tissues stained with basic fuchsin to those stained with cresyl violet. This would enable one to demonstrate if the data collected in the cresyl violet samples were significantly different in comparison with the basic fuchsin samples; hence, concluding whether cresyl violet is a stoichiometric stain. All tissues-liver, sperm, plasmodia, and myxamoeba-were examined in this way in order to help validate the results. Table 9 summarizes the results of this analysis and includes: tissue type, expected ploidy level, variance of mean, p-value, and significance. Looking at table 9, it is evident that cresyl violet was significantly comparable to basic fuchsin in its stoichiometric ability in all tissue types except the liver tissue, which is accounted for by the variability of the liver cells in different stages of activity in the cell cycle.

Table 9

One-Way Analysis of Variance (ANOVA)

Table 9

Tissue	Variance of Mean	P-value	Sig. Diff.=(*) No Sig. Diff=(#)
Liver (2N)	0.8541237	P=<10 (-6)	*+
Sperm (1N)	0.00601387	0.09185243	#
Plasmodium (2N)	0.08989334	0.08318585	#
Hon 7A² (1N)	0.000079632	0.7374864	#

	df D	F	Mean Variance
Liver (2N)	198	91.111150	0.9375584
Sperm (1N)	198	2.868662	0.2150912
Plasmodium (2N)	198	3.032102	0.2964297
Hon 7A² (1N)	198	.112665	0.0704262

+ due to cell cycle phase differences.

Note: significant level of probability is equal to 0.05.

Chapter 4

Discussion

A study involving quantitative comparison between basic fuchsin and cresyl violet was conducted in order to determine if cresyl violet could provide quantitative measurement of DNA similar to basic fuchsin in its stoichiometric, staining, and attachment properties. In order to accomplish this, quantitative microspectrophotometry was employed in conjunction with the Feulgen reaction. Kurnich (1955) demonstrated that the Feulgen reaction must satisfy the following requirements for cytophotometric analysis of DNA: 1) the reaction must be stoichiometric, 2) the dye must be suitable for photometry, and 3) the chromophore must be specific for localization of the nucleic acid and remain stable in the process. Basic fuchsin, when used properly in the Feulgen reaction, yields reliable localization and quantitative nuclear DNA measurements (Ely and Ross, 1949; Swift, 1950, 1953; Swift and Rasch, 1956; Leuchtenberger, 1958; Kasten, 1960, 1964; Yemma and Stroh, 1991). It is used as an control throughout all experiments in this study.

An important requirement of the Feulgen reaction is to obtain maximum dye development, for the amount of dye bound is proportional to the relative amount of nuclear DNA present. Therefore, it is imperative that all factors which

affect the stain intensity be controlled for both basic fuchsin and cresyl violet. Both stains were prepared from fresh stock solutions and subjected to identical conditions. Some of the problematic factors which affect maximum stain intensity are: 1) choice and length of fixation (Kurnich, 1955; Swift, 1966; Deitch, 1967; Kelley, 1984), 2) length of time, temperature, and concentration of acid hydrolysis (Ely and Ross, 1949; DiStefano, 1948; Patau, 1952; Jordanov, 1963; DeCosse and Aiello, 1966), 3) sulphur dioxide source and concentration (DeTomasi, 1936; Kasten, 1959; Bedi and Horobin, 1980), 4) dye impurities, and 5) length of time in the potassium metabisulphite solutions (Kasten, 1960, 1964). Thus, all factors which effect the intensity of the Feulgen reaction were adjusted, such that to effectively validate any data collected and minimize possible error. By exercising stringent controls over all experiments and subjecting both stains to identical conditions, it was possible to compare the data with good validity.

The two-wavelength method of microspectrophotometry, used in this study, requires an absorption curve for each chromophore. The absorption curves generated in this study are presented in Figures 3 and 4. Basic fuchsin steadily rises to a maximum absorbance at 565 nm while cresyl violet exceeds to a maximum absorbance of 630 nm. From these curves, one can see there is a dramatic difference between their spectrum. Since both absorption curves used liver tissue treated in the same manner and displayed replicatable

results, it has been demonstrated, as suspected, that the two chromophores, differing in chemical structure, exhibit resonance at different wavelengths.

The specificity of cresyl violet for nuclear DNA was demonstrated by Kasten (1959), who noted that it had metachromatic properties under certain conditions, for it stained cartilage a violet color in certain tissues. The results of experiment-1 in this study support the work of Kasten. However, metachromatic staining was not exhibited regarding the staining of DNA, due to specificity of binding. When experimental slides were stained with cresyl violet extreme specificity was observed regarding coloration, only nuclei were stained. It is obvious from this experiment that cresyl violet does not show extracellular staining properties when employed in connection with the Feulgen reaction and is specific for nuclear DNA. Though cresyl violet demonstrated DNA specificity, the specific attachment mechanism(s) to the DNA molecule remained unanswered according to available published literature (Kasten, 1959; Floyd, 1990).

An examination of this problem was accomplished in experiment-2. Kasten (1958, 1959, 1960) postulated that a basic stain having at least one free amine, in the presence of sulphur dioxide, could react with hydrolytic formed aldehyde groups on the DNA molecule. To confirm this, experiment-2 made use of a powerful aldehyde blocking agent, thiosemicarbizide. The results of this experiment verified

that cresyl violet is specific for attachment to aldehyde groups, for untreated liver slides demonstrated nuclear coloration, while treated liver slides were absent of any stain. Likewise, basic fuchsin, which has been documented to complex with aldehydes on the hydrolyzed DNA (Kasten, 1959), was also inhibited by the aldehyde blocking agent. It is reasonable to conclude that cresyl violet does complex with aldehydes in the Feulgen reaction. It is possible that other sites of stain attachment exist. For example, Dutt (1971b) stated that cresyl violet may complex with phosphate groups of depolymerized DNA. However, depolymerization of DNA did not take place in the present study. Seperate experiments would have to be carried out in order to verify these findings. Such work was not pertinent to this study, since as previously stated, depolymerized form of DNA was not present in whole cells, as verified by Feulgen staining which does not stain depolymerized DNA (Yemma, 1972, 1985, 1991).

The staining properties and dye characteristics of cresyl violet were an important consideration in the present study. The determination of its stoichiometric ability in comparison to basic fuchsin was however a major consideration in this study. The quantitative properties of basic fuchsin have been shown on many occasions to be very reliable (Ely and Ross, 1949; Kasten, 1960, 1964; Yemma, 1985, 1991), and it would be used as a control for this study. All sorts of tissues were previously studied. For

example, liver, sperm, plasmodium, and myxamoeba tissues were used to measure nuclear DNA quantitativly. With differences in ploidy level and differing periods of cellular development, these tissues are ideal for correlating quantitative data. The mean DNA values of the tissues stained with cresyl violet were analyzed by statistical analysis and related to the corresponding values of the tissues stained with the control, basic fuchsin. In this way, it was possible to draw important conclusions.

The liver tissue, being primarily diploid in nature, was expected to have a mean DNA value approximately twice as high as the haploid sperm tissue. The basic fuchsin controls (Fig. 5 and 6) demonstrated this well, whereas cresyl violet (Fig. 7 and 8) showed a margin of difference relatively greater than what was expected. This margin can be explained by the nature of liver tissue, and stages exhibited throughout the cell cycle, where template activity and DNA replication may vary. More specifically, the liver sample in Figure 8 has two peaks. One resides at a DNA value equal to 4.7, which coincides with the control sample (Fig. 6), and another peak at 7.8. This second maximum peak represents cells that are in the synthetic and G_2 phase of the cell cycle respectively. The presence of polyploid cells serve to distort the mean DNA concentration as expected, and serves to explain variance recorded for the sample, thus accounting for slight differences in the results. This is further confirmed by comparing both sperm

tissue samples (Fig. 5 and 7). From these histograms it is evident that both samples are nearly identical. Upon analyzing their means, no significant difference is found (Table 9), unlike the liver tissues. Here metabolic differences reflected in template activity, as well as DNA synthetic activity, is minimal. Regarding the latter condition, this does not occur in mature sperm. Sperm tissue is normally in G_1 arrest, the variability is very low, and the representing data is easily recognized upon comparison as their being in the same ploidy level.

To further confirm the quantitative ability of cresyl violet, myxomycete tissues were tested. Given the hypothesis that cresyl violet is stoichiometric, this experiment would strengthen the support for its reliable use in different species. As expected, basic fuchsin verified its stoichiometric abilities (Fig. 9 and 10). The diploid plasmodium sample was double the mean of the haploid myxamoeba sample. Both samples show very low variability, which is very characteristic of their species, which are largely G_2 cell as previously explained. Likewise, the s samples stained with cresyl violet (Fig. 11 and 12) duplicates these results. The comparison between both plasmodial populations (Fig. 10 and 12) show no stat' significant difference between the means (Table 9), peaks at relatively the same values. The same ho' for the myxamoeba models (Fig. 9 and 11). With results in mind, it is logical to conclude tha'

violet is comparable in its stoichiometric staining properties to basic fuchsin. Also, it has been shown to be sensitive in monitoring changes in the stages of the cell cycle and template activity, as noted in the liver samples.

In conclusion, cresyl violet, which is a basic dye in the oxazine dye class, has been shown to specifically bind to nuclear DNA at hydrolytic formed aldehyde groups. In addition, microspectrophotometric data and statistical analysis has confirmed in this study that cresyl violet does possess stoichiometric capabilities. This dye offers some advantages over basic fuchsin such as: 1) increased shelf-life (Floyd, 1990) 2) ease of compounding 3) time efficiency, and 4) cost effectiveness. It is also important to note that basic fuchsin is a known carcinogen, whereas cresyl violet has not been shown to have this property. With these advantages in mind, it is advantageous to use cresyl violet in place of basic fuchsin in the Feulgen reaction.

Although this study has shed some light on the characteristics and capabilities of cresyl violet, more research is warranted, such as the effects of different hydrolytic times and fixatives on the staining properties of cresyl violet. Also, any other mechanism(s) of attachment to the DNA molecule need to be studied in more detail. Therefore, a measure of the extent of the shelf-life and stability of the dye is currently taking place in our lab. Overall, the properties of cresyl violet have been shown to

be worthy of additional scientific use in nuclear DNA staining.

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