QUANTITATIVE CYTOPHOTOMETRIC ANALYSIS OF THE EFFECTS OF VARIOUS FIXATIVES AND TISSUE TYPES ON HYDROLYSIS TIMES AND STAINING INTENSITIES USING THE BLUE FEULGEN REACTION

by

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ABSTRACT

QUANTITATIVE CYTOPHOTOMETRIC ANALYSIS OF THE EFFECTS OF VARIOUS FIXATIVES AND TISSUE TYPES ON HYDROLYSIS TIMES AND STAINING INTENSITIES USING THE BLUE FEULGEN REACTION

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Three fixatives, ten percent buffered formalin, Carnoy's fixative, and a new fixative, Streck's Tissue Fixative (STF), were used to fix four tissue types--murine liver and sperm, myxomycete plasmodium and myxamoeba--to determine their influence on hydrolysis times and staining intensities in the Blue Feulgen reaction. Relative amounts of DNA per nucleus were determined using the two wavelength method of quantitative microspectrophotometry.

Liver fixed in formalin and Carnoy's fixatives exhibited similar peak hydrolysis plateaus, while the hydrolysis plateau for STF-fixed liver cells began later. Liver fixed in all three fixatives exhibited more intense staining after 60 minutes in the stoichiometric cresyl violet stain than after 20 minutes, so all tissues were stained for 60 minutes.

All three fixatives proved to be effective for use with the Blue Feulgen reaction, as all three provided approximate 2:1 ratios between DNA content of the diploid tissues (liver and plasmodium) and their respective haploid tissues (sperm and myxamoeba). Carnoy's fixative, however, is not recommended for use with the Blue Feulgen reaction because it provided lower stain intensities for all four tissues. The alcohol-based Carnoy's fixative is known to extract some Feulgen-stainable material. Ten percent buffered formalin and STF provided excellent staining, with STF exhibiting 10 to 20 percent stain enhancement over

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formalin or Carnoy's fixative for all four tissue types. The new fixative, STF, also exhibited great sensitivity to changes in DNA content during the cell cycle and is highly recommended for use with Blue Feulgen microspectrophotometry.

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SYMBOL	DEFINITION	UNITS
A	Area	$\mathcal{M}$
C	Correction factor for unoccupied space: $C = (2-Q)^{-1}\ln(Q-1)^{-1}$	
E	Extinction	
I_o	Flux of photons on chromophore	
I_s	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_1	Wavelength of one half maximum extinction	nm
λ_2	Wavelength of maximum extinction	nm

INTRODUCTION

Over seventy years ago, Feulgen and Rossenbeck (1924) first proposed their method for localizing deoxyribonucleic acid (DNA) in cells. The broad applicability of the Feulgen reaction to cellular biology and medicine was quickly recognized, as evidenced by the publication of thousands of papers on the use of the technique within ten years of its publication (Kasten, 1964). During the Feulgen reaction, hydrolysis with hydrochloric acid removes purine bases from the DNA, leaving exposed aldehyde groups. Aldehydes then react with a dye-sulfurous acid mixture (the Schiff reagent) to produce a bright, quantitative, cytophotometrically measurable color.

Localization and quantitation of DNA is of paramount importance in understanding the biochemical changes that occur during the cell cycle because the changes that occur are reflected and accompanied by changes in nuclear DNA (Yemma and Therrien, 1972). Therefore, many researchers have studied the various aspects of the Feulgen reaction and have determined its reliability.

The quantitative usefulness of the procedure was questioned by Stedman and Stedman (1943, 1947) and Ely and Ross (1949) who found that acid hydrolysis allows for the loss of some stainable material. Swift (1950, 1956) showed that the Feulgen reaction can be used to indicate relative amounts of DNA per cell since there will be an equal loss of material from all cells in a sample that are simultaneously subjected to the same reaction procedures. Swift's study shows remarkable constancy in dye regeneration over many tissue types. Swift's 1956 microspectrophotometric study of the DNA content of murine somatic and spermatid nuclei showed a 2:1 ratio, respectively, between these mouse tissues. This ratio was in agreement with determinations of bovine somatic and spermatid DNA content performed by Boivin, Vendrely, and Vendrely (1948) and Vendrely and Vendrely (1948, 1949) using suspension counts. The quantitative capability of the Feulgen reaction with microspectrophotometry was no longer in doubt.

Development of the two-wavelength method of microspectrophotometry independently by Ornstein (1952) and Patau (1952) further established a reliable method

for quantitative measurement of DNA. Microspectrophotometric determinations of mean nuclear DNA content also reveal immediately whether or not a cell population is uniform in DNA content (Leuchtenberger, 1958), and their extreme sensitivity allows the detection of even slight changes in DNA, as occurs, for example, during template activity (Yemma and Stroh, 1991). Gross analysis of nuclear suspensions provides only one average amount of DNA for a large number of nuclei. The microspectrophotometric mean, on the other hand, is based upon a number of analyses done on individual nuclei and is much more sensitive to variations in DNA (Leuchtenberger, 1958).

Localization of DNA by the Feulgen reaction has been proven by Stowell (1945), Ely and Ross (1949), Sibatani (1953), Lessler (1953) and Kasten (1960) who demonstrated the specificity of Schiff's reagent for DNA. The use of 4% trichloroacetic acid to remove DNA from control slides or aldehyde-blocking agents such as thiosemicarbazide and hydroxylamine on control cells resulted in no staining, showing the specificity of Schiff's reagent for aldehyde groups on DNA.

Today it is widely agreed that the Feulgen reaction can provide reliable quantitation of nuclear DNA when the procedure is carefully controlled. Variables such as the length of hydrolysis and acid strength, the type of dye used, and the method of fixation have all been shown to influence the results of the Feulgen procedure (Pressly, 1978; Kelley, 1984).

Mild acid hydrolysis, to remove purines and leave exposed aldehyde groups on the DNA, is the defining step in the Feulgen reaction. Without proper hydrolysis, dye molecules will not attach to DNA and staining will not occur. Therefore, it is important to select the optimum acid strength, temperature, and length of hydrolysis time. Classically, before 1950, researchers hydrolyzed tissues in 1N HCl at 60^0 C. When hydrolysis time is plotted versus dye regeneration, this "hot" hydrolysis produces hydrolysis curves with a rapid rise to optimum stain intensity, then a marked drop in staining as hydrolysis continues. The shape of the hydrolysis curve indicates progressive depurination of the DNA up to the optimal hydrolysis time. Post-optimal hydrolysis, however, promotes

depolymerization of the DNA and possible loss of the thymic acid from the nucleus (diStefano, 1948; Andersson and Kjellstrand, 1975), resulting in the loss of dye-binding sites. The transition between depurination and depolymerization is rapid with hydrolysis at high temperatures (DeCosse and Aiello, 1966), so the optimum hydrolysis time must be carefully chosen. Later studies found hydrolysis at room temperature in more concentrated acid to be much more practical (Itikawa and Ogura, 1954). The optimum hydrolysis time in 5N HCl at room temperature will depend upon the tissue and the fixative used (diStefano, 1948; Deitch, et al., 1967; Bancroft, 1975), but typical times range from 20 minutes to 60 or even 120 minutes providing optimum staining (DeCosse and Aiello, 1966; Deitch, et al., 1967; Dutt, 1975, 1976). Hydrolysis curves for room temperature hydrolysis in 5N HCl typically show an initial rise in staining followed by an extended plateau in optimal stain intensity (Jordanov, 1963; DeCosse and Aiello, 1966; Pressly, 1978), indicating that depolymerization of the DNA is significantly delayed. Jordanov (1963) and DeCosse and Aiello (1966) determined that it is heat, not acid, that primarily promotes the further loss of Feulgen stainable material during post-optimum hydrolysis. Deitch, Wagner, and Richart (1968) also found that Feulgen values may be 5 to 30 percent higher with room temperature hydrolysis than with "hot" hydrolysis due to the loss of some DNA at 60° C. In light of the extended period of optimum Feulgen staining and the delay of depolymerization of the DNA, most researchers today recommend hydrolysis in 5N HCl at room temperature over hydrolysis in 1N HCl at 60° C for the Feulgen procedure (Dutt, 1976).

The dye chosen for the Feulgen reaction has also been the subject of much research. In order for quantitative cytophotometric measurements of DNA to be made, the stain used with the Feulgen reaction must satisfy three criteria (Kurnick, 1955):

1) The absorbing chromophore must be specific for DNA, must localize DNA, and must remain stable.

2) The reaction between the chromophore and the apurinic DNA must be stoichiometric.

3) The chromophore must be suitable for photometry.

Classically, Schiff's reagent, basic fuchsin, has been the stain of choice for the Feulgen reaction. This dye fulfills the above requirements, and the amount of chromophore bound is proportional to the DNA content of a given cell type (Deitch, *et al.*, 1967, 1968; Andersson and Kjellstrand, 1975). However, the Feulgen reaction does have limitations. An important one is that the duration of stain storage is critical. Orosz (1992) found the storage time of two weeks in a cold environment to be the maximum length of time the fuchsin stain gave reliable quantitative results. More lengthy storage times failed to give reliable results.

Kasten (1958) was the first to make a search of Schiff-type dyes to replace basic fuchsin in the Feulgen reaction. He recommended 24 different stains, all containing a primary amine group, for use in the Feulgen procedure, including the oxazine dye cresyl violet which was used in this study. Dutt (1976) worked with many of the same stains as well as some that did not contain a primary amino group (such as thionine blue and methylene blue) and found many of them suitable for the Feulgen reaction. Dutt's (1970, 1971) investigation with cresyl violet on pig liver nuclei produced quantitative distribution of DNA (2N:4N) although some anomalies were observed. Additional research in our laboratories by Bidinotto (1993) with rat liver and sperm and *Didymium iridis* plasmodium and myxamoebal nuclei established that cresyl violet is a stoichiometric stain and retains its quantitative characteristics over a long period of time.

The modified Blue Feulgen reaction developed by Floyd (1990) employing cresyl violet has several advantages over the standard fuchsin reaction. Cresyl violet produces deep blue nuclei and is much simpler to prepare than basic fuchsin. Basic fuchsin requires repeated filtering and eight to twelve hours of shaking. Incomplete filtering with activated charcoal can result in dye impurities contaminating the stained nuclei (Kasten, 1964). Cresyl violet, on the other hand, can be used immediately after being dissolved in cold water and decoloration with sodium dithionite. Additionally, Floyd has noted that the

cresyl violet dye solution has a shelf-life of years as opposed to a few weeks for the standard Schiff's reagent (Dutt, 1979; Orosz, 1992).

The fixative employed on the tissue to be stained with the Feulgen procedure influences the intensity of the regenerated dye (Swift, 1950) and, therefore, must be chosen carefully. An appropriate fixative must exhibit several general properties (Bancroft, 1975):

1) The fixative must not cause the tissue to swell or shrink significantly,

2) the fixative must stop autolytic enzyme activity,

3) the fixative must not remove other enzymes from the tissue, and

4) the fixative must denature the cellular proteins.

More specifically for the Feulgen reaction, the fixative must denature DNA to the fullest extent possible (Dutt, 1971) in order to provide the largest number of aldehyde groups for dye binding.

Ten percent buffered formalin is often recommended for use with the Feulgen procedure (Dutt, 1976). Formalin readily forms methylene bridges between proteins which stabilize and denature the proteins (Pearse, 1961). Also, formalin forms cross links between proteins and DNA which arrest depolymerized fragments of DNA and prevent the loss of potential dye-binding sites (Giroud and Montmasson, 1989). Hydrolysis and washing during the Feulgen procedure remove the cross links and leave the majority of active groups available for reaction with other reagents (Pearse, 1961). Giroud and Montmasson (1989) note that formalin-fixed nuclei exhibit medium stain intensity, staining better than several other fixatives, including Carnoy's, yet not as intensely as some.

Carnoy's fixative, for use with the Feulgen procedure, has proponents and opponents. Porcelli (1972), Karalova (1980), and Giroud and Montmasson (1989) all describe Carnoy's fixative as ineffective for Feulgen staining because of the low stain intensities regenerated. Swift (1950), however, found that although stain intensities were much lower for Carnoy's fixative than for 50% formaldehyde, the ratios between nuclear

classes remained the same. Therefore, Carnoy's fixative may be used to demonstrate relative DNA values.

In this study a recently developed and largely untested new fixative, Streck's Tissue Fixative (STF), will be compared with ten percent formalin and Carnoy's fixative for stain intensity and maintenance of nuclear class ratios.

Research on the Feulgen reaction has also studied its applicability to different tissue types (Swift, 1950; Kelley, 1984). Four different tissue types were used for this study: mammalian liver and sperm (obtained from a rat) and slime mold myxamoebae and plasmodium cells.

Mammalian somatic cells exhibit several nuclear DNA classifications during interphase. Swift (1950) designates these as unreplicated 2C, S phase, and replicated 4C values for diploid cells in interphase. After mitosis is completed, the cell enters the G_1 stage of interphase--the pre-replication stage--when the DNA content can be classified as 2C (diploid, unreplicated). Replication of the DNA in preparation for the next mitotic division occurs during the S phase. After replication, the somatic cell enters G_2 and the DNA content can be classified as 4C (diploid, replicated). Liver cells are especially good for demonstrating somatic DNA nuclear classes because of their high mitotic rate. Polyploid (8C) cells are common in liver cell populations as well (Ris and Mirsky, 1949; Swift, 1950).

Sperm cells provide excellent contrast to liver cells because the mature spermatozoa should always be haploid, unreplicated (DNA content classification of 1C). The distinct flagellated shape of the mature sperm develops after meiosis is complete and the haploid spermatids have differentiated (Alberts, *et al.*, 1989). Therefore, DNA content ratios between liver and sperm nuclei will be 2:1 or higher.

The slime mold *Didymium iridis* is an excellent organism for cytological studies because of its ease of culturing and quick growth. This lower organism has distinct haploid (myxamoeba) and diploid (plasmodium) phases. The life cycle of *D. iridis* is shown in Figure 1. Like liver cells, myxamoebal and plasmodial nuclei exhibit changes in DNA

FIGURE 1

The generalized life cycle of the Myxomycete.

Didymium iridis follows this reproductive cycle, fusing into the diploid plasmodium stage when opposite myxamoebal mating types meet.

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Spore

A

Germination

B

Myxamoeba

C

Cell division

Swarm cell

G

D

Plasmogamy

MEIOSIS

Fructifications

L

DIPLOPHASE   HAPLOPHASE

K

Beginning
of
fruiting

J

Older
plasmodium

I

Young
plasmodium

H

E

F

Karyogamy
(zygote)
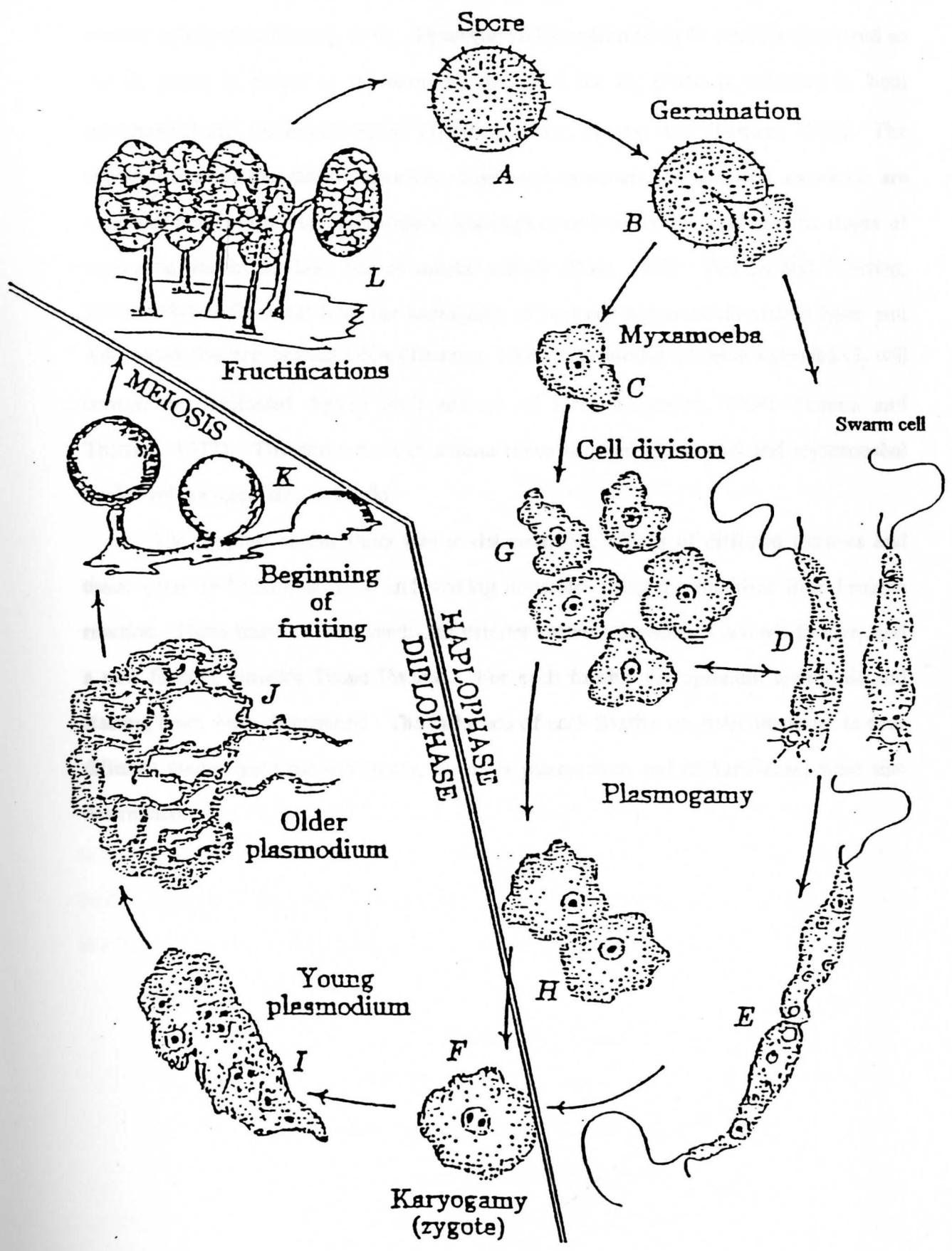
content as they pass from G_1 to G_2. However, DNA replication in *D. iridis* is very rapid so the G_1 phase is absent or of short duration, and the G_2 phase is extended in both myxamoebal and plasmodial nuclei (Therrien, 1966; Yemma and Therrien, 1972). The myxamoebae, as logarithmic amoebae, flagellated swarmers, or encysted amoebae, are replicated haploid (2C) in DNA content, although some have been found in other stages of replication due to the high rate of mitotic activity (Ross, 1967; Yemma and Therrien, 1972). Plasmodia result from the karyogamy of opposite myxamoebal mating types and will contain the sum of their DNA (Therrien, 1966). Plasmodial nuclei in extended G_2 will contain the replicated diploid (4C) amount of DNA (Therrien, 1966; Yemma and Therrien, 1972). Therefore, nuclear content ratios between plasmodial and myxamoebal nuclei will demonstrate a 2:1 ratio.

The purpose of this study was to determine the effects of different fixatives and tissue types on hydrolysis times and staining intensities using the modified Blue Feulgen reaction. Three fixatives were used: ten percent buffered formalin, Carnoy's fixative, and a new fixative, Streck's Tissue Fixative. For each fixative the optimum hydrolysis and staining times were determined. The influence of each fixative on stain intensities in four different tissues (rat liver and sperm, *D. iridis* plasmodium and myxamoebae) were also determined.

MATERIALS AND METHODS

I. Mammalian Tissues

A. Fixation

Two male Long-Evans rats were obtained from the Youngstown State University colony and were sacrificed by asphyxiation in a carbon dioxide chamber. The liver and testes were immediately excised and washed in large amounts of cold Hank's solution for 1 to 2 minutes to remove excess blood. The liver was then cut with a scalpel into 1 cm³ blocks and the tissue pieces distributed to beakers containing large volumes of the three different fixatives to be studied--10% buffered formalin, Carnoy's fixative, and Streck's Tissue Fixative (STF). See Table 1 for compositions of these three fixatives. Each testicle was cut open along one side and one testis placed in each of the three fixatives. Both liver and testes were fixed for 24 hours at room temperature, regardless of fixative used, and subsequently post-fixed in 70% ethanol.

B. Preparation of Liver Slides

Following 24 hours of fixation in either 10% formalin, Carnoy's fixative, or STF, each piece of liver tissue was placed in a labeled plastic tissue cassette. All liver tissue was treated simultaneously from this point on. The liver was dehydrated and paraffin infused in a Tissue Tek II automatic tissue processor set to the following schedule:

70% ethanol--24 hours

90% ethanol (twice)--1 hour each

absolute ethanol (twice)--1 hour each

xylene (twice)--1 hour each

paraffin (50-55°C)--1 hour

TABLE 1

FIXATIVE COMPOSITION

10% Buffered Formalin*

Formaldehyde (40%)--10 ml
distilled water--90 ml
Sodium hydrogen phosphate (anhydrous) --350 mg
Disodium hydrogen phosphate (anhydrous)--650 mg

*from Bancroft, 1975 Histochemical Techniques

Carnoy's Fixative*

glacial acetic acid	--10 ml
absolute ethanol	--60 ml
chloroform	--30 ml

*from Humason, 1962 Animal Tissue Techniques

Streck's Tissue Fixative (STF)*

Diazolidinyl Urea (CAS # 78491-02-8)
2-bromo-2-nitropropane-1,3 diol (CAS # 52-51-7)
Zinc Sulfate (CAS # 7446-20-0)
Sodium Citrate (CAS # 68-04-2)

*information provided by Streck Laboratories

The liver was immediately removed from the paraffin bath and embedded in paraffin to create a tissue block. These blocks were cooled and stored at room temperature until needed.

Slides of the liver tissue were prepared by cutting 4 micron sections on an American Optical Company "820" microtome and mounting on albuminized microscope slides. Slides were heated at $40^{\circ}C$ on a slide warmer to set the tissue and evaporate excess water.

C. Preparation of Sperm Slides

Following 24 hours of fixation in either 10% formalin, Carnoy's fixative, or STF, the testes were transferred to 70% ethanol for 24 hours to remove excess fixative. Each testis was then placed in a small dish and minced with a scalpel and teased with sharp probes in fresh 70% ethanol until a milky suspension of sperm and ethanol was obtained. The suspension was placed in 12 ml centrifuge tubes and centrifuged for 10 minutes at 3700 RPM in a Damon IEC HN-SII swinging bucket centrifuge. The supernatant containing the sperm was drawn off and 3 to 4 drops placed on each albuminized slide. The slides were placed on a slide warmer for several hours to set the sperm.

II. MYXOMYCETE TISSUES

A. Cultivation of *Didymium iridis*

Myxamoebal isolates of the myxomycete *Didymium iridis* used in this study were provided by Dr. John Yemma, Youngstown State University, from stock cultures maintained on half-strength cornmeal agar slants. Two compatible mating strains of *D. iridis* were used for this study. They were designated Honduran $7A^2$ (Hon $7A^2$) and Panamanian $2\text{-}21A^{7b}$ (Pan $2\text{-}21A^{7b}$).

The myxamoebal clones (Hon $7A^2$ and Pan $2\text{-}21A^{7b}$) were maintained on half-strength cornmeal (cm/2) agar plates that were previously inoculated with *Escherichia coli* according to the methods described by Yemma and Therrien (1972). *E. coli* was grown

on nutrient agar slants, then washed and transferred into sterile water. The cm/2 agar plates were then flooded with approximately 2 ml of the bacterial solution, and the plate uniformly covered using a sterile bent glass rod.

A 1 cm x 1 cm agar plug containing cells of either clone was then placed cell-side down on the cm/2 agar plate. The plates were stored inverted at room temperature in a Bioflow hood for 4 to 6 days to achieve log-phase growth of the cells. Once log-phase growth had been established, fresh cultures of Hon $7A^2$ or Pan 2-21A^{7b} could be obtained by repeating the above procedures.

The diploid plasmodium form of *D. iridis* was obtained by placing 1 cm² agar plugs of both Hon $7A^2$ and Pan 2-21A^{7b} side by side on a cm/2 agar plate inoculated with *E. coli*. Plates were stored inverted at room temperature and checked daily for formation of plasmodium. When plasmodial growth was evident, the plug containing the plasmodium was cut out and transferred immediately to plasmodium agar, plasmodium-side up. Compositions of both half-strength cornmeal agar and plasmodium agar can be found in Table 2. The plasmodium was then allowed to grow for another 4 to 5 days before fixation.

B. Fixation and Preparation of Myxamoebal Slides

After achieving log-phase growth of the myxamoeba, 30 plates of each strain were fixed, 10 in each of the three fixatives to be studied: 10% formalin, Carnoy's fixative, or Streck's Tissue Fixative.

Each plate was flooded with 15 to 20 ml of fixative for 24 hours. The fixative was then drawn off with a pipette and the plate flooded with 70% ethanol for 1 hour. That washing was drawn off and a fresh bath of 70% ethanol added for 24 hours. The myxamoebal cells were then removed from the agar by gentle washing of the agar surface with a pipette. Care was taken not to disrupt the medium itself.

The ethanol washings containing the myxamoebal cells were then centrifuged for 20 minutes at 1500 RPM in a Damon IEC HN-SII centrifuge. The ethanol supernate was drawn off and discarded, and the pellet resuspended with 70% ethanol and recentrifuged.

TABLE 2

MEDIA

Half-Strength Cornmeal Agar (cm/2):

8.0 g   Difco Agar
8.5 g   Difco Cornmeal Agar
1.0 L   distilled water

Plasmodium Agar:

15.0 g   Difco Bacto-agar
1.0 g   Difco Peptone
1.0 g   Lactose
1.0 L   distilled water

All media were autoclaved at $121^{o}C$ and 15 psi for 15 minutes. Approximately 25 ml aliquots were dispensed into sterile petri dishes, then refrigerated until use.

Several drops of supernate were drawn from the lower third of the centrifuge tube and placed on albuminized microscope slides. The slides were allowed to dry on a slide warmer at 40°C.

C. Fixation and Preparation of Plasmodium Slides

Once the plasmodium had grown on plasmodium agar for 4 to 5 days, the cm/2 plug was removed and the plasmodia fixed in the same manner as the myxamoebal cells: 1) flood plate with 15 to 20 ml fixative (10% formalin, Carnoy's, or STF) and fix for 24 hours; 2) draw off fixative and flood plate with 70% ethanol for 1 hour; 3) draw off ethanol and flood plate with fresh 70% ethanol for 24 hours. Approximately 5 plates were prepared for each fixative.

The fixed plasmodia were "floated" off the agar by gentle washing with a disposable pipette. This process tended to break the plasmodium into smaller pieces which could be drawn into the pipette and then dispensed onto albuminized slides. Several slides could be prepared from each plasmodium. These slides were allowed to dry on a slide warmer.

III. STAINING

A. Hydrolysis Curves

It was found that each fixative has an optimum hydrolysis time when employed in the Feulgen reaction (Pressly, 1978; Kotelnikov and Litinskaya, 1981; Kelley, 1984). Therefore, the following procedures were employed to determine the best hydrolysis period for each of the three fixatives under study--10% formalin, Carnoy's fixative, and Streck's Tissue Fixative (STF).

Twelve slides of liver tissue fixed in each of the three fixatives and cut to 4 micron widths were prepared and were treated simultaneously through the following procedures.

1. Slides were deparaffinized and hydrated through a graded ethanol series (xylene, absolute ethanol, 90% ethanol, 70% ethanol, water), 10 minutes each.

2. Hydrolysis was performed in 5N HCl at room temperature (Jordanov, 1963; DeCosse and Aiello, 1966; Dietch, et al., 1968) for the following times: 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, and 90 minutes respectively. As each slide reached its designated hydrolysis time it was transferred to distilled water until all slides were hydrolyzed.

3. All slides were stained simultaneously in Cresyl Violet stain for 20 minutes (Floyd, 1990; Bidinotto, 1993).

4. Two successive 10 minute sulfite bleach rinses followed to remove extraneous stain.

5. Slides were dehydrated by reversing the hydration sequence (Step 1) and coverslips mounted with Permount.

Fifteen cells from each fixative and hydrolysis time were read on the microspectrophotometer (see Section IV on Microspectrophotometric Methods for details) to establish each fixative's hydrolysis curve.

B. Staining Curves

Dutt (1976) states that the standard practice of staining with basic fuchsin for 1 to 2 hours in the Feulgen procedure is unnecessary, as all aldehyde groups of hydrolyzed DNA are stained within 15 minutes, yet many workers continue to stain for extended periods of time (Kotelnikov and Litinskaya, 1981; Giroud and Montmasson, 1989). Cresyl violet has not been used as widely as the Feulgen reaction and has only recently been discovered to be a quantitative stain (Bidinotto, 1993). Thus optimal staining time has not been previously investigated regarding this stain. Therefore, this study included development of a staining curve for each fixative studied.

Five slides of liver cells, cut in 4 micron widths, were prepared for each of the three fixatives. All slides were treated simultaneously, beginning with deparaffinization through a xylene-ethanol series as mentioned previously. Formalin and Carnoy's-fixed liver sections were hydrolyzed for 45 minutes in 5N HCl at room temperature, while STF-fixed

slides received 60 minutes of hydrolysis (these were the optimum hydrolysis times determined previously).

After a water rinse, slides were placed in cresyl violet stain for 20, 30, 40, 50, 60 and 90 minutes (STF only), respectively. Two successive 10 minute sulfite bleach rinses followed, then the slides were dehydrated through an ethanol to xylene series and coverslipped with Permount.

Fifteen cells from each slide were read on a microspectrophotometer (see Section IV of Methods for details) to determine the staining curve for each fixative.

C. Staining Protocol

Once the optimum hydrolysis and staining times had been determined, slides of all four tissue types (liver, sperm, myxamoeba, and plasmodium) were treated according to the modified Feulgen protocol found in Table 3. STF-fixed tissues were hydrolyzed for 60 minutes, while Carnoy's and formalin-fixed tissues were hydrolyzed for 45 minutes. All slides were stained for 60 minutes. The hydration sequence was followed only for paraffin-embedded liver tissue. All other tissues began treatment with 5N HCl hydrolysis.

IV. MICROSPECTROPHOTOMETRIC METHODS

This study employed the two-wavelength method of microspectrophotometry (Patau, 1952; Ornstein, 1952; Mendelsohn, 1961). This method is particularly useful because nuclei with heterogeneous distribution of DNA can be used as well as homogeneous nuclei. Also, measurement of nuclear area is not necessary (Mayall and Mendelsohn, 1970).

All readings were taken on a Zeiss Type 01 microspectrophotometer using a Planachromat oil immersion objective, N.A. 1.30 x 100. The wavelength of light was controlled using a Zeiss continuous interference-filter monochrometer. The linearity and alignment of the light source were checked each time any reading were taken.

In order to measure the relative amounts of absorbing material present, the two-wavelength method depends upon accurately establishing the wavelength of maximum

TABLE 3

MODIFIED BLUE FEULGEN REACTION PROTOCOL

Hydration sequence: (paraffinized slides only)

xylene	-- 10 min.
absolute ethanol	-- 10 min.
90% ethanol	-- 10 min.
70% ethanol	-- 10 min.
distilled water	-- 10 min.

Hydrolysis: (at room temperature) 5N HCl

formalin or Carnoy's-fixed	-- 45 min.
STF-fixed	-- 60 min.
distilled water	-- rinse

Staining:

cresyl violet stain	-- 60 min.
sulfite bleach	-- 10 min.
sulfite bleach	-- 10 min.

Dehydration sequence:

distilled water	-- 10 min.
70% ethanol	-- 10 min.
90% ethanol	-- 10 min.
absolute ethanol	-- 10 min.
xylene	-- 10 min.

Coverslips were immediately adhered with Permount.

TABLE 4

STAIN AND SULFITE BLEACH COMPOSITIONS

Cresyl Violet Stain

0.02 g cresyl violet acetate*
1.0 L distilled water

-- Stir mechanically until dissolved.
-- Store in a dark bottle in refrigerator until use.
-- Add 1.0 g sodium dithionite per 50 ml stain to decolor and fortify before use.

*SIGMA Chemical Company C-1791

Sulfite Bleach

10 ml	10% potassium metabisulfite
10 ml	1N HCl
200 ml	distilled water

--Mix up fresh before each use.

absorption (λ_2) for the particular dye-fixative complex being employed, and then determining the wavelength (λ_1) which will provide one-half the extinction (E) of λ_2. The extinction is the ratio of the log of the intensity of background light (I_0) to the intensity of light transmitted through the specimen (I_s), where $E = \log(I_0/I_s)$. Thus, λ_1 is found so that the extinctions form a 2:1 ratio. For this study a separate absorption curve was established for each of the three fixatives (formalin, Carnoy's, and STF) using liver cells. One curve was sufficient for each fixative since all tissues for each fixative were hydrolyzed and stained under the same conditions (Patau, 1952; Kelley, 1984).   Ten-percent formalin had $\lambda_2 = 625$ nm and $\lambda_1 = 540$ nm. Carnoy's fixative had $\lambda_2 = 620$ nm and $\lambda_1 = 535$ nm. Streck's Tissue Fixative had $\lambda_2 = 630$ nm and $\lambda_1 = 555$ nm. These wavelengths are listed in Table 5.

Once the two wavelengths were established for the particular fixative, four transmission readings were taken for each cell: 1) aperture centered on the nucleus at maximum wavelength (I_s at λ_2), 2) the nucleus at half-maximum wavelength (I_s at λ_1), 3) background (I_0) at λ_1, and 4) background at λ_2. The transmission (T) at each wavelength is then $T_1 = I_0/I_s$ at λ_1 and $T_2 = I_0/I_s$ at λ_2. Furthermore, the absorbance (L) at each wavelength is $L_1 = 1-T_1$ and $L_2 = 1-T_2$. The aperture, which adjusts the photometric field, was selected to encompass the entire nucleus while minimizing the unstained space surrounding it. One hundred cells were read at random for each tissue and fixative combination.

To correct for distributional error due to unoccupied space, the ratio between the absorbencies (Q) was calculated, where $Q = L_2/L_1$. The Q value is used to determine C, the correction factor between the two wavelengths. $C = 1/(2-Q) \ln 1/(Q-1)$, but Patau's 1952 paper lists the values of C as a function of Q so actual computation of C was unnecessary.

The amount of absorbing material, or chromophore, (M) present in the measuring field (A) of each nucleus may be determined using the equation $M = kAL_1C$, where k is the extinction coefficient of the dye-DNA complex at the particular wavelength. Extinction

TABLE 5

ABSORPTION WAVELENGTHS FOR CRESYL VIOLET

Fixative	Tissue	λ_{max}	$\lambda_{1/2max}$
10% buffered formalin	liver	625 nm	540 nm
Carnoy's fixative	liver	620 nm	535 nm
Streck's Tissue Fixative	liver	630 nm	555 nm

coefficient values were omitted from these calculations, however, since DNA values presented in relative (arbitrary) units have been proven to be quantitatively related to absolute DNA values and were sufficient for this study. For the sake of accuracy, all computations of relative DNA values were performed on an IBM 470 Model B-5 mainframe computer at Youngstown State University by a program developed by Dr. John Yemma.

RESULTS

I. Spectral Absorption Curves

The two wavelength method of microspectrophotometry (Ornstein, 1952; Patau, 1952) requires the establishment of wavelengths of maximum and one-half maximum extinction for the stain being used. The fixative employed may affect stain binding (Swift, 1950; Dutt, 1971, 1976; Giroud and Montmasson, 1989), so it is necessary to establish the absorption maxima and, thus, the two wavelengths for each fixative-chromophore combination under study in order that quantitative measurements may be made (Patau, 1952; Ornstein, 1952). Figures 2-4 show the absorption curves for the chromophore cresyl violet on liver cells fixed in 10% formalin, Carnoy's fixative, and the new fixative Streck's Tissue Fixative (STF).

Figure 2 shows the maximum extinction for liver cells fixed with 10% formalin and stained with cresyl violet to occur at 625 nm. One-half this extinction occurs at wavelength 540 nm. The absorption curve for cresyl violet-stained cells prepared with Carnoy's fixative (Figure 3) demonstrates a plateau between 570 nm and 640 nm and a maximum extinction at 620 nm. One-half maximum extinction occurs at 535 nm for cresyl violet with Carnoy's fixative. Cresyl violet, in combination with STF (Figure 4), shows a maximum extinction at wavelength 630 nm and one-half maximum extinction at 555 nm. These results are summarized in tabular form in Table 5. Each fixative-chromophore combination exhibited a characteristic plateau, or shoulder, around the wavelength of maximum absorption. This also occurs when similar data is plotted for the Feulgen reaction (using basic fuchsin) (Giroud and Montmasson, 1989; Yemma and Therrien, 1972). The plateau is the area of maximum absorption.

Statistical analysis (using analysis of variance) showed no significant difference between the maximum wavelengths of absorption for these three fixatives. All microspectrophotometric readings could have been made at the same wavelength, but the individual maximum wavelengths were used for the sake of accuracy.

FIGURE 2

Spectral absorption curve for cresyl violet stain on liver cells fixed with 10% buffered formalin.
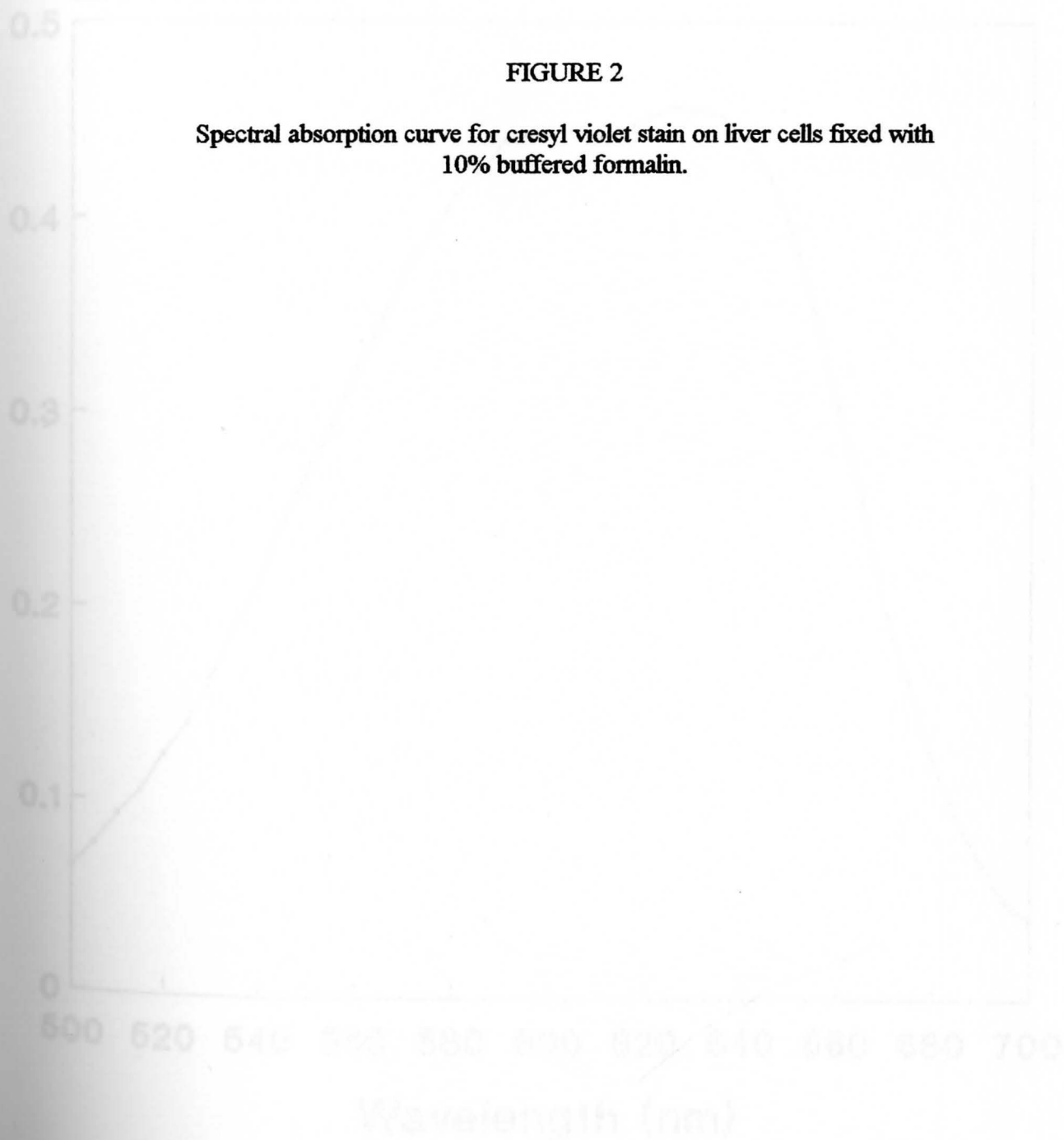
Absorption Curve
10% Formalin

FIGURE 3

Spectral absorption curve for cresyl violet stain on liver cells fixed with
Carnoy's fixative.
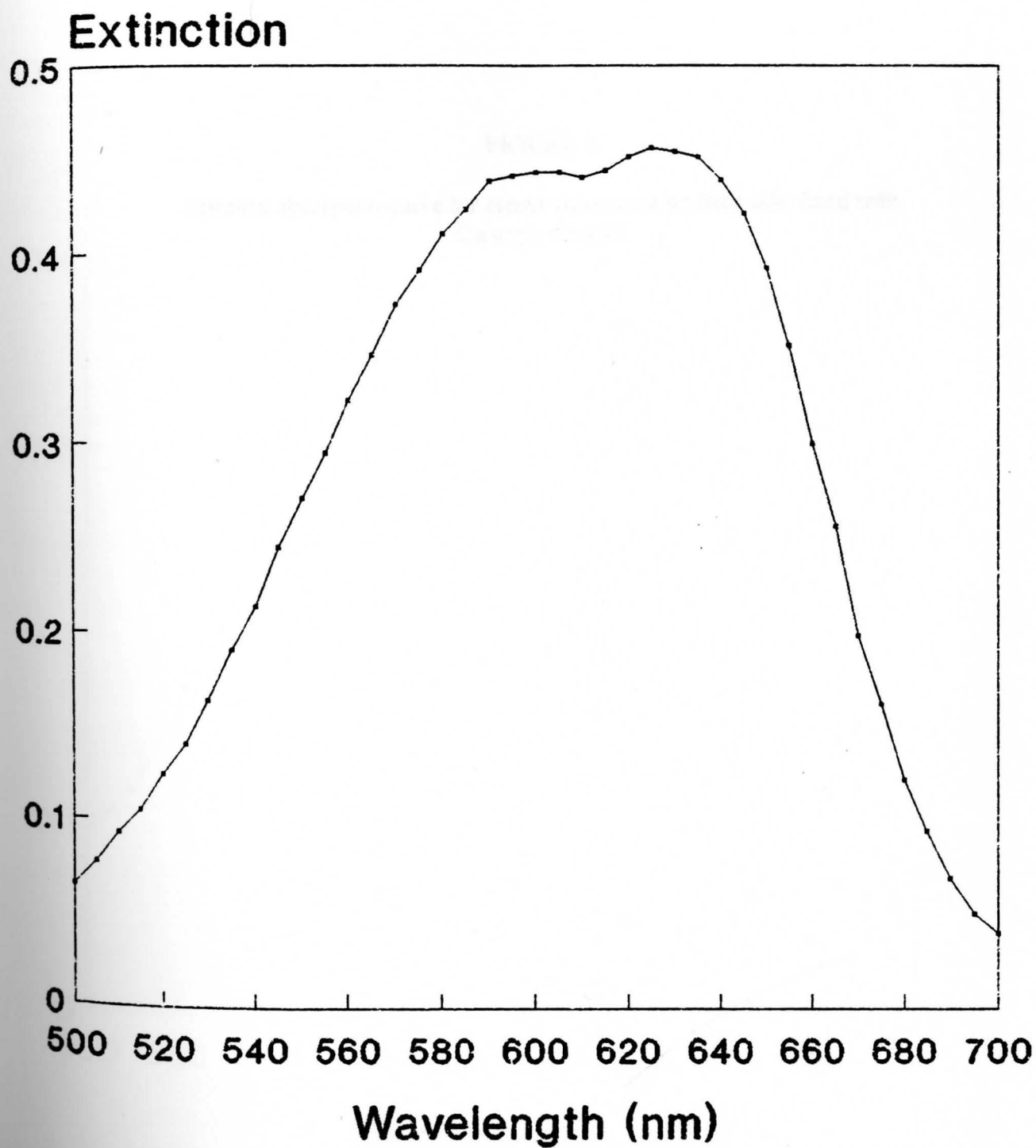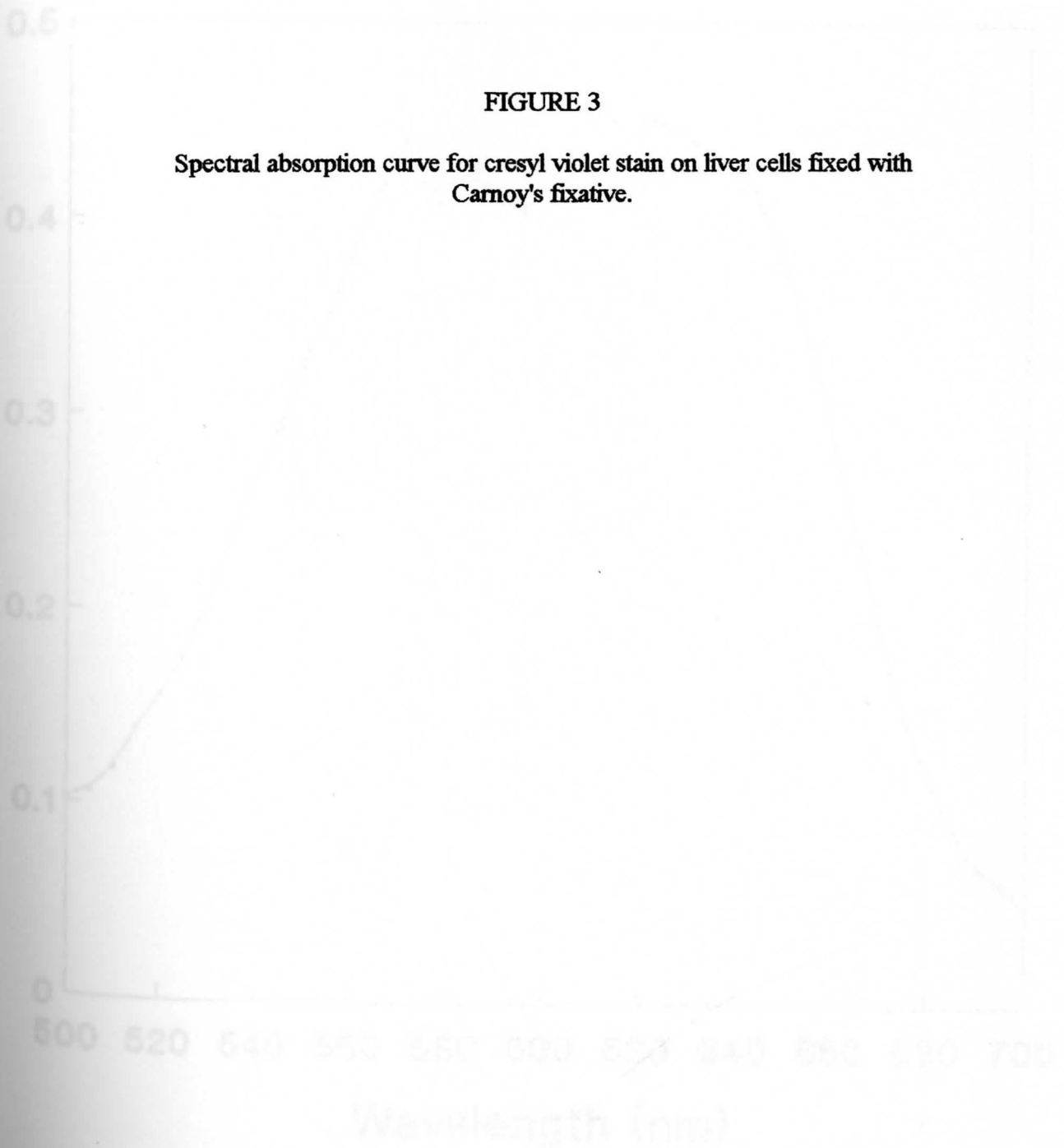
Absorption Curve
Carnoy's
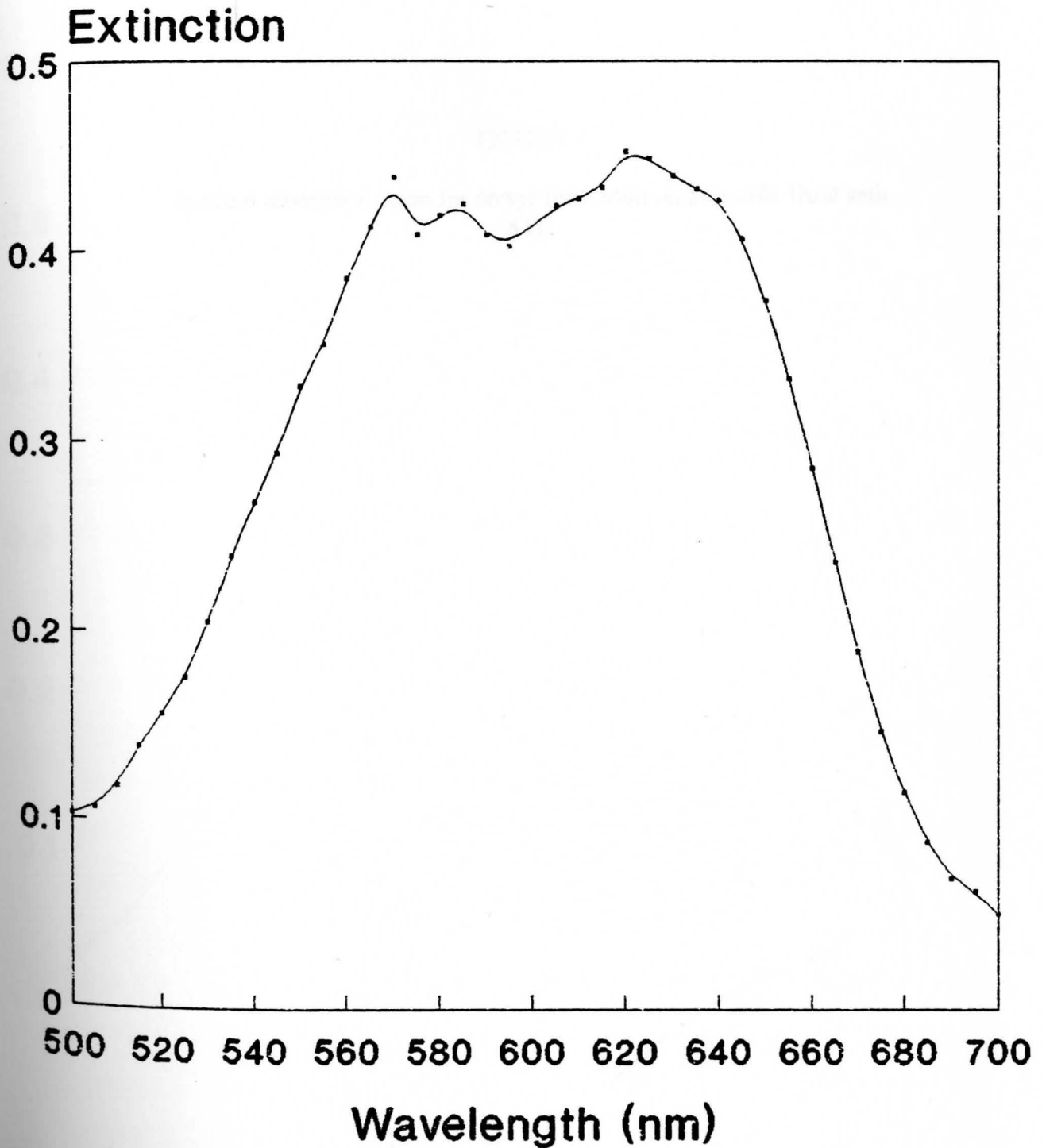
FIGURE 4

Spectral absorption curve for cresyl violet stain on liver cells fixed with STF.
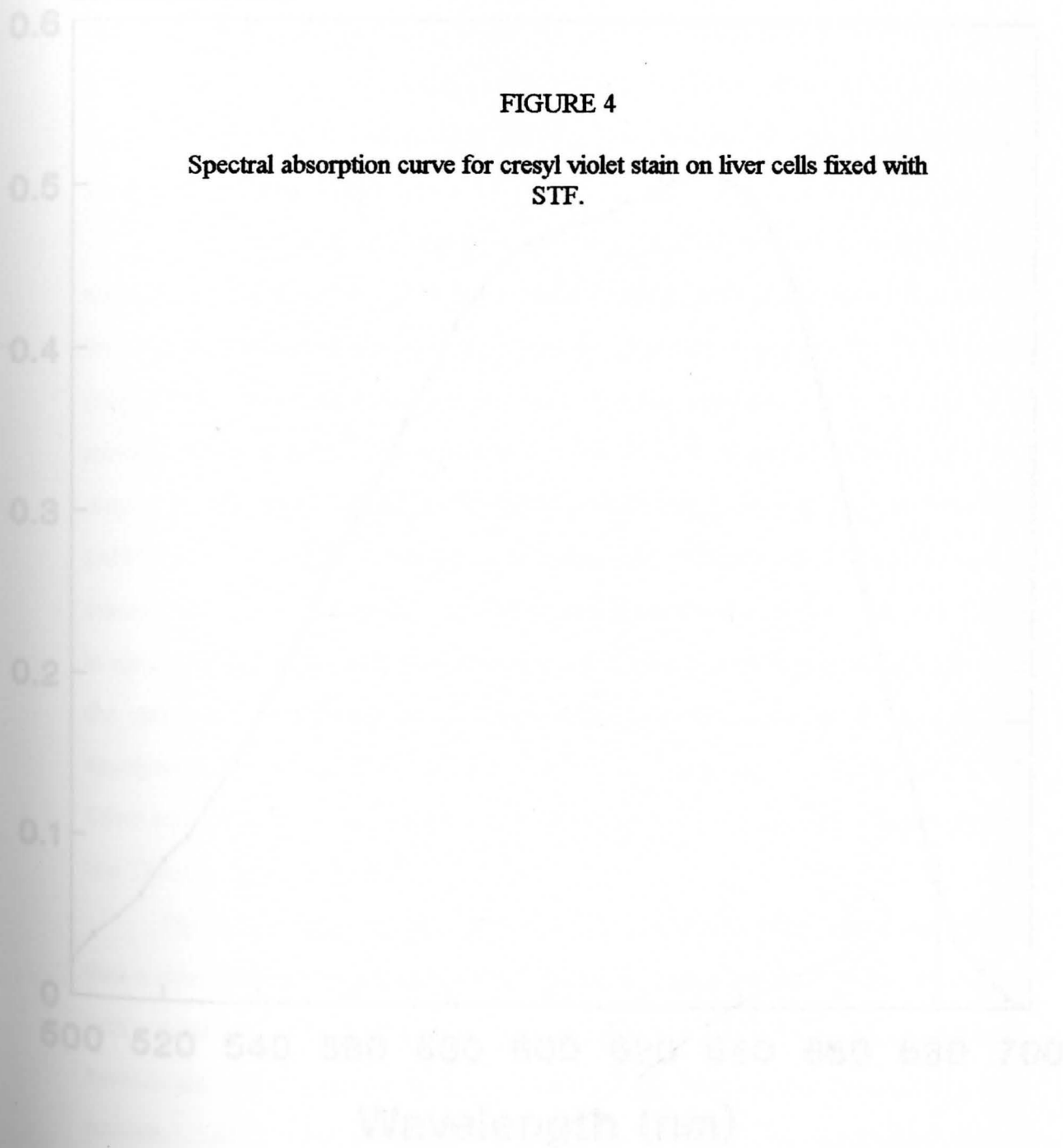
Absorption Curve
STF
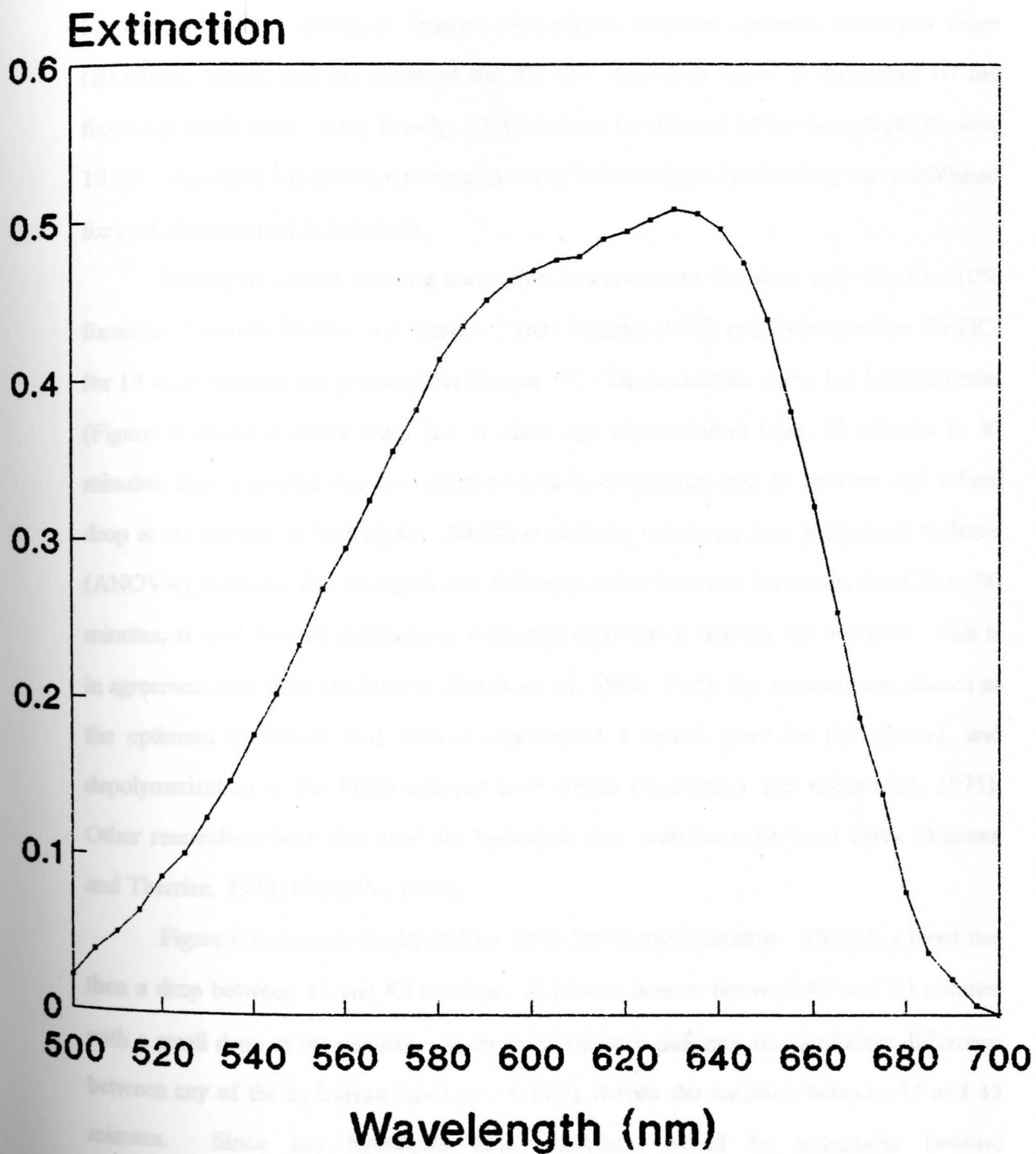
II. Hydrolysis Curves

The development of maximum staining intensity in the Feulgen reaction is dependent upon adequate hydrolysis time for depurination of the DNA. However, excessive hydrolysis must be avoided as progressive depolymerization and loss of DNA may occur during the post-optimum hydrolysis period (diStefano, 1948; Andersson and Kjellstrand, 1975). Different fixatives will exhibit different optimum hydrolysis times (diStefano, 1948), and the shape of the 5N HCl hydrolysis curve is dependent on the fixative (Deitch, *et al.*, 1968; Pressly, 1978) and can be affected by the tissue type (Pressly, 1978). Therefore, an hydrolysis curve showing Blue Feulgen dye binding was established for each fixative used in this study.

Hydrolysis curves showing mean dye concentrations for liver cells fixed in 10% formalin, Carnoy's fixative, and Streck's Tissue Fixative (STF) and hydrolyzed in 5N HCl for 15 to 90 minutes are presented in Figures 5-7. The hydrolysis curve for 10% formalin (Figure 5) shows a rather steep rise in mean dye concentration from 15 minutes to 30 minutes, then a gradual rise to a plateau between 45 minutes and 80 minutes and a final drop at 90 minutes of hydrolysis. Statistical analysis, using one way analysis of variance (ANOVA), indicates that no significant difference exists between the means from 30 to 90 minutes, at 0.05 level of significance, indicating a plateau in staining has occurred. This is in agreement with data obtained by Deitch, *et al.*, 1968. Forty five minutes was chosen as the optimum hydrolysis time since it represented a central point on this plateau, and depolymerization of the DNA will not have begun (Andersson and Kjellstrand, 1975). Other researchers have also used this hydrolysis time with formalin-fixed tissue (Yemma and Therrien, 1972; Bidinotto, 1993).

Figure 6 represents the hydrolysis curve for Carnoy's fixative. There is a rapid rise then a drop between 15 and 45 minutes. A plateau is seen between 45 and 80 minutes with a small drop at 90 minutes. Analysis of variance indicates no significant difference between any of the hydrolysis times ($p = 0.069$), despite the variation between 15 and 45 minutes. Since any hydrolysis time, therefore, would be acceptable because

FIGURE 5

Hydrolysis curve for liver cells fixed in 10% buffered formalin.

Hydrolysis was performed in 5N HCl at room temperature. Dye concentration is recorded in arbitrary units.
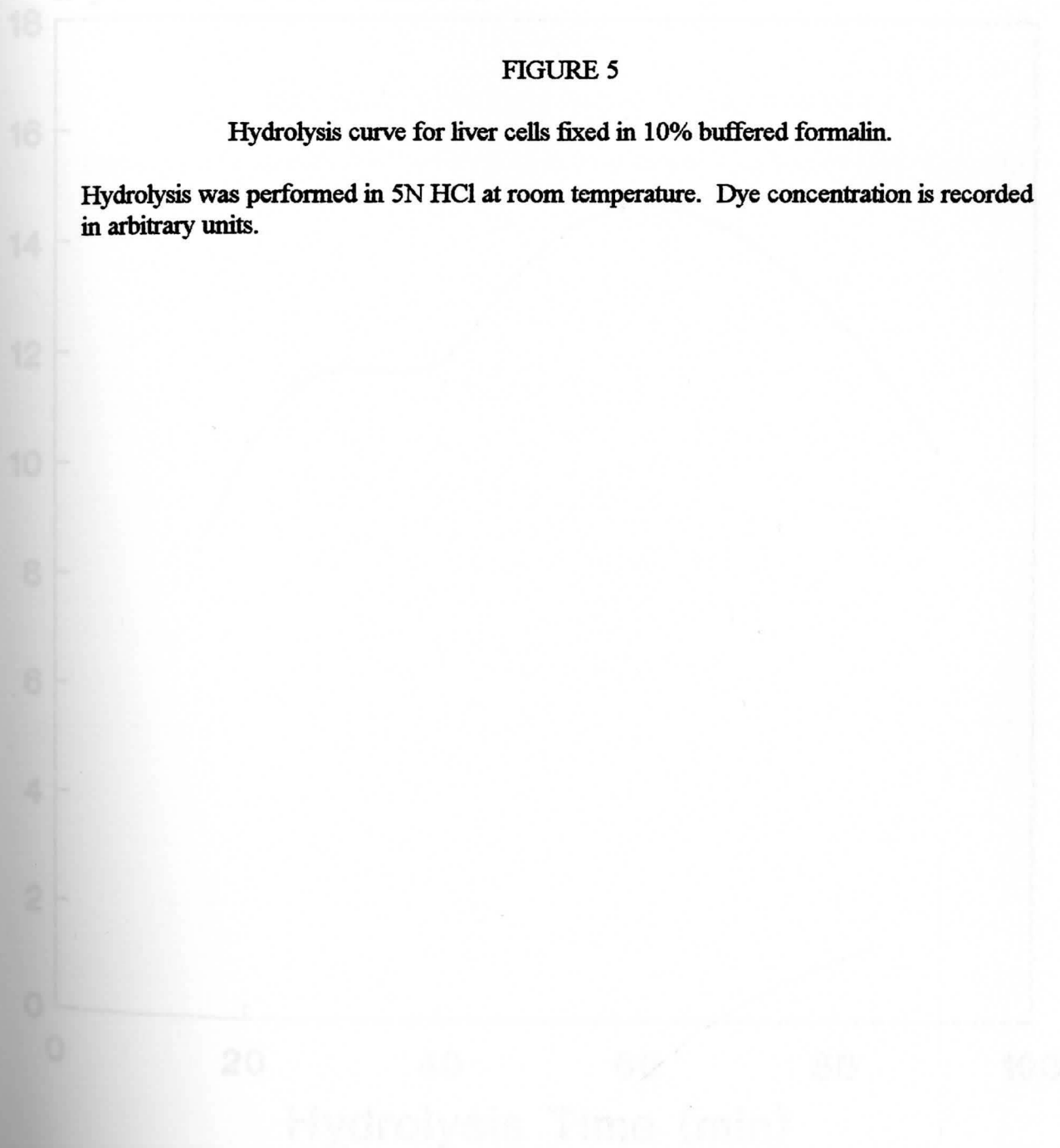
Hydrolysis Curve
10% Formalin

Dye Concentration

Hydrolysis Time (min)

FIGURE 6

Hydrolysis curve for liver cells fixed in Carnoy's fixative.

Hydrolysis was performed in 5N HCl at room temperature. Dye concentration is recorded in arbitrary units.
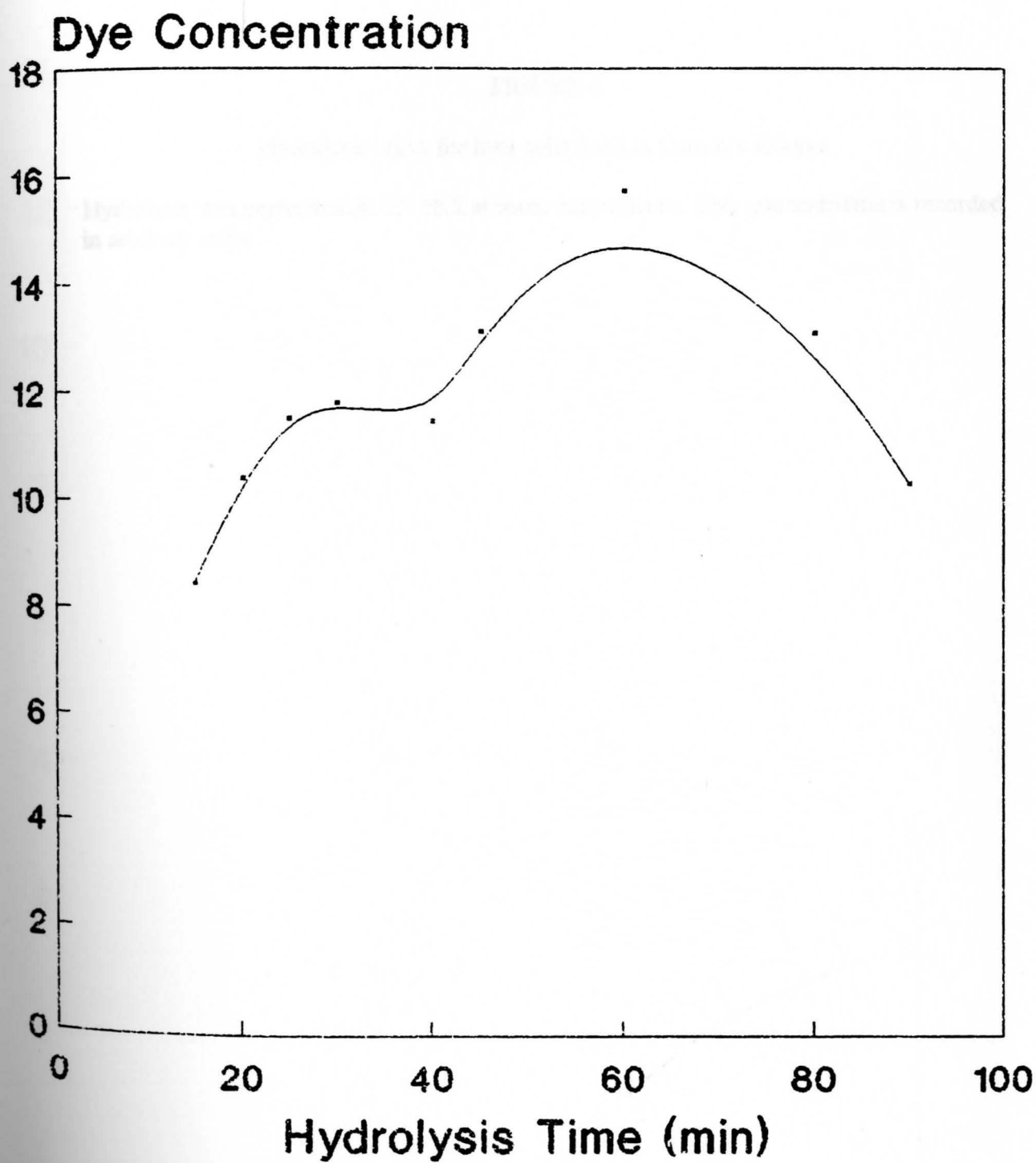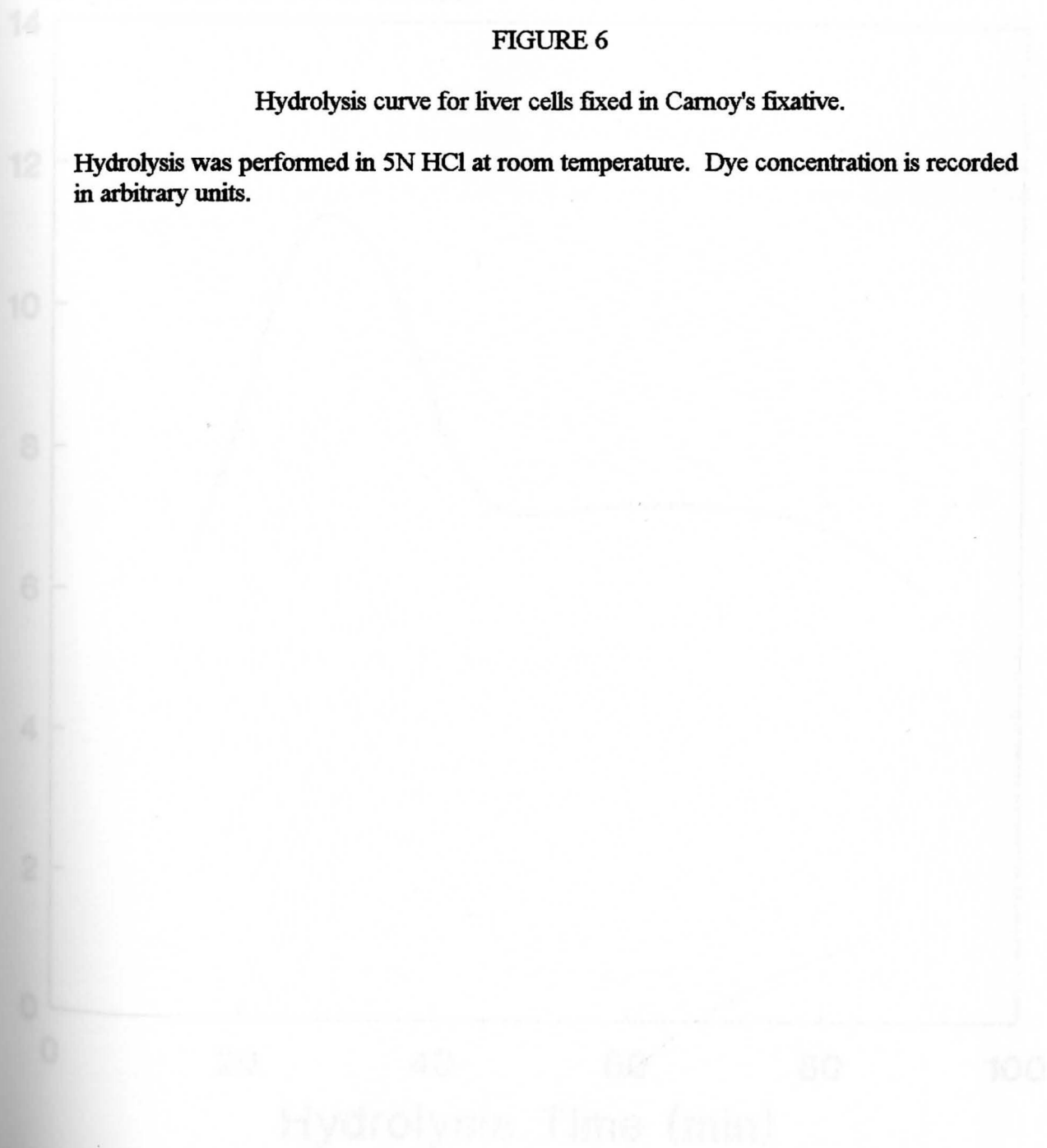
Hydrolysis Curve
Carnoy's
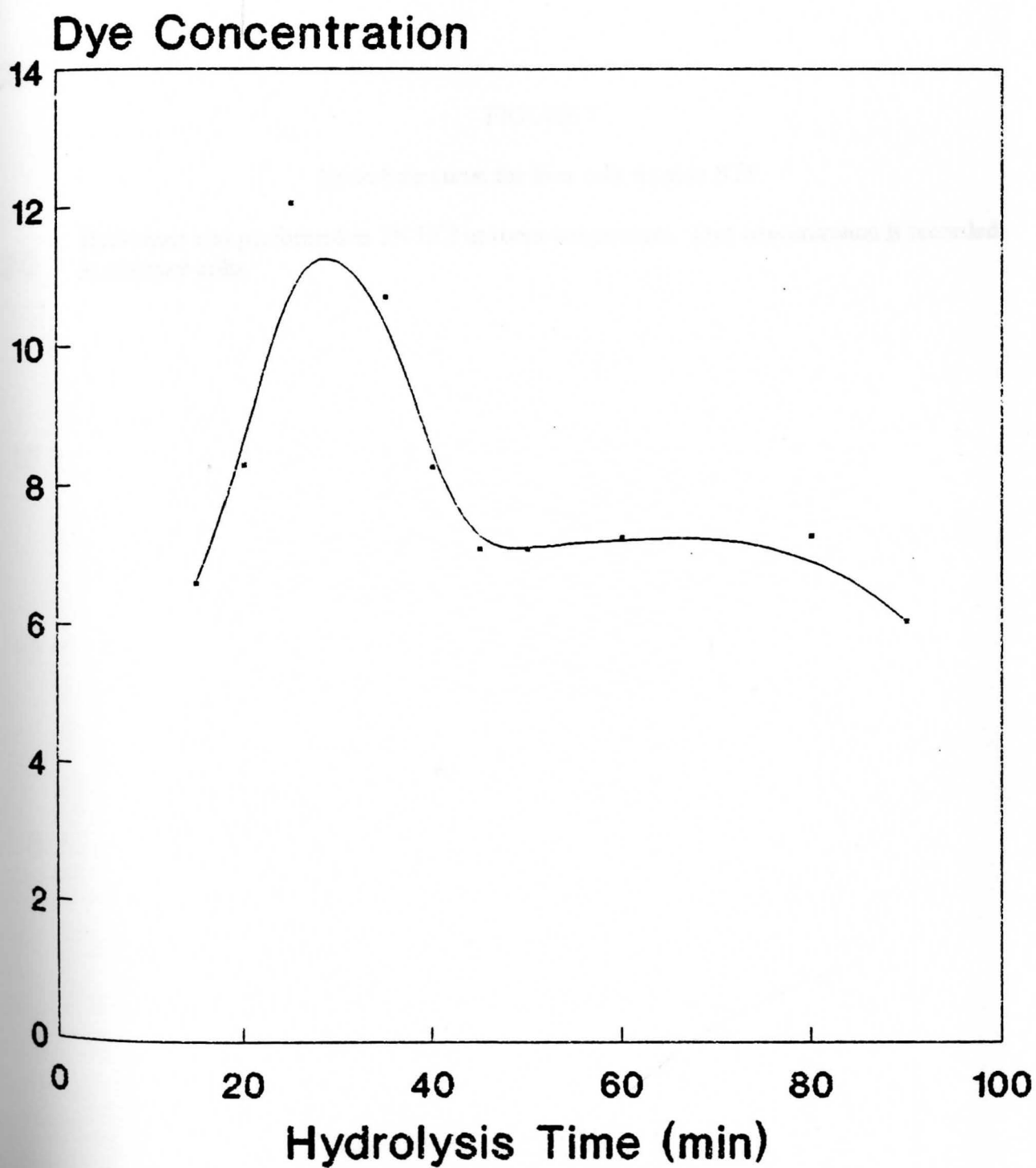
FIGURE 7

Hydrolysis curve for liver cells fixed in STF.

Hydrolysis was performed in 5N HCl at room temperature. Dye concentration is recorded in arbitrary units.
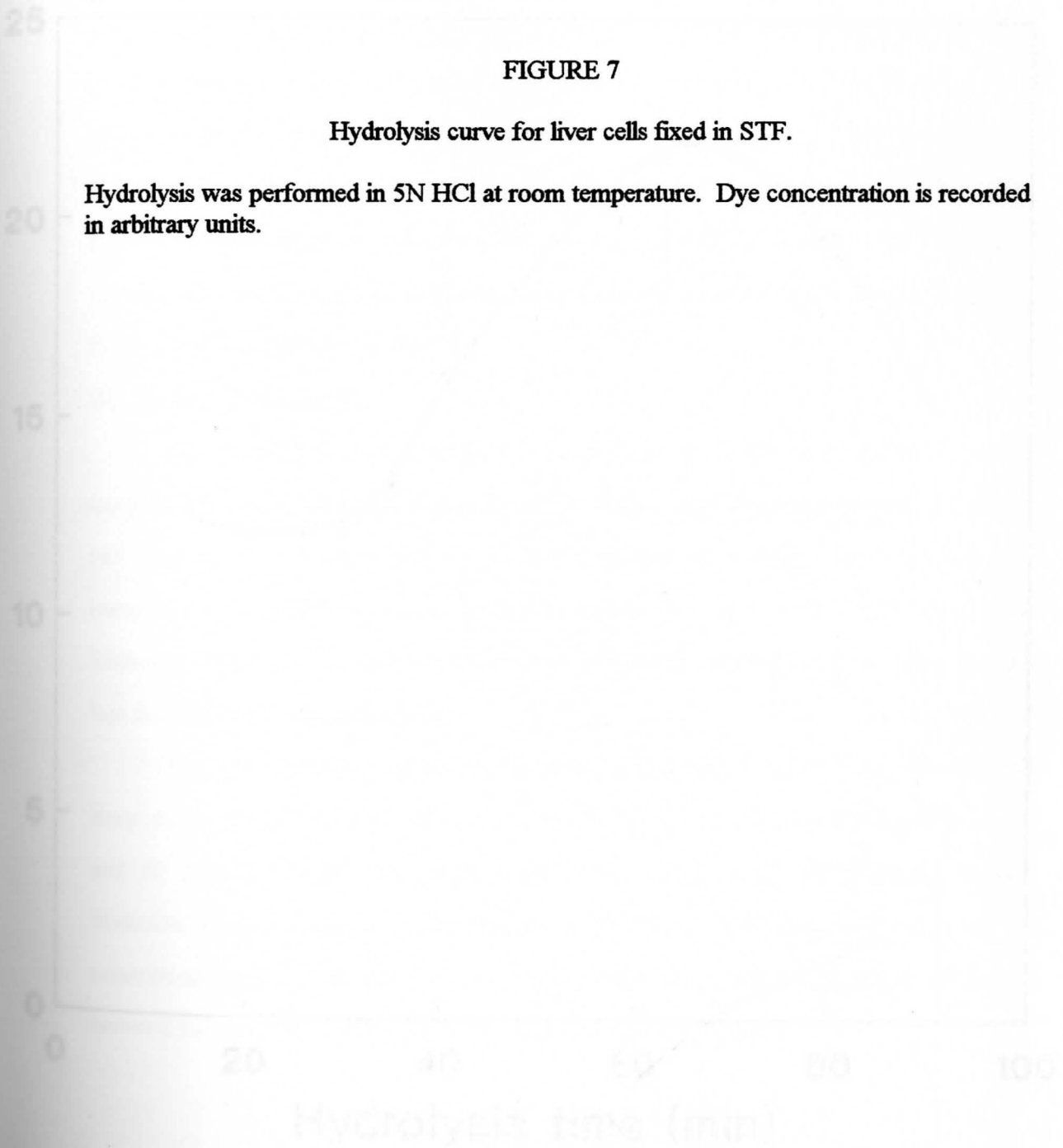
Hydrolysis Curve
STF
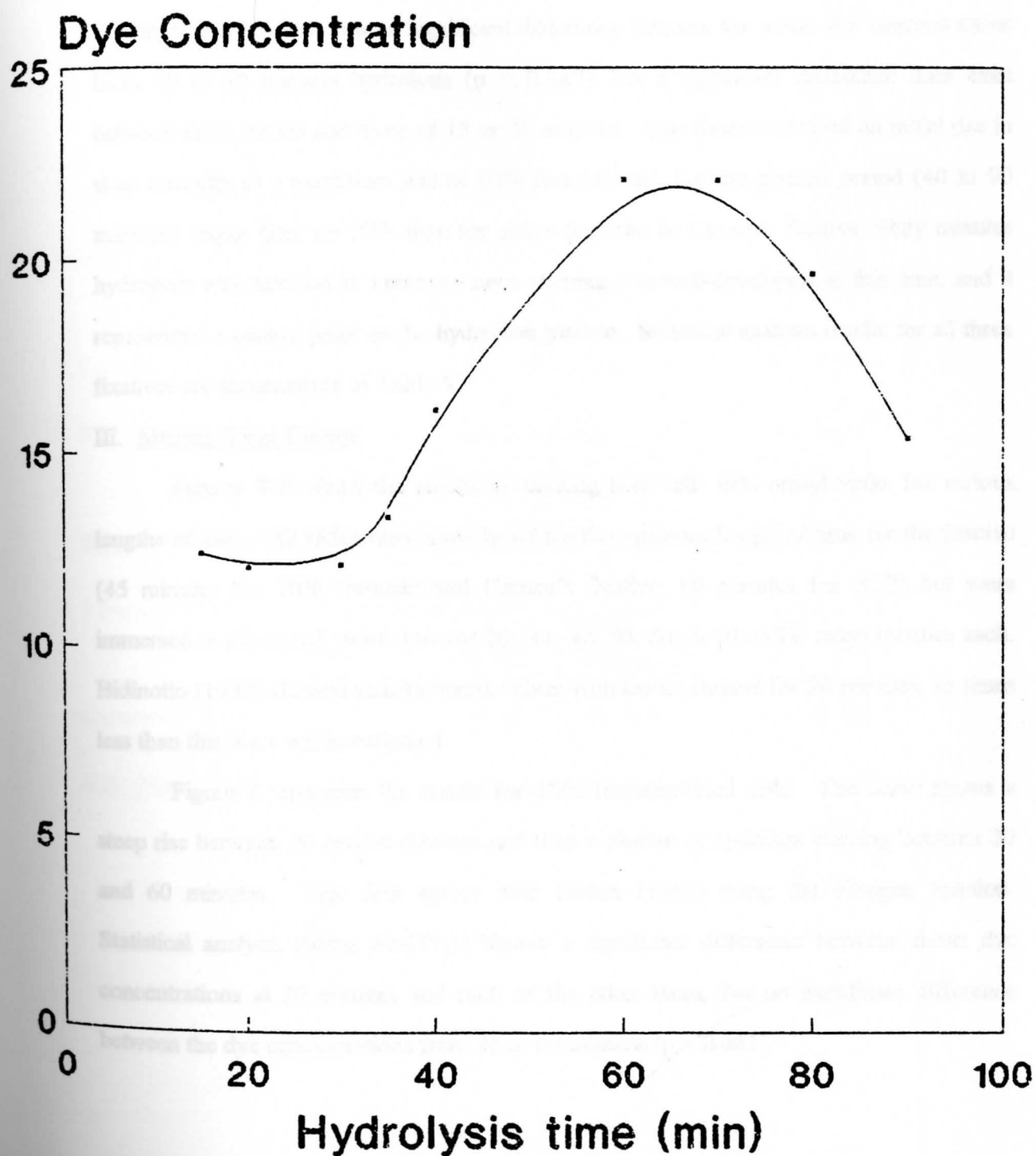
depolymerization has not begun, a point along the extended plateau (45 minutes) was chosen as the optimum hydrolysis time.

The hydrolysis curve for STF is shown in Figure 7. This fixative showed a short plateau between 15 and 30 minutes, then a steep rise in dye concentration to 60 minutes. A short plateau occurred between 60 and 80 minutes followed by a decline at 90 minutes. Statistical analysis indicates no significant difference between the mean dye concentrations from 40 to 90 minutes hydrolysis ($p = 0.147$), but a significant difference does exist between these means and those of 15 or 35 minutes. This fixative showed an initial rise in stain intensity to a maximum just as 10% formalin did, but the plateau period (40 to 90 minutes) began later for STF than for either formalin or Carnoy's fixative. Sixty minutes hydrolysis was selected as optimum since staining was well-developed at this time, and it represented a central point on the hydrolysis plateau. Statistical analysis results for all three fixatives are summarized in Table 6.

III. Staining Time Curves

Figures 8-10 show the results of staining liver cells with cresyl violet for various lengths of time. All slides were hydrolyzed for the optimum length of time for the fixative (45 minutes for 10% formalin and Carnoy's fixative, 60 minutes for STF) but were immersed in the cresyl violet stain for 20, 30, 40, 50, 60, or 90 (STF only) minutes each. Bidinotto (1993) showed stoichiometric values with tissues stained for 20 minutes, so times less than this were not investigated.

Figure 8 represents the results for 10% formalin-fixed cells. The curve shows a steep rise between 20 and 30 minutes and then a plateau of optimum staining between 30 and 60 minutes. This data agrees with Deitch (1968) using the Feulgen reaction. Statistical analysis (using ANOVA) shows a significant difference between mean dye concentrations at 20 minutes and each of the other times, but no significant difference between the dye concentrations from 30 to 60 minutes ($p = 0.461$).

TABLE 6

STATISTICAL ANLYSIS OF HYDROLYSIS AND STAINING CURVES

Statistical Results using one way Analysis of Variance (ANOVA)

HYDROLYSIS CURVES	TIMES	CONFIDENCE LEVEL
Formalin (Figure 5)	15, 45, 60 min.	p = 0.041 *
	30, 40, 45, 60, 80, 90 min.	p = 0.335
Carnoy's (Figure 6)	15 through 90 min.	p = 0.069
STF (Figure 7)	15, 40, 60 min.	p = 0.014 *
	40, 60, 70, 80 90 min.	p = 0.147

STAIN INTENSITY CURVES	TIMES	CONFIDENCE LEVEL
Formalin (Figure 8)	20, 30, 40, 50, 60 min.	p = 0.0254 *
	30, 40, 50, 60 min.	p = 0.461
Carnoy's (Figure 9)	20, 40, 50, 60 min.	p = 0.252
STF (Figure 10)	60 & 90 min.	p = 0.0052 *
	30, 40, 50, 60 min.	p = 0.184

*significant difference (p ≤ 0.05)

FIGURE 8

Stain Intensity Curve for 10% Buffered Formalin

Mean dye concentrations are presented in arbitrary units for liver cells fixed in 10% buffered formalin and stained in cresyl violet for various times. All cells were hydrolyzed for 45 minutes.
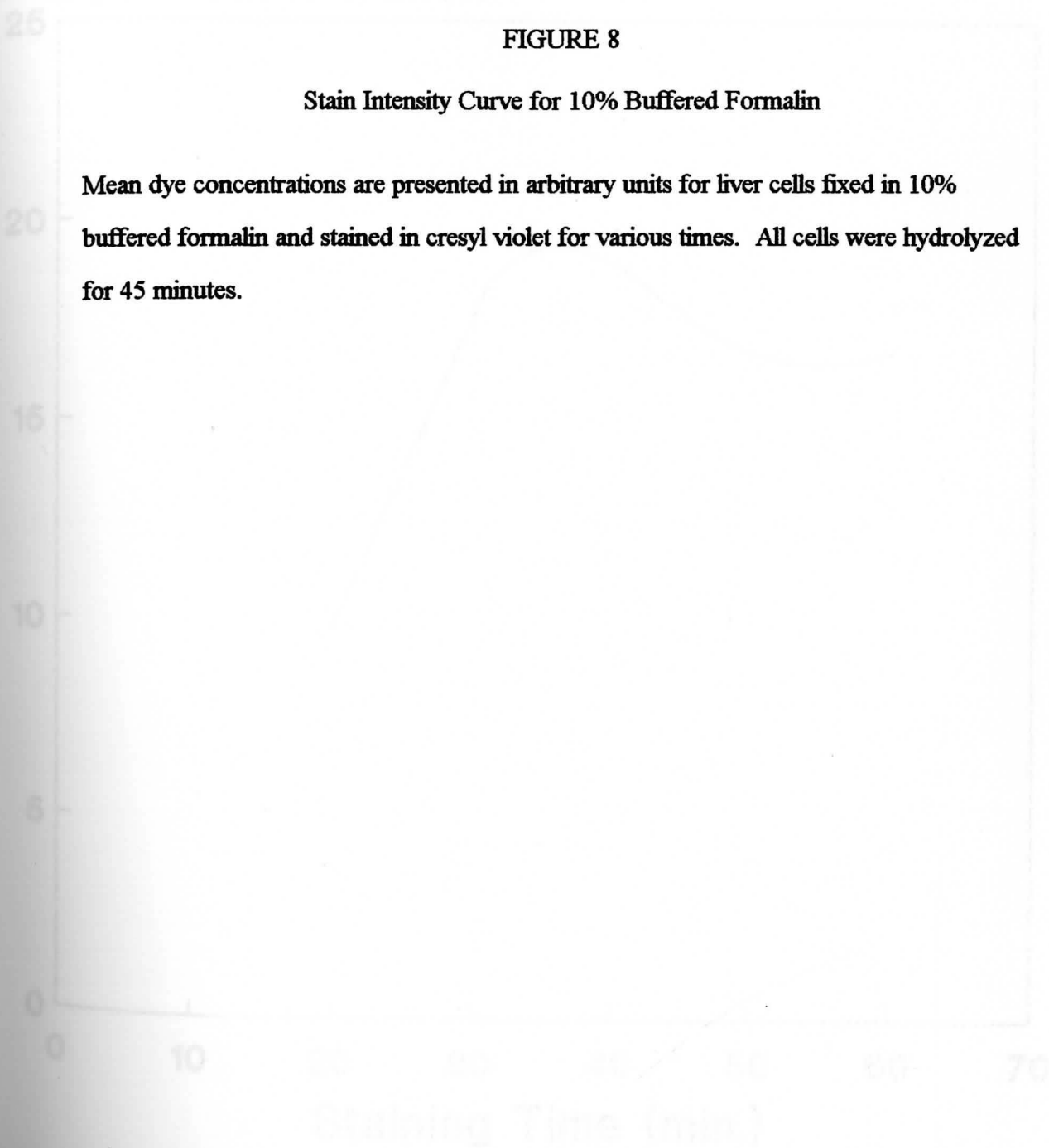
Stain Intensity
10% Formalin

Dye Concentration

Staining Time (min.)

n = 15
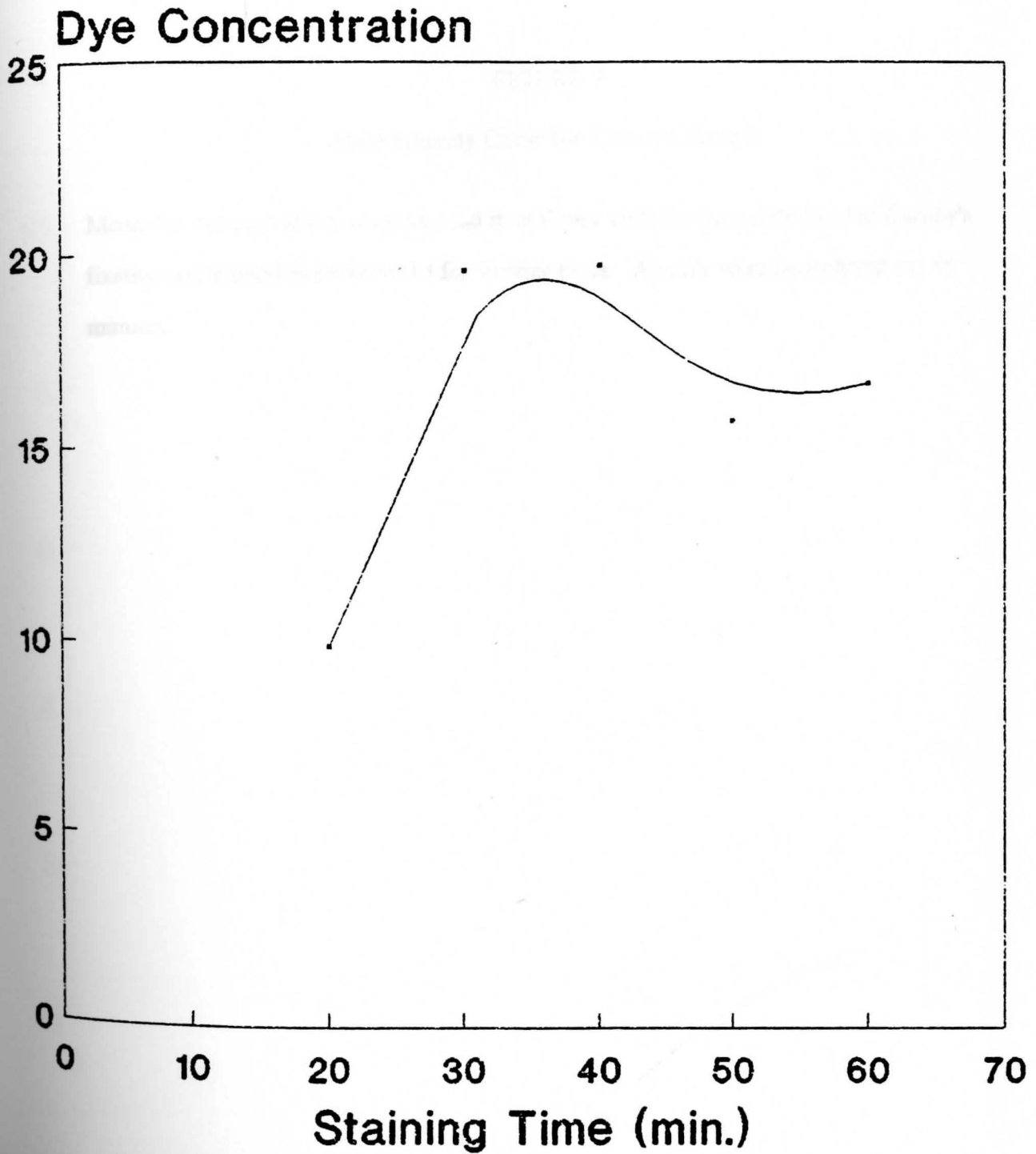
FIGURE 9

Stain Intensity Curve for Carnoy's Fixative

Mean dye concentrations are presented in arbitrary units for liver cells fixed in Carnoy's fixative and stained in cresyl violet for various times. All cells were hydrolyzed for 45 minutes.
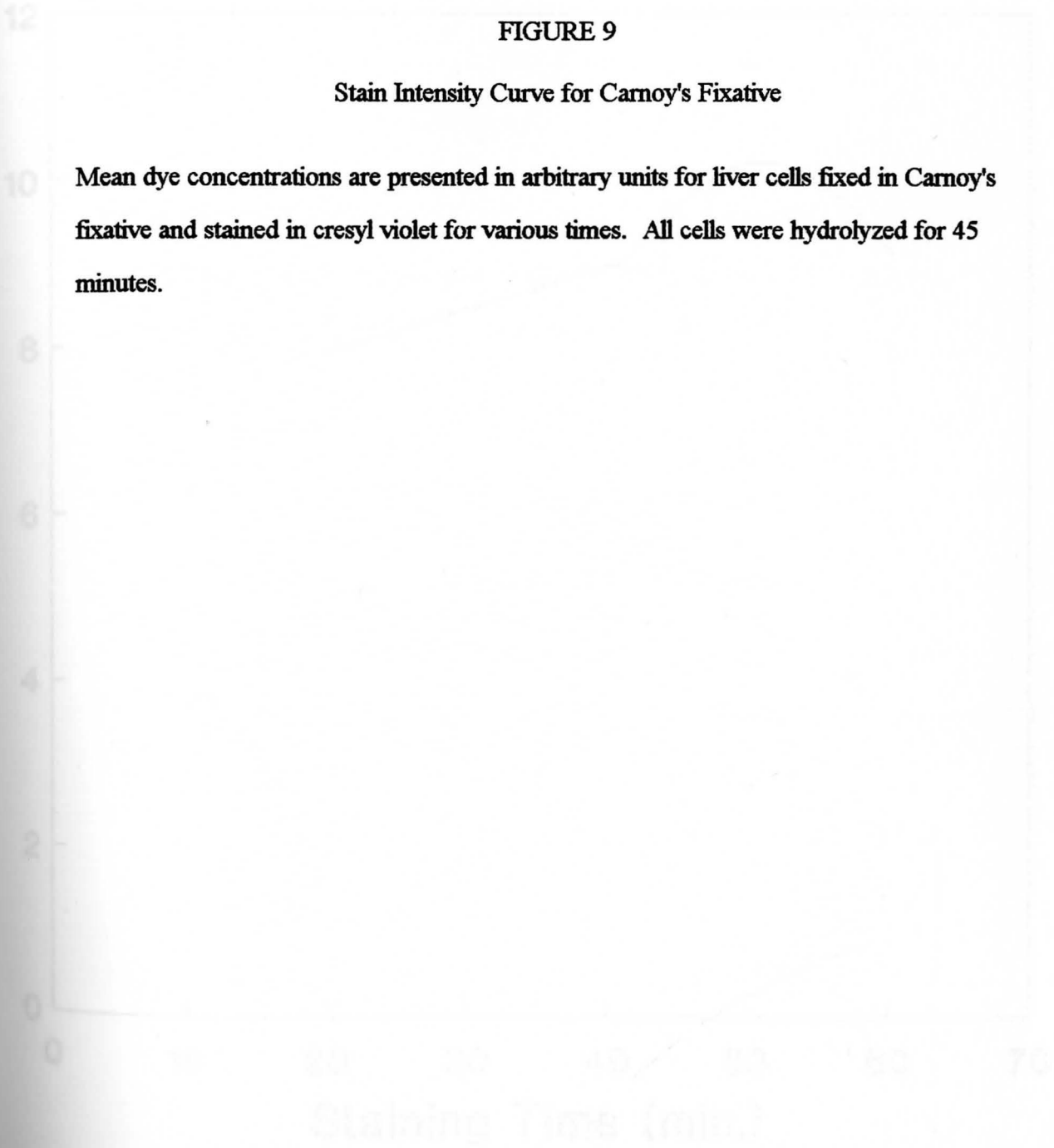
Stain Intensity
Carnoy's

Dye Concentration

Staining Time (min.)

n = 15
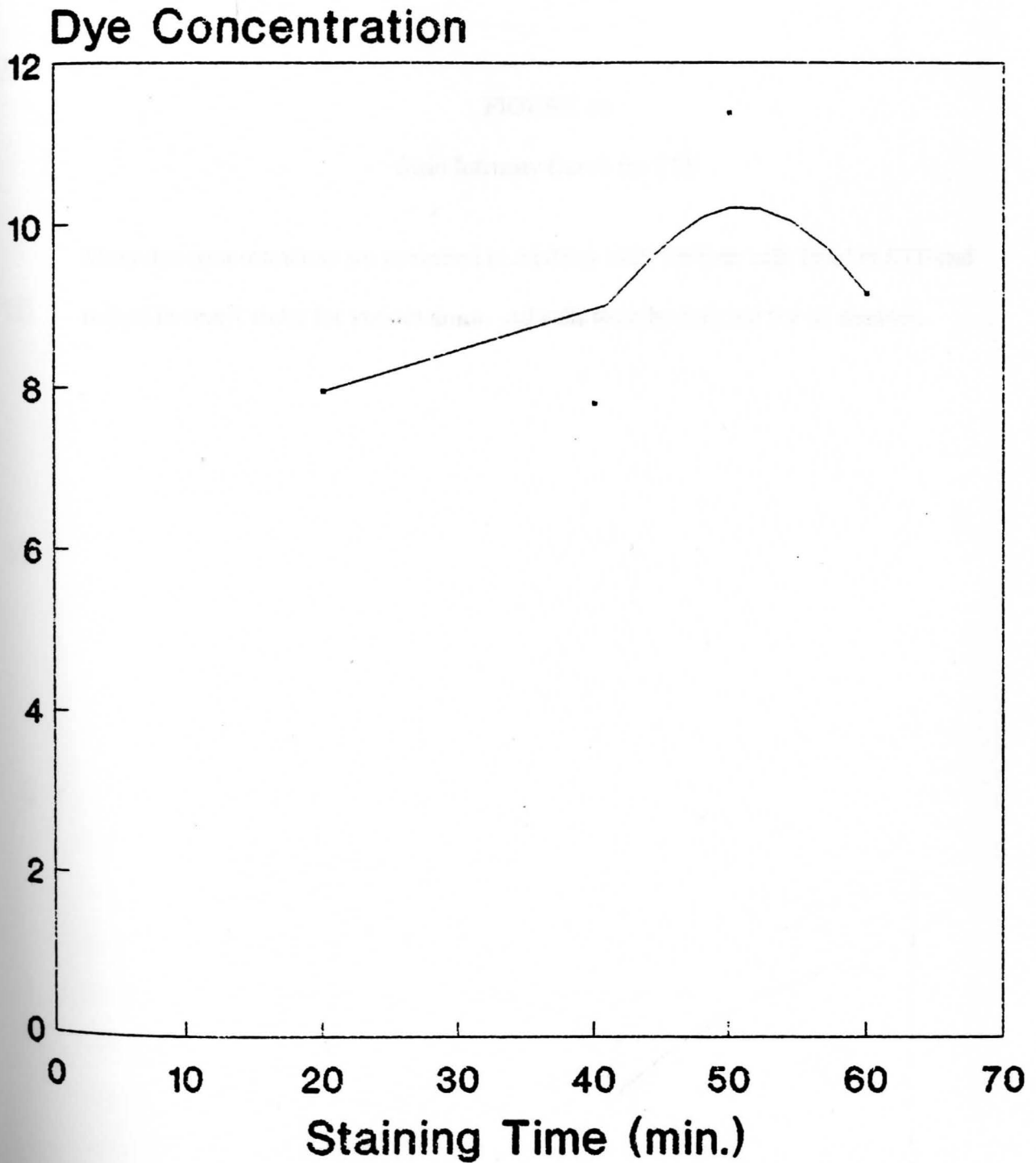
FIGURE 10

Stain Intensity Curve for STF

Mean dye concentrations are presented in arbitrary units for liver cells fixed in STF and stained in cresyl violet for various times. All cells were hydrolyzed for 60 minutes.
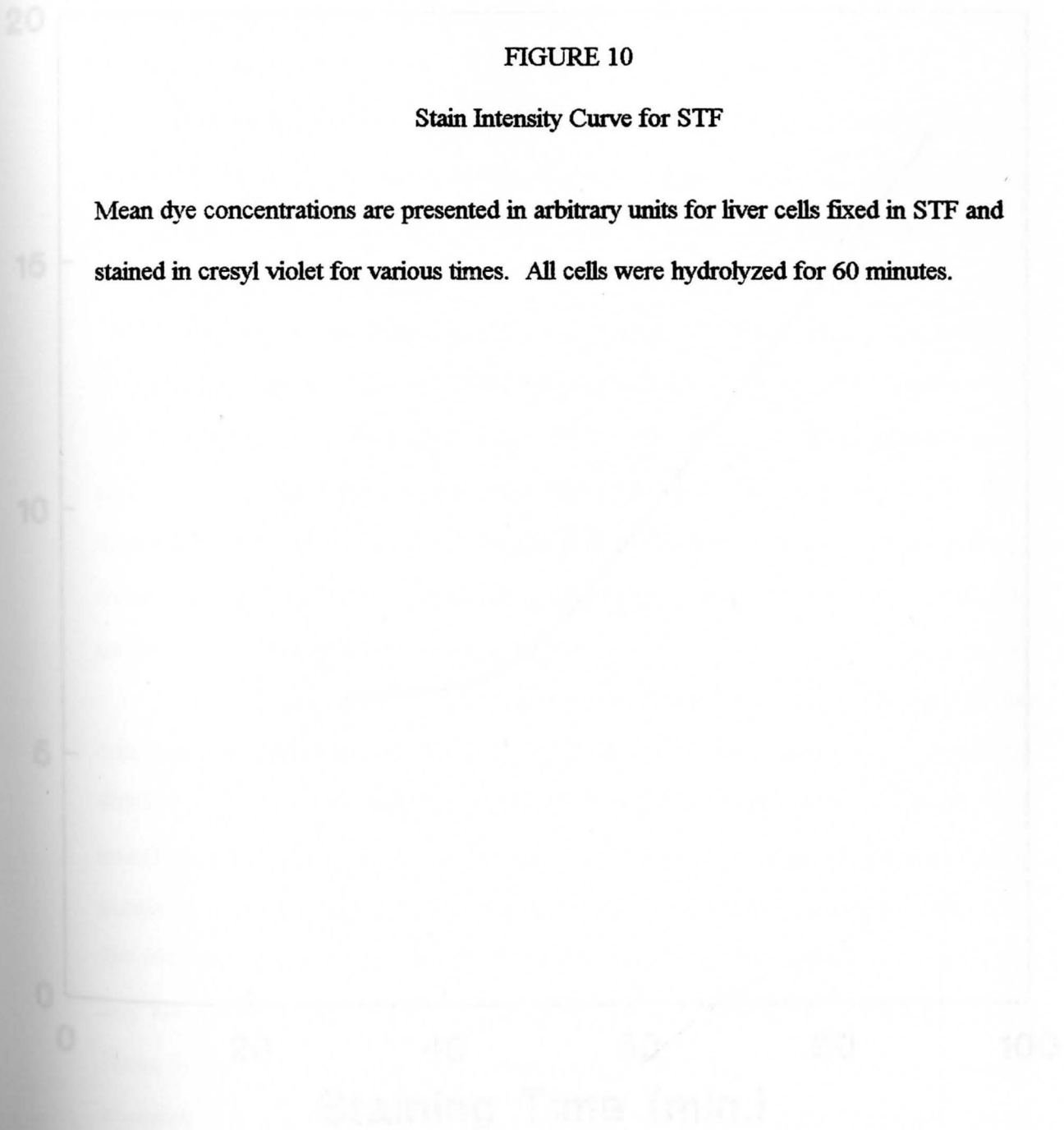
Stain Intensity
STF

Dye Concentration

n • 15
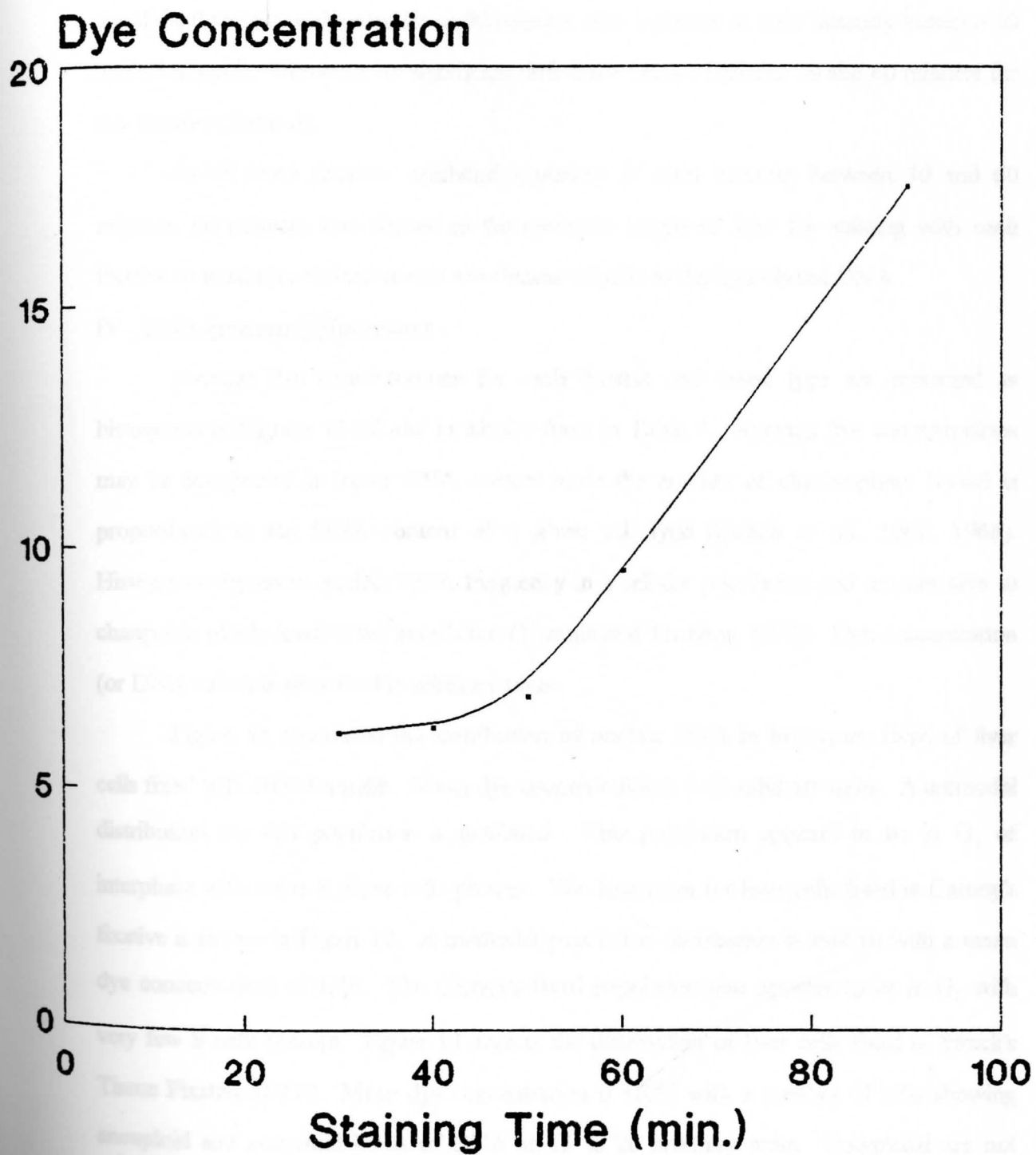
The staining curve for Carnoy's-fixed cells (Figure 9) shows a gradual rise in stain intensity over the staining period. Statistical analysis shows no significant difference in staining at any of these times ($p = 0.252$).

The liver cells fixed with STF (Figure 10) also showed a gradual increase in stain intensity up to 60 minutes in the stain. Figure 10 also demonstrates a rapid rise to a significantly higher stain intensity at 90 minutes after a plateau in stain intensity between 30 and 60 minutes. However, no significant difference existed between 30 and 60 minutes for this fixative (Table 6).

As all three fixatives exhibited a plateau of stain intensity between 30 and 60 minutes, 60 minutes was chosen as the optimum length of time for staining with each fixative to maximize stoichiometric attachment of stain to the hydrolyzed DNA .

IV. DNA Frequency Histograms

Average dye concentrations for each fixative and tissue type are presented as histograms in Figures 11-22 and in tabular form in Table 7. Average dye concentrations may be interpreted as mean DNA content since the amount of chromophore bound is proportional to the DNA content of a given cell type (Deitch et al., 1967, 1968). Histograms represent nuclear DNA frequency in a cellular population and are sensitive to changes in ploidy level of the population (Yemma and Therrien, 1972). Dye concentration (or DNA value) is presented in arbitrary units.

Figure 11 represents the distribution of nuclear DNA in histogram form of liver cells fixed with 10% formalin. Mean dye concentration is 5.65 arbitrary units. A unimodal distribution for this population is exhibited. This population appears to be in G_1 of interphase with some S phase cells present. The histogram for liver cells fixed in Carnoy's fixative is shown in Figure 12. A unimodal population distribution is evident with a mean dye concentration of 4.28. The Carnoy's-fixed population also appears to be in G_1 with very few S cells present. Figure 13 depicts the distribution of liver cells fixed in Streck's Tissue Fixative (STF). Mean dye concentration is 10.52 with a number of cells showing aneuploid and polyploid levels of DNA at 15 to 20 arbitrary units. Polyploids are not

FIGURE 11

Histogram representing Feulgen DNA values for liver cells fixed in 10% buffered formalin.

Dye concentration (DNA content) is presented in arbitrary units.

Liver
10% Formalin

Number of Nuclei
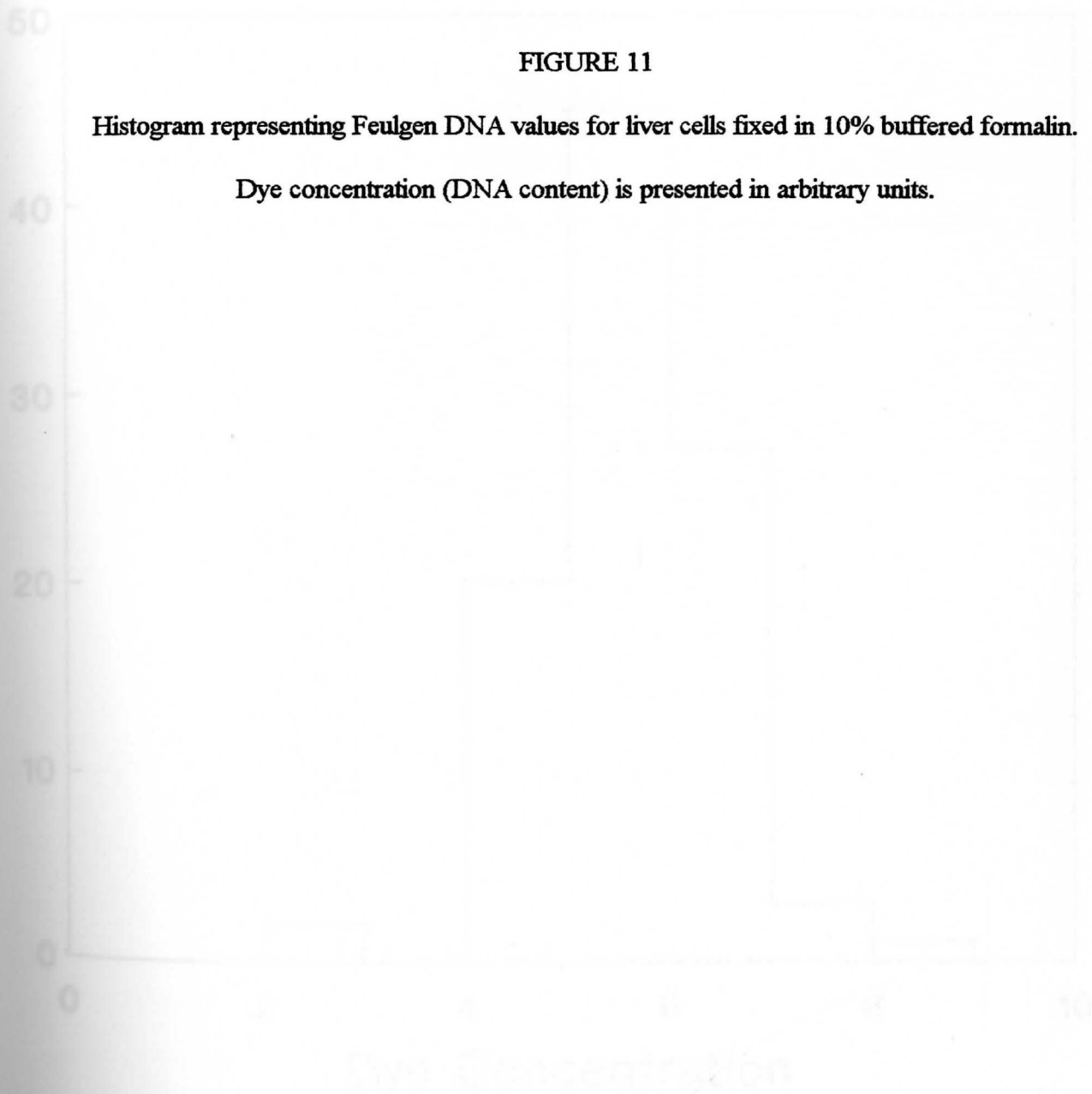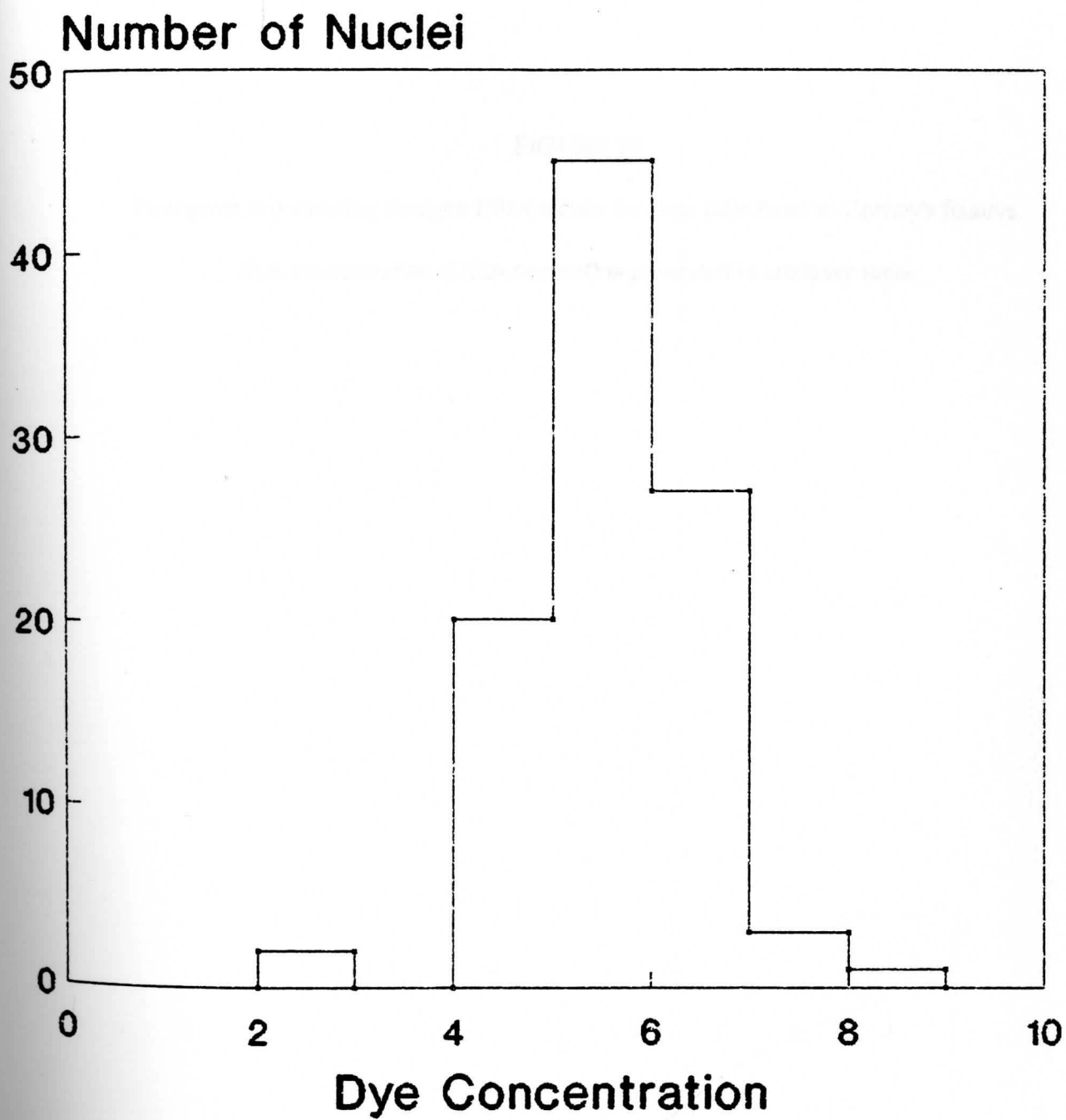
Dye Concentration

mean dye conc. = 5.65
n = 100
S.D. = 0.83

FIGURE 12

Histogram representing Feulgen DNA values for liver cells fixed in Carnoy's fixative.

Dye concentration (DNA content) is presented in arbitrary units.

Liver
Carnoy's

Number of Nuclei

Dye Concentration

mean dye conc. = 4.28
n = 100
S.D. = 0.82

FIGURE 13

Histogram representing Feulgen DNA values for liver cells fixed in STF.

Dye concentration (DNA content) is presented in arbitrary units.
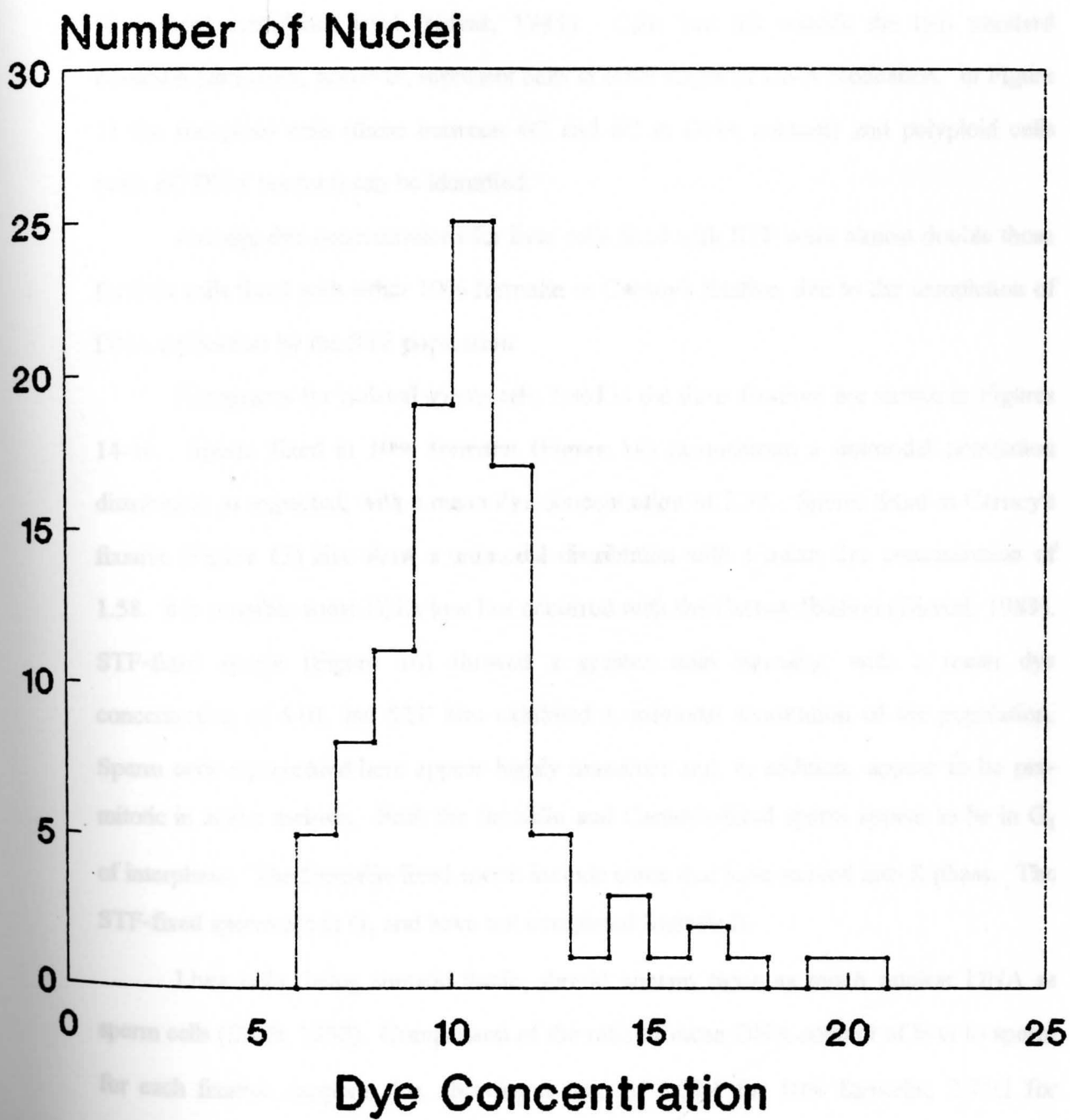
uncommon among liver cells, resulting in the DNA distribution exhibited (Swift, 1950; Yemma and Penza, 1987). This population of liver cells is in G_2 of interphase. The stage of DNA replication of outlying cells on a histogram may be determined by taking two standard deviations on either side of the mean. Cells that fall within two standard deviations of the mean represent the same stage of interphase as those at the mean, at the 98 percent confidence level (Novak, 1985). Cells that fall outside the two standard deviation parameter, however, represent cells in other stages of DNA replication. In Figure 13 the aneuploid cells (those between 4C and 8C in DNA content) and polyploid cells (with 8C DNA content) can be identified.

Average dye concentrations for liver cells fixed with STF were almost double those for liver cells fixed with either 10% formalin or Carnoy's fixative, due to the completion of DNA replication by the STF population.

Histograms for isolated sperm cells fixed in the three fixatives are shown in Figures 14-16. Sperm fixed in 10% formalin (Figure 14) demonstrate a unimodal population distribution as expected, with a mean dye concentration of 2.71. Sperm fixed in Carnoy's fixative (Figure 15) also show a unimodal distribution with a mean dye concentration of 1.58. It is possible some DNA loss has occurred with the Carnoy fixation (Giroud, 1989). STF-fixed sperm (Figure 16) showed a greater stain intensity, with a mean dye concentration of 6.01, but STF also exhibited a unimodal distribution of the population. Sperm cells represented here appear highly immature and, in addition, appear to be pre-mitotic in active meiosis. Both the formalin and Carnoy's-fixed sperm appear to be in G_1 of interphase. The formalin-fixed sperm include some that have moved into S phase. The STF-fixed sperm are in G_2 and have not completed Meiosis II.

Liver cells, being somatic tissue, should contain twice as much nuclear DNA as sperm cells (Swift, 1950). Comparison of the ratio of mean DNA content of liver to sperm for each fixative supports this, showing a ratio of 2.08:1 for 10% formalin, 2.71:1 for Carnoy's fixative and 1.75:1 for STF. These ratios are listed in Table 7 (page 73).

FIGURE 14

Histogram representing Feulgen DNA values for sperm cells fixed in formalin.

Dye concentration (DNA content) is presented in arbitrary units.

Sperm
10% Formalin

Number of Nuclei

Dye Concentration
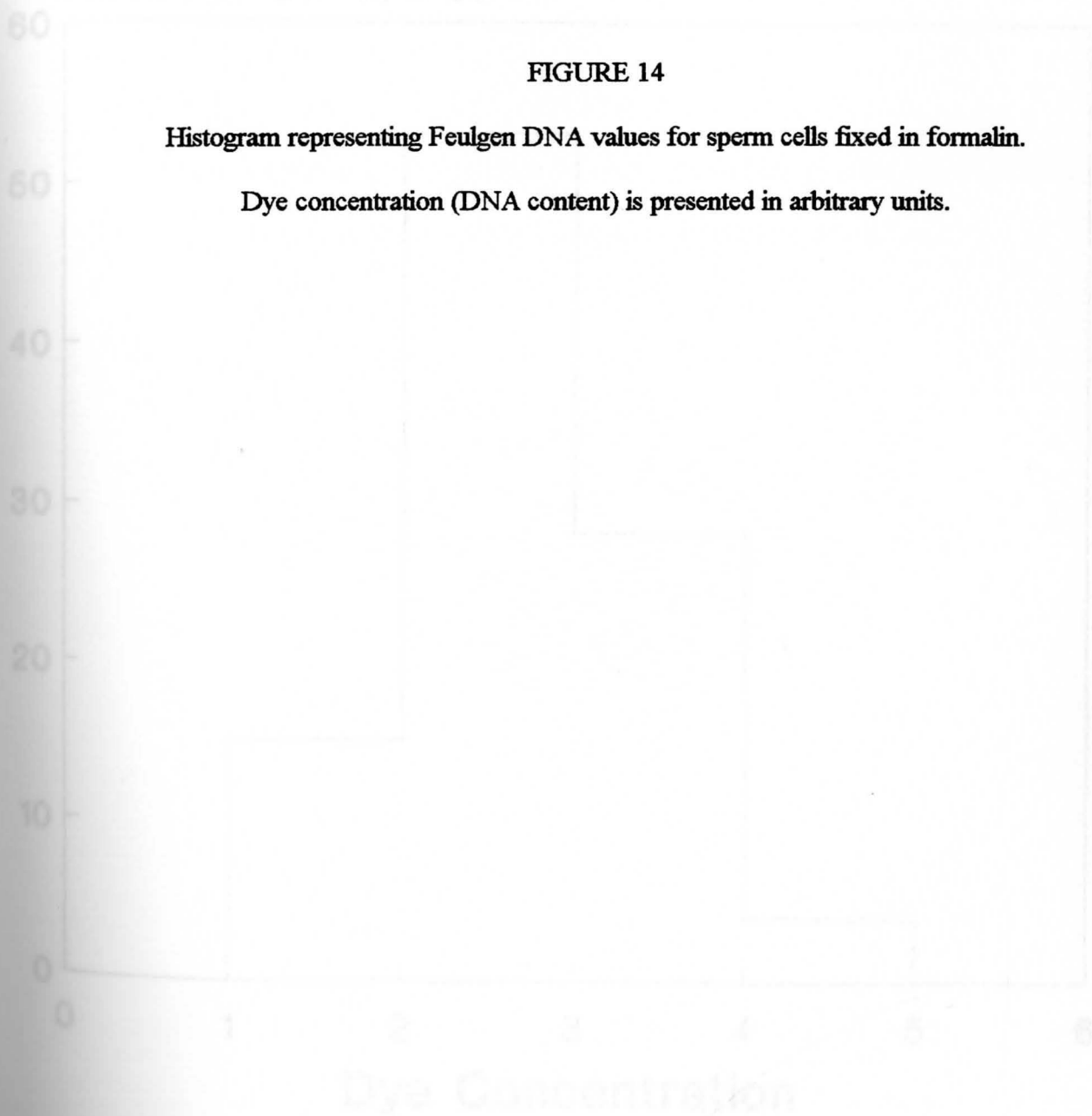
mean dye conc. • 2.71
n • 100
S.D. • 0.68

FIGURE 15

Histogram representing Feulgen DNA values for sperm cells fixed in Carnoy's fixative.

Dye concentration (DNA content) is presented in arbitrary units.

Sperm
Carnoy's

Number of Nuclei

Dye Concentration
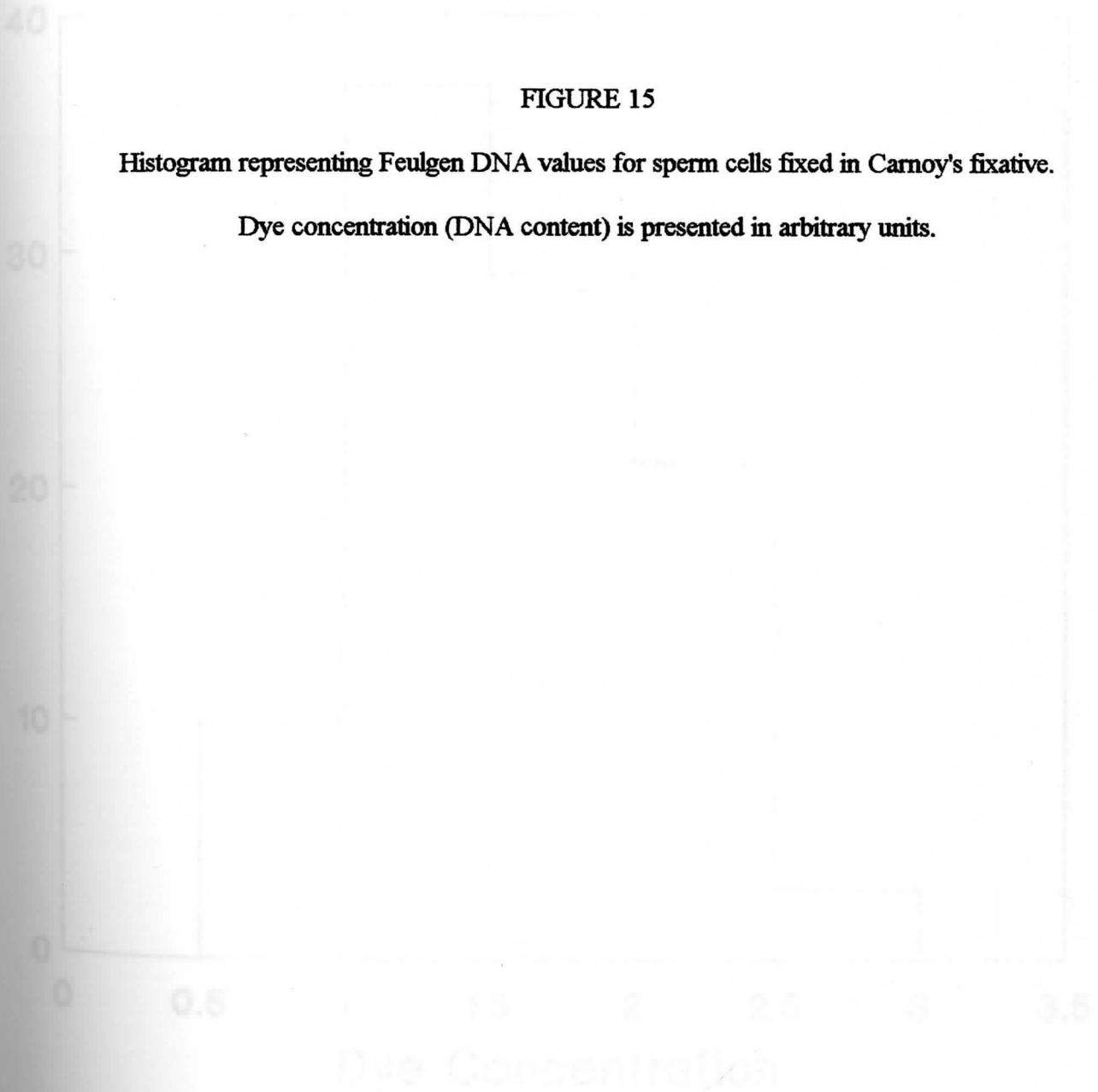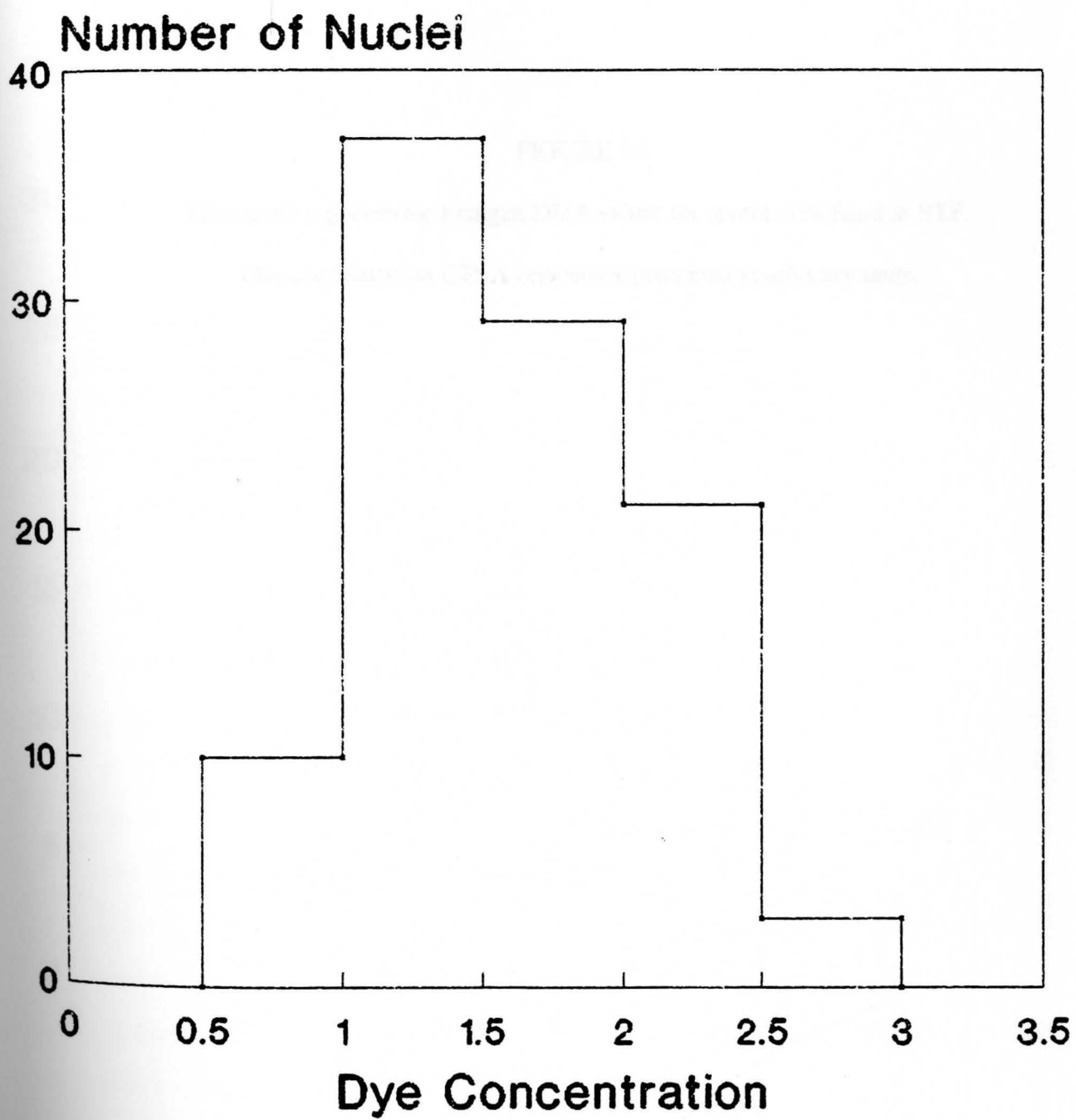
mean dye conc. = 1.58
n = 100
S.D. = 0.48

FIGURE 16

Histogram representing Feulgen DNA values for sperm cells fixed in STF.

Dye concentration (DNA content) is presented in arbitrary units.

Sperm
STF

Number of Nuclei
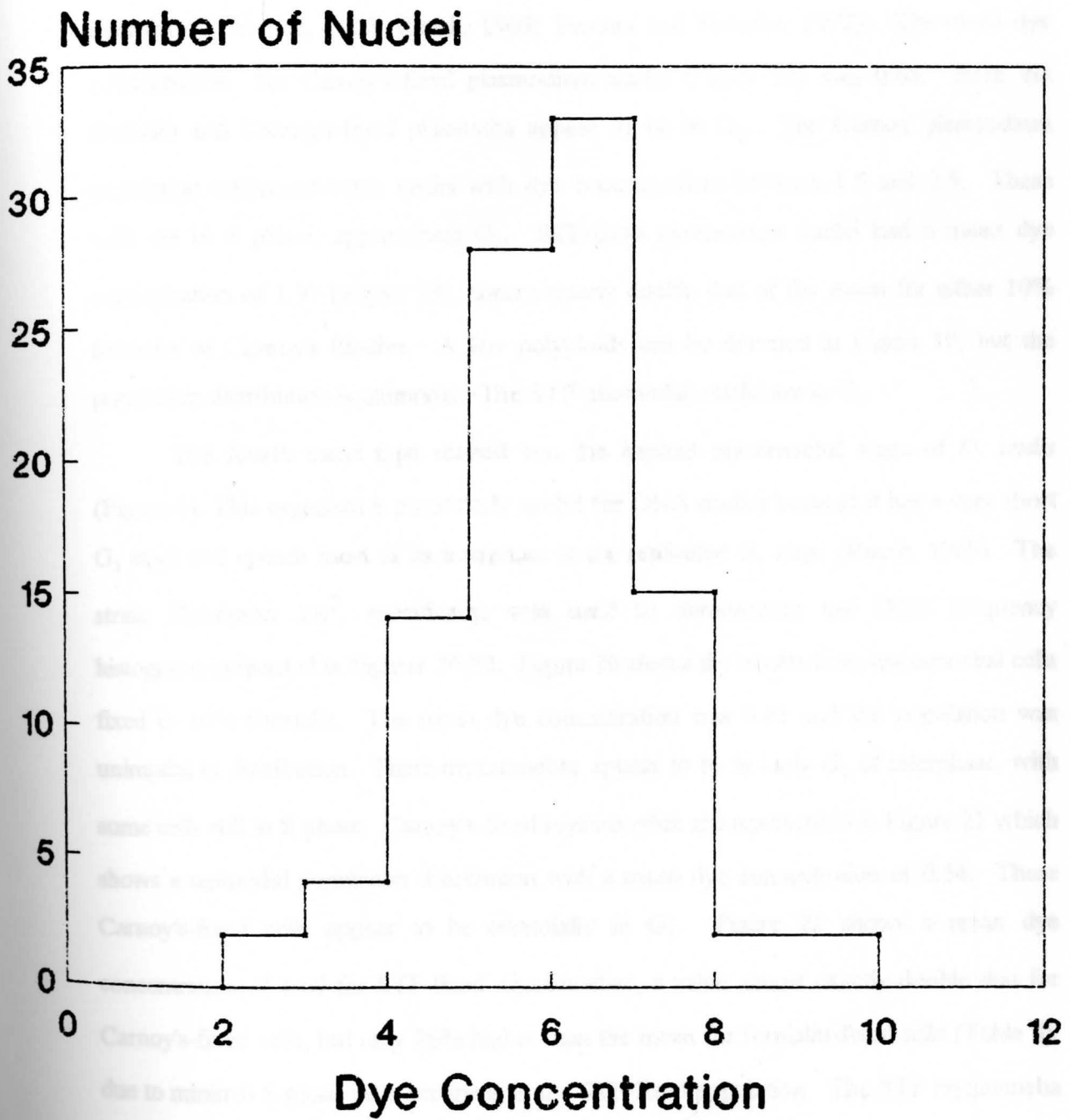
Dye Concentration

mean dye conc. = 6.01
n = 100
S.D. = 1.25

Histograms showing the frequency of nuclear DNA for the diploid plasmodium of *D. iridis* are presented in Figures 17-19. In Figure 17, formalin-fixed plasmodium cells had a mean dye concentration of 0.80. The population was unimodal, showing only one peak on the histogram. Unimodal population distributions are expected of *D. iridis* plasmodial histograms due to their synchronous mitoses and extended G_2 period of interphase (Therrien, 1966; Rusch, 1969; Yemma and Therrien, 1972). The mean dye concentration for Carnoy's-fixed plasmodium nuclei (Figure 18) was 0.88. Both the formalin and Carnoy's-fixed plasmodia appear to be in G_1. The Carnoy plasmodium population evidenced some nuclei with dye concentrations between 1.5 and 2.5. These cells are in S phase, approaching G_2. STF-fixed plasmodium nuclei had a mean dye concentration of 1.97 (Figure 19), approximately double that of the mean for either 10% formalin or Carnoy's fixative. A few polyploids can be detected in Figure 19, but the population distribution is unimodal. The STF plasmodial nuclei are in G_2.

The fourth tissue type studied was the haploid myxamoebal stage of *D. iridis* (Figure 1). This organism is particularly useful for DNA studies because it has a very short G_1 stage and spends most of its interphase in the replicated G_2 stage (Rusch, 1969). The strain Honduran 7A^2, specifically, was used to demonstrate the DNA frequency histograms presented in Figures 20-22. Figure 20 shows the results from myxamoebal cells fixed in 10% formalin. The mean dye concentration was 0.81 and the population was unimodal in distribution. These myxamoebae appear to be in early G_2 of interphase, with some cells still in S phase. Carnoy's-fixed myxamoebae are represented in Figure 21 which shows a unimodal population distribution with a mean dye concentration of 0.54. These Carnoy's-fixed cells appear to be essentially in G_1. Figure 22 shows a mean dye concentration of 1.09 for STF-fixed myxamoebae, a value almost exactly double that for Carnoy's-fixed cells, but only 26% higher than the mean for formalin-fixed cells (Table 8), due to minimal S phase cells occurring in the STF-fixed population. The STF myxamoeba population is essentially unimodal and represents cells in G_2 of interphase.

FIGURE 17

Histogram representing Feulgen DNA values for plasmodium nuclei fixed in 10% buffered formalin.

Dye concentration (DNA content) is presented in arbitrary units.

Plasmodium
10% Formalin
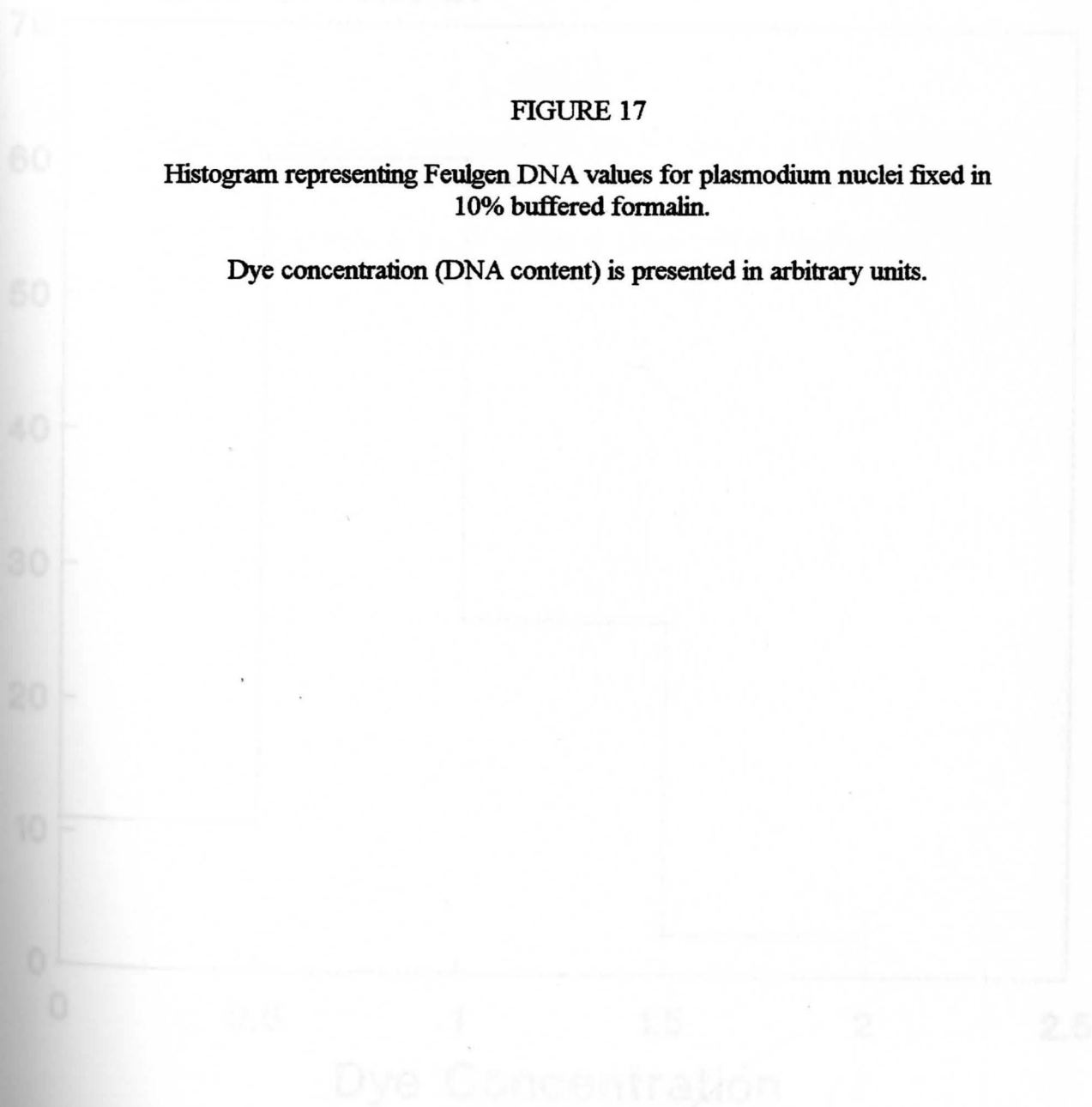
mean dye conc. ▪ 0.80
n ▪ 100
S.D. ▪ 0.31

FIGURE 18

Histogram representing Feulgen DNA values for plasmodium nuclei fixed in Carnoy's fixative.

Dye concentration (DNA content) is presented in arbitrary units.

Plasmodium
Carnoy's

Number of Nuclei
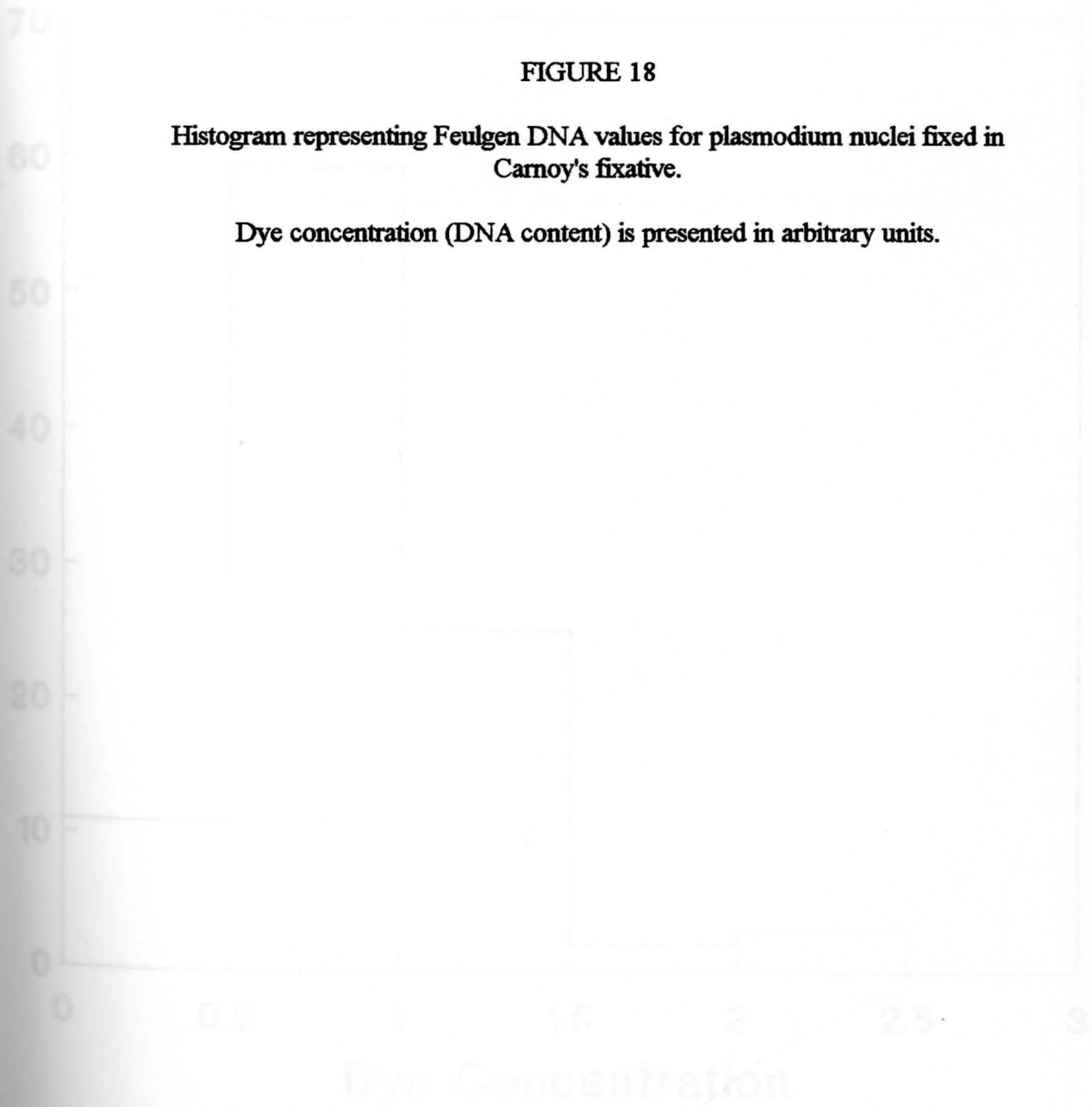
Dye Concentration

mean dye conc. = 0.88
n = 100
S.D. = 0.37

FIGURE 19

Histogram representing Feulgen DNA values for plasmodium nuclei fixed in STF.

Dye concentration (DNA content) is presented in arbitrary units.

Plasmodium
STF

Number of Nuclei
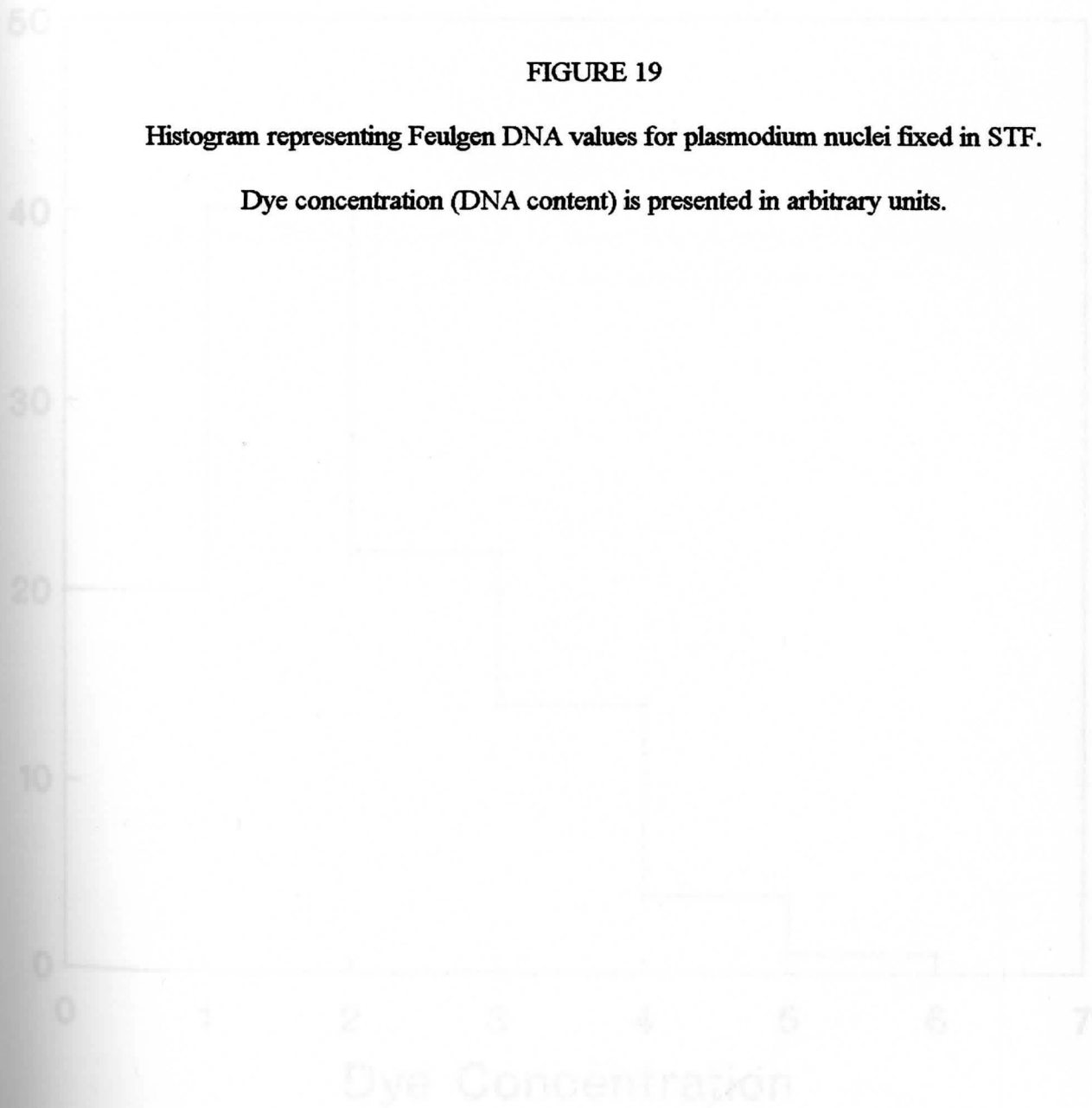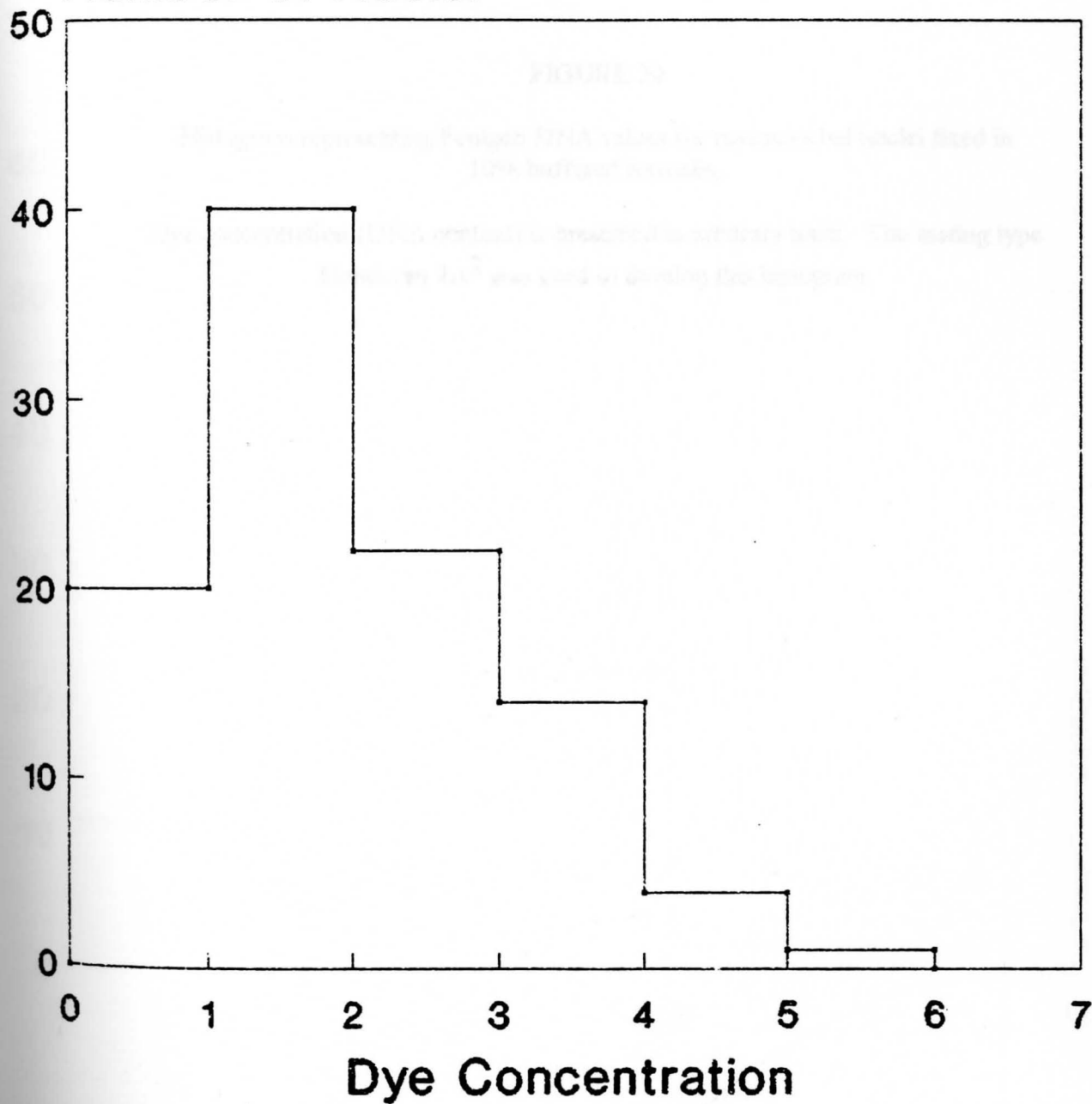
Dye Concentration

mean dye conc. = 1.97
n = 101
S.D. = 1.06

FIGURE 20

Histogram representing Feulgen DNA values for myxamoebal nuclei fixed in 10% buffered formalin.

Dye concentration (DNA content) is presented in arbitrary units. The mating type Honduran $7A^2$ was used to develop this histogram.

Myxamoeba
10% Formalin

Number of Nuclei
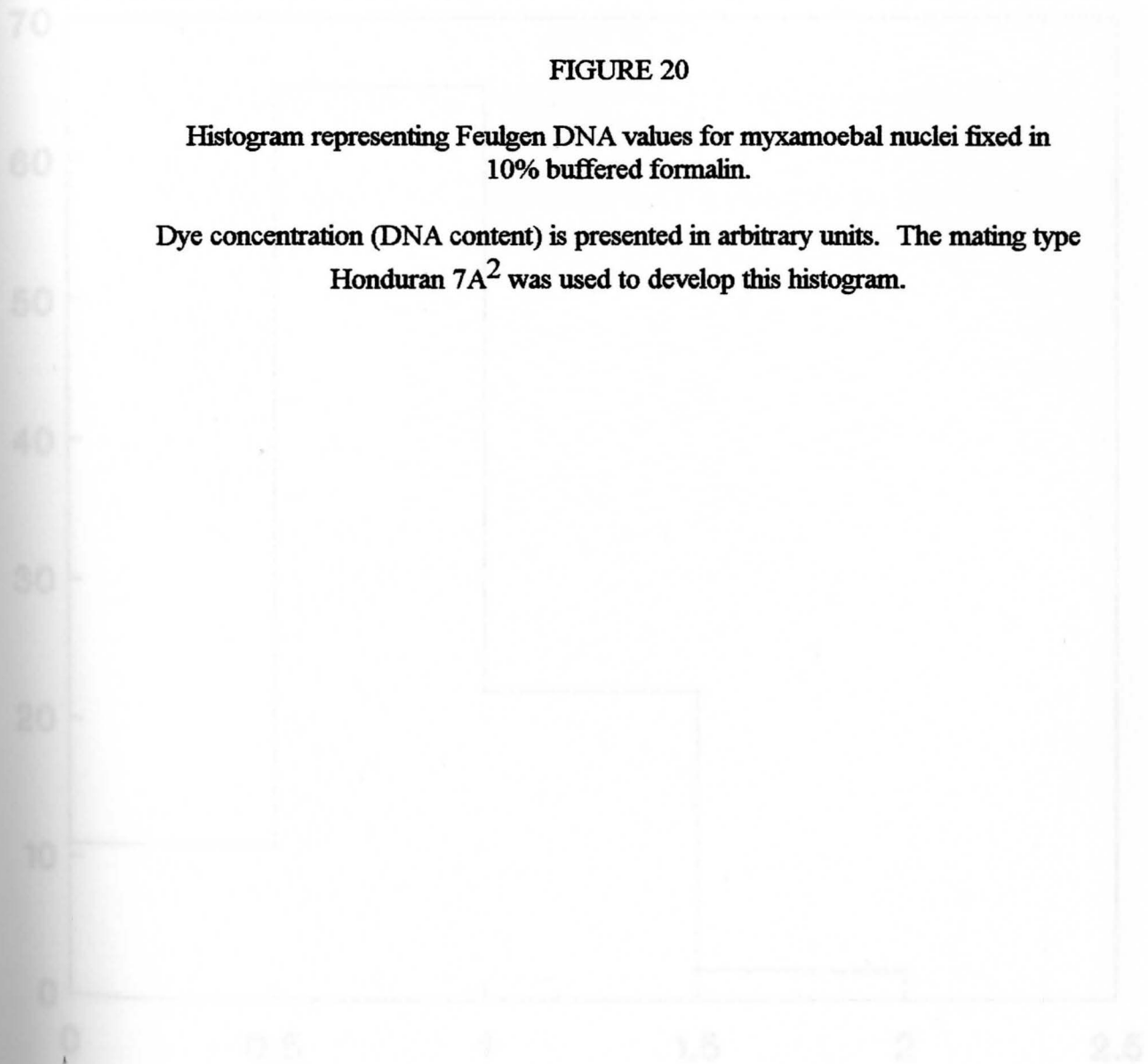
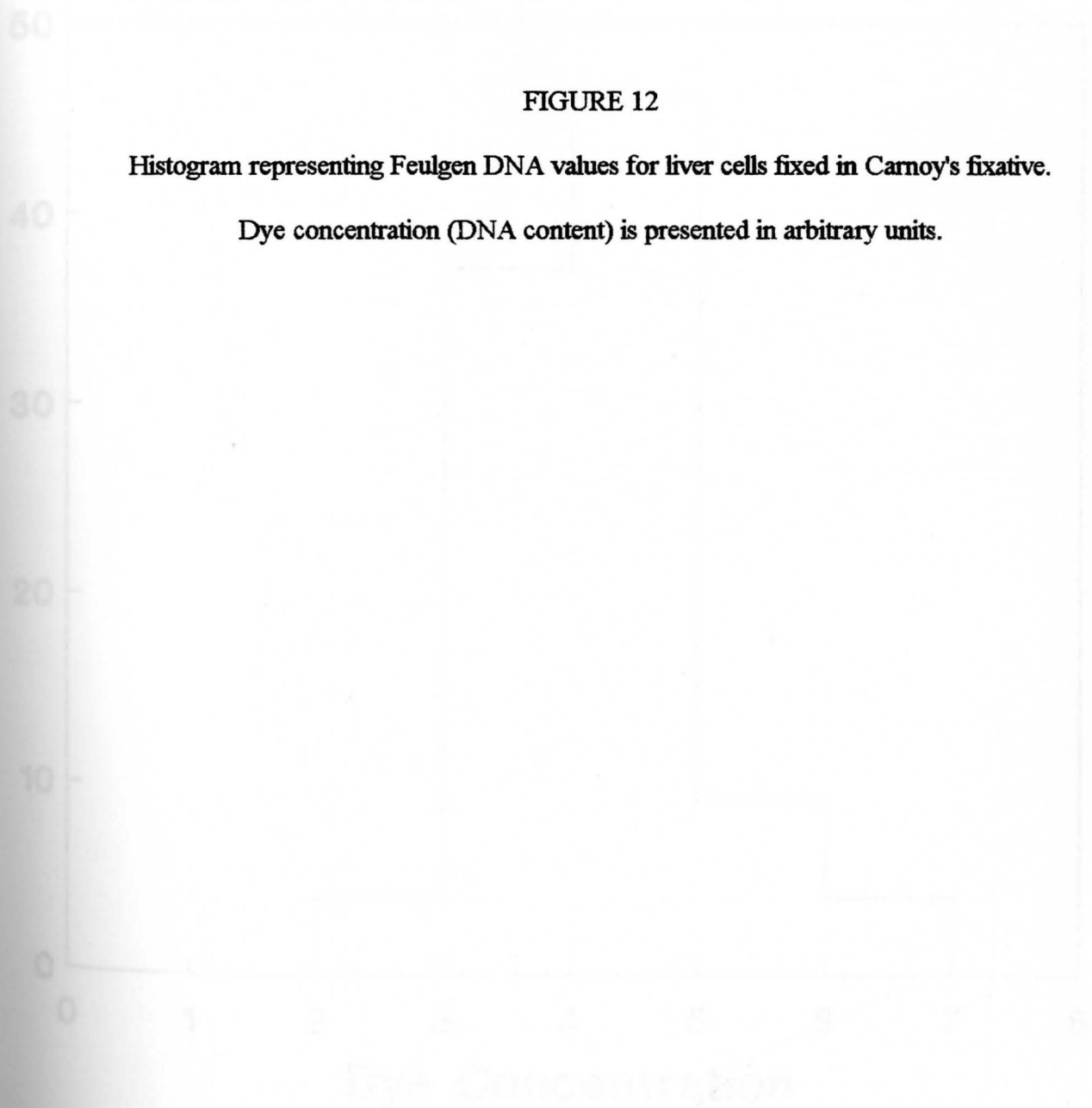

Dye Concentration

mean dye conc. = 0.81
n = 100
S.D. = 0.26

FIGURE 21

Histogram representing Feulgen DNA values for myxamoebal nuclei fixed in
Carnoy's fixative.

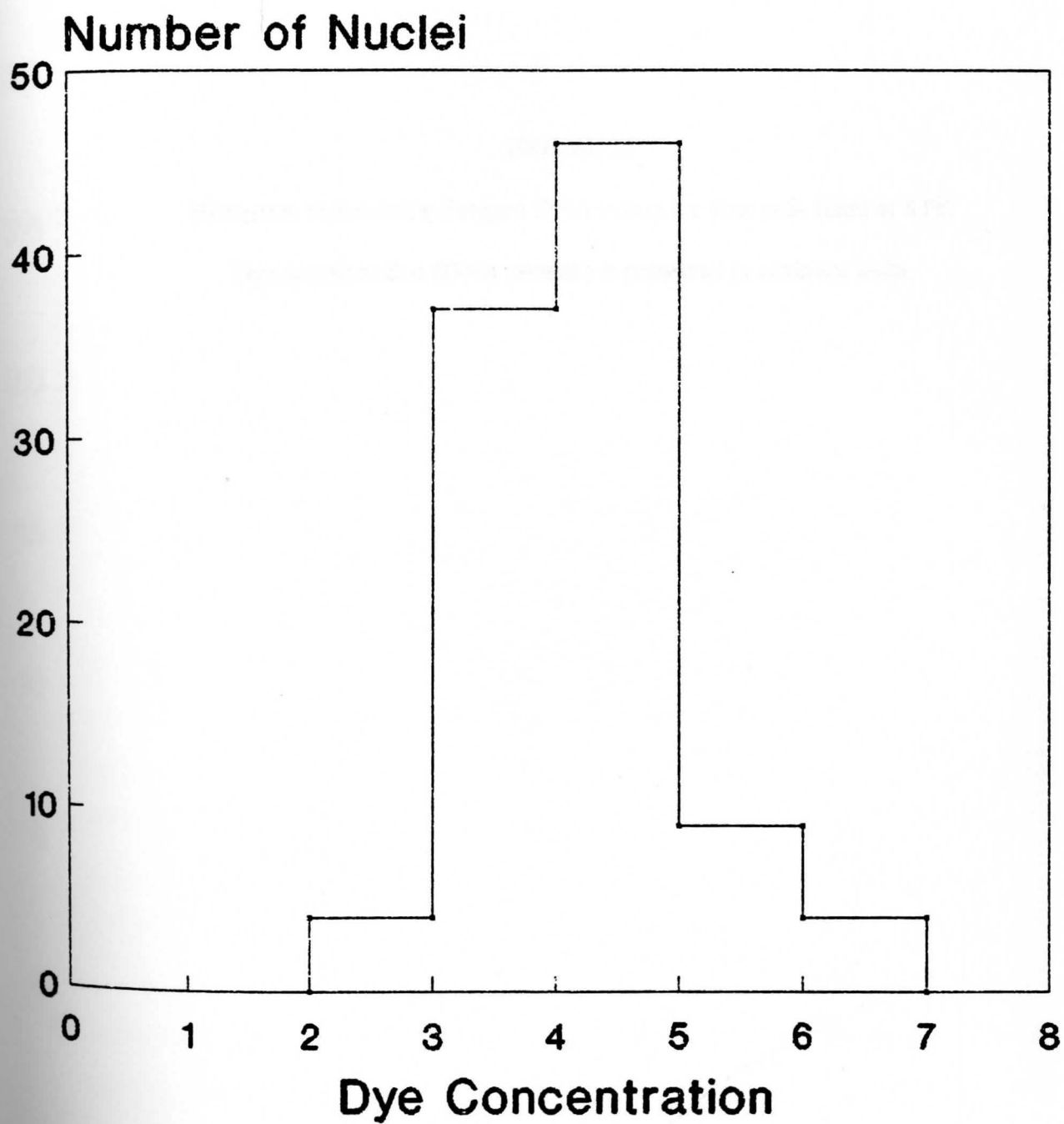Dye concentration (DNA content) is presented in arbitrary units. The mating type
Honduran $7A^2$ was used to develop this histogram.
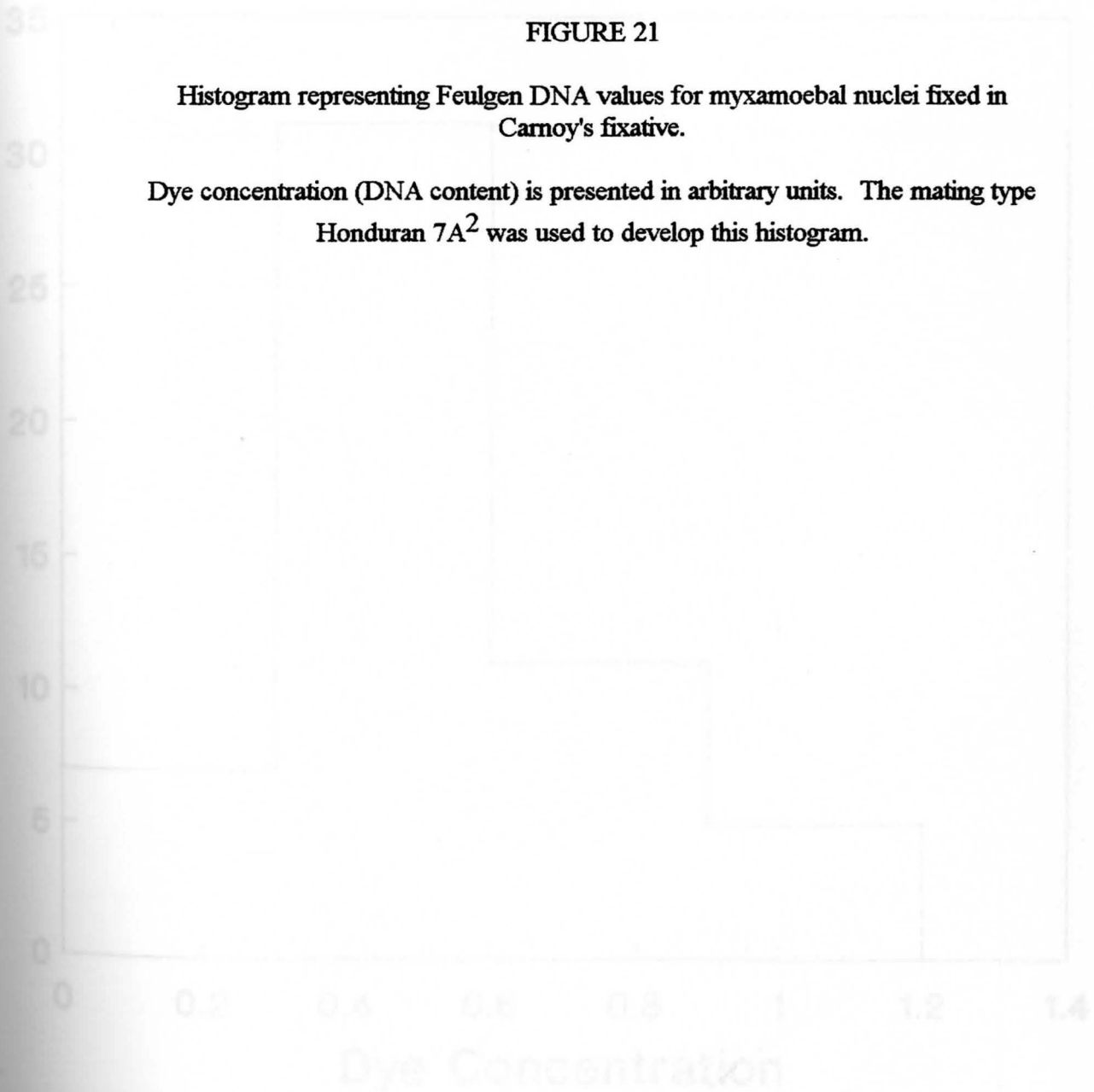
Myxamoeba
Carnoy's

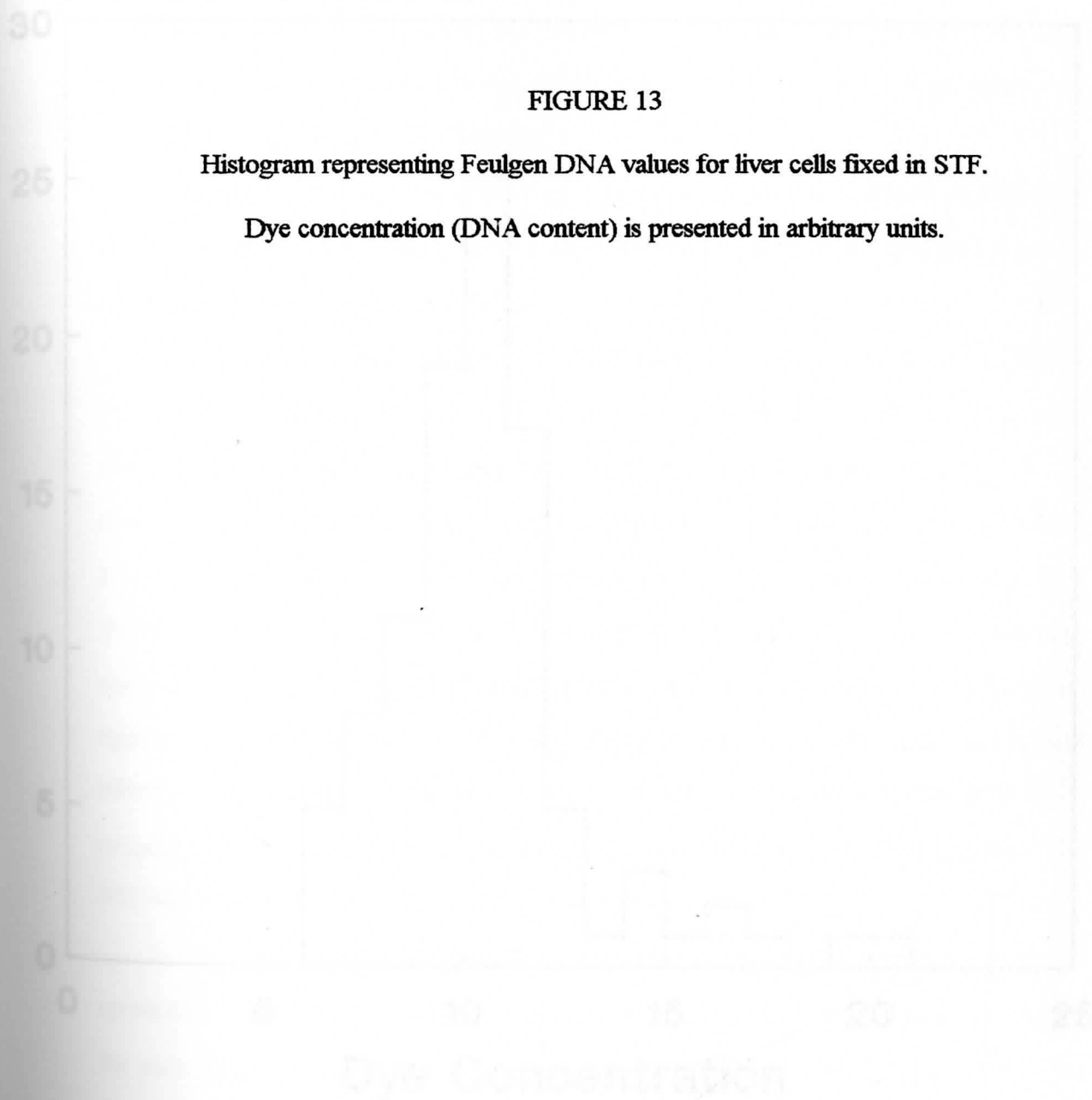**Number of Nuclei**
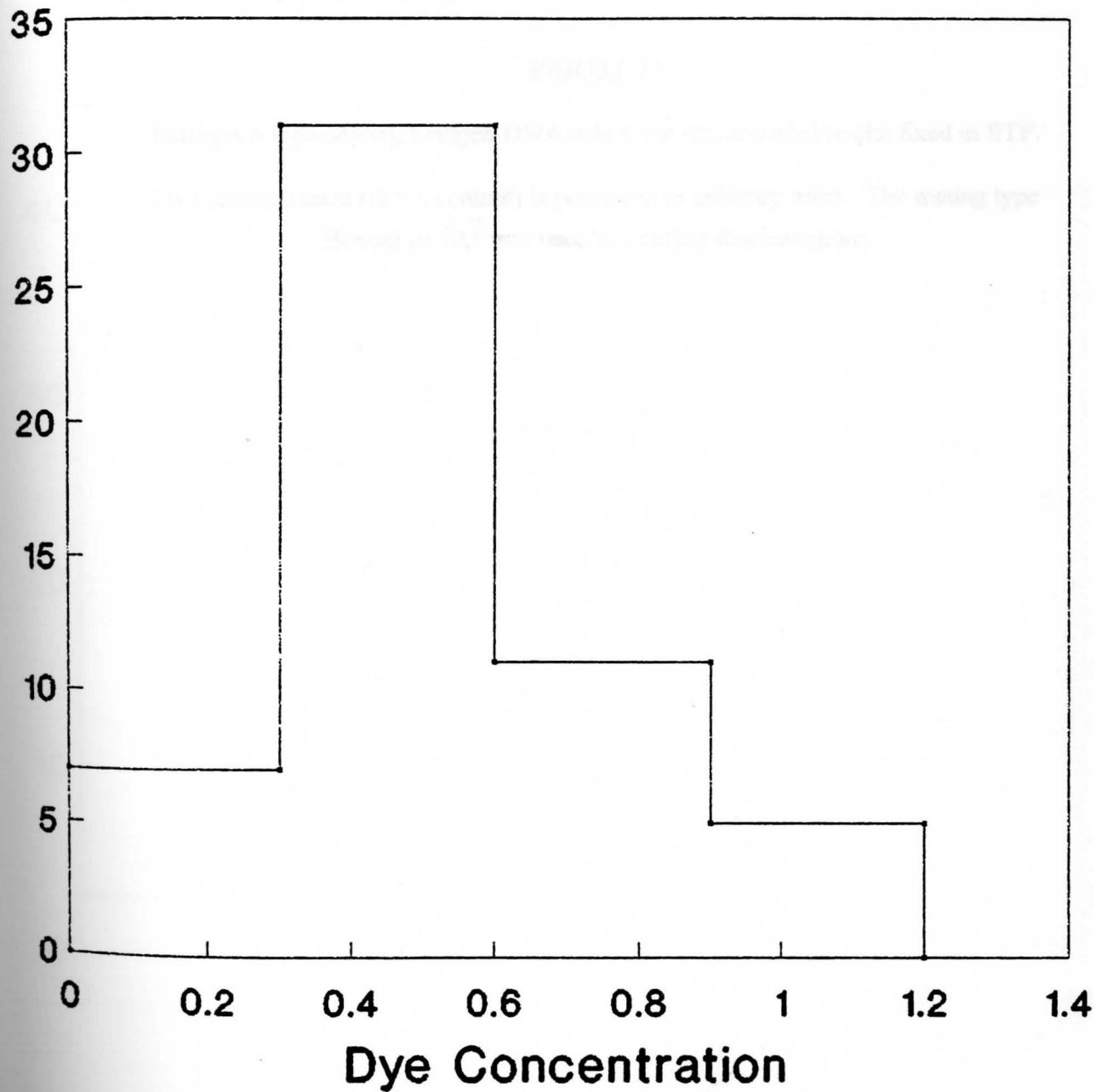
mean dye conc. = 0.54
n = 54
S.D. = 0.23

FIGURE 22

Histogram representing Feulgen DNA values for myxamoebal nuclei fixed in STF.

Dye concentration (DNA content) is presented in arbitrary units. The mating type Honduran $7A^2$ was used to develop this histogram.

Myxamoeba
STF

Number of Nuclei
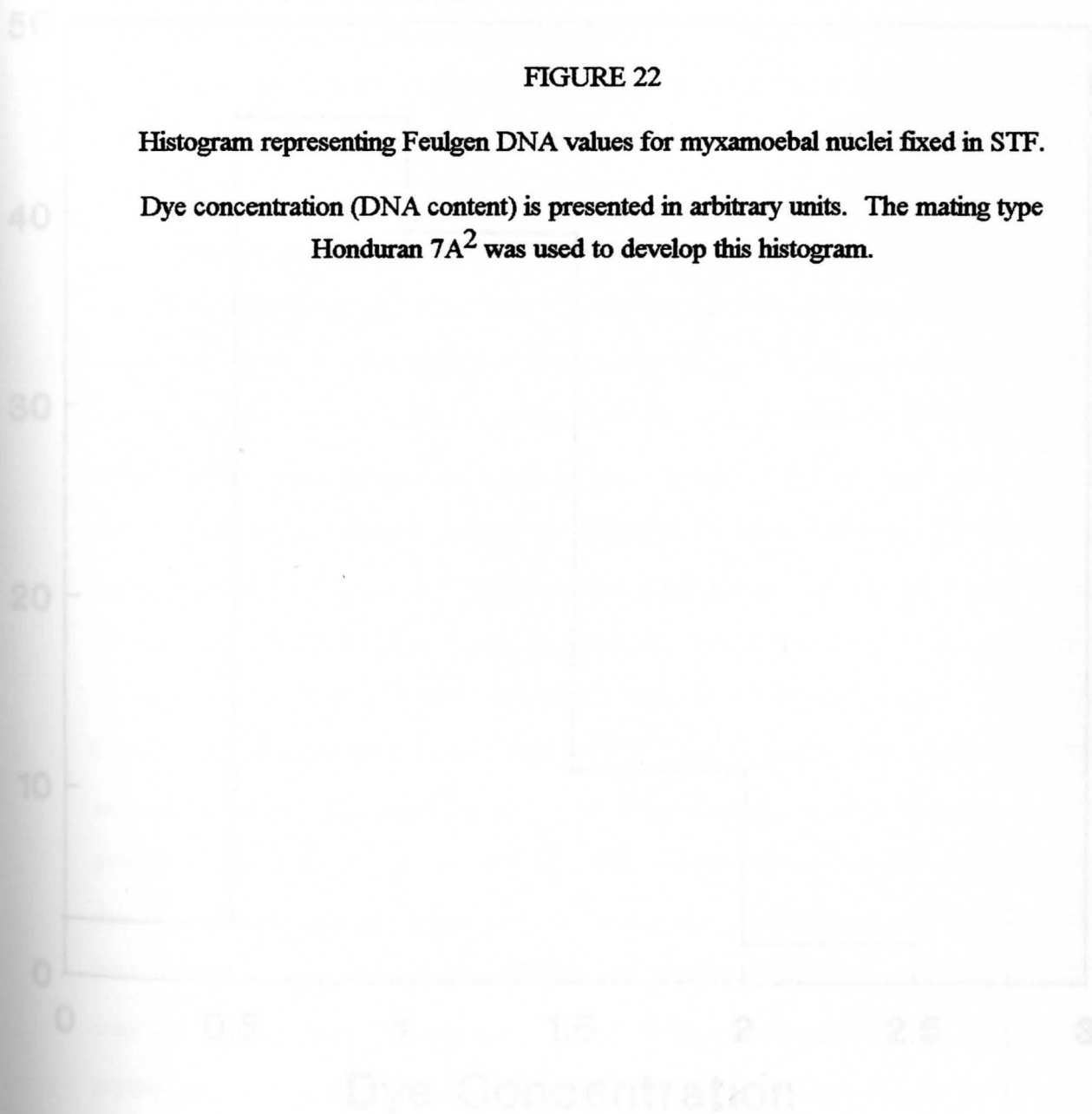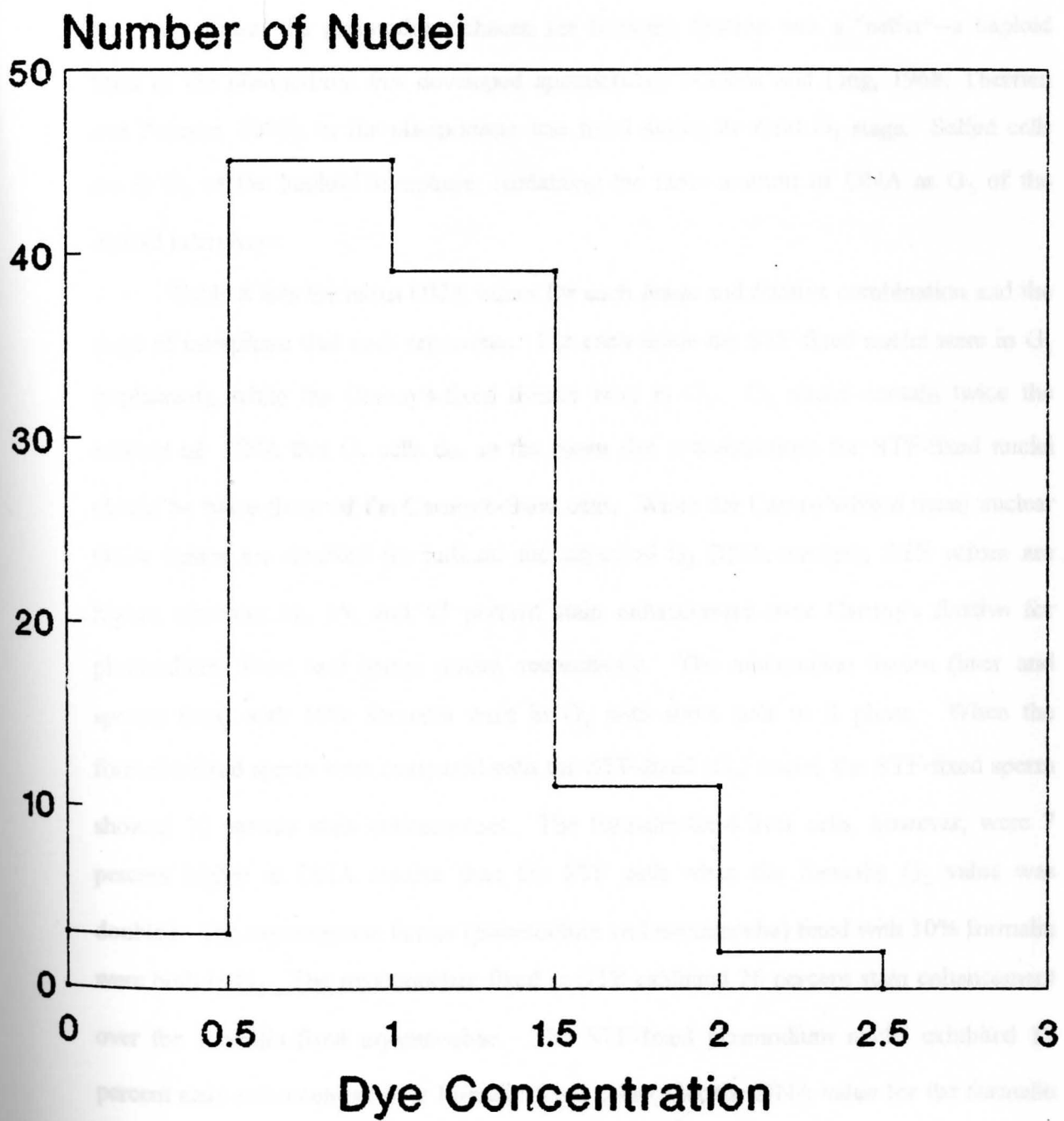
mean dye conc. = 1.09
n = 100
S.D. = 0.37

Yemma and Therrien (1972) established that the cross plasmodial stage of *D. iridis* is diploid and the myxamoebal stage is haploid. Relative DNA values between plasmodium and myxamoebal nuclei prepared in the same fixative should exhibit a 2:1 ratio. The ratio for STF-fixed plasmodium and myxamoebae was 1.81:1 (Table 7). The ratio for Carnoy's-fixed *D. iridis* nuclei was 1.65:1. The ratio for formalin-fixed cells, however, was 1:1. Either the plasmodium chosen for formalin fixation was a "selfer"--a haploid form of the plasmodium that developed apomictically (Collins and Ling, 1968; Therrien and Yemma, 1975), or the plasmodium was fixed during its short G_1 stage. Selfed cells are in G_2 of the haploid interphase, containing the same amount of DNA as G_1 of the diploid interphase.

Table 8 lists the mean DNA values for each tissue and fixative combination and the stage of interphase that each represents. For each tissue the STF-fixed nuclei were in G_2 (replicated), while the Carnoy's-fixed tissues were in G_1. G_2 nuclei contain twice the amount of DNA that G_1 cells do, so the mean dye concentrations for STF-fixed nuclei should be twice those of the Carnoy's-fixed cells. When the Carnoy's-fixed mean nuclear DNA values are doubled (to indicate the expected G_2 DNA content), STF values are higher, showing 11, 19, and 47 percent stain enhancement over Carnoy's fixative for plasmodium, liver, and sperm nuclei, respectively. The mammalian tissues (liver and sperm) fixed with 10% formalin were in G_1 with some cells in S phase. When the formalin-fixed sperm were compared with the STF-fixed (G_2) nuclei, the STF-fixed sperm showed 10 percent stain enhancement. The formalin-fixed liver cells, however, were 7 percent higher in DNA content than the STF cells when the formalin G_1 value was doubled. The myxomycete tissues (plasmodium and myxamoeba) fixed with 10% formalin were both in G_2. The myxamoebae fixed in STF exhibited 26 percent stain enhancement over the formalin-fixed myxamoebae. The STF-fixed plasmodium nuclei exhibited 19 percent stain enhancement over formalin-fixed cells (after the DNA value for the formalin plasmodium (a haploid selfer) was doubled).

TABLE 7

MEAN DNA CONCENTRATIONS AND RATIOS

TISSUE

FIXATIVE	Liver	Sperm	Ratio	Plasmodium	Myxamoeba	Ratio
Formalin	5.65	2.71	2.08:1	0.80	0.81	1:1
Carnoy's	4.28	1.58	2.71:1	0.88	0.54	1.65:1
STF	10.52	6.01	1.75:1	1.97	1.09	1.81:1

TABLE 8

MEAN DNA CONTENT AND STAGE OF INTERPHASE

FIXATIVE	TISSUE / PHASE			
	Liver (%)[*]	Sperm (%)	Plasmodium (%)	Myxamoeba (%)
Formalin	5.65 (107) G_1/S	2.71 (90) G_1/S	0.80 (81) G_2-selfer	0.81 (74) G_2
Carnoy's	4.28 (81) G_1	1.58 (53) G_1	0.88 (89) G_1/S	0.54 (99) G_1
STF	10.52 (100) G2	6.01 (100) G2	1.97 (100) G2	1.09 (100) G2

[*] % = percent stain provided by this fixative/tissue combination when the phases are equalized (G_1 value x 2 = expected G_2).

$$\frac{(\text{expected G2})}{(\text{STF G2 value})} \times 100\% = \% \text{ stain}$$

V. Statistical Analysis

Analysis of variance (Zar, 1984) was performed on the raw data collected at the wavelength of half maximum absorbance (λ_1). The difference between each reading on the nucleus and off the nucleus at this wavelength was calculated for each fixative and tissue combination. Using the squares of the sums of the differences, an F value was calculated to indicate if treatments of a particular tissue with each of the three fixatives were statistically the same. For all four tissues, there was significant variation between the means for the fixatives, indicating that all the fixatives did not behave identically. F values are listed in Table 9. Subsequently, Tukey's test for unequal sample sizes was run to determine for each tissue if each fixative treatment varied from the other two. Results are presented in Tables 10-13.

For liver (Table 10), Tukey's test indicates that all three treatments are different. Table 11 indicates that the three fixatives differ significantly for sperm, as well, although formalin and Carnoy's fixation are very close. In Table 12, STF fixation of plasmodium nuclei differs significantly from the other two fixatives, but the formalin and Carnoy's treatments do not differ significantly. For the myxamoeba (Table 13), STF and formalin fixation produced almost identical results, while Carnoy's fixation was significantly different from the other two.

TABLE 9

ANALYSIS OF VARIANCE OF DATA AT λ_1

TISSUE	CONFIDENCE LEVEL (F ≥ 3.73 shows variance)
Liver	F = 125.736
Sperm	F = 27.367
Plasmodium	F = 40.871
Myxamoeba	F = 141.108

F values indicate variance between fixative treatments for all four tissues.

TABLE 10

TUKEY'S TEST ON LIVER DATA

	STF	FORMALIN	CARNOY'S
Total	1435.1	1282.9	990.3
number	100	100	104
mean	14.4	12.8	9.5

Tukey multiple comparison with unequal sample size

	S.E.	q [*]	Conclusion
Carnoy vs. STF	0.221	-21.887	significantly different
Carnoy vs. Formalin	0.221	-14.989	significantly different
Formalin vs. STF	0.223	-6.832	significantly different

[*] $q \geq 3.314$ is significant, confidence level 0.05.

TABLE 11

TUKEY'S TEST ON SPERM DATA

	STF	FORMALIN	CARNOY'S
Total	810.1	511.1	324.9
number	100	100	98
mean	8.1	5.1	3.3

Tukey multiple comparison with unequal sample size

	S.E.	q *	Conclusion
Carnoy vs. STF	0.485	-9.859	significantly different
Carnoy vs. Formalin	0.485	-3.699	significantly different
Formalin vs. STF	0.483	-6.191	significantly different

*$q \geq 3.314$ is significant, confidence level 0.05.

TABLE 12

TUKEY'S TEST ON PLASMODIUM DATA

	STF	FORMALIN	CARNOY'S
Total	1124.0	623.4	708.6
number	96	96	96
mean	11.7	6.5	7.4

Tukey multiple comparison for equal sample size

	S.E.	q *	Conclusion
Carnoy vs. STF	0.443	-9.772	significantly different
Carnoy vs. Formalin	0.443	2.004	NOT significantly different
Formalin vs. STF	0.434	-12.019	significantly different

*$q \geq 3.314$ is significant, confidence level 0.05.

TABLE 13

TUKEY'S TEST ON MYXAMOEBA DATA

	STF	FORMALIN	CARNOY'S
Total	581.3	591.0	188.5
number	100	101	50
mean	5.8	5.9	3.5

Tukey multiple comparison with unequal sample size

	S.E.	q *	Conclusion
Carnoy vs. STF	0.244	-9.524	significantly different
Carnoy vs. Formalin	0.244	-9.682	significantly different
Formalin vs. STF	0.199	0.193	NOT significantly different

*$q \geq 3.314$ is significantly different, confidence level 0.05.

DISCUSSION

Cytologists today generally agree that the Feulgen reaction can be used to efficiently localize and quantify DNA in the nuclei of cells provided the conditions under which the reaction is run are carefully controlled. A variety of factors will influence the results of the Feulgen reaction, and in this study we have looked at the influence of three different fixatives (10% buffered formalin, Carnoy's fixative, and Streck's Tissue Fixative, an untried, new fixative) and four tissue types on the Blue Feulgen reaction.

Investigation of the effect of each fixative on the absorption of cresyl violet indicated that the peak wavelength of absorbance was not significantly affected by the fixative employed. However, as the extinction of the wavelength of one-half maximum extinction is dependent upon the specific peak chosen and the width of the absorption curve, it is necessary to determine the two best wavelengths for each fixative-chromophore combination for use with the Feulgen quantitative microspectrophotometric method.

The 5N HCl hydrolysis curves developed for the three fixatives in this study indicate that each has an extended period of time during which hydrolysis may be terminated and still provide optimum Feulgen staining. For 10% formalin, hydrolysis plateaued from 30 to 90 minutes, while for Carnoy's fixative, statistically, any hydrolysis time between 15 and 90 minutes could be chosen. The hydrolysis plateau for STF began later than for either of the other two fixatives, at 40 minutes, but also extended until 90 minutes. Although the curves for all three fixatives appear to drop at 90 minutes, statistically this decline is not significant for any of them (Table 6) and does not indicate the onset of depolymerization of the DNA (Andersson and Kjellstrand, 1975). Ten percent formalin has been shown to stablize tissues up to 120 minutes during hydrolysis at room temperature (DeCosse and Aiello, 1966; Dutt, 1975, 1976). Further study is necessary to determine when depolymerization begins with Carnoy's fixative and STF. For this study, however, 45 minutes in 5N HCl at room temperature provided optimal hydrolysis for 10% formalin and Carnoy's-fixed tissues and did not promote depolymerization of the DNA.

For STF-fixed tissues 60 minutes of hydrolysis was necessary to ensure maximal depurination of the DNA without promoting depolymerization.

Previous investigations with the Blue Feulgen reaction indicate that immersion of tissues in cresyl violet stain for only 20 minutes gave an elevated staining intensity (Floyd, 1990; Bidinotto, 1993). Stain curves developed in this investigation (Figures 8-10) indicate that a longer period of staining is desirable. For Carnoy's fixative there is no statistically significant difference in stain development between 20 and 60 minutes, but the trend of the mean dye concentrations does rise. Ten percent formalin does show significantly more dye development after 30 minutes of staining than at 20 minutes. STF also exhibited a slight rise in stain intensities with longer immersion in the stain, up to 60 minutes. However, extending the period of immersion in the cresyl violet stain to several hours is not recommended. Figure 10 indicates that unexpected increases in dye intensity may occur after lengthy exposure (90 minutes or more) to the stain. These unexpected increases are likely due to depolymerization caused by the acidic cresyl violet stain. Methyl green has been shown to cause additional depolymerization in DNA after extended staining (Taft, 1951) and cresyl violet appears to act in the same way (Figure 10). Hydrolytic depolymerization causes a reduction in stain intensity with many stains because some DNA fragments may be lost from the nucleus before staining (diStefano, 1948). It is also reasonable to assume in this case that depolymerization while in the stain will provide more potential dye sites and is so rapid as to enhance staining with very little loss of nuclear DNA. It should be noted that hydrolysis in the Feulgen reaction does not remove every purine in the DNA helix, only those in the areas where template activity is occuring and the DNA is diffuse (Garcia, 1969). The number of these active sites is relatively constant in any particular tissue (Garcia, 1969; Yemma and Penza, 1987), providing the stoichiometric aspect of the reaction. Depolymerization and depurination of non-diffuse DNA will destroy the stoichiometry of the Feulgen reaction and should be avoided.

Inspection of the mean dye concentrations listed in Tables 7 and 8 indicates that Streck's Tissue Fixative provides somewhat enhanced staining during the Blue Feulgen

reaction, and that this new fixative is highly sensitive to the changes in DNA ploidy level that occur during the cell cycle.

Although stain enhancement in the Feulgen reaction is desirable, it is most important that the fixative maintains the DNA ratios between nuclear classes (Swift, 1950). Staining can only occur in diffuse regions of DNA where the genes are active (Yemma and Penza, 1987). Some fixatives may be more sensitive to these sites, and therefore provide enhanced staining, but the relative number of genes active at any one time will be constant, and even a less sensitive fixative should demonstrate the proper ratios between nuclear classes (Pressly, 1978). From this standpoint any of the three fixatives tested in this study would be acceptable for use with the Blue Feulgen reaction.

As Table 7 indicates, all three fixatives demonstrated an approximate 2:1 ratio between the diploid and haploid mammalian tissues, liver and sperm. Ten percent buffered formalin (usually the fixative of choice) demonstrated an almost perfect 2:1 ratio between liver and sperm, reinforcing this fixative's usability with the Blue Feulgen reaction (Bidinotto, 1993) as well as the standard fuchsin procedure (Dutt, 1976). The ratio was somewhat high for Carnoy's-fixed liver and sperm, but this appears to be due to the extraction of histone, of which sperm has a high concentration, by the alcohol-based fixative (Porcelli, 1972; Karalova, 1980; Giroud and Montmasson, 1989). This would result in lower DNA values for sperm due to the loss of Feulgen stainable material and a ratio greater than 2:1. Conversely, the ratio for STF-fixed liver and sperm was slightly lower than 2:1, but this may be due to an active cellular cycle and therefor the presence of unreplicated liver cells in G_1 or S. The histogram for STF-fixed liver cells (Figure 13) is not completely unimodal, indicating the presence of cells in several stages of replication, including a rather large population of aneuploid and polyploid cells. It is important to note that this is normal for liver tissue (Yemma and Penza, 1987).

Ratios between the diploid plasmodial nuclei and the haploid myxamoebae (Therrien, 1966; Yemma and Therrien, 1972) also exhibited the expected 2:1 DNA content. Both STF and Carnoy's fixative produced an approximate 2:1 ratio for plasmodium:myxamoebal

nuclei. These two fixatives may be registering slightly lower than 2:1 due to the strain of myxamoeba used for measurements. Opposite mating types of *D. iridis* myxamoebae have slightly different amounts of DNA (Therrien, 1966), but the sum of the two equals the amount of DNA found in the cross plasmodium. Since only one mating type was used for readings on myxamoebal nuclei (Hon 7A[2]), that appears to have been the mating type with more than half the DNA seen in the cross plasmodium. This would shift the ratio between plasmodium and myxamoeba to slightly less than 2:1. Formalin, however, produced an unexpected 1:1 ratio. This can be explained by the occurrence of a selfed plasmodium or by the plasmodium being in G_1 while the myxamoebae are in G_2. However, selfers are more probable since G_1 for plasmodium is of extremely short duration (Yemma and Therrien, 1972). The appearance of *D. iridis* plasmodia by apogametic replication has been well documented (Collins and Ling, 1968; Yemma and Therrien, 1972; Therrien and Yemma, 1975). These selfed plasmodia are replicated haploid (2C) (Therrien and Yemma, 1975), and have the same DNA content as the myxamoebal mating type they arose from. In this investigation a single plasmodium was used for each fixative type, so the occurrence of a selfed plasmodium would provide the unimodal distribution seen in Figure 18 and the 1:1 DNA content ratio between the plasmodium and myxamoeba nuclei.

The three fixatives used in this study provide insight into the variations in the stage of the cell cycle encountered with each tissue. Only interphase nuclei were measured for this investigation, so the nuclear populations should be in one of three states of DNA replication: G_1--unreplicated, S--in the process of replication (some cells complete, some incomplete), or G_2--replicated. Using Swift's (1950) designations of C, 2C, or 4C to indicate DNA content, the haploid tissues (sperm and myxamoeba) will be C if in G_1 and 2C if in G_2. The diploid tissues (liver and plasmodium) will be in 2C if in G_1 and 4C if in G_2, although in liver tissue polyploid cells can commonly be found (Swift, 1950; Yemma and Penza, 1987). On a histogram, cells in the same stage of DNA replication will fall within two standard deviations of the mean (Novak, 1985).

The liver cells fixed in formalin and Carnoy's fixative appear to be in G_1, while the population fixed in STF are in G_2 (Table 8), explaining the doubled stain intensity for STF-fixed cells. Some of the cells in the formalin population are in S phase, making the mean DNA content somewhat higher than that of the Carnoy's-fixed population. Also, Carnoy's fixation causes some loss of nuclear DNA, resulting in lower stain intensities (Giroud and Montmasson, 1989).

With sperm the new fixative, STF, again shows substantially higher levels of DNA content than either 10% formalin or Carnoy's fixative. The STF population has twice the DNA content of the formalin-fixed sperm, and almost three times that of the Carnoy's-fixed sperm. The formalin and Carnoy's populations can be interpreted to be in G_1, especially since Carnoy's fixative may remove some stainable material as suggested by Giroud and Montmasson (1989). The formalin-fixed sperm may also include some cells in S phase, which includes immature sperm cells, which would increase the mean DNA concentration of that population. These two populations represent predominantly post-meiotic sperm with 1C DNA content. The STF population, however, is 2C and must represent pre-meiotic or meiotic and immature sperm.

Interpretation of the myxamoebal populations suggests that both the STF and formalin-fixed nuclei are in G_2. Since the myxamoebae were pooled from several plates, some of the formalin cells likely come from a culture that was in G_1 or S, reducing the mean DNA content somewhat. The Carnoy's-fixed myxamoebae have half the DNA of the STF nuclei, and are in G_1. *D. iridis* myxamoebae have a very short G_1 phase and an extended G_2 (Yemma and Therrien, 1972), so the Carnoy's population must have been fixed just after a mitotic division.

The formalin-fixed plasmodium was a selfer in G_2 and therefore had only half the DNA of the STF population. The STF-fixed plasmodium nuclei were in G_2 with a replicated diploid DNA content of 4C. The Carnoy's-fixed plasmodium also had half the DNA of the STF population, indicating that these nuclei were in G_1 with an unreplicated diploid content of 2C. Both the formalin and Carnoy's-fixed plasmodium populations

contained the 2C amount of DNA, but the formalin selfer cells were haploid, replicated (Therrien and Yemma, 1975), while the G_1 crossed plasmodium nuclei were diploid, unreplicated.

Tukey's tests (Tables 10-13), run on the raw data presented in Appendix A, reinforce the interpretation of various stages of interphase presented in Table 8.

For liver tissue (Table 10) Tukey's test indicates that all three populations are different. Carnoy's-fixed liver cells are in G_1, formalin-fixed cells are moving from G_1 to S, and the STF-fixed cells are in G_2.

For sperm cells (Table 11), Tukey's test indicates that formalin and Carnoy's-fixed cells are very similar populations (both are in G_1), but the STF-fixed population is significantly different (G_2).

Table 12 indicates that the formalin and Carnoy's-fixed plasmodium populations can be seen as identical in DNA content. This correlates with the G_1 Carnoy's-fixed plasmodium and G_2 (selfed) formalin-fixed plasmodium which are both 2C in DNA content. Again, the STF population (which is 4C in DNA content) is significantly different from the other two.

Tukey's test on the myxamoeba data (Table 13) indicate that the STF and formalin-fixed populations can be viewed as identical. Both are G_2 populations. The Carnoy's-fixed cells, however, are significantly different and are in G_1.

The influence of a fixative on dye regeneration in the Feulgen reaction is recognized by many researchers. Swift (1950) reported that stain intensities were doubled in nuclei fixed in 50% formalin over the same tissue type fixed in Carnoy's fixative. Dutt (1971, 1976) observed enhanced staining with 40% buffered neutral formaldehyde over 10% formalin and less intense staining with acetic acid alcohol than with 10% formalin. Giroud and Montmasson (1989) observed lower stain intensity in Carnoy's-fixed tissue than with formalin fixation. They attributed this to the presence of ethanol in Carnoy's fixative which is known to extract histones and thus induce a loss of Feulgen-stainable

material. For this reason they describe Carnoy's fixative as ineffective for Feulgen staining (also Porcelli, 1972 and Karalova *et al.*, 1980).

In this investigation the new fixative, STF, appears to have provided somewhat enhanced stain binding over 10% buffered formalin and Carnoy's fixative for almost every tissue. To make the comparison, however, the stage of interphase that each tissue was in had to be equalized. The STF-fixed tissues were in G_2 of interphase while most of the Carnoy's and formalin-fixed tissues were in G_1. To equalize these DNA values, the G_1 values were doubled to indicate their expected G_2 DNA content (Table 8). This "expected G_2" value was then compared to the STF G_2 value to determine the percent of stain enhancement provided by the new fixative. Enhancement ranged from 1 to 47 percent, but most tissues showed 10 to 20 percent stain enhancement when fixed with STF (Table 8).

Liver nuclei fixed with 10% formalin, however, appeared to give 7 percent higher stain intensities than STF when converted from G_1 to G_2. However, the population of liver cells fixed in 10% formalin contained some cells in S phase (as indicated by its slightly higher mean DNA value than Carnoy's-fixed liver cells), so doubling the mean DNA value resulted in a value larger than the "expected G_2" value for that population.

The stain enhancement provided by STF may, however, be due to some depolymerization of the DNA by the acidic cresyl violet stain. The staining curve generated for STF (Figure 10) shows an initial rise at 60 minutes of exposure to the stain, followed by the rapid increase in stain intensity to 90 minutes. Although the stain intensity at 60 minutes is not significantly different from those at 30, 40, or 50 minutes (Table 6), the increase at 60 minutes could represent the beginning of depolymerization. This small amount of depolymerization could raise stain intensities by 10 to 20 percent, as seen in Table 8.

The role of a fixative is to denature proteins and DNA to prevent autolysis by tissue enzymes (Bancroft, 1975). For the Feulgen reaction, a "good" fixative will denature DNA to the largest extent possible to expose a larger proportion of aldehyde for dye binding. Therefore, enhanced staining by 40% and 50 % formaldehyde and STF over 10% formalin

and Carnoy's fixative may be due to a greater denaturation of DNA by these fixatives (Dutt, 1971). Swift (1950) noted that treatment with desoxyribonuclease sufficient to remove most or all of the Feulgen-positive material from Carnoy-fixed sections had little effect on 50% formalin-fixed sections. This indicates that the DNA is denatured differently by various fixatives.

The results of this investigation indicate that any of the three fixatives, 10% buffered formalin, Carnoy's fixative, and Streck's Tissue Fixative, may be used with the Blue Feulgen reaction. Ten percent formalin has been successfully proven by other investigators to work well with the cresyl violet procedure (Kasten, 1959; Dutt, 1970, 1971b; Bidinotto, 1993), and the results here reinforce their results. Formalin fixation provided excellent staining and demonstrated the proper ratios between nuclear DNA classes. Carnoy's fixative produced acceptable results, demonstrating the proper ratio between tissues of different ploidy level, but with somewhat reduced stain intensity on all tissue types. Carnoy's fixative would not, then, be the first fixative of choice when running the Blue Feulgen reaction. Streck's Tissue Fixative (a previously untried fixative) proved to be easy to work with, had an inoffensive smell, and produced excellent Feulgen results. While maintaining the proper ratio between tissues of different ploidy level, it also provided excellent staining. STF fixation appeared to produce 10 to 20 percent deeper staining than Carnoy's fixative and 10% formalin for all four tissue types. In addition, Streck's Tissue Fixative proved to be sensitive enough to elucidate different stages of the cell cycle. STF and 10% buffered formalin are highly recommended for use with the Blue Feulgen reaction for microspectrophotometric preparations.

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APPENDIX A

Microspectrophotometer Readings On and Off Each Nucleus at λ_1

Liver Tissue

Liver/Formalin			Liver/STF			Liver/Carnoy		
On /	Off	Peak/2	On /	Off	Peak/2	On /	Off	Peak/2
82.0	98.1	16.1	75.3	90.5	15.2	82.2	93.7	11.5
80.6	97.7	17.1	80.7	91.3	10.6	79.0	92.5	13.5
79.4	94.8	15.4	77.5	92.5	15.0	81.6	92.8	11.2
81.2	95.2	14.0	77.3	92.2	14.9	83.1	90.7	7.6
82.1	94.3	12.2	78.9	91.4	12.5	81.7	92.1	10.4
80.6	96.0	15.4	81.9	92.7	10.8	81.9	91.6	9.7
82.4	96.0	13.6	77.9	92.9	15.0	82.7	91.3	8.6
82.4	97.5	15.1	78.4	91.7	13.3	83.0	91.9	8.9
81.9	96.2	14.3	78.0	92.9	14.9	82.4	91.8	9.4
81.7	95.7	14.0	80.9	93.1	12.2	86.4	93.0	6.6
82.4	95.6	13.2	78.1	89.9	11.8	80.8	92.9	12.1
82.5	95.7	13.2	79.1	92.3	13.2	82.6	92.3	9.7
82.6	94.6	12.0	78.4	92.3	13.9	83.8	92.4	8.6
82.2	95.1	12.9	80.1	91.5	11.4	82.9	91.9	9.0
81.3	95.5	14.2	78.7	92.7	14.0	81.9	91.8	9.9
81.4	95.7	14.3	78.9	92.1	13.2	81.6	92.1	10.5
80.9	95.6	14.7	79.3	92.3	13.0	79.5	91.3	11.8
84.9	95.7	10.8	90.6	90.8	0.2	81.9	92.9	11.0
81.8	95.6	13.8	79.2	93.2	14.0	82.0	91.6	9.6
81.6	95.2	13.6	76.3	91.8	15.5	83.1	90.7	7.6
80.3	91.9	11.6	81.1	92.2	11.1	81.1	91.8	10.7
78.2	92.1	13.9	78.2	91.5	13.3	83.1	92.1	9.0
79.8	91.6	11.8	75.0	92.3	17.3	83.0	91.9	8.9
80.6	94.0	13.4	79.7	90.3	10.6	82.5	91.7	9.2
82.0	93.6	11.6	77.4	92.1	14.7	82.4	90.9	8.5
81.5	94.9	13.4	79.3	91.3	12.0	85.2	93.0	7.8
86.4	93.4	7.0	67.8	91.6	23.8	82.5	91.2	8.7
83.0	94.9	11.9	76.3	91.8	15.5	81.4	90.3	8.9
81.7	95.7	14.0	78.0	90.5	12.5	83.5	92.1	8.6
81.7	93.0	11.3	77.8	90.4	12.6	82.6	92.3	9.7
83.8	97.0	13.2	77.9	92.0	14.1	81.1	91.6	10.5
83.4	95.3	11.9	79.3	91.9	12.6	81.6	92.1	10.5
81.7	95.7	14.0	77.6	90.8	13.2	83.9	91.6	7.7
81.3	94.7	13.4	76.4	90.9	14.5	81.2	91.3	10.1
81.8	95.0	13.2	75.6	90.7	15.1	81.4	90.6	9.2
82.6	94.5	11.9	77.1	91.0	13.9	82.0	92.7	10.7
81.0	95.1	14.1	78.4	91.6	13.2	83.2	92.6	9.4
80.7	94.4	13.7	76.2	91.4	15.2	79.6	89.4	9.8
80.8	92.6	11.8	76.5	91.1	14.6	84.6	91.6	7.0
81.4	93.4	12.0	76.5	90.7	14.2	81.1	91.8	10.7
81.3	93.3	12.0	77.7	89.4	11.7	81.7	91.5	9.8
83.1	94.8	11.7	76.4	91.1	14.7	85.2	93.0	7.8

Liver

80.3	93.2	12.9	75.8	90.5	14.7	83.5	92.1	8.6
81.5	93.7	12.2	76.7	90.3	13.6	82.0	92.7	10.7
79.9	93.2	13.3	75.2	90.8	15.6	82.4	90.8	8.4
80.8	93.7	12.9	76.1	90.3	14.2	81.8	91.0	9.2
80.6	93.4	12.8	74.6	90.6	16.0	81.7	91.5	9.8
82.3	95.1	12.8	75.9	89.7	13.8	81.5	90.6	9.1
81.2	93.2	12.0	75.5	90.1	14.6	79.8	91.1	11.3
79.9	93.6	13.7	67.3	89.8	22.5	81.1	91.0	9.9
79.6	94.0	14.4	75.1	88.1	13.0	78.4	91.9	13.5
80.5	93.2	12.7	78.7	89.6	10.9	78.3	89.5	11.2
80.7	94.0	13.3	74.1	88.8	14.7	75.3	88.5	13.2
81.4	93.6	12.2	74.1	88.1	14.0	80.6	88.2	7.6
81.3	95.6	14.3	74.3	89.0	14.7	76.6	87.7	11.1
79.1	90.9	11.8	75.1	89.2	14.1	78.1	88.6	10.5
78.6	93.0	14.4	74.4	90.6	16.2	81.5	90.6	9.1
80.2	92.7	12.5	77.3	90.0	12.7	81.9	88.7	6.8
80.9	92.3	11.4	74.5	88.0	13.5	81.2	89.1	7.9
81.2	94.5	13.3	73.3	88.4	15.1	80.6	88.2	7.6
79.4	94.6	15.2	76.4	86.5	10.1	77.8	88.6	10.8
79.2	92.1	12.9	69.6	87.9	18.3	77.7	88.7	11.0
80.4	90.8	10.4	77.9	88.3	10.4	77.2	88.0	10.8
81.2	94.0	12.8	77.0	88.5	11.5	78.4	88.9	10.5
77.7	89.5	11.8	74.1	87.4	13.3	81.9	88.7	6.8
78.0	91.6	13.6	73.8	89.1	15.3	80.0	88.5	8.5
77.8	91.5	13.7	73.2	88.5	15.3	79.1	89.5	10.4
78.8	90.8	12.0	72.3	88.1	15.8	78.8	87.9	9.1
78.3	91.6	13.3	73.6	87.4	13.8	79.3	88.0	8.7
78.8	93.1	14.3	77.8	87.9	10.1	77.2	88.0	10.8
78.8	90.6	11.8	70.8	88.2	17.4	78.3	88.3	10.0
78.8	90.9	12.1	73.8	89.3	15.5	80.3	88.5	8.2
78.6	92.2	13.6	66.0	86.3	20.3	78.8	87.9	9.1
81.7	93.1	11.4	74.2	87.2	13.0	80.7	89.1	8.4
79.5	93.1	13.6	75.1	88.3	13.2	76.3	88.4	12.1
79.6	93.4	13.8	72.3	88.4	16.1	80.7	89.1	8.4
82.2	93.4	11.2	75.2	88.6	13.4	78.8	89.4	10.6
79.8	92.7	12.9	72.7	86.9	14.2	80.5	88.3	7.8
79.2	93.0	13.8	73.2	87.3	14.1	75.1	88.9	13.8
80.6	94.1	13.5	64.2	88.7	24.5	76.0	87.1	11.1
80.7	92.4	11.7	72.7	87.5	14.8	76.3	88.4	12.1
79.6	91.5	11.9	64.2	88.4	24.2	76.5	87.1	10.6
80.3	92.7	12.4	72.5	87.9	15.4	78.4	88.1	9.7
80.0	93.0	13.0	65.0	86.3	21.3	78.0	87.7	9.7
80.3	93.5	13.2	72.8	87.1	14.3	80.4	89.0	8.6
83.8	94.5	10.7	71.9	86.6	14.7	78.9	87.9	9.0
81.2	92.5	11.3	73.2	87.3	14.1	79.9	88.9	9.0
80.2	93.5	13.3	71.5	86.1	14.6	77.8	88.3	10.5

81.6	95.2	13.6	73.9	87.8	13.9	80.5	88.3	7.8
79.8	93.9	14.1	73.5	86.7	13.2	81.2	86.5	5.3
81.7	95.7	14.0	71.8	86.9	15.1	79.9	87.2	7.3
80.2	95.2	15.0	72.1	87.1	15.0	81.0	87.2	6.2
79.2	90.7	11.5	72.3	86.7	14.4	81.7	89.0	7.3
84.1	90.4	6.3	73.0	86.1	13.1	76.6	87.4	10.8
78.9	89.4	10.5	72.7	87.2	14.5	80.4	89.0	8.6
79.8	88.6	8.8	71.4	87.2	15.8	82.2	88.9	6.7
78.1	88.0	9.9	73.6	86.0	12.4	80.8	87.7	6.9
80.3	91.4	11.1	71.7	87.2	15.5	80.2	88.1	7.9
78.3	92.1	13.8	72.5	86.2	13.7	74.8	88.3	13.5
77.9	91.4	13.5	65.7	86.3	20.6	79.9	88.9	9.0
		1282.9			1435.1	80.1	88.4	8.3
		100			100	79.1	89.0	9.9
N	304					76.0	88.3	12.3
	3708.3					77.8	88.3	10.5
C	45235.1608							990.3
SSi	16716.87			21534.53				104
								9725.63
SS	47977.03							

TSS   2741.86918

GSS1   16458.3241
GSS2   20595.1201
GSS3   9429.75087

46483.1951
GG   1248.03424   F=   125.736   ***

Source of Variation	SS	DF	MS
Total	2741.9	303	
Groups	1248.0	2	624.017122
Error	1493.8	301	4.96290676

F0.05(1),2,301   3.03
Rej Ho

Sperm Tissue

Sperm/Formalin			Sperm/STF			Sperm/Carnoy		
On /	Off	Peak/2	On /	Off	Peak/2	On /	Off	Peak/2
90.4	94.0	3.6	86.1	93.2	7.1	90.4	94.1	3.7
81.3	86.9	5.6	83.2	90.4	7.2	88.9	93.0	4.1
89.0	94.4	5.4	85.0	90.5	5.5	91.0	94.1	3.1
82.3	86.1	3.8	87.4	92.6	5.2	90.1	94.3	4.2
85.8	92.1	6.3	84.4	90.0	5.6	90.2	94.9	4.7
78.2	84.6	6.4	80.5	89.3	8.8	90.0	94.0	4.0
84.9	90.7	5.8	84.9	92.5	7.6	90.6	95.3	4.7
88.1	93.9	5.8	83.6	91.7	8.1	89.5	93.4	3.9
84.1	90.3	6.2	85.6	91.8	6.2	90.6	95.5	4.9
88.0	93.9	5.9	83.3	89.9	6.6	91.0	94.7	3.7
82.4	86.3	3.9	86.5	93.1	6.6	90.9	94.4	3.5
81.3	86.1	4.8	83.9	90.3	6.4	90.2	95.0	4.8
82.6	86.4	3.8	86.2	92.7	6.5	90.5	95.0	4.5
83.4	86.8	3.4	86.1	91.6	5.5	91.1	95.5	4.4
86.5	88.9	2.4	84.1	88.4	4.3	91.5	95.3	3.8
83.5	86.1	2.6	85.5	92.4	6.9	91.0	95.7	4.7
84.6	90.1	5.5	85.8	92.4	6.6	91.2	95.0	3.8
83.5	88.2	4.7	83.8	91.6	7.8	90.4	94.7	4.3
79.7	84.4	4.7	85.9	93.2	7.3	91.1	94.2	3.1
84.3	90.3	6.0	86.3	91.3	5.0	90.6	94.5	3.9
82.4	87.0	4.6	81.3	89.2	7.9	91.2	95.0	3.8
84.0	87.9	3.9	83.6	92.3	8.7	90.4	95.3	4.9
83.5	89.3	5.8	85.5	92.4	6.9	90.2	94.7	4.5
84.8	89.1	4.3	85.0	93.1	8.1	91.4	94.9	3.5
84.6	88.7	4.1	85.0	92.6	7.6	90.8	95.1	4.3
87.4	93.6	6.2	84.8	92.4	7.6	91.4	94.5	3.1
81.7	86.9	5.2	84.9	92.3	7.4	91.8	94.5	2.7
84.3	89.3	5.0	85.0	92.3	7.3	91.0	94.8	3.8
94.6	98.9	4.3	83.6	90.9	7.3	88.9	93.0	4.1
90.8	96.8	6.0	84.3	92.1	7.8	91.8	94.5	2.7
85.6	89.8	4.2	84.6	91.5	6.9	92.0	95.2	3.2
84.5	88.8	4.3	83.8	90.9	7.1	91.8	94.5	2.7
91.7	95.9	4.2	81.8	90.0	8.2	90.7	93.7	3.0
91.2	96.3	5.1	82.9	91.7	8.8	93.2	96.4	3.2
89.3	95.2	5.9	82.6	90.9	8.3	89.6	92.7	3.1
86.1	94.2	8.1	84.6	91.2	6.6	90.8	95.9	5.1
85.8	91.1	5.3	82.9	90.3	7.4	91.0	94.7	3.7
89.6	93.0	3.4	83.4	90.4	7.0	87.5	91.5	4.0
88.1	94.0	5.9	82.3	89.9	7.6	89.8	92.1	2.3
87.1	92.1	5.0	82.9	90.4	7.5	89.9	93.4	3.5
89.5	94.9	5.4	81.5	88.1	6.6	91.2	95.0	3.8
88.9	94.3	5.4	81.7	89.1	7.4	93.2	95.7	2.5

Sperm

92.1	95.8	3.7	83.0	91.3	8.3	93.6	96.1	2.5
89.2	96.4	7.2	84.0	93.1	9.1	93.1	95.5	2.4
89.2	94.2	5.0	82.5	90.1	7.6	92.7	95.1	2.4
90.8	95.6	4.8	81.8	89.1	7.3	93.5	96.3	2.8
91.7	99.4	7.7	80.8	89.2	8.4	90.2	94.7	4.5
91.1	95.5	4.4	82.9	90.3	7.4	93.2	95.5	2.3
89.0	95.1	6.1	84.3	90.0	5.7	92.6	96.3	3.7
91.6	97.9	6.3	80.2	87.6	7.4	92.3	94.6	2.3
90.4	96.8	6.4	81.2	89.5	8.3	90.4	94.3	3.9
90.1	92.8	2.7	82.0	88.9	6.9	93.1	95.7	2.6
89.2	95.1	5.9	80.6	88.9	8.3	93.2	95.8	2.6
91.5	96.7	5.2	81.3	88.9	7.6	92.2	95.6	3.4
86.7	91.4	4.7	81.0	85.7	3.7	90.7	93.7	3.0
87.4	93.4	6.0	82.5	89.1	8.5	92.5	94.9	2.4
89.6	94.7	5.1	79.9	86.2	4.9	92.4	95.6	3.2
86.8	89.8	3.0	78.9	86.7	5.7	92.5	95.7	3.2
90.6	95.5	4.9	80.1	86.4	3.9	91.8	96.0	4.2
90.2	95.8	5.6	82.2	88.8	8.9	92.0	95.0	3.0
90.4	95.5	5.1	82.8	89.6	10.7	91.0	94.6	3.6
89.1	93.9	4.8	82.6	89.5	9.4	92.7	95.1	2.4
85.8	93.0	7.2	83.2	89.2	7.0	92.9	95.6	2.7
88.7	94.0	5.3	82.3	89.3	6.5	92.3	95.1	2.8
85.2	89.8	4.6	82.5	83.3	5.7	91.5	95.7	4.2
86.7	91.6	4.9	82.5	89.2	6.0	92.1	95.1	3.0
91.8	98.2	6.4	82.3	88.6	6.3	91.2	93.9	2.7
88.1	93.9	5.8	81.7	89.2	6.7	90.4	94.3	3.9
88.5	94.5	6.0	80.7	87.7	5.2	91.3	95.0	3.7
87.3	92.5	5.2	81.0	87.7	5.4	92.4	94.6	2.2
92.6	98.6	6.0	81.5	89.3	7.6	91.0	94.1	3.1
88.9	94.2	5.3	81.6	88.6	7.9	92.5	94.1	1.6
89.9	94.5	4.6	81.2	89.3	8.3	91.0	94.0	3.0
88.9	93.7	4.8	81.8	88.8	7.3	91.4	93.8	2.4
86.7	92.2	5.5	81.3	87.8	6.2	91.3	93.4	2.1
84.2	87.4	3.2	81.6	89.3	8.1	90.0	92.6	2.6
92.7	99.4	6.7	78.1	87.6	5.8	90.7	94.5	3.8
96.3	101.5	5.2	82.0	89.6	8.3	90.7	94.3	3.6
89.1	93.2	4.1	81.6	88.9	7.3	91.6	94.2	2.6
87.1	92.1	5.0	80.4	89.7	11.6	91.2	94.6	3.4
89.7	95.2	5.5	81.3	89.3	7.3	90.3	93.7	3.4
84.6	90.3	5.7	80.6	88.1	6.5	91.8	93.6	1.8
87.2	91.1	3.9	81.3	89.4	9.0	92.2	94.2	2.0
87.9	93.3	5.4	81.1	89.2	7.9	91.8	93.9	2.1
81.8	84.9	3.1	82.2	88.9	8.3	89.8	93.1	3.3
84.3	90.3	6.0	81.8	89.0	7.7	89.8	93.5	3.7
87.5	92.1	4.6	81.4	88.9	7.8	90.4	93.4	3.0

Sperm

89.2	94.0	4.8	81.1	88.8	7.0	91.0	93.4	1.9
86.3	92.2	5.9	81.2	88.5	7.1	89.9	92.9	3.9
88.0	94.3	6.3	80.2	88.2	88.2	89.3	93.8	4.7
89.3	93.1	3.8	79.2	87.3	6.2	91.2	94.0	3.1
82.0	86.4	4.4	81.9	90.1	8.9	91.0	94.3	3.5
84.0	90.5	6.5	80.7	88.2	8.0	91.2	94.5	2.9
88.1	93.9	5.8	81.6	88.9	9.7	91.7	94.1	2.9
84.2	89.1	4.9	79.2	89.4	7.5	92.3	94.6	1.8
84.5	90.0	5.5	82.5	88.9	8.2	91.5	94.1	2.4
84.2	89.3	5.1	81.1	88.3	6.7	91.3	93.9	1.9
86.7	92.2	5.5	81.1	90.3	11.1	89.7	93.2	3.5
83.6	88.5	4.9	82.3	89.2	6.7			
95.2	100.1	4.9	79.2	89.4	8.3			

	511.1		810.1		
	100		100		
N	298				
	1646.1				324.9
C	8913.30661				98
SSi	2726.21		13222.99		1144.09
SS	17093.29				

Sperm Tissue

Source of Variation	SS	DF	MS
Total	8180.0	298	
Groups	1276.5	2	638.272842
Error	6903.4	296	23.3224247

TSS   8179.98339

GSS1   2612.2321
GSS2   6562.6201
GSS3   1015.0001

10189.8523

GG   1276.54568   Rej Ho   F=   27.367   ***

F0.05(1),2,296   3.73

Plasmodium Tissue

Plasmodium/Formalin			Plasmodium/STF			Plasmodium/Carnoy		
On / Off Peak/2			On / Off Peak/2			On / Off Peak/2		
85.4	94.3	8.9	83.4	97.5	14.1	82.0	84.9	2.9
88.2	94.4	6.2	88.9	101.2	12.3	82.0	84.9	2.9
87.3	94.9	7.6	85.3	98.4	13.1	87.7	90.0	2.3
85.5	94.6	9.1	84.8	96.0	11.2	80.5	84.9	4.4
85.1	94.8	9.7	79.0	91.0	12.0	82.4	86.4	4.0
88.0	94.8	6.8	80.5	99.0	18.5	80.5	84.9	4.4
86.3	95.0	8.7	80.7	98.1	17.4	77.6	87.0	9.4
86.6	92.4	5.8	82.9	97.1	14.2	83.6	91.3	7.7
88.3	93.0	4.7	86.6	93.8	7.2	80.2	90.6	10.4
88.6	94.5	5.9	81.0	100.7	19.7	77.6	87.0	9.4
87.3	94.9	7.6	67.4	90.8	23.4	85.2	89.6	4.4
84.7	94.7	10.0	78.2	91.3	13.1	82.4	86.4	4.0
89.5	94.0	4.5	70.4	87.5	17.1	83.8	90.0	6.2
85.6	94.5	8.9	73.4	95.2	21.8	85.1	95.0	9.9
87.4	92.5	5.1	72.5	95.0	22.5	82.3	87.0	4.7
86.6	92.1	5.5	67.7	87.5	19.8	83.6	91.3	7.7
85.7	91.8	6.1	79.3	97.6	18.3	82.1	89.8	7.7
87.8	94.0	6.2	90.3	94.2	3.9	84.4	89.5	5.1
85.0	94.5	9.5	70.9	92.1	21.2	83.8	90.0	6.2
85.7	93.9	8.2	70.8	94.1	23.3	74.3	92.0	17.7
83.8	94.0	10.2	86.8	97.1	10.3	82.3	87.0	4.7
81.5	93.7	12.2	81.0	100.3	19.3	82.4	91.8	9.4
81.8	92.2	10.4	80.5	97.9	17.4	78.6	89.0	10.4
85.5	94.2	8.7	90.3	96.4	6.1	80.8	91.1	10.3
85.7	93.4	7.7	90.5	94.4	3.9	78.1	90.3	12.2
87.2	93.6	6.4	89.6	94.0	4.4	84.5	91.3	6.8
89.8	93.8	4.0	80.1	95.3	15.2	73.1	83.5	10.4
90.3	93.5	3.2	84.3	97.4	13.1	79.8	88.4	8.6
86.3	93.0	6.7	82.5	96.0	13.5	87.7	93.3	5.6
84.6	91.0	6.4	85.4	95.6	10.2	84.5	91.6	7.1
84.8	91.9	7.1	76.3	92.7	16.4	81.8	89.6	7.8
84.2	92.0	7.8	79.1	85.7	6.6	82.9	91.9	9.0
85.1	93.3	8.2	81.8	91.8	10.0	88.5	95.0	6.5
83.7	93.8	10.1	80.3	90.2	9.9	78.3	88.7	10.4
87.9	93.6	5.7	80.8	90.9	10.1	78.9	86.8	7.9
82.5	93.8	11.3	87.7	90.6	2.9	80.2	86.0	5.8
85.8	92.7	6.9	81.8	91.5	9.7	86.3	97.0	10.7
88.5	93.5	5.0	82.1	91.1	9.0	81.9	93.5	11.6
89.0	93.7	4.7	81.3	92.8	11.5	78.0	86.0	8.0
84.0	94.0	10.0	82.5	89.0	6.5	87.7	94.8	7.1
89.0	93.3	4.3	75.0	93.7	18.7	86.1	92.2	6.1
87.4	92.8	5.4	88.5	97.0	8.5	80.8	87.2	6.4

Plasmodium

85.4	92.0	6.6	82.7	89.2	6.5	85.2	88.4	3.2
89.2	93.1	3.9	75.0	85.7	10.7	82.3	91.4	9.1
88.7	93.3	4.6	86.7	97.1	10.4	80.5	93.2	12.7
87.6	92.6	5.0	84.8	95.6	10.8	84.0	91.7	7.7
88.4	93.1	4.7	79.1	91.4	12.3	86.6	95.7	9.1
88.7	93.3	4.6	87.2	95.3	8.1	80.6	89.4	8.8
87.6	92.6	5.0	89.7	97.5	7.8	84.0	92.0	8.0
88.4	93.1	4.7	79.2	93.2	14.0	88.0	96.4	8.4
89.0	93.6	4.6	92.4	100.3	7.9	75.9	85.5	9.6
90.1	93.4	3.3	94.0	99.4	5.4	86.6	95.0	8.4
89.9	93.8	3.9	95.3	100.4	5.1	89.6	94.0	4.4
88.9	92.3	3.4	88.1	99.9	11.8	80.2	85.2	5.0
82.1	92.3	10.2	93.9	103.1	9.2	83.1	90.0	6.9
87.3	92.9	5.6	94.7	98.3	3.6	83.6	92.4	8.8
86.8	92.8	6.0	95.4	103.9	8.5	79.9	91.0	11.1
88.3	93.6	5.3	89.2	97.6	8.4	91.3	97.8	6.5
88.4	93.2	4.8	92.6	96.8	4.2	82.4	89.3	6.9
84.6	91.4	6.8	83.1	99.4	16.3	79.4	89.5	10.1
89.1	92.4	3.3	81.3	98.2	16.9	77.7	90.4	12.7
89.1	92.4	3.3	74.1	97.3	23.2	81.8	87.1	5.3
83.3	88.4	5.1	88.6	100.3	11.7	73.6	80.4	6.8
86.6	93.0	6.4	94.5	99.4	4.9	69.0	81.0	12.0
87.1	93.4	6.3	96.7	102.9	6.2	87.1	90.1	3.0
88.3	91.8	3.5	93.7	99.9	6.2	84.7	89.0	4.3
87.7	92.0	4.3	79.4	101.1	21.7	83.5	91.9	8.4
87.8	92.6	4.8	86.6	98.0	11.4	91.0	96.0	5.0
83.1	92.4	9.3	91.2	94.8	3.6	84.8	90.4	5.6
86.1	91.3	5.2	89.9	98.8	8.9	90.8	96.9	6.1
84.9	91.1	6.2	90.6	97.2	6.6	84.4	90.2	5.8
89.5	91.8	2.3	83.7	97.1	13.4	87.7	93.4	5.7
86.6	91.1	4.5	91.3	99.9	8.6	76.0	84.5	8.5
84.7	90.2	5.5	92.4	100.3	7.9	86.2	91.7	5.5
87.2	90.5	3.3	85.4	99.8	14.4	74.5	85.5	11.0
88.6	91.5	2.9	93.1	98.8	5.7	88.9	92.5	3.6
88.7	91.2	2.5	81.6	97.6	16.0	81.6	89.8	8.2
87.5	91.1	3.6	89.7	96.5	6.8	79.4	88.4	9.0
80.4	91.4	11.0	87.0	100.0	13.0	87.2	93.0	5.8
82.8	92.1	9.3	88.7	99.7	11.0	87.0	90.8	3.8
82.2	92.0	9.8	83.0	94.8	11.8	90.6	94.5	3.9
82.6	92.2	9.6	87.9	98.3	10.4	88.6	93.8	5.2
86.0	94.5	8.5	76.5	89.3	12.8	82.7	90.9	8.2
89.0	92.8	3.8	83.7	98.2	14.5	85.7	94.0	8.3
82.2	93.3	11.1	92.0	97.1	5.1	87.7	93.3	5.6
82.1	89.7	7.6	93.2	97.0	3.8	76.4	91.0	14.6
86.3	94.0	7.7	90.7	102.1	11.4	84.5	91.3	6.8

Plasmodium

85.5	94.4	8.9	89.4	97.8	8.4	89.0	92.9	3.9
90.1	94.2	4.1	75.8	88.8	13.0	86.2	93.4	7.2
86.4	94.4	8.0	90.9	96.1	5.2	90.9	95.4	4.5
79.0	86.5	7.5	77.3	91.8	14.5	85.7	90.6	4.9
76.6	88.1	11.5	81.3	94.8	13.5	89.5	92.2	2.7
83.3	88.4	5.1	83.1	96.5	13.4	88.3	93.3	5.0
79.3	87.0	7.7	79.0	99.6	20.6	74.3	92.0	17.7
82.5	87.6	5.1	79.0	98.0	19.0	87.0	91.8	4.8
83.2	87.6	4.4	84.6	94.8	10.2	80.8	91.1	10.3

		623.4		1124.0		
		96		96		
N		288				
		2443.5				
C	19640.435					696.1
SSi	4596.68			15841.84		96
						6105.78
SS	26544.3					

Plasmodium

Source of Variation	SS	DF	MS
Total	6903.9	287	
Groups	1538.8	2	769.392639
Error	5365.1	285	18.824841

TSS   6903.86497

GSS1   3886.2756

GSS2   12633.76

GSS3   4659.18471

        21179.2203

GG   1538.78528   F=   40.871   ***

            F0.05(2),2,285   3.73

                Rej Ho

Honduran Myxamoeba

Hon/Formalin On / Off Peak/2			Hon/STF On / Off Peak/2			Hon/Carnoy On / Off Peak/2		
83.7	93.1	9.4	86.7	93.7	7.0	87.1	93.2	6.1
85.5	91.7	6.2	88.0	93.7	5.7	88.9	96.6	7.7
90.9	94.0	3.1	86.3	91.0	4.7	90.3	92.8	2.5
87.0	94.1	7.1	88.1	91.9	3.8	86.6	88.9	2.3
88.9	93.3	4.4	88.4	93.6	5.2	78.1	80.2	2.1
85.6	93.0	7.4	89.7	94.4	4.7	85.3	88.8	3.5
87.8	92.9	5.1	90.8	93.5	2.7	84.4	86.4	2.0
87.2	92.8	5.6	86.9	94.6	7.7	84.4	88.1	3.7
87.3	92.2	4.9	90.3	94.0	3.7	87.1	90.9	3.8
86.9	92.6	5.7	87.3	92.4	5.1	88.6	90.0	1.4
86.5	93.5	7.0	85.8	93.3	7.5	83.7	86.7	3.0
81.6	92.3	10.7	86.9	92.2	5.3	84.8	88.0	3.2
87.5	93.4	5.9	82.5	92.7	10.2	85.8	89.8	4.0
88.6	93.3	4.7	86.6	89.1	2.5	85.4	90.7	5.3
84.4	93.5	9.1	83.2	90.2	7.0	85.5	88.5	3.0
86.5	92.9	6.4	84.4	90.7	6.3	85.2	89.9	4.7
85.9	89.8	3.9	86.7	88.6	1.9	84.6	90.1	5.5
85.1	90.8	5.7	84.4	92.7	8.3	83.7	88.8	5.1
80.7	87.4	6.7	88.4	93.3	4.9	86.3	89.9	3.6
81.5	89.9	8.4	84.8	92.3	7.5	85.5	88.5	3.0
87.2	92.4	5.2	89.7	92.2	2.5	81.9	84.7	2.8
84.0	93.0	9.0	82.4	88.3	5.9	86.6	89.3	2.7
85.6	92.6	7.0	78.5	88.8	10.3	77.5	81.6	4.1
85.9	92.0	6.1	84.6	91.1	6.5	78.9	84.0	5.1
83.4	87.7	4.3	85.6	90.5	4.9	82.7	87.1	4.4
86.1	92.0	5.9	82.8	89.6	6.8	84.4	86.1	1.7
85.3	89.9	4.6	82.1	87.4	5.3	81.4	85.1	3.7
87.7	93.6	5.9	86.3	92.3	6.0	82.4	84.3	1.9
87.1	92.8	5.7	86.3	92.2	5.9	83.1	85.8	2.7
87.3	94.9	7.6	82.7	88.8	6.1	84.0	85.5	1.5
84.5	90.4	5.9	85.4	93.4	8.0	84.2	87.8	3.6
86.5	89.7	3.2	83.8	91.8	8.0	83.6	86.3	2.7
83.8	89.7	5.9	85.0	91.1	6.1	82.5	86.7	4.2
87.3	94.2	6.9	81.2	89.6	8.4	83.8	85.1	1.3
87.8	92.9	5.1	86.6	91.4	4.8	85.5	88.5	3.0
90.2	95.1	4.9	84.7	90.3	5.6	83.5	87.3	3.8
83.7	92.2	8.5	84.7	90.4	5.7	80.5	83.7	3.2
88.0	92.9	4.9	84.6	89.0	4.4	84.4	86.1	1.7
88.3	93.0	4.7	86.6	89.9	3.3	87.1	90.9	3.8
88.2	91.4	3.2	89.3	93.8	4.5	81.6	85.5	3.9
87.5	93.9	6.4	88.4	93.8	5.4	80.7	86.3	5.6
85.7	94.5	8.8	85.9	93.9	8.0	81.5	86.2	4.7

87.0	93.6	6.6	87.9	93.6	5.7	83.7	86.7	3.0
88.8	91.5	2.7	87.8	94.3	6.5	77.5	81.9	4.4
86.3	92.4	6.1	90.2	94.1	3.9	77.9	80.6	2.7
88.9	93.7	4.8	87.7	92.7	5.0	78.1	80.2	2.1
84.0	88.5	4.5	88.2	92.6	4.4	77.1	81.1	4.0
85.5	90.7	5.2	87.0	91.8	4.8	78.4	80.0	1.6
85.1	90.1	5.0	86.0	92.3	6.3	78.8	81.5	2.7
85.9	90.2	4.3	87.1	93.0	5.9	78.4	83.2	4.8
85.5	91.7	6.2	85.3	91.5	6.2	79.3	81.7	2.4
85.3	91.4	6.1	86.4	92.9	6.5	77.5	80.2	2.7
88.7	93.1	4.4	84.9	92.9	8.0	77.0	84.0	7.0
86.0	93.4	7.4	82.2	92.8	10.6	78.5	82.0	3.5
87.1	92.8	5.7	87.3	92.0	4.7			
83.8	94.4	10.6	86.0	89.1	3.1			
85.9	94.2	8.3	81.8	90.4	8.6			
88.7	92.1	3.4	84.8	89.4	4.6			
89.6	93.7	4.1	85.2	89.5	4.3			
88.5	93.3	4.8	85.2	90.4	5.2			
89.5	93.8	4.3	85.8	92.7	6.9			
83.0	92.0	9.0	80.2	86.4	6.2			
84.1	91.4	7.3	86.5	91.1	4.6			
88.2	91.6	3.4	86.1	90.0	3.9			
86.2	93.5	7.3	84.0	89.2	5.2			
87.6	92.0	4.4	85.9	90.1	4.2			
87.2	92.5	5.3	82.4	90.8	8.4			
89.1	93.5	4.4	84.0	91.0	7.0			
83.2	92.0	8.8	86.0	92.6	6.6			
87.4	91.1	3.7	85.8	89.8	4.0			
86.0	93.4	7.4	82.4	92.2	9.8			
82.9	90.4	7.5	82.9	91.6	8.7			
85.4	93.5	8.1	86.8	91.2	4.4			
87.8	93.7	5.9	80.3	88.3	8.0			
87.7	92.7	5.0	82.2	89.0	6.8			
88.7	93.9	5.2	80.3	87.1	6.8			
88.1	94.4	6.3	83.6	88.8	5.2			
84.4	90.2	5.8	82.8	87.8	5.0			
86.7	92.8	6.1	84.6	89.0	4.4			
86.8	91.7	4.9	84.7	88.9	4.2			
87.9	93.7	5.8	80.7	86.1	5.4			
87.7	95.0	7.3	81.1	87.0	5.9			
86.8	92.2	5.4	81.0	88.8	7.8			
87.1	91.4	4.3	80.9	87.4	6.5			
86.8	90.0	3.2	87.0	92.4	5.4			
85.7	89.5	3.8	86.5	91.9	5.4			
83.5	88.3	4.8	84.7	92.3	7.6			

Myxamoeba

88.8	92.1	3.3	86.3	91.0	4.7
84.3	90.9	6.6	87.5	93.5	6.0
85.5	92.7	7.2	82.7	89.5	6.8
87.5	92.1	4.6	78.7	86.1	7.4
88.4	93.7	5.3	85.2	91.3	6.1
86.0	90.5	4.5	84.3	88.7	4.4
87.4	95.0	7.6	80.5	85.2	4.7
82.9	88.0	5.1	84.3	87.3	3.0
85.4	90.2	4.8	83.1	90.1	7.0
83.4	92.8	9.4	85.7	90.3	4.6
88.2	92.7	4.5	86.7	90.4	3.7
88.3	92.2	3.9	87.5	91.5	4.0
84.6	91.4	6.8	85.3	91.5	6.2
83.6	89.0	5.4			

	591.0		581.3	
	101		100	
	251			
N	1360.8			
	6091.37053			188.5
C	3754.88		3683.85	50
SSi				760.97
	8199.7		**Honduran Myxamoeba**	

Source of Variation	SS	DF	MS
Total	2108.3	250	
Groups	1122.2	2	561.096312
Error	986.1	248	3.97635827

SS 2108.32947

TSS

GSS1 3492.81

GSS2 3379.0969

GSS3 341.65625

7213.56315

GG 1122.19262

$F=$ 141.108 ***

F0.05(2),2,248 3.73

Rej Ho