THE EFFECTS OF MANUFACTURING AND CONCENTRATION OF BASIC FUCHSIN ON THE QUANTITATIVE ASPECTS OF THE FEULGEN REACTION

by

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ABSTRACT

THE EFFECTS OF MANUFACTURING AND CONCENTRATION OF BASIC FUCHSIN ON THE QUANTITATIVE ASPECTS OF THE FUELGEN REACTION

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Master of Science

Youngstown State University, 1985

Four separate preparations of basic fuchsin were compared in order to study the effects if any that manufacturing and concentration may have on the quantitative aspects of the Feulgen reaction. The two wavelength method of microspectrophotometry was employed to analyze maximal DNA staining of chicken erythrocytes, myxamoeba, and plasmodium.

It was determined that all preparations of the stains were able to yield valid quantitative data in relative values regardless of concentration, whereas maximum staining was affected by both differences in chemical makeup of the individual stains and differences in the manufacturing processes.

To obtain accurate quantitative absolute values of DNA, the stain of choice should have the color index number of 42510 and should be manufactured by Fisher Scientific.

ACKNOWLEDGEMENTS

The author would like to sincerely thank Dr. John J. Yemma for without his dedication, guidance, and encouragement this study would not have been possible. A special thanks also goes to Joseph Duda and Len Perry for their assistance.

Finally, this thesis is dedicated to the author's parents, John and Freida Penza; sister, Marla Penza; and his girlfriend, Elizabeth Schiffer, for their continual support and encouragement.

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SYMBOL	DEFINITION	UNITS OF REFERENCE
A	Area	μ
C	Correction factor for unoccupied space: $C = (2-Q)^{-} \ln(Q-1)^{-1}$	
E	Extinction	
I_{os}	Flux of photons on chromophore	
I_{o0}	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	
λ	Wavelength	nm
α	Confidence interval	

CHAPTER I

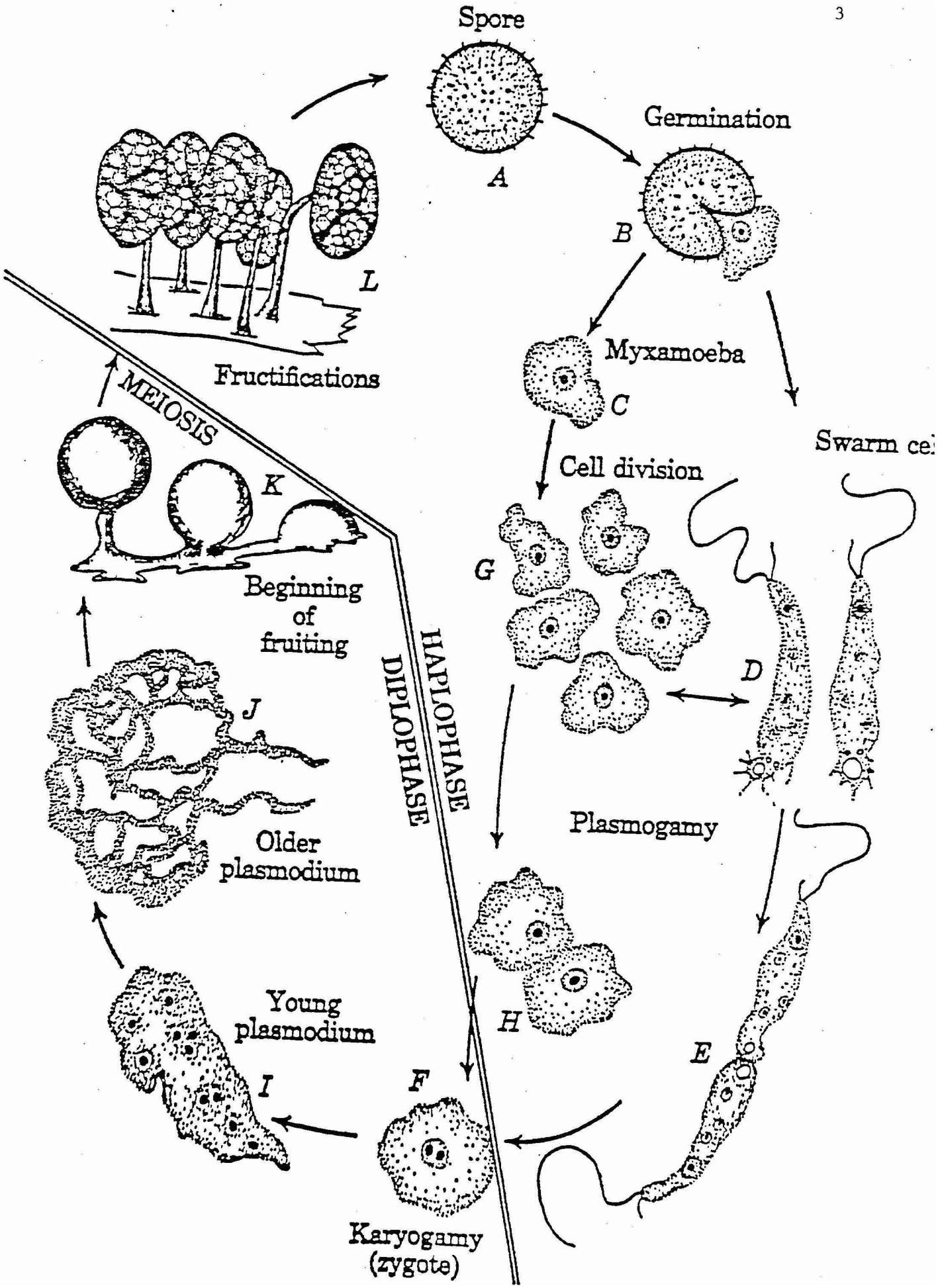
Introduction

The myxomycete represents a group of ideal "model organisms" for research in cell biology. Its relatively short life cycle has both plant and animal characteristics. In addition, it presents several interesting and distinct morphological forms and is easily cultured and maintained upon artificial media; therefore, it allows one to perform various studies on its growth and differentiation throughout its cell cycle. The life cycle of Didymium iridis, used throughout this study, involves an alternation of unicellular haploid and multinucleate diploid stages and has been well defined and discussed in the literature by Guttes, Guttes and Rusch (1961), Alexopoulos and Koevenig (1962), and Gray and Alexopoulos (1968) (see Figure 1).

Germination of a spore into a single protoplast signifies the emergence of the myxamoebal stage (Collins, 1961). This myxamoebal form is readily interconverted to a biflagellated form known as a swarmer. This change is solely dependent upon the presence or absence of an aqueous environment. The myxamoeba feed by ingestion through

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

pseudopodia and utilize as a food source various bacterial strains such as Escherichia coli and Aerobactor aerogenes. When the food source becomes depleted the cells are capable of encystment until environmental conditions once again become favorable. Plasmodia are formed in heterothallic strains, including D. iridis, by crossing sexually compatible myxamoebal clones. Crossing competancy requires that cells be in the log phase of growth and that at least 10^5 cells of each mating type be present in culture (Yemma, 1985 in press Cytobios). Following cell fusion (syngamy) between pairs of myxamoeba, karyogamy occurs and the zygotes develop and grow into plasmodia (Yemma, 1985 in press Cytobios). After plasmodia1 formation, the organism will continue to grow with synchronous nuclear divisions (Rausch, 1969) until it encounters unfavorable environmental conditions. When this occurs the plasmodium develops into either a hard-walled resistant form known as a sclerotium, or it may encyst. It will remain in this dormant stage until more favorable conditions are once again obtained. This phenomenon is also observed throughout the true fungi taxonomy (Moore-Landecker, 1982). The cycle is completed when the plasmodia give rise to spores (location where meiosis occurs). Sporulation is essential to the maintenance of the myxomycete species in

that it allows for genetic reassortment and gamete formation. Spores can remain viable for months or even years. The haploid spores, when dispersed to favorable growth environments by wind or water, can germinate to produce more myxamoeba; therefore, the life cycle continues (Alexopoulos 1962, 1963 and Gray and Alexopoulos 1968).

Myxamoeba differentiate into plasmodia by one of two modes: sexual or clonal. Sexual reproduction in the myxomycete life cycle is subdivided into two categories: heterothallism (those possessing a compatibility system) and homothallism (those lacking a compatibility system) (Therrien et al., 1977). Clonal reproduction, on the other hand, is non-sexual. The mechanism of clonal plasmodium formation has been shown to be either homothallic or apomictic (Gray and Alexopoulos, 1968). In this study, an apomictic plasmodium was utilized and presented several distinct experimental advantages. Therefore, hetero-thallism and homothallism will only briefly be explained.

Heterothallic mating is genetically controlled by a single locus on a chromosome and contains multiple allelic possibilities (Collins, 1963; Collins and Ling, 1968). This process involves syngamy and karyogamy (nuclear fusion) between two sexually compatible mating types of myxamoeba. The resulting diploid zygote undergoes

mitotic nuclear divisions without corresponding cytokinesis. This continued growth, accompanied by nuclear divisions, results in the formation of a multinucleated plasmodium (Therrien and Collins, 1976).

Homothallism is observed when monosporous cultures of myxamoeba undergo syngamy and karyogamy prior to the formation of plasmodia; consequently, myxamoeba which arise from such cultures need not bear different mating alleles. For example, any two cells from a single clone may fuse to form a zygote which will then form a plasmodium (Therrien and Yemma, 1974). A later paper by Yemma, Therrien and Jakupcin (1980) refers this to be a condition by which some myxamoeba replicate their DNA but do not undergo subsequent divisions.

Apogamic development of plasmodia can result in an anomalous cell ploidy. Apomictic development of plasmodia in D. iridis has been well documented in the literature (Therrien and Collins, 1976; Anderson, Cooke, and Dee, 1976; and Mulleavy and Collins, 1979). Periodically, mitotic karyogamy in myxamoeba results in an uneven division of chromosomes through non-disjunction; therefore, producing aneuploid cells. For example, some cells may be N - X and others may be N + X. If non-disjunction involves the mating locus, plasmodia will develop without benefit of

cross. The resulting clonally-formed plasmodia are thus haploid (Mohberg and Rusch, 1971; Mohberg et al., 1973; Cooke and Dee, 1974). In sexual plasmodium formation, haploid myxamoeba yield diploid plasmodia. Therefore, a distinction between sexual and clonally formed plasmodium can be made. In a homothallic isolate, the myxamoebal nuclei contain one-half the amount of DNA as do the diploid plasmodia1 nuclei. In contrast, an apogamic isolate would not display an alternation of haploid and diploid generations; thus, the DNA in apogamic plasmodia have the same DNA content as the myxamoeba (Therrien et al., 1977).

DNA content is measured by means of the Feulgen reaction. This reaction is a quantitative cytochemical procedure. The procedure requires the utilization of techniques which permit the localization and quantitative measurement of macromolecules in an individual cell in a cytological preparation (DiStefano, 1948). For the cytochemical determination of a substance certain conditions must be fulfilled: (A) The substance must be immobilized at its original location. (B) The substance must be identified by a procedure that is specific for it, or for the chemical group for which it belongs. This identification can be made by: (1) chemical reactions

similar to those used in analytical chemistry, but adapted to tissue, (2) reactions that are specific for certain groups of substances, and (3) physical methods (DeRobertis and DeRobertis, 1980).

The vast and frequent application of the Feulgen reaction for microscopic localization and quantitative cytochemical determination of deoxyribonucleic acid (DNA) by cytophotometry attests to the general acceptance and importance of this colorimetric reaction in biological research (Kasten, 1960). The cytological method was developed by Feulgen and Rossenbeck (1924), as an application of the former's discovery that the product of mild acid hydrolysis of thymus nucleic acid gives a positive Schiff's test for aldehydes (general property of aldehydes to regenerate the color of fuchsin-sulfurous acid) (DiStefano, 1948). The chemical basis for the breaking of only the purine-sugar bond as the result of mild acid hydrolysis comes from the old discovery of Osborne and Heyl (1908). The treatment of nucleic acids with a two percent solution of H_2SO_4 removed specifically and quantitatively the purine bases, while the pyrimidine-sugar linkage was broken only after very drastic acid hydrolysis with 20 percent sulfuric acid at 150 degrees Centigrade. Despite the overwhelming evidence

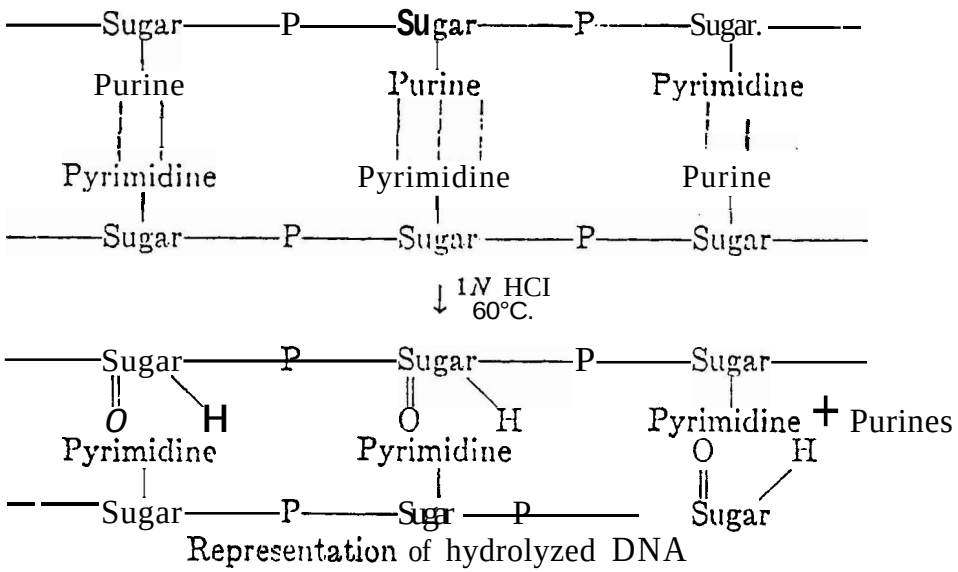
supporting the cytochemical specificity and quantitative application of the reaction (summarized in Swift, 1955 and Leuchtenberger, 1958), the reaction mechanism is complex and incompletely understood (Kasten, 1960).

According to Andersson and Kjellstrand (1972), nucleic acids possess two types of easily hydrolyzed chemical bonds: the glycosidic linkage between the purines and the sugars and the phosphodiester linkage between the sugars. Schramm et al. (1961) demonstrated that the former bond type broke about 120 times faster than the latter in DNA. Others (Andersson and Kjellstrand, 1972) have shown that the breakage of the glycosidic linkage between the purine and deoxyribose is a macromolecular reaction.

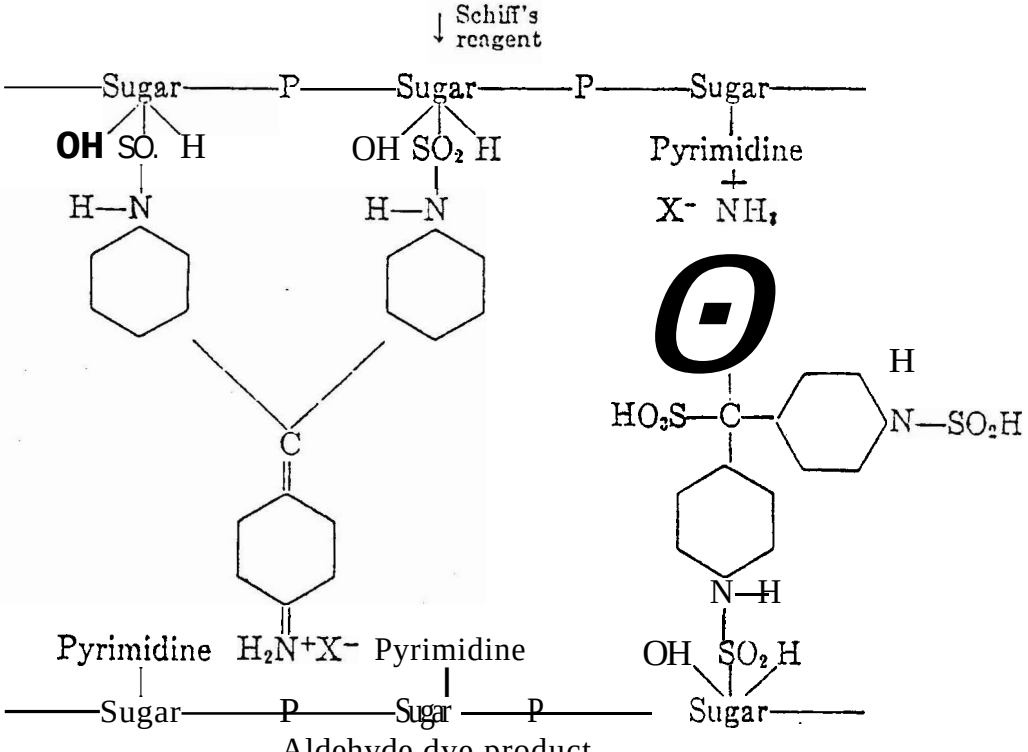
The accepted view of the general mechanism of the Feulgen reaction (DiStefano, 1948; Lessler, 1953; Kasten, 1960) is that the reaction occurs in two separate and distinct steps (Figure 2). First, acid hydrolysis removes the purines at the level of the purine-deoxyribose glucosidic bond of DNA (depurination); thus, unmasking the aldehyde groups of deoxyribose, wherein lies the specificity of the reaction. The second step involves a chemical reaction between the exposed aldehyde group and the Schiff's reagent (leukofuchsin), which results in the synthesis of a new dye compound.

Figure 2.

Mechanism of Feulgen Reaction, by F. H. Kasten, 1960, The Chemistry of Schiff's Reagent, Intern. Rev. Cytol., Vol. 10, p.69.



Representation of hydrolyzed DNA



Aldehyde dye product

Mechanism of Feulgen reaction

When the Feulgen nucleal reaction is used as a quantitative cytochemical determination of DNA, as is the case in the present study, it must be shown (1) that the reaction is specific for localizing DNA within the cell, (2) that the intensity of colored product produced is proportional to the amount of DNA present, and (3) that the microspectrophotometric determinations are accurate.

In the past, many papers have been published which challenged the specificity of the Feulgen reaction for DNA (Carr, 1945; Stedman and Stedman, 1943, 1947). However, the vast majority of investigators have proven the stain's specificity for DNA to exist (Stowell, 1945; Ely, 1949; Sibatani, 1953; Lessler, 1953). At present, most cytochemists agree that under proper conditions with appropriate controls, the reliability of the Feulgen reaction for localizing DNA within the cell can be universally accepted as a valid procedure.

Skepticism concerning the quantitative phase of the Feulgen reaction has arisen (Ely, 1949; Stedman and Stedman, 1947) despite the wealth of evidence supporting the usefulness of the reaction in this regard (Swift, 1955; Leuchtenberger, 1958). Ris and Mirsky (1949) demonstrated that in nuclei, the intensity of the nucleal reaction was proportional to the DNA content. This was accomplished by

comparing the absorption of nuclei at 546 nm with the DNA per nucleus as determined by independent chemical determination on a known number of nuclei. Another study which further substantiates the stain's quantitative capability was carried out by Swift (1950), who measured the nuclear DNA content in mice and other animal nuclei. He showed that the murine spermatid nuclei possessed half the DNA content of murine somatic nuclei. As a result of these studies and others (Pollister et al., 1949; Moore, 1951; Lessler, 1953), it is generally agreed that under properly controlled conditions the intensity of the Feulgen colored product is proportional to the amount of DNA present in the nucleus.

DiStefano (1948) and Ris and Mirsky (1949) have provided convincing evidence concerning the accuracy of microspectrophotometric analysis by measuring the relative DNA content in several different species of animals using both biochemical and spectrophotometric methods. The two methods yielded values which fell within ten percent of one another. Working independently, Ornstein (1952) and Patau (1952) established the validity of the two-wavelength method of microspectrophotometry which was used exclusively in this study.

A number of conditions are known to influence the intensity of the Feulgen reaction or final dye product(s). Jordanov (1963) and others (Ithawa and Ogura, 1953; DeCosse and Aiello, 1966) have substituted the conventional hydrolysis in 1N hydrochloric acid at 60 degrees Centigrade with hydrolysis in 5N hydrochloric acid at room temperature. This prolongs the period of maximal Feulgen intensity. Obtaining maximum color development for the reaction product is necessary since the dye bound is thought to be proportional to the DNA content of a given type of cell (Deitch et al., 1968). Microspectrophotometric analysis of nuclear DNA is performed at the point of acid hydrolysis yielding maximal color intensity of the Feulgen dye product (Deitch et al., 1968). The type of fixative used and its effect on tissues is another procedural detail affecting the intensity of the Feulgen reaction (Swift, 1955; Dietch et al., 1968; Kelley, 1984). Studies have been carried out which have demonstrated that fixatives do interact with polynucleotides and nucleic acids (Haselkorn and Doty, 1961). Pearse (1968) has noted that changes occur in the physical state of nucleic acids using different methods of fixation. In view of this, the best fixatives for Feulgen reaction procedures would be ones that render high

chromatin stability toward acid hydrolysis, thus allowing maximal staining intensity over extended periods of hydrolysis. In Feulgen microspectrophotometry, formalin fixation of tissue has been recommended by several authors to accomplish these objectives (Kelley, 1984; Kurnich, 1955; Ruch, 1966; Swift, 1966). Formalin fixation involves the formation of a network between fibrous proteins of the cell nuclei and amino groups of the DNA bases. This stabilizes the molecule to a much greater degree than most fixatives. Experimentation by Greenwood and Berlyn (1968) also showed the ability of formalin to stabilize chromatin. In addition to the choice of fixative, other preparation procedures are known to influence the intensity of the Feulgen reaction. The pH and sulfite content of the Schiff's reagent, the presence of dye impurities, the length of time in the bleach, dehydration steps, and different sequences of washing stained cells are some factors known to affect its intensity (Swift, 1955; Kasten 1960, 1964). To minimize such errors all slides containing cells which were to be quantitatively studied and compared were simultaneously subjected to the staining procedure.

The objective of this investigation was to make a quantitative comparison between four different and separate dye preparations of Schiff's reagent at optimal staining

intensity in order to determine if there were significant differences in their dye binding properties. To accomplish this, absorption microspectrophotometry was employed to measure and compare quantitatively the nuclear deoxyribonucleic acid content of myxamoeba, plasmodium, and chicken erythrocyte smears. All were stained using the Feulgen method.

CHAPTER II

Materials and Methods

Tissue Preparation

All isolates of *Didymium iridis* used in this investigation were supplied by Dr. John J. Yemma, Department of Biology, Youngstown State University. The isolates used were designated as Honduran 1-2 A^1 (Hon 1-2) and Panamanian 2-7 A^8 (Pan 2-7).

Cultivation of Amoeba

The original single spore isolates of *Didymium iridis* were permitted to germinate and provided clones of myxamoeba. These myxamoebal clones were grown on a lawn of *Escherichia coli* that had been previously inoculated upon a solid growth media consisting of half strength (2%) cornmeal agar (Table 1) according to the methods of Yemma and Therrien, 1972. In this two-membered culture system, the *E. coli* metabolize the cornmeal while the myxamoeba utilize the bacteria as a food source.

Myxamoebal cultures were obtained by inoculating CM/2 agar with two drops of dilute *E. coli* suspension and spreading it over the entire media surface with a sterile glass rod. The media was then incubated in an inverted

TABLE 1

MEDIA*

Growth Media (Half-Strength 2% Cornmeal Agar - CM/2)

8.0 grams Difco Agar
8.5 grams Difco Cornmeal Agar
1.0 liter Distilled Water
(Collins, 1963; Yemma et al., 1974)

Plasmodium Media

5.0 grams Difco Agar
1.0 gram Difco Peptone Agar
1.0 gram Lactose
1.0 liter Distilled Water

*All media was autoclaved at 121°C at 15 psi for 15 min and then dispensed in approximately 25 ml aliquots to sterile petri plates. The plates were then refrigerated until needed.

position in a 21°C incubator for 24 hours to ensure a confluent bacterial growth or lawn. The media was then inoculated with the respective myxamoebal clones from the original stock culture slants and incubated at 21°C. The myxamoeba from the initial stock isolate transfers were then allowed to attain the log phase of growth in order to subculture them to several other CM/2 agar plates previously inoculated with *E. coli*. Subcultures of the two clones to fresh media were made every 5-7 days post-transfer. All myxamoebal cultures were maintained in an incubator at 21°C (Yemma and Therrien, 1972; Yemma et al., 1974).

Cultivation of Plasmodium

The plasmodia1 cultures were obtained by taking agar plugs (~ 1 cm x 1 cm) containing myxamoeba of the Honduran 1-2 A^1 and Panamanian 2-7 A^8 subcultures and placing them in close proximity on CM/2 growth media previously inoculated with *E. coli*. The plate was then incubated at 21°C until a significant plasmodia1 growth was obtained (usually 6 to 8 days). The plasmodia was then further subcultured onto plasmodia1 growth media (Table 1) which was sprinkled with flakes of sterilized oats. This method permitted the plasmodium to grow into large workable organisms within 7 to 10 days incubation. Pooled Plasmodia for Stains A, B, C and D were all subcultured from

one original plasmodium. Plasmodium for Stain D was subcultured though at a later date than plasmodia for Stains A, B, and C.

Cultures of both the amoeba and plasmodium were periodically examined microscopically for fungal and bacterial contaminants. Any plates that were contaminated were immediately discarded.

Slide Preparation for Cytochemical Study

The procedure of harvesting, fixating and preparing the slides was identically performed upon the myxamoeba and plasmodium and was as follows:

1) The plates were flooded with commercially prepared 10% buffered formalin (pH 7.0).
2) The cells were "washed" from the media surface by suction of a pasteur pipet and gentle agitation by a rubber policeman.
3) The cells were transferred to conical centrifuge tubes and spun at 750 rev/min for 10 min on an Adams Dynac swinging bucket centrifuge.
4) The supernatant (containing mostly bacteria) was discarded and the pellet was again washed 2 or 3 times more in order to completely

clear the pellet from bacterial contamination.

5) The cells were then allowed to fix for 24 hours in 10% buffered formalin (pH 7.0).

6) After fixation, the cells were again centrifuged but now at 2,500 rev/min for 5 min in order to re-wash the cells 3 times in 70% ethanol.

7) After the third washing, the cells were allowed to post-fix in 70% ethanol for 12 hours.

8) After post-fixation, the cells were centrifuged at 2,500 rev/min for 5 min and the supernatant discarded.

9) Finally 70% ethanol was added a little above the pellet and the suspension was thoroughly mixed.

10) A drop of the suspension was then applied to labeled albuminized slides.

Cytochemical Methods

The myxamoebal and plasmodia1 slides were divided into 4 sets, each set being stained by a different preparation of Schiff's reagent. The reagents differed with regard to the commercial source and the concentration

of the basic fuchsin. The stains used were labeled as follows and are listed with their respective concentrations and certification numbers (CI):

Stain	Total Dye Content	C.I.	Commercial Source
A	99%	42510	Harleco
B	93%	42510	Fisher Scientific
C	91%	42500	Fisher Scientific
D	88%	42500	Manufacturing Chemists

Most basic fuchsins are mixtures of pararosanalin, rosanalin, and magenta II (see Figure 3). Many of the basic fuchsins prove to be fairly pure pararosanalin, whereas others are deeper in hue than rosanalin and presumably contain appreciable quantities of magenta II. This is accountable to the general rule regarding alkyl substitution in which pararosanalin is the lightest, rosanalin is darker, and magenta II is the darkest. Pararosanalin (triaminotriphenylmethane chloride) is the chief constituent of all basic fuchsins which have the color index number of 42500. Basic fuchsins which have the CI number of 42510 have rosanalin (magenta I) as the major constituent with magenta II probably being present in market samples of the dye (Conn, 1977). The basic fuchsin for Stain D even though

it had the CI number of 42500 had triaminotriphenylcarbinol chloride as a slight modification from triaminotriphenylmethane chloride.

Before slides are stained, it is first essential to find the optimum hydrolysis time that would yield the greatest stain intensity. As previously described, this is obtained when all the purine bases have been removed thereby exposing the reactive aldehyde groups (DiStefano, 1948). In addition, to facilitate DNA measurements for the production of hydrolysis curves, a cell type that does not undergo DNA synthesis was needed to avoid a wide dispersion of values. Finally, it was beneficial to choose a cell type that could be standardized against the myxamoeba and plasmodium for future studies that may require absolute DNA values. Chicken erythrocytes satisfied these requirements since nucleated mature avian erythrocytes are not engaged in DNA synthesis (Andersson and Kjellstrand, 1975). As a result there is both a constant and similar amount of nuclear DNA material in these erythrocytes.

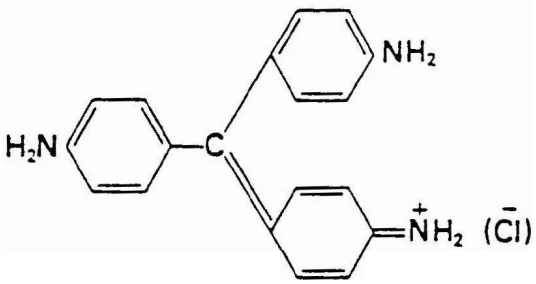
Hydrolysis curves were carried out at predetermined time intervals of 30, 45, 60, and 90 min in 5 N hydrochloric acid (HCl) at room temperature. The 5N hydrochloric acid hydrolysis at room temperature was used as a substitute for the more conventional "hot" hydrolysis in 1N hydrochloric

Figure 3.

Formulations of pararosanalin, rosanalin, and magenta II; all are constituents of basic fuchsin.

PARAROSANILIN (MAGENTA O) C.I. 42500
(C.I. Basic **Red** 9)

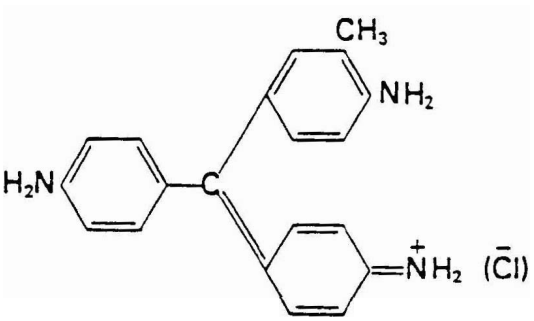
triaminotriphenylmethane chloride.



$C_{19}H_{18}N_3Cl$; mol. **wt.** 323.828

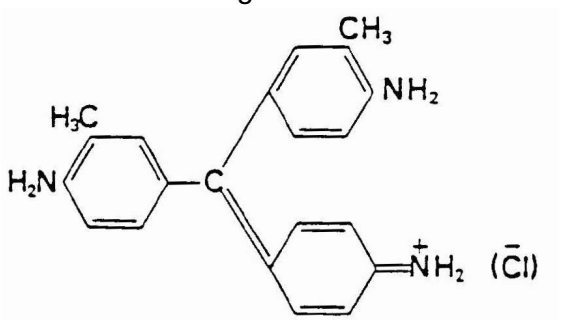
ROSANILIN (MAGENTA I) MAGENTA II C.I. 42510
(C.I. **Basic Violet** 14)

Rosanilin



$C_{20}H_{20}N_3Cl$; mol. **wt.** 337.855

Magenta II



$C_{21}H_{22}N_3Cl$; mol. wt. 351.883

acid at 60°C in order to prolong the period of maximal Feulgen intensity (Itikawa and Ogura, 1953; Jordanov, 1963; and DeCosse and Aiello, 1966). Chicken red blood cells obtained from the Cleveland Clinic were used in order to generate hydrolysis curves for Stains B and C (see Figure 8). In addition, because Stains B and C were produced by the same manufacturer, smears of the chicken red blood cells were also stained along with the myxamoeba and plasmodium so that an additional comparison could be made relating to dye concentration (see Figure 13).

Feulgen Reaction

The Feulgen nuclear reaction (Feulgen and Rossenbeck, 1924; as modified by Bryant and Howard, 1969) was used to localize and quantify deoxyribonucleic acid (DNA) in both the myxamoebal, plasmodia1 and chicken red blood cells. The reaction involves basically two stages:

1) Mild acid hydrolysis removes the purines at the level of the purine - deoxyribose glycosidic bond of DNA, thereby unmasking the aldehyde groups of deoxyribose.
2) Schiff's reagent reacts with the free aldehyde groups to yield a colored complex.

Schiff's reagent is made by changing a red dye basic fuchsin, which generally consists of a mixture of pararosanalin, rosanalin, and magenta II (Conn, 1977) to the colorless compound bis-N aminosulfonic acid by the addition of sulfurous acid. This colorless compound is better known as Schiff's reagent or leucofuchsin (DeRobertis & DeRobertis, 1980). The Schiff's reagent, upon combining with the free aldehyde groups of the deoxyribose sugar, forms a deep magenta color which can be measured by absorption spectrophotometry. The quantitative basis for the Feulgen reaction is due to the stable bond formed in a stoichiometric ratio between the Schiff's reagent and the polyaldehyde nature of hydrolyzed DNA. The bond between the dye and the deoxyribose sugar has been shown to be of an alkyl-sulfonic acid nature (Nauman, West, et al., 1960). This bond stability allows for the quantitation of relative DNA content in individual nuclei (Kasten, 1967; Ris and Mirsky, 1949).

Staining Procedure

Control slides, hydrolyzed for 15 minutes at 90 degrees Centigrade in five percent trichloroacetic acid (TCA) in order to remove the DNA, were run through the staining procedure along with the cells to be stained to ensure that the stain was always DNA specific. The control

slides did not stain indicating the absence of DNA and the specificity of the Feulgen stain for DNA.

The staining procedure carried out simultaneously on all sets of smears was as follows:

1) The smears were rinsed in distilled water prior to and after being hydrolyzed in 5N HCl for 60 min at room temperature.
2) The smears were stained 1 hour in Schiff's reagent (Lillie, 1951) freshly fortified with 10 percent potassium metabisulfite in a ratio of 1 to 4 with the reagent (10 ml to 40 ml of Schiff's reagent). Each set of smears was stained with a different preparation of Schiff's reagent (see Table 2).
3) The smears were rinsed three times for 5 minutes each in a sulfurous acid rinse solution (see Table 2).
4) The smears were rinsed in distilled water and dehydrated in a graded ethanol series.
5) Finally, the smears were cleared in xylene and mounted in permount.

Cytophotometric Determinations

A Zeiss Type 01 microspectrophotometer was used for all cytophotometric determinations employing a Planachromat

and staining were done at the same time (Swift and Pasch

oil immersion objection, N.A. 1.30 X 100 at an optovar setting of 1.25 x. A Zeiss continuous interference filter monochromator (No, 47 43 10) was used for isolation of chosed wavelengths of light, Instrument alignment and linearity of the phototube were checked each time prior to the instrument's use.

The two-wavelength method for quantitative DNA measurement (Patau, 1952; Ornstein, 1952; Mendelsohn, 1961) was used in this investigation. This method minimizes the distributional error in optical density measurements that result from heterogeneous staining within a specimen. Elimination of the need for direct measurement of the nuclear area is also provided for when employing this method (Mayall and Mendelsohn, 1970). The reliable estimation of the absorbing material is ensured by the selection of the two wavelengths. These wavelengths were chosen for each stain by making several absorption curves for the Feulgen stained material representing the different preparations of Feulgen stain (see Figures 4-7). The optical density (OD) at λ_2 = 2 X O D of λ_1. The wavelengths should give specific absorptivities at a 2:1 ratio since all hydrolysis and staining were done at the same time (Swift and Rasch, 1956). Heterogeneous dye distributions can be measured once the proper wavelengths are chosen.

TABLE 2

STAINING REAGENTS

Schiff's reagent - Add 2.0 g Basic Fuchsin and 4.4 g K-metabisulfite to 200 ml of 0.2 N HCl, stir mechanically for 2 hours, let stand overnight in a dark place, then mix with activated charcoal and filter. Use approximately 200 mg of activated charcoal (Norit A) for each 100 ml of Schiff's reagent. The Schiff's reagent must be water clear. If it is not water clear after the first filtering, add more Norit A and refilter. Store Schiff's reagent in a dark bottle at 5°C in a refrigerator. It is stable for approximately 2 weeks.

2. 10% K-metabisulfite - 10 g K-metabisulfite and bring volume to 100 ml with dH_2O.

3. Sulfurous Acid Rinse - 10 ml of 1N HCl
   10 ml of 10% K-metabisulfite solution
   200 ml of dH_2O

The two wavelengths (λ_1 and λ_2) were chosen conforming to the extinction requirements of the method. The photometric field was adjusted to circumscribe the nucleus with a minimal amount of unoccupied space around its border, and the absorbing molecules were measured by transmission at the two reference wavelengths. The amount of chromophore (M) to be determined within the measured area (A) was calculated using the equation $M = KAL_1Q$. The constant, K, was eliminated from this investigation since only arbitrary values of M, and not absolute values, were necessary. Transmissions (T_1 and T_2) were taken at wavelengths 1 and 2 for each nucleus. $T_1 = I_s/I_o$ and $T_2 = I_s/I_o$ where I_o is the transmitted flux and I_s is the flux of photons on the chromophore. From these values, L_1 and L_2 were calculated by the equations $L_1 = (1 - T_1)$ and $L_2 = (1 - T_2)$. The ratio, L_2/L_1, which corresponds to Q can be used to determine C, the correction factor for the distributional error (Swift and Rasch, 1956; Leuchtenberger, 1958), where $C = (2 - Q)^{-1} \ln (Q - 1)^{-1}$. The C value and corresponding Q value were found in a table formulated by Patau (1952).

Statistical Methods and Computations

To ensure accuracy all relative DNA calculations and statistical analyses were carried out on the main computer at

Youngstown State University through a program written by Dr. John J. Yemma.

CHAPTER III

Results

Spectral Absorption Curves for Feulgen-DNA Stains

The two wavelength method of microspectrophotometry requires the establishment of absorption curves for each particular chromophore-molecular complex being investigated. In this study, stained chicken red blood cell nuclei were used to establish the spectral absorption curves for each of the four stains since DNA values for these cells are relatively constant and upon maturity represent a non-dividing population of cells. The two wavelengths were calculated for each of the individual stains as previously described from the absorption curves (Figures 4-7) and are listed as follows:

Commercial Source	C.I. Number	λ at max. Absorption (nm)	λ at 1/2 max. Absorption (nm)
Stain A (Harleco)	42510	560	500
Stain B (Fisher)	42510	560	500
Stain C (Fisher)	42500	555	495
Stain D (MCB)	42500	560	497

It is interesting to note from the above data the relationship of the CI numbers with the calculated

Figure 4.

Absorption Spectrum of Feulgen stained chicken red blood cell nuclei using Stain A.

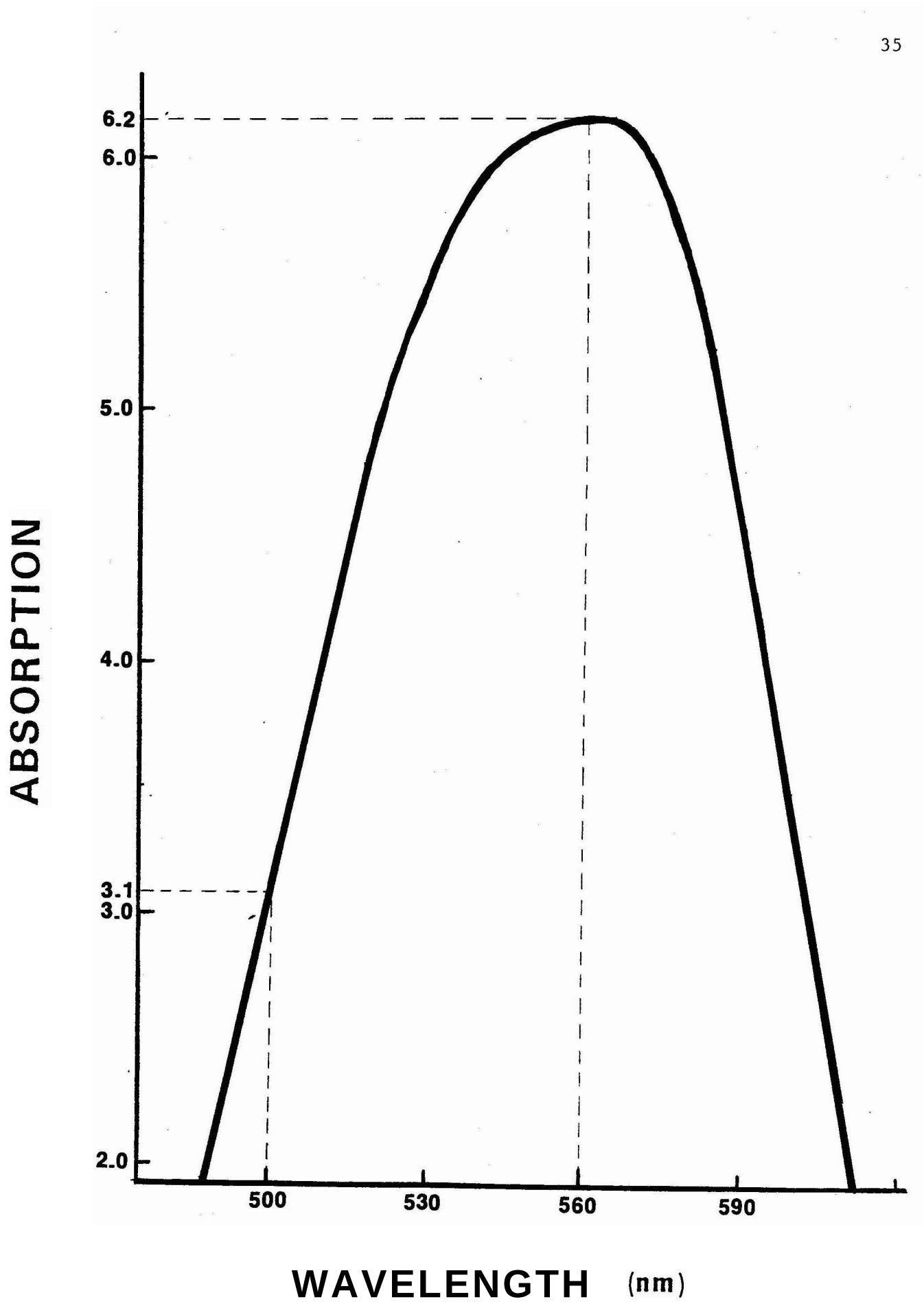

ABSORPTION
6.2
6.0
5.0
4.0
3.1
3.0
2.0
500
530
560
590
WAVELENGTH (nm)

Figure 5.

Absorption Spectrum of Feulgen stained chicken red blood cell nuclei using Stain B.

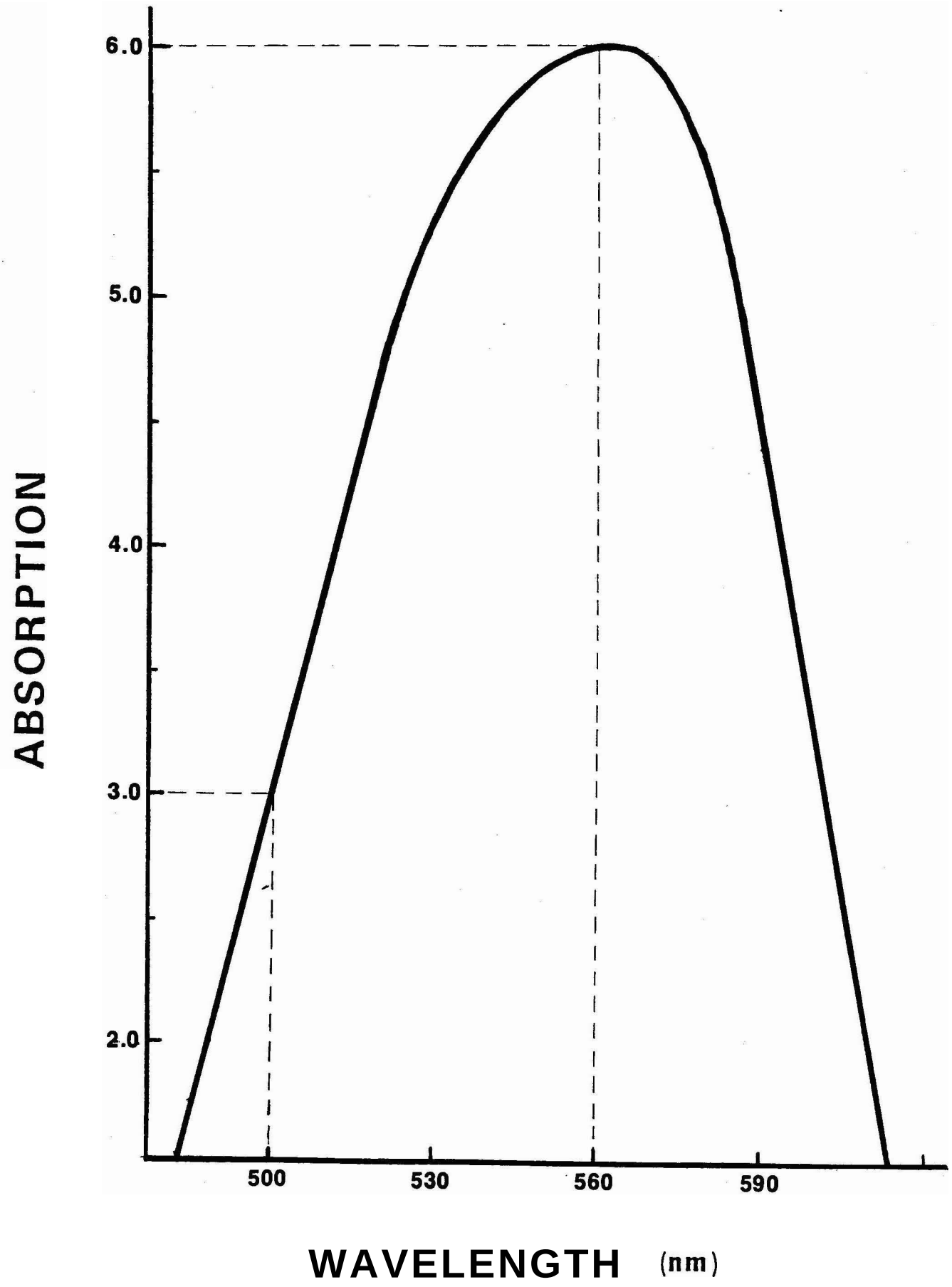

ABSORPTION
6.0
5.0
4.0
3.0
2.0
500
530
560
590
WAVELENGTH (nm)

Figure 6.

Absorption Spectrum of Feulgen stained chicken red blood cell nuclei using Stain C.

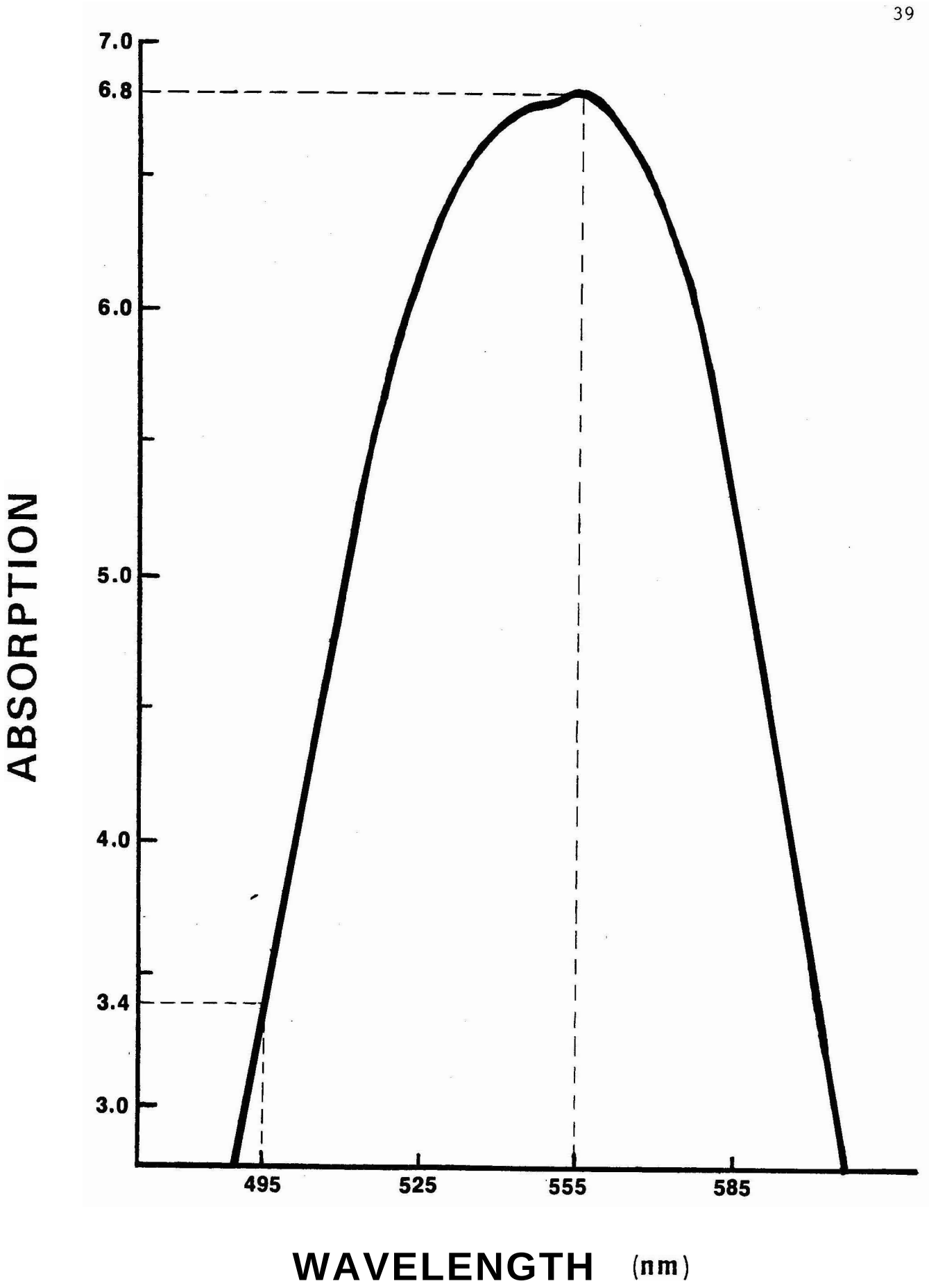
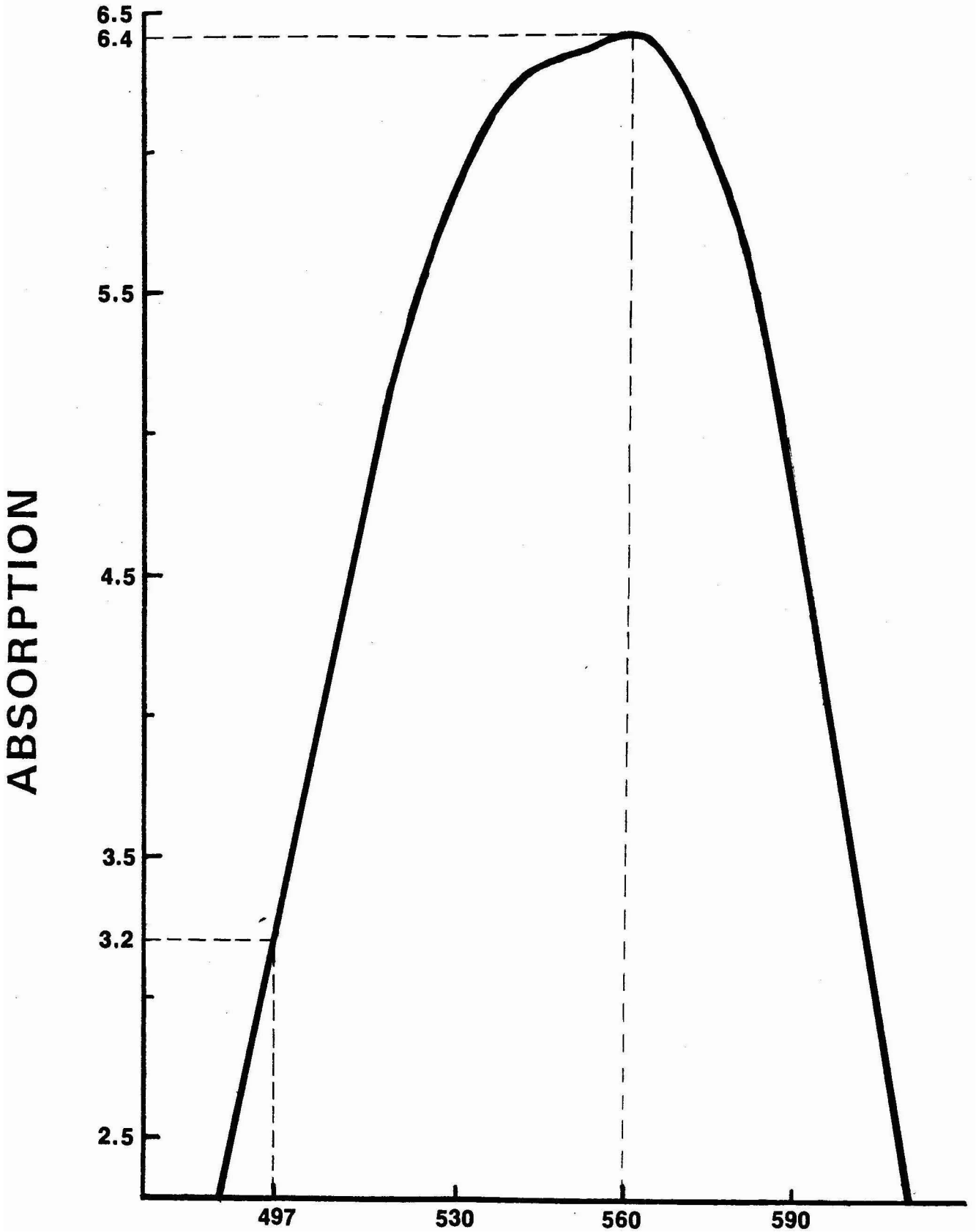

7.0
6.8
6.0
5.0
4.0
3.4
3.0
ABSORPTION
495
525
555
585
WAVELENGTH (nm)

Figure 7.

Absorption Spectrum of Feulgen stained chicken red blood cell nuclei using Stain D.


6.5
6.4
5.5
4.5
3.5
3.2
2.5
ABSORPTION
497
530
560
590
WAVELENGTH (nm)

wavelengths. Specifically note that even though Stains B and C had the same manufacturer, the two wavelengths calculated were not identical. In addition, note that Stain D produced two wavelengths that are different from that of Stain C.

The results of this study are presented in either tabular form or graphically as histograms and hydrolysis curves. All measurements were limited to interphase nuclei (no mitotic figures were scored) and were reported in arbitrary units as relative amounts of DNA.

Hydrolysis Curves

The hydrolysis curves illustrate not only maximal intensity attained but also the time period that will yield maximal intensity. The hydrolysis curves were performed upon chicken red blood cell nuclei utilizing Stains B and C. In the generation of the curves, the relative mean amount of cellular dye binding was plotted on the ordinate, and the time period of hydrolysis (in minutes) was plotted on the abscissa (see Figure 8). Each point on the hydrolysis curve represents the relative mean DNA value of 25 scored chicken red blood cell nuclei and is graphed with a 95% confidence interval. The mean DNA value for each time period is accompanied by its standard deviation and standard error in Table 3. In addition, a two sample

Figure 8.

Hydrolysis Curves representing Feulgen stained chicken red blood cell nuclei using Stains B and C.

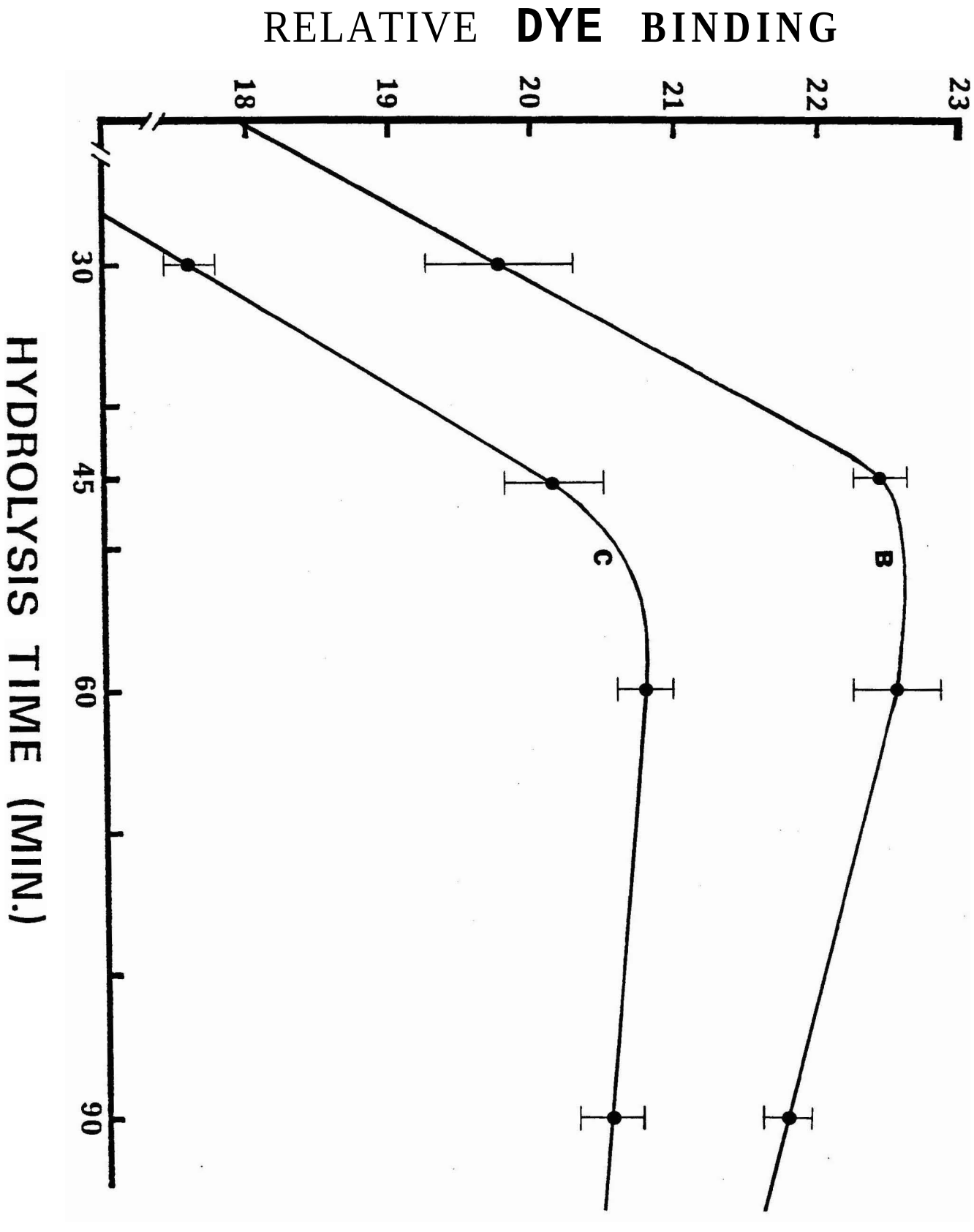

RELATIVE DYE BINDING
18
19
20
21
22
23
30
45
60
90
HYDROLYSIS TIME (MIN.)
B
C

TABLE 3

Standard Deviations and Standard Errors of Mean DNA Values for Hydrolysis Curves

	Hydrolysis time (min)	Mean DNA	Standard Deviation	Standard Error
Stain B	30	19.77	1.54	.30
	45	22.43	.53	.11
	60	22.56	.92	.18
	90	21.73	.51	.10
Stain C	30	17.56	.54	[illegible]
	45	20.12	1.09	.21
	60	20.76	.60	.12
	90	20.50	.66	.13

t-distribution comparison was performed on the time periods to the immediate left and right of the time period which yielded maximal absorption (Table 4). This was performed in order to determine which points represented the plateau of the curve, and thus the maximum hydrolysis period.

Microspectrophotometric Analysis of Nuclear DNA in Didymium iridis.

The analyses of nuclear DNA in the myxamoeba and plasmodium of D. iridis are illustrated as histograms for each of the individual stains. Histograms are convenient, for they represent nuclear DNA frequency distributions of cell populations and facilitate the observation of even minor shifts of DNA that may be due to the diffuse condition or template activity, synthetic activity, or changes in ploidy level. The number of nuclei scored were plotted on the ordinate and the relative dye concentrations of Feulgen stained DNA were correspondingly scored on the abscissa. One hundred nuclei were scored in order to give an adequate random representation of the cell population. Analyses of Stains A, B, C and D are represented by Figure **9**, 10, **11** and 12 respectively. The mean DNA values, standard deviations, and standard errors are also recorded.

Swift **(1950)** designated a system for the description of ploidy levels: C, 2C, and 4C corresponding

TABLE 4

Two Sample t-Distribution Comparison of Mean DNA Values for Hydrolysis Curves

	Hydrolysis Periods Compared (Min.)	t-Value	Significance Denoted by *
Stain B	45 60	.62	
	60 90	4.03	*
	45 90	4.71	*
Stain C	45 60	2.65	*
	60 90	1.47	
	45 90	1.54	

df = 48
= .1
critical value of t at .1 = 1.679

to unreplicated haploid, replicated haploid, and replicated diploid respectively. In addition, the designations of Howard and Pelc (1953) are used to describe the nuclei in either the presynthesis (G_1) or postsynthesis (G_2) phase of the mitotic interphase.

In each case, the mean DNA content of the myxamoeba is equivalent to the haploid 2C value for D. iridis. Unimodal histograms were observed for the myxamoeba indicating that they are in G_2 since according to Guttes, Guttes, and Rusch (1961) the myxamoebal population spends 12 hours (out of a cell cycle of about 12.5 to 13.0 hours) in G_2 with no appreciable G_1. In other words, these myxamoeba can be said to be in G_2 arrest with the G_1 stage being very brief or totally absent.

Generally, plasmodia formed from heterothallic isolates of D. iridis, such as those utilized in this study, are diploid (Therrien, 1966) and the nuclei are in the replicated diploid state (4C) 99% of the time (Yemma and Therrien, 1972). In this study though, it was observed that the plasmodia had the same ploidy DNA content as the myxamoeba. This observation has been explained in the literature by Yemma and Therrien (1974) in which they had reported that self-sterile clones on occasion gave rise to haploid plasmodia without the benefit of cross; 13.2 percent

in Honduran 1-2 and 10.0 percent in Panamanian 2-7. Thus, plasmodia in this study were considered to have developed apomictically and were not the results of fusion between cells to form zygotes. Therefore, the plasmodia are also in the haploid G_2 state.

Analysis of Stain A

In Stain A, both the myxamoeba and plasmodia histograms show a unimodal distribution which is expected since the population is essentially in G_2 of the cell cycle. Note that the myxamoeba has a slightly wider range of values and forms almost a perfect normal bell-shaped curve; indicating that the myxamoeba are a non-synchronously dividing population while the division of nuclei in the plasmodia divide synchronously (Guttes, Guttes, and Rusch, 1961). In addition, observe that the myxamoeba had a slightly higher mean DNA content (higher by 0.22 relative DNA units) than the plasmodia. Both means indicate rather clearly that the representative cellular populations are of the same ploidy level.

Analysis of Stain B

The histograms of the myxamoeba and plasmodia of Stain B likewise demonstrate a unimodal distribution. In this case, the myxamoeba population demonstrated a

Figure 9.

Histograms representing Feulgen-DNA values using Stain A for: (A) Honduran 1-2 A^1 myxamoeba (B) Plasmodia formed from the cross of Honduran 1-2 A^1 and Panamanian 2-7 A^8.

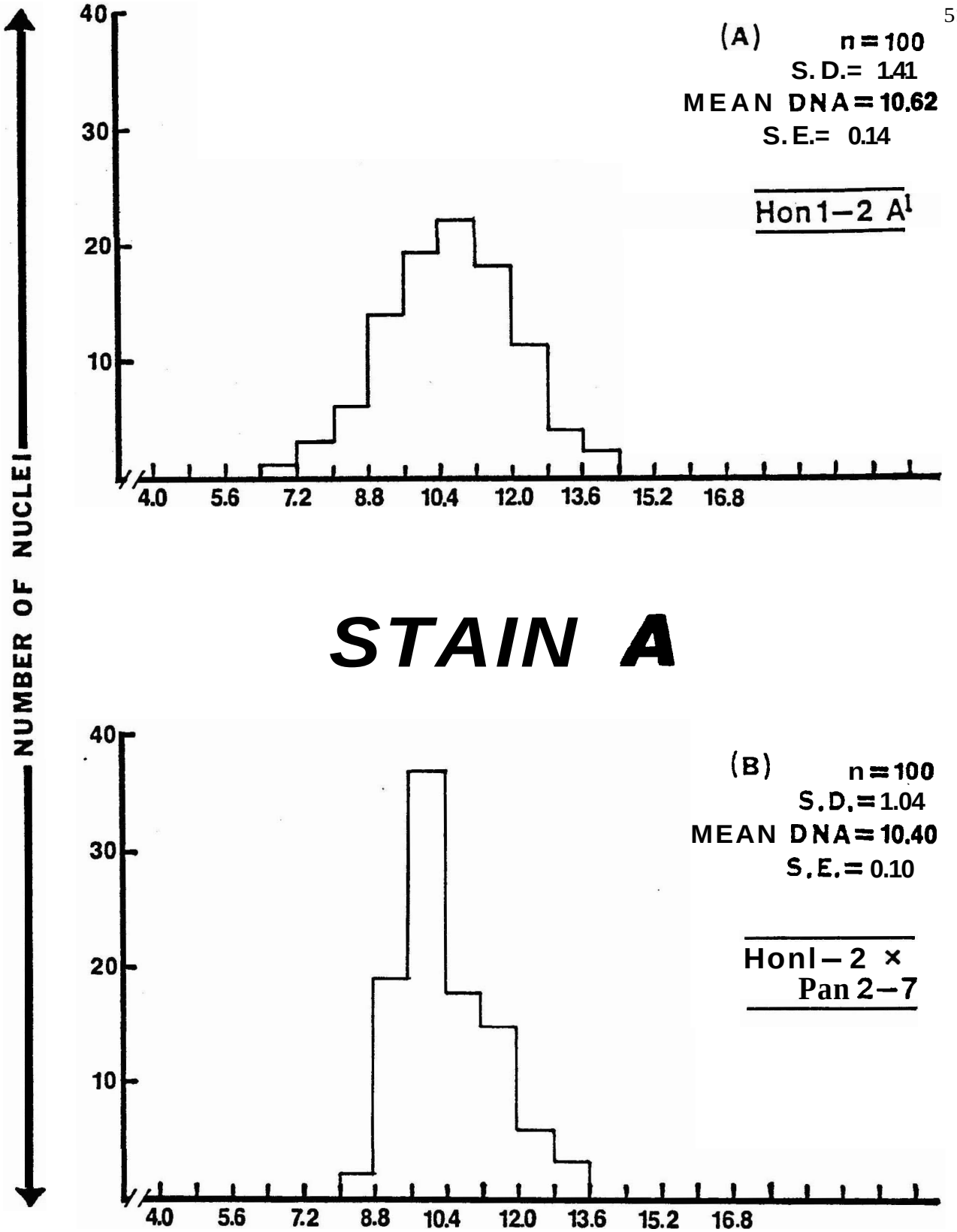

NUMBER OF NUCLEI
40
30
20
10
(A) n=100
S.D.= 1.41
MEAN DNA=10.62
S.E.= 0.14
Hon1–2 A1
4.0
5.6
7.2
8.8
10.4
12.0
13.6
15.2
16.8
STAIN A
40
30
20
10
(B) n=100
S.D.=1.04
MEAN DNA=10.40
S.E.=0.10
Honl–2 ×
Pan 2–7
4.0
5.6
7.2
8.8
10.4
12.0
13.6
15.2
16.8
RELATIVE AMOUNTS 0F DNA

Figure 10.

Histograms representing Feulgen-DNA values using Stain B for: (A) Honduran 1-2 A^1 myxamoeba (B) Plasmodia formed from the cross of Honduran 1-2 A^1 and Panamanian 2-7 A^8.

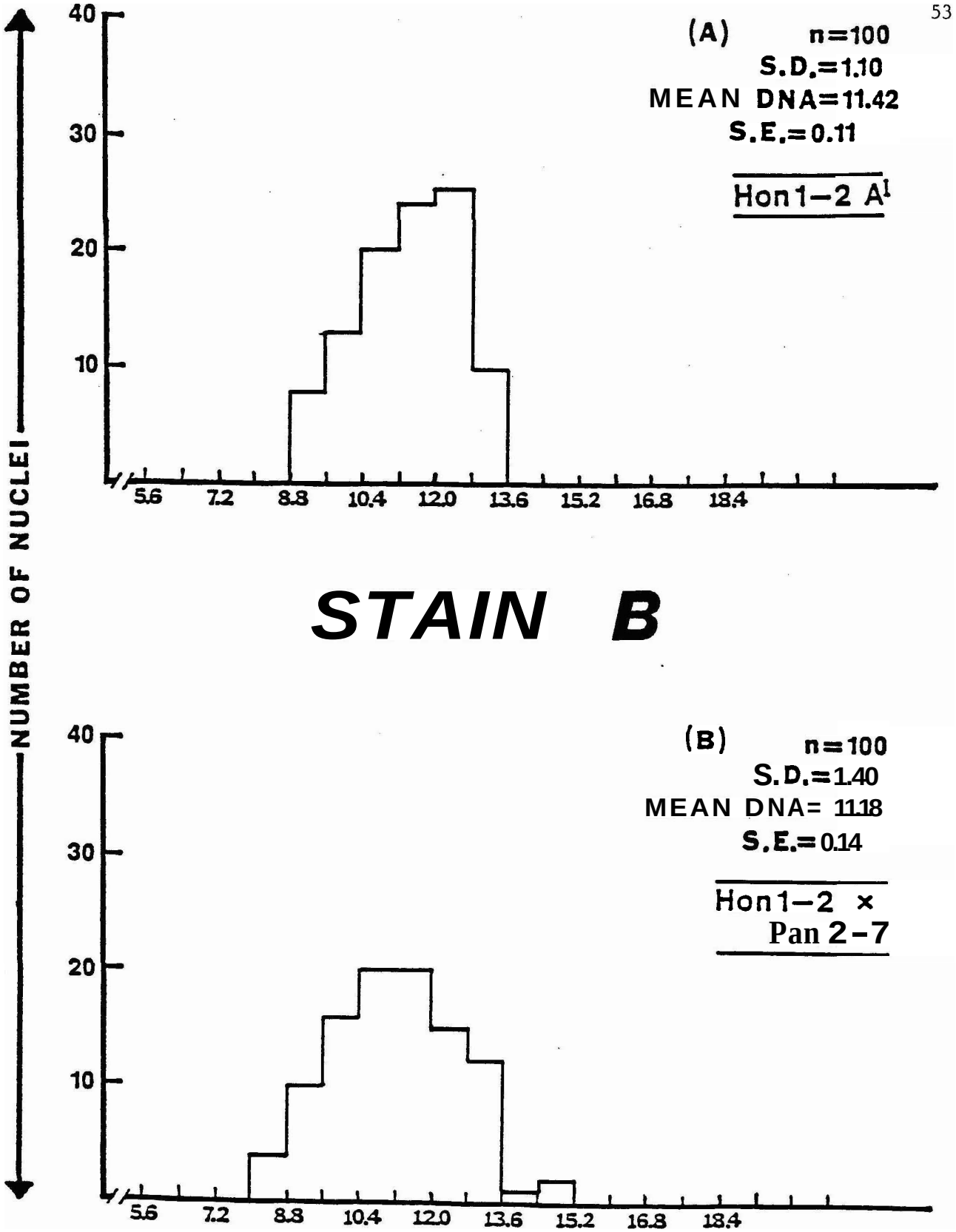
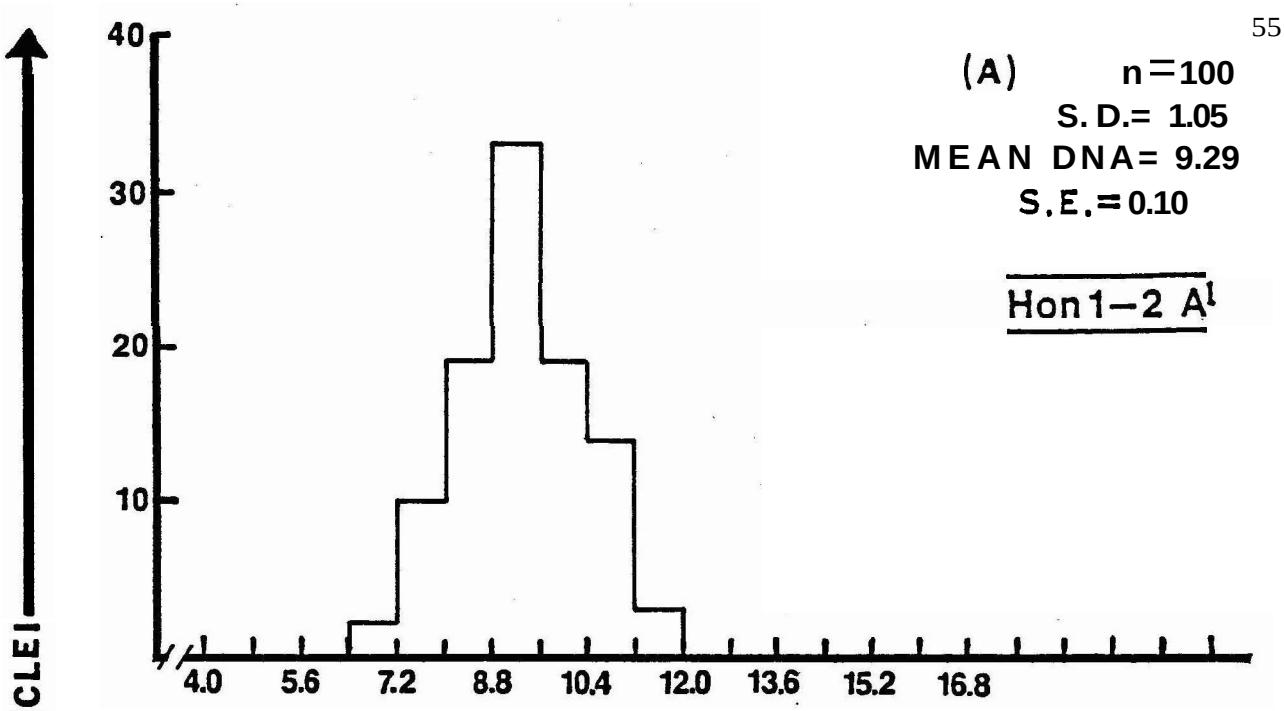
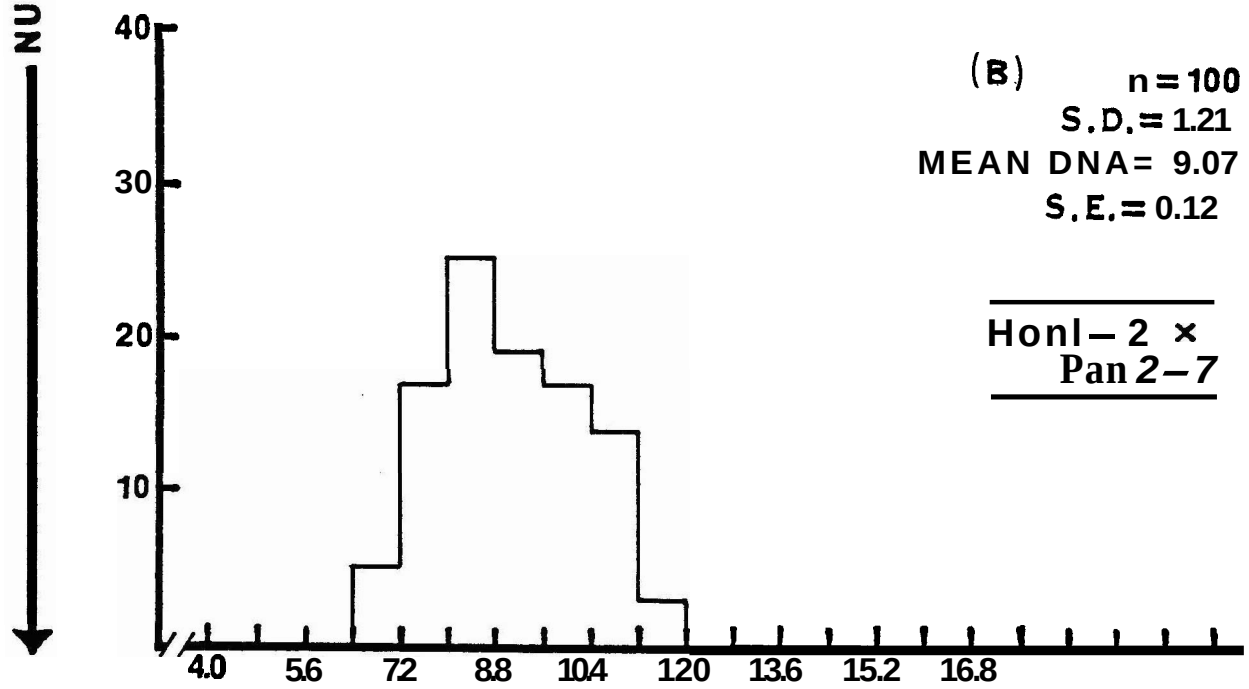

(A) n=100
S.D.=1.10
MEAN DNA=11.42
S.E.=0.11
Hon1–2 A1
STAIN B
(B) n=100
S.D.=1.40
MEAN DNA= 11.18
S.E.=0.14
Hon1–2 × Pan 2–7
NUMBER OF NUCLEI
RELATIVE AMOUNTS OF DNA
40
30
20
10
5.6
7.2
8.8
10.4
12.0
13.6
15.2
16.8
18.4

Figure 11.

Histograms representing Feulgen-DNA values using Stain C for: (A) Honduran 1-2 A^1 myxamoeba (B) Plasmodia formed from the cross of Honduran 1-2 A^1 and Panamanian 2-7 A^8.



STAIN C

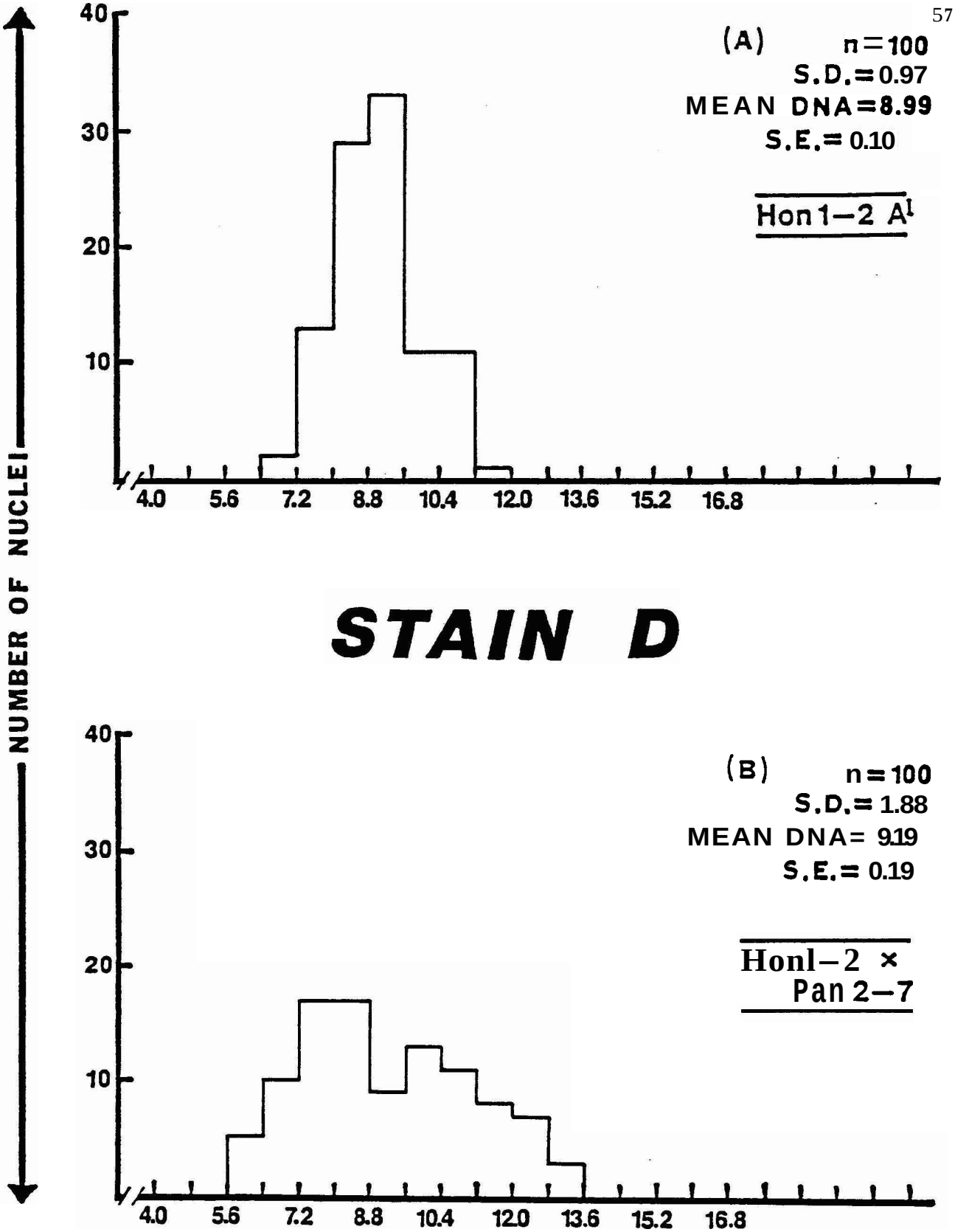


NUMBER OF NUCLEI

RELATIVE AMOUNTS OF DNA

Figure 12.

Histograms representing Feulgen-DNA values using Stain D for: (A) Honduran 1-2 A^1 myxamoeba (B) Plasmodia formed from the cross of Honduran 1-2 A^1 and Panamanian 2-7 A^8.


(A)
n=100
S.D.=0.97
MEAN DNA=8.99
S.E.=0.10
Hon1–2 A1
40
30
20
10
4.0
5.6
7.2
8.8
10.4
12.0
13.6
15.2
16.8
NUMBER OF NUCLEI
STAIN D
(B)
n=100
S.D.=1.88
MEAN DNA= 9.19
S.E.= 0.19
Honl–2 ×
Pan 2–7
40
30
20
10
4.0
5.6
7.2
8.8
10.4
12.0
13.6
15.2
16.8
RELATIVE AMOUNTS OF DNA

histogram with less variability regarding nuclear DNA than the plasmodia, But as in Stain A, the myxamoeba had a slightly higher mean DNA content (higher by 0.24 relative DNA units) than the plasmodia.

Analysis of Stain C

Similarly, Stain C showed unimodal distributions for the myxamoeba and plasmodia, as did Stains A and B. The spread of values for both histograms are similar. Stain C also follows the same pattern as Stains A and B in that the myxamoeba had a slightly higher mean DNA content (higher by 0.22 relative DNA units) than the plasmodia.

Analysis of Stain D

Results from Stain D were observed to be different from the previous three stains in that here the plasmodia had a slightly higher mean DNA content (higher by 0.20 relative DNA units) than the myxamoeba. Note also that whereas the myxamoeba had a unimodal distribution with a narrow range of values the plasmodia nuclear DNA describe a unimodal distribution with greater DNA variability, and thus has a wider dispersion of values. This could be due to the population of nuclei in plasmodia synthesizing DNA before preparing to divide - DNA synthesis may not be synchronous although division is (Therrien and Yemma, 1975). Recall that it was the plasmodia which were stained with

Stain D that had come from a later subculture than Stains A, B, and C. This accounts for the variation observed in the plasmodia of Stain D as compared to Stains A, B, and C.

Comparison of Stain B and Stain C

Figure 13 clearly shows the comparison among Stain B and Stain C using chicken erythrocytes. Because Stain B and Stain C were produced by the same manufacturer, it was possible to compare these two stains relative to the differences in CI number and concentration. This additional comparison between CI number and concentrations correlates well with the differences obtained using the myxamoeba and plasmodia. Note how narrow the range of values are in these unimodal histograms. In addition, the standard deviations and standard errors are also remarkably small.

An analysis of variance was carried out on the four stains through a SAS statistical program on the main computer at Youngstown State University. The results show that the comparison between Stain C and Stain D in both the myxamoeba and plasmodia is not significant at the 0.05 level of confidence. All other comparisons are significant at the 0.05 level. The results of these statistical analyses are listed in Table 5 for the myxamoeba and Table 6 for the plasmodia.

Figure 13.

Histograms representing Feulgen-DNA values of chicken red blood cell nuclei comparing Stains B and C.

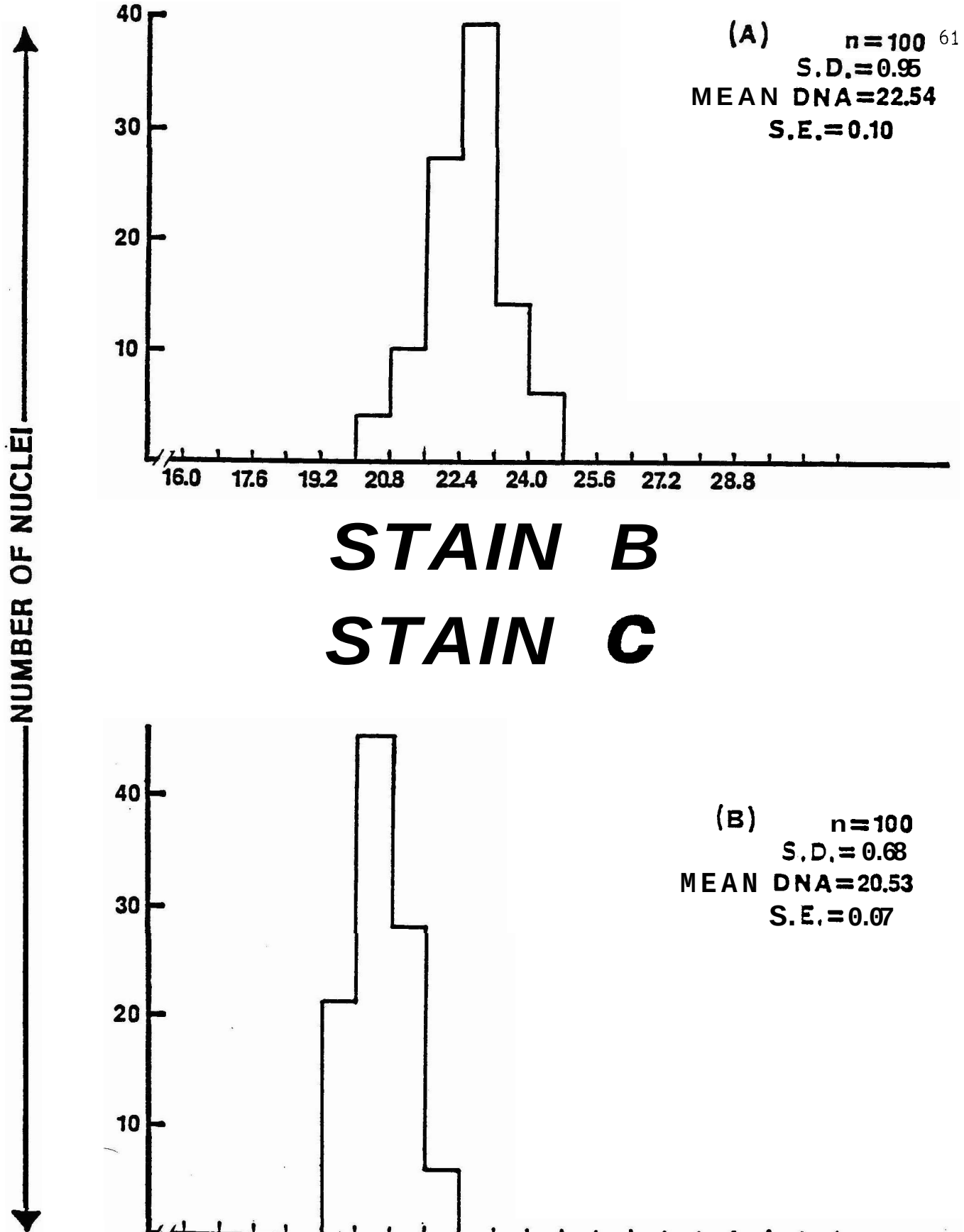

(A)
n=100
S.D.=0.95
MEAN DNA=22.54
S.E.=0.10
40
30
20
10
16.0
17.6
19.2
20.8
22.4
24.0
25.6
27.2
28.8
STAIN B
STAIN C
(B)
n=100
S.D.=0.68
MEAN DNA=20.53
S.E.=0.07
40
30
20
10
16.0
17.6
19.2
20.8
22.4
24.0
25.6
27.2
28.8
NUMBER OF NUCLEI
RELATIVE AMOUNTS OF DNA

TABLE 5

SAS

ANALYSIS OF VARIANCE PROCEDURE

TUKEY'S STUDENTIZED RANGE (HSD) TEST FOR VARIABLE: DYECONC
NOTE: THIS TEST CONTROLS TRE TYPE I EXPERIMENTWISE ERROR RATE

ALPHA=0.05 CONFIDENCE=0.95 DF=396 MSE=2.0251
CRITICAL VALUE OF STUDENTIZED RANGE=3.649
MINIMUM SIGNIFICANT DIFFERENCE=0.519224

COMPARISONS SIGNIFICANT AT *THE* 0.05 LEVEL ARE INDICATED BY '***'

STAIN COMPARISON	SIMULTANEOUS LOYER CONFIDENCE LIMIT	DIFFERENCE BETWEEN MEANS	SIMULTANEOUS UPPER CONFIDEICE LIMIT	
BPLAS - APLAS	0.258	0.777	1.296	***
BPLAS - DPLAS	1.473	1.992	2.511	***
BPLAS - CPLAS	1.592	2.111	2.631	***
APLAS - BPLAS	-1.296	-0.777	-0.258	***
APLAS - DPLBS	0.696	1.215	1.735	***
APLAS - CPLAS	0.815	1.335	1.854	***
DPLAS - BPLAS	-2.511	-1.992	-1.473	***
DPLAS - APLAS	-1.735	-1.215	-0.696	***
DPLAS - CPLAS	-0.400	0.119	0.638	
CPLAS - BPLAS	-2.631	-2.111	-1.592	***
CPLAS - APLAS	-1.854	-1.335	-0.815	***
CPLAS - DPLAS	-0.638	-00119	0.400	

TABLE 6

SAS

ANALYSIS OF VARIANCE PROCEDUBE

TUKEY'S STUDENTIZED *RANGE* (HSD) TEST FOR VARIABLE: DYECONC
NOTE: THIS TEST CONTROLS THE TYPE I EXPERIMENTWISE ERROR RATE

ALPHA=0.05 CONFIDENCE=0.95 DF=396 MSE=1.32519
CRITICAL VALUE OF STUDENTIZED RANGE=3.649
MINIMUM SIGNIFICANT DIFFERENCE=0.42002

COMPARISONS SIGNIFICANT AT THE 0.05 LEVEL ARE INDICATED BY '***'

STAIN COMPARISON	SIMULTANEOUS LOWER CONFIDENCE LIMIT	DIFFERENCE BETWEEN MEANS	SIMULTANEOUS UPPER CONFIDENCE LIMIT	
BAMOE - AAMOE	0.379	0.799	1.219	***
BAMOE - CAMOE	1.713	2.133	2.553	***
BAMOE - DAMOE	2.008	2.428	2.848	***
AAMOE - BAMOE	-1.219	-0.799	-0.379	835
AAMOE - CAMOE	0.914	1.334	1.754	***
AAMOE - DAMOE	1.209	1.629	2.049	***
CAMOE - BAMOE	-2.553	-2.133	-1.713	***
CAMOE - AAMOE	-1.754	-1.334	-0.914	***
CAMOE - DAMOE	-0.125	0.295	0.715	
DAMOE - BAMOE	-2.848	-2.428	-2.008	***
DAMOE - AAMOE	-2.049	-1.629	-1.209	***
DAMOE - CAMOE	-0.715	-0.295	0.125	

CHAPTER IV

Discussion

A quantitative comparison between four batches of Schiff's reagent was done in order to determine whether there are any significant differences in their dye binding properties. In order to accomplish this, quantitative cytophotometry was employed in conjunction with the Feulgen reaction. According to Kurnich (1955), the Feulgen reaction must satisfy the following requirements for cytophotometric quantitation of DNA: 1) the absorbing chromophore must be specific for nucleic acids and must localize it and remain stable, 2) the reaction must be stoichiometric, and 3) the chromophore must be suitable for photometry. In the past, the literature has provided abundant support concerning the application of the Feulgen reaction for microscopic localization and quantitative cytochemical determination of DNA by cytophotometry (Ely and Ross, 1949; Swift, 1950, 1953; Swift and Rasch 1956; Leuchtenberger, 1958; and Kasten, 1960; 1964).

Obtaining maximum chromophore development for the reaction product is essential since the amount of chromophore bound is proportional to the DNA content of a given cell type (Deitch et al, 1967, 1968). Thus, it is not

only imperative that the conditions which yield maximum staining intensity be fulfilled but it is also of utmost importance that the stains employed be subjected to identical conditions. This posed a problem regarding this study for a number of conditions and factors are known to influence the maximum stain intensity and therefore maximum absorption of the Feulgen reaction. Some of the conditions or factors which are known to affect the quantitation of the Feulgen reaction are: 1) choice and length of fixation (Swift, 1966; Kurnich, 1955; Deitch, 1967), 2) temperature, length and acid concentration of hydrolysis (Ely, 1949; Patau, 1952; DiStefano, 1948; Jordanov, 1963; DeCosse and Aiello, 1966), 3) sulphite content, 4) impurities present and 5) length of time in bleach and dehydration steps (Kasten, 1960, 1964). Therefore, all conditions and factors which are known to affect the quantitation of the Feulgen reaction were precisely regulated and controlled in this study, in order to most effectively maximize the validity of any comparisons made. Likewise, all stains were made from fresh stock reagents and all slides were prepared and run through the staining procedure simultaneously.

The absorption curves experimentally generated (Figures 4-7) demonstrated a great deal of similarity among stains having the same CI number. Harleco 99% and Fisher 93%

had the same CI number of 42510; likewise, Fisher 91% and MCB 88% had the same CI number of 42500. Correspondingly, the two wavelengths that were calculated in order to permit use of the two wavelength method of microspectrophotometry for the stains having the CI number of 42510 were both 560/500. Because Fisher 91% and MCB 88% also shared a common **CI** number it would be expected that both stains would also have the same wavelengths of maximum absorption and one-half maximum absorption. This however was not the case. Fisher 91% had wavelengths of 555/495, whereas MCB had wavelengths of 560/497. The difference can be attributed more than likely to the differing chemical makeup of the MCB 88% stain which contained triaminotriphenylcarbinol chloride whereas the Fisher 91% stain contained triaminotriphenylmethane chloride. The small drop just prior to maximum absorption of the stains with the CI numbers of 42500 is unexplainable. Note though that it did not occur with the stains having the **CI** number of 42510. This indicates that the stains having rosanalin and/or magenta II have chemical properties that differ from pararosanalin. The hydrolysis curves also support this view concerning the differences between stains with CI numbers of 42500 and 42510, stains having pararosanalin and rosanalin respectively.

Acid hydrolysis is an essential part of the Feulgen reaction since unhydrolyzed tissues do not stain (Kasten, **1960)**. Similarly, tissues treated with deoxyribonuclease do not stain. The ascending slope of the hydrolysis curve represents a period of minimal hydrolysis where part of the DNA remains untransformed into apurinic acid and therefore few aldehyde groups are available for dye binding (Jordanov, **1963)**. The absorption peak occurs during that time period of hydrolysis when the number of exposed aldehyde groups is at a maximum, creating a plateau (Greenwood, **1968)**. Leveling of the curve at maximum stain intensity signifies depurinization without significant depolymerization of the DNA (Andersson and Kjellstrand, **1975)**. This prolonging of the period of maximal Feulgen intensity can be attributed to the ability of formalin to render high chromatin stability toward acid hydrolysis (Kurnich, **1955**; Ruch, **1966**; Swift, **1966)**. It is further enhanced by the use of **5N** HCl at room temperature rather than 1N HCl at 60°C, which was used in many previous studies (Jordanov, **1963**; Ithawa and Ogura, **1953**; DeCosse and Aiello, **1966)**. Since optimum hydrolysis times are affected by various factors such as acid concentration, temperature and fixation (Kasten, **1960)**; all stains were subjected to identical treatment.

The hydrolysis curves clearly demonstrate that the Fisher stain having the higher concentration and the CI number of 42510 is far superior than that having the lower concentration and the CI number of 42500. A reasonable question then arises, namely, whether it is the concentration or the specific chemistry of the stain that causes differences in staining patterns between the two stains. It is reasonable, in view of the data presented, to conclude that because there is only a 2% difference in concentration between the two Fisher stains the most critical factor responsible for the differences observed can be attributed to the specific chemical components composing each stain. Thus, rosanalin (CI 42510) has a greater affinity to bind to the hydrolyzed DNA than pararosanalin (CI 42500).

An observation that further supports the view that concentration of the stains is of secondary importance regarding its role in the DNA differences obtained is seen in the parallel ascending slopes for the hydrolysis curves. It is pertinent to recall that, as the ascending slopes of the hydrolysis curves are generated, only a small proportion of the DNA has been hydrolyzed; therefore, only a small proportion of reactive aldehyde groups is exposed (Kelley, 1984). The stains because of the high concentration of dye

molecules are almost certainly capable of saturating all of these binding sites: therefore, during this period the concentrations of the stains are not a factor and the differences in DNA content must be exclusively due to the specific dye binding capabilities of the pararosanalin **(CI** 42500) and the rosanalin and magenta II **(CI** 42510).

Another interesting observation is that the 45 min. and 60 min. time periods for the Fisher 93% stain were statistically not significantly different while the 45 min. and 60 min. time periods for the Fisher 91% stain were significantly different and were not statistically the same until the 60 min. and 90 min. time periods were compared. This indicates that the rate at which the plateau and absorption peak was obtained was faster for the stain containing rosanalin and magenta II than for the stain containing pararosanalin. Thus, the concept of rosanalin having a greater affinity and therefore binding more rapidly and effectively to the hydrolyzed DNA than pararosanalin is well supported by the data presented in this study.

The analyses of the stained myxamoeba and plasmodia from the four different preparations of Schiff's reagent also illustrates that the stains which have rosanalin and magenta II consistently demonstrated higher levels of DNA detection than those containing pararosanalin. As was

the case in the hydrolysis curves, it appears that the concentration of the stain does not have a major effect on the dye binding. This is exemplified by the observation that the Fisher 93% stain showed higher levels of DNA content than the Harleco 99% stain. This situation can most likely be attributed to the differences in the manufacturing processes. The Fisher stain must have a slightly higher specificity for DNA than the Harleco stain and is therefore capable of exhibiting the subtle variations in measurable nuclear DNA content. The differences in DNA content between the myxamoeba and the apomictic plasmodia for each specific stain should not be considered to have different nuclear DNA contents, but rather have a difference in the measurable DNA, or more specifically in the number of dye binding sites. These subtle variations in measurable DNA in cells of identical ploidy levels have been observed by Garcia **(1969)** and Noeske **(1971)** to be due to functional genetic differences among the cell types of a species. These functional genetic differences relate to the condensed or diffuse nature of the chromatin material. Condensed chromatin material (heterochromatin) provides less RNA template activity and therefore fewer dye binding sites are exposed. Diffuse chromatin (euchromatin) correspondingly provides greater template activity and thus enables

greater dye binding. Littau et al. **(1964)** using autoradiographic studies also demonstrated that diffuse chromatin provided a more efficient template for **RNA** transcription than condensed chromatin.

The only two stains that statistically demonstrate similar **DNA** levels for the myxamoeba and plasmodia are the Fisher **91%** stain and the MCB 88% stain. This more than likely indicates that the substitution of triaminotri-phenylcarbinol chloride causes its staining actions to be similar to the pararosanalin of the Fisher **91%** stain, assuming manufacturing processes are similar and the differences in concentration of the two stains as already described, is of no consequence. It is important however, that one not eliminate the possibility of it being far superior or far inferior to pararosanalin and it is compensated for by the differences in the manufacturing process.

This study effectively demonstrates that the differences of manufacturing and chemical composition of the basic fuchsins are most critical in the quantitative aspects of the Feulgen reaction, whereas the differences in concentration from one stain to another appear to have little or no effect on the measurable **DNA.** The most significant factor one must consider when attempting

quantitative studies involving the Feulgen reaction is the selection of the proper chemical composition of the basic fuchsins. Stains containing mostly rosanalin and/or magenta II (CI number of 42510) are far superior in detecting measurable DNA than stains containing mostly pararosanalin (CI number of 42500). This suggests a higher specificity or affinity for DNA in the stains having the CI number of 42510 as compared to stains having the CI number of 42500. Fisher Scientific manufacturing company obtained the highest levels of DNA so therefore it is the manufacturer of choice for quantitative studies involving basic fuchsins.

In conclusion, it appears that all the stains studied are capable of yielding quantitative and accurate relative DNA measurements. It also appears that in order to obtain quantitatively accurate absolute values, the stain of choice should be of CI 42510 and be manufactured by Fisher Scientific. It is also pertinent that controls be run in order to assure constant staining capabilities.

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