THE EFFECTS OF AGING ON PRE-PREPARED AND POST-PREPARED BASIC FUCHSIN DYES USED IN THE FEULGEN NUCLEAL REACTION, FOR QUANTITATIVE CYTOPHOTOMETRIC MEASUREMENTS

by

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ABSTRACT

THE EFFECTS OF AGING ON PRE-PREPARED AND POST-PREPARED BASIC FUCHSIN USED IN THE FEULGEN NUCLEAL REACTION, FOR QUANTITATIVE CYTOPHOTOMETRIC MEASUREMENTS

Elizabeth J. Orosz

Master of Science

Youngstown State University, 1992

The objective of this investigation was to determine the effects of the aging of pre-prepared (hydrated) Basic Fuchsin on the quantitative cytophotometric properties of the Feulgen nucleal reaction. This was done by comparing six separate preparations of Schiff's reagent prepared from Basic Fuchsins manufactured in 1972, 1974, 1976, 1985 and 1987 respectively.

Changes in reactivity of post-prepared Schiff's reagents over time were analyzed in order to determine the stability, and thus the effect of dye ageing on quantitative determinations of cellular DNA content. The establishment of the time period (duration) for stability is important as part of determination of reaction parameters best suited to producing optimal Feulgen intensities.

To accomplish these objectives the two wavelength method of quantitative microspectrophotometry was utilized (Patau 1952). Measurements were made using

myxamoebae and plasmodia of the slime mold Didymium iridis, since both haploid and diploid cells are known to regularly occur in this organism.

The analysis of nuclear DNA is illustrated as histograms for each stain (number of nuclei vs. relative dye concentration of DNA). The mean values, standard deviations and standard errors are also recorded.

Upon analysis of the data obtained it was determined that the shelf-life of the undissolved stain over a long length of time does not affect its quantitative staining ability, as long as the dyes are properly stored unhydrated and handled properly. The intensity of the dye concerning nuclear uptake and subsequent stoichiometric color change developed appears to be initially influenced by the chemical composition of the stain rather than the age of the unhydrated original dye. Basic Fuchsins with CI number of 42500 have higher initial affinity for DNA than stains having CI number 42510 according to the measured DNA content.

Post-prepared hydrated dye can be used safely for at least two weeks, and give optimal quantitative results during this period of time. Following this time, however the staining intensity of the nuclei produced by the dye is gradually diminished. However, the stain is still capable of stoichiometric DNA measurements in arbitrary units.

ACKNOWLEDGEMENT

I would like to thank Dr. J. Yemma for the plan for this study, guidance in carrying out the work, and writing as well as encouragement received in the developement of this thesis.

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LIST OF SYMBOLS

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

Introduction

The Feulgen reaction can serve to quantitatively measure the nuclear DNA content of cells. The Feulgen reaction in its application is a modified form of a reaction originally proposed by Schiff (Feulgen and Rosenbeck, 1924). When cellular nuclei are subjected to the Feulgen reaction, which includes hydrolysis of deoxyribonucleic acids with 5 normal HCl for a short time, aldehyde groups are liberated which react with Schiff's reagent in a quantitative manner, and produce magenta color.

Nucleic acids possess two types of bonds: the glycosidic linkage between the purines and the sugars and the phosphodiester bond between the sugars (Andersson and Kjellstrand, 1972). The first type of bond can be broken much easier than the latter one.

As early as 1908 Osborne and Heyl (Osborne and Heyl, 1908) described the chemical basis for the breaking of the purine-sugar bond as the result of mild ascid hydrolysis (2% sulfuric acid) in contrast with the difficult breakage of the pyrimidine-sugar linkage (20% sulfuric acid at 150 degrees C).

The specificity of the Feulgen reaction depends on the hydrolysis, since unhydrolyzed tissues do not stain

(Kasten, 1960). In the hydrolysis phase of the Feulgen reaction, the DNA is converted to a macromolecular polyaldehyde - apurinic acid. During the initial part of Feulgen hydrolysis, purines are removed and stainable aldehydes are developed. The purines are removed in a way very similar to a first order reaction pattern (a constant fraction of the remaining aldehydes is removed during a constant time). At the same time the removal of the DNA and the apurinic acid begins. This removal follows an S-shaped pattern. The overall extraction rate pattern is bell-shaped (Kjellstrand, 1978). When treated with Schiff's reagent, these aldehydes become the reactive site for the aminosulfinic groups in the colorless leuco forms of fuchsin, giving colored acid-fuchsin products.

Schiff's reagent of the leucofuchsin form is a colorless compound made by changing the red color basic fuchsin into bis N-aminosulfonic acid by the addition of sulfurous acid (DeRobertis and DeRobertis, 1980). Basic fuchsin is a mixture of pararosanilin, rosanilin and magenta II (Conn, 1977). Basic fuchsin containing pararosanilin (triaminotriphenylmethane chloride) as chief component is the lightest in color; while magenta II will give a darker color to the compound.

The reaction creating the stainable groups is a first order reaction: the purines are removed by the

breaking of a single bond. The removal of the stainable intermediate is more complex. Kjellstrand and Lamn (1976) developed a model and proposed that apurinic acid was extracted in chains whose length determined the extraction pattern. DNA was regarded as a chain-formed polymer whose units are connected by bonds that are randomly broken during hydrolysis. Fragments shorter than the critical length become soluble and are extracted. Using this model the authors showed that the prevention of the diffusion of the DNA fragments is one factor determining the outcome of the hydrolysis. Other influencing factors are the length of the fragments and the coupling between the depurination and the chain breaking reaction.

The ratio between the amount of phosphorus in the DNA and the number of bound pararosaniline molecules is the complex is a parameter for the stoichiometry of the conversion of Dna to apurinic acid-Schill complex (Handonk and Van Duijn, 1964). This complex is not homogeneous, but it is composed of 2 to 3 components, depending on the s taining procedure followed (Duijndam et al., 1973). Even when it is assumed that the aldehyde group liberated has the sam properties, it was demonstrated that one aldehyde group and one amino group of the pararosaniline may give rise to several products. (Layer, 1963; Mander, Mander and Carmichael 1968). On the other hand, the pararosaniline carries

three amino groups of which all are able to react with aldehydes. The reaction is further complicated by the possible influence of competing reactions. Amino groups from sources other than the stain may compete with the pararosaniline for the available aldehydes. The chromatin compactness also has an influence on the stoichiometry of the conversion of the DNA to apurinic acid-Schiff chromophore. By using polyacrylamide model films Duijndam and Van Duijn (1975) demonstrated that while the depurination rate of the various model systems differed, the conversion of the aldehyde to the Schiff chromophore had the same stoichiometry.

For all practical purposes two reactions determine the number of aldehyde groups available for staining during hydrolysis: a) depurination and b) depolymerization and extraction. The purines are removed by the breaking of a single bond, whose breaking intensity is constant. On the other hand, the removal of the suitable intermediate seems to follow a more complax pattern. Andersson and Kjellstrand (1971) suggested that the apurinic acid was extracted in chains whose length determined the extraction pattern.

The relative speed and the interaction between the depurination and the depolymerization will determine the final result of the hydrolysis (Kjellstrand, 1980). Depurination is the aldehyde-producing reaction and depolymerization is the aldehyde-removing reaction. The

use of cells labeled with different DNA precursors were used by Andersson and Kjellstrand (1971) to measure depurination and depolymerization dependent extractions and to calculate the number of aldehydes present at any moment during hydrolysis. In 1975 these authors concluded that there was a constant ratio between the amount of dye measured and the calculated number of exposed aldehyde groups.

For absolute DNA measurements strictly defined reaction conditions are necessary to keep pH, dye concentration, sulfite concentration and state of oxidation of the sulfite under control. The well-standardized conditions are necessary also because the control of leakage of material from the cells during the Feulgen procedure strongly depends on the type of fixation and the conditions chosen for acid hydrolysis (Groub, Auer and Zetterberg, 1975). Swiff (1966) believes that for relative DNA determinations the strict control of all the conditions is desirable but not always critical.

The most important procedural details affecting the intensity of the Feulgen reactions are: the hydrolysis procedure, fixatives employed, pH and SO2 (!) content of the Schiff reagent, the presence of dye impurities and the length of time in the bleach, wash and dehydration steps. From this list of factors fixatives and the hydrolysis procedure have a major influence on the

developing color intensity (Pressly - unpublished Y.S.U. thesis).

Pearse (1968) showed that changes in the physical state of nucleic acids occur using any method of fixation. The best fixative is the one which gives high chromatin stability toward hydrolysis and will only slightly alter the DNA's conformation (Kjellstrand, 1975). In the present study 10% formalin was used which involves the formation of a network between fibrous proteins of the cell nuclei and amino groups of the DNA bases (Kurmich, 1955.) According to Deitch (1967) 18-24 hours fixation are recommended. Formaldehyde stabilizes the chromatin (Greenwood and Berlyn, 1968), which will slow down DNA depolymerization and provide optimal Feulgen staining intensities over extended hydrolysis time.

Swift (1950) stated that the physical state of the nucleoprotein is alered following formalin fixation. Chambers (1968) favors the use of 10% buffered formalin in quantitative cytophotometric studies because of its chemical stability and rapid penetrating of tissues. Fixation by formalin at room temperature also blocks DNAse action (Swift, 1966). It is also worth mentioning that overfixation should be avoided, because it can cause the loss of detectable DNA (Chambers, 1968).

As early as 1933 Bauer realized that the maximum staining in the Feulgen reaction required an optimal

hydrolysis time. The process of hydrolysis depends on the following factors: temperature, acid concentration and the stability of the chromatin (Kasten, 1960). A number of studies dealing with the effect of temperature and acid concentration on the hydrolysis have been presented (Bohm, 1968; Decosse, 1966; Fand, 1970; Morgatroyd, 1968). It had been demonstrated that the traditional 1M HCl 60 degree C hydrolysis system is not the most desirable and has been replaced by higher concentrations and lower temperatures. The period of maximal staining intensity is prolonged by 5N HCl hydrolysis at room temperature (Itikawa and Ogura, 1953; Jordanov, 1963, Mayall, and Mendelssohn, 1970). It was also shown that if the hydrolysis procedure is performed at room temperature using a more concentrated acid solution, the maximum production of aldehyde groups occurs over a wider range of time (30-60 minutes - according to Deitch, Wagner and Richart, 1967). The plateau of optimal hydrolysis results extends for at least 24 minutes, lessening the risks of under- or over-hydrolysis (Kjellstrand, 1977). The control of hydrolysis temperature is critical to achieving maximal staining intensity. A 5 degree C increase approximately doubles the reaction rate (depurination rate). For practical reasons lower temperatures are preferred because they will give the reaction moderate speed - so that the time control will not be a crucial factor.

Increasing the hydrogen ion concentration while hydrolysing at a temperature of 56 degree C speeds up the reaction but shortens the duration of optimum hydrolysis (Murgatroyd, 1968). During hydrolysis some stainable material is lost (Stedman and Stedman, 1943 and 1947), but Swift (1966) felt that there would be an equal loss of DNA following this procedure, so DNA measurements in arbitrary units, rather than absolute values, are preferred.

Besides these major factors the Feulgen intensity is also determined by the dye pararosaniline (basic component of basic fuchsin) and the prepared Schiff's solution (Pateau, 1952). The maximum staining intensity is affected by defferences in basic fuchsin (Yemma and Penza, 1987).

The staining power of the prepared, stored Feulgen reagent will not be retained indefinitely (Ely and Ross, 1949). This is perhaps true for the unprepared dye as well.

The objectives of this investigation are: to determine the effects of the aging of prepared Basic Fuchsin on the quantitative cytophotometric properties of the Feulgen reaction. Information on the effects of date of manufacture prior to preparation will also be presented.

In addition, any changes in reactivity of pre-prepared Schiff's reagents over time will be

Figure 1

COSTITUENTS OF BASIC FUCHSIN

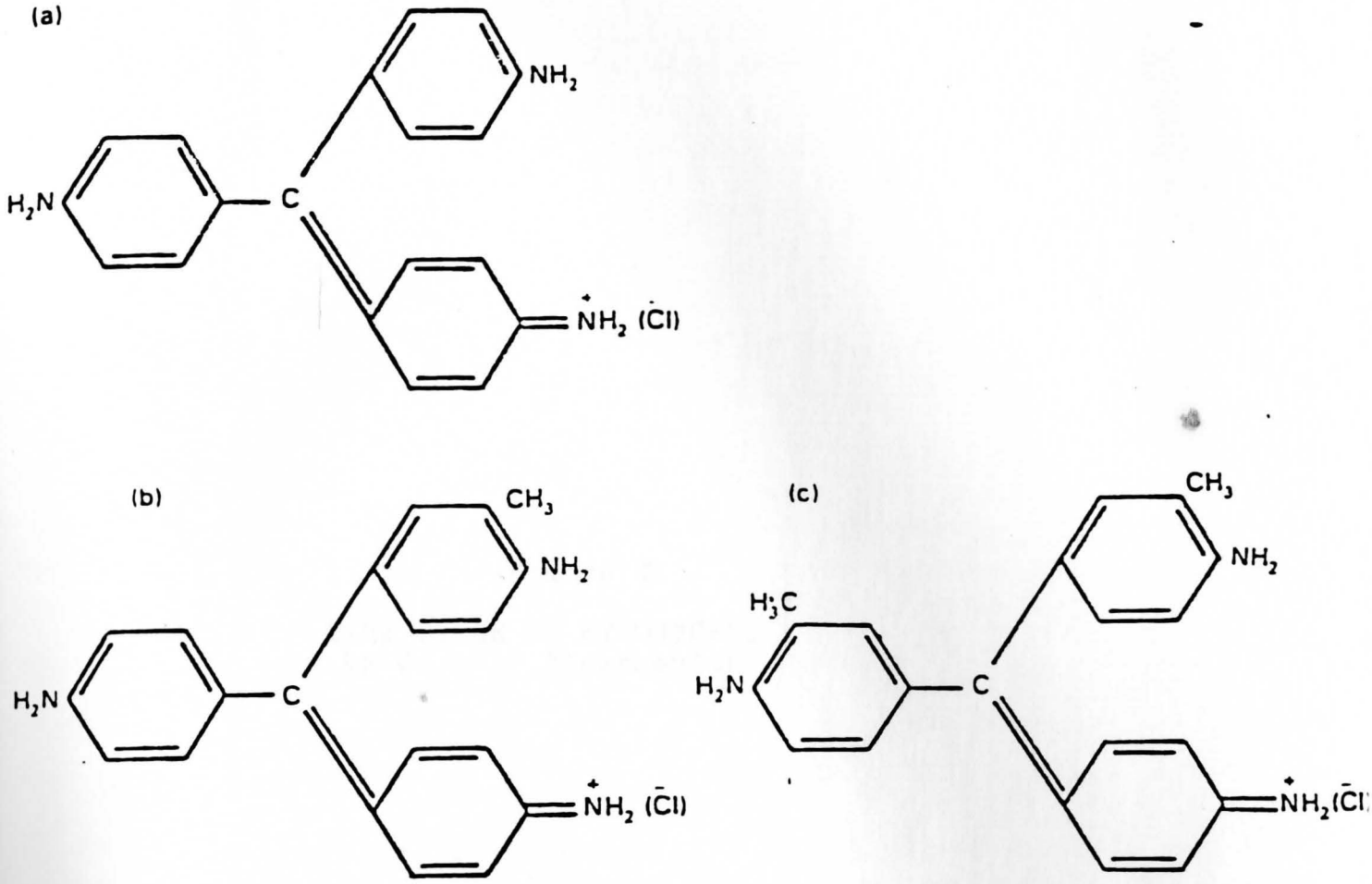
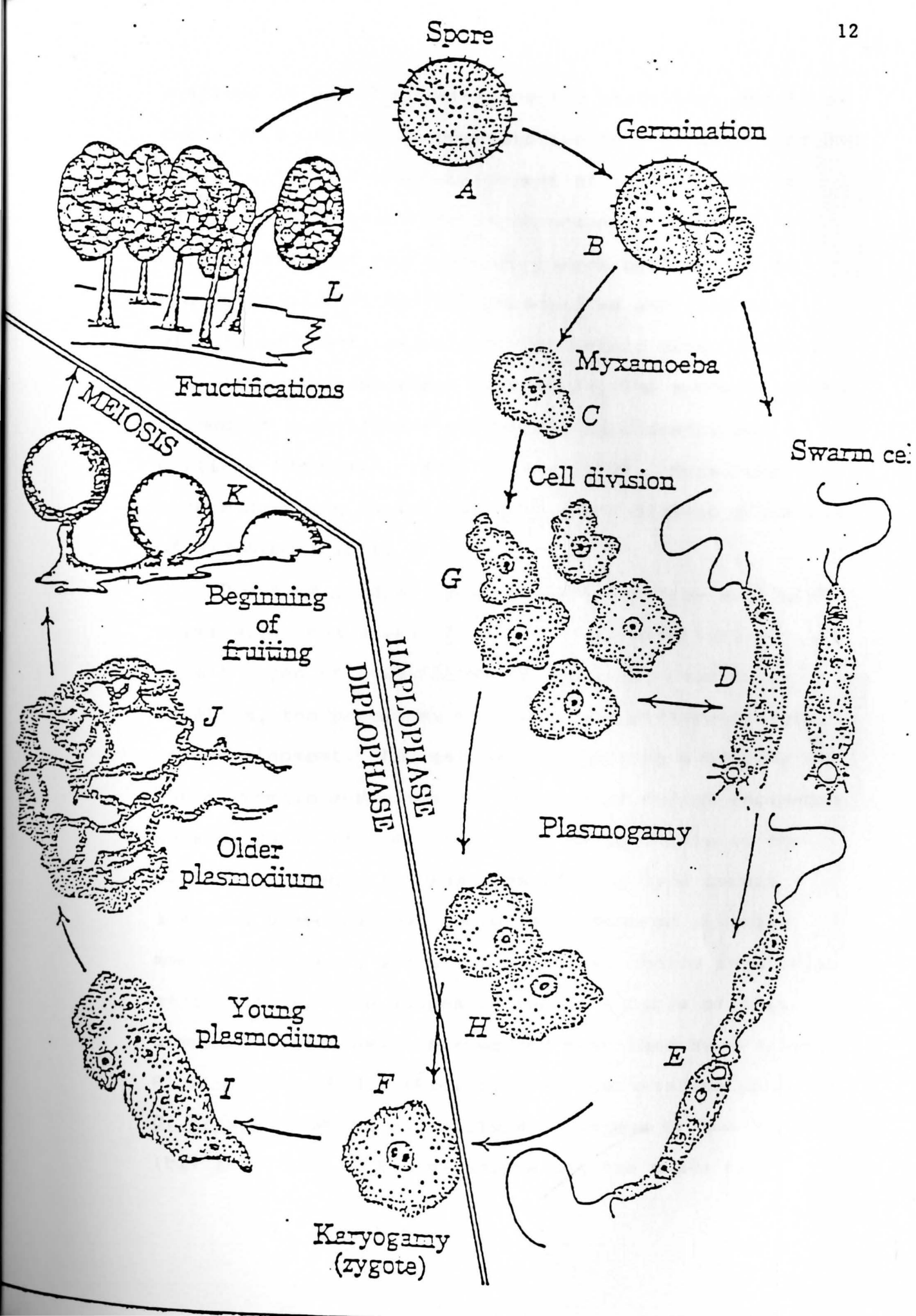


Figure 1 Formulae of (a) pararosanilin (magenta O) or triaminotriphenylmethane chloride, $C_{19}H_{18}N_3Cl$, having a molecular wt of 323,828 daltons, and a colour index of 42500 (Cl basic red 9); (b) rosanilin (magenta I), $C_{20}H_{20}N_3Cl$, with a molecular wt of 337,855 daltons, and a colour index of 42510 (Cl basic violet 14); and (c) magenta II, $C_{21}H_{22}N_3Cl$, having a molecular wt of 351,883 daltons, and a colour index of 42510 (Cl basic violet 14).

Figure 2

LIFE CYCLE OF MYXOMYCETE.
by C. J. Alexopoulos


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

analyzed in order to determine the stability, and thus the effect of time on quantitative determinations of DNA (shelf-life). The establishment of the time period (duration) for stability is important as part of determination of reaction parameters best suited to producing optimal Feulgen intensities and thus the effects on quantitative cytophotometric measurements.

To accomplish these objectives, the two-wavelength method of quantitative microspectrophotometry was utilized (Ornstein, 1952, Patau, 1952). Measurements were made using heploid myxamobae and diploid plasmodia of Didymium iridis.

Didymium iridis - a myxomycete, or true acellular slime mold, has proven to be an invaluable tool for use in all types of investigations involving cell cycle kinetics, the processes of growth and differentiation, and development. The naturally occurring synchrony of DNA synthesis and mitosis, which is of normal incidence in some parts of the life cycle, particularly in the plasmodial stage, in organisms of this type permit analyses based on what could be considered a single macroscopic cell, and so is a logical choice in studies of this type. The stages in the life cycle of this group have been well defined and described by Gray and Alexopoulos (1968) (Fig. 1.) Each germinating spore of D. iridis produces one haploid myxamoeba or swarm cell (biflagellated). The myxamoeba and the swarm cell

are interconvertible, the latter being dependent on aquaous environmental conditions. (Collins, 1961) The population of cells then increases in size by successive mitotic divisions until achieving the log phase of growth. Myxamoeba can then serve as gametes in sexual fusions (Yemma, 1985). Plasmodial production requires that fusing cells must be of compatible mating types. This organism has a one locus-multiple allelic system. (Collins, 1963). It has 12 different strains which are heterothallic.

The diploid plasmodium grows and achieves the multinucleate condition through a series of nuclear divisions (Rausch, 1969) or by coalescence with oither zygotes. Through differentiation, the plasmodium gives rise to the sporangium and then spores by the process of sporogenesis. This process happens when there is a natural depletion of nutrients.

Many studies involving the cell cycle during growth and differentiation have already been done using D. iridis (Therrien, 1966; Therrien and Yemma, 1972, 1974 and 1975 ; Therrien and Jakupcin, 1980; Yemma and Perry, 1985). Studies involving the Feulgen reaction are far more numerous; however, the effect of the age of the unprepared stain, as well as the length of time the prepared stain can be retained and still permits quantitative measurements, has never been determined.

Materials and Methods

The Costa Rican - IIA" and Costa Rican 5-5A[5] isolates of Didymium iridis used in this study were supplied by Dr. J. Yemma, Dept. of Biological Sciences, Youngstown State University.

Cultivation and Preparation of Material for Cytochemical Studies

In these experiments myxamoebal cells were removed from the solid agar media, and recultured in liquid media in order to achieve maximal cell numbers. The primary culture medium for the plasmodium was half-strength cornmeal agar. This solid medium was prepared by dissolving 8.5 grams of Difco cornmeal Agar and 8.0 grams of Difco Agar in 1 litre of distilled water, then autoclaved (Collins 1963, Yemma, Therrien and Ventura, 1974).

The myxamoebal clones of D. iridis were grown in liquid culture of distilled water containing E. coli as a food source. Cells used in this investigation were grown to log phase and fixed with 10% buffered formalin. After an 18-hour fixation period, the cells were subjected to washing with 70% alcohol. The remaining suspension was centrifuged at 750 rpms for 10 minutes in an Adams Dunac swinging bucket centrifuge. The

supernatant was removed. Washing with 70% alcohol and centrifugation were repeated. The cells were allowed to post-fix for 12 hours in the alcohol. The cells were then smeared on previously labeled and albuminized slides and placed on a warming tray overnight.

To obtain diploid plasmodium the two compatible mating types (CR 5 A^{5} and CR IIA") of amoeba were crossed on the agar. The plates were incubated at 21 C degrees until significant plasmodial growth was obtained in approximately 8 days. Sterilized oatmeal was sprinkled on the surface of the agar for feeding purposes.

The formed plasmodia were washed from the media surface with 10% buffered formalin. The washings were collected in a sterile conical centrifuge tube, then spun down as noted for the amoeba. The washings were repeated several times to obtain a bacteria-free plug.

Fixed plasmodia were dehydrated in a graded ethanol series (absolute, 90%, 70% ethanol, then water) to xylene and imbedded in paraffin. Sections were cut at 4 microns, and affixed to previously labeled, albuminized slides.

Cytochemical Methods

Six separately purchased basic fuchsin dyes, with different manufacturing dates, were used to prepare the Feulgen reagents used in this study. These stains were labeled as follows:

A 1972 - Baker Chemical Co. -Basic Fuchsin C. I. 42510

B 1974 - MC/B - Basic Fuchsin C. I. 42510 Dye content 97%. (Manufacturing Chemists)

C 1976 - Fisher Scientific - Basic Fuchsin F-98 C. I. 42510 Lot 762172 Dye content 88%

D 1985 - Fisher Scientific - Pararosaniline - Basic Fuchsin C. I. 42500 Lot 792591-A Dye content 97%

E 1986 - Fisher Scientific - Basic Fuchsin F 98 C. I. 42510 Lot 762172 Dye content 88%

F 1987 - Fisher Scientific - Pararosaniline - Basic Fuchsin C. I. 42500 Lot 792591-B Dye content 97%

Basic fuchsins with C. I. (color index) numbers of 42510 have as chief constituents rosaniline (Conn, 1977), on the other hand, basic fuchsins with C.I. number 42500 contain pararosaniline (Yemma and Penza, 1987).

Six hundred ml of each stain solution was made

according to the following method:

> 1 g basic fuchsin and 2.2 f K-metabisulfite are added to 100 ml of 0.2 N HCl. The solution is stirred mechanically for 2 hours. Activated charcoal is added (approximately 200 mg of activated charcoal for each 100 ml of Schiff's reagent), then it is filtered. The final solution, after repeated filtering, must be water clear.

The prepared solutions were stored in dark bottles at 5 degree C prior to use.

The previously prepared slides were divided into six groups - each group containing 32 slides (16 amoeba and 16 plasmodium slides).

Prior to staining, paraffin sections of plasmodia were cleared in xylene, and hydrated through a graded ethanol series to distilled water. The myxamoebal smears were placed in water one hour prior to staining. Control slides, hydrolyzed for 15 minutes at 90 degree C in trichloroacetic acid in order to remove the DNA, were run simultaneously through the staining procedure along with the experimental tissues (in order to check DNA specificity of the stain).

Five normal HCl acid hydrolyses at room temperature were used as suggested by Itikawa and Ogura (1953) instead of the "hot" hydrolysis. Hydrolysis time was 45 minutes.

For each stain (A through F) and each staining period (first through fourth) three amoeba and three

plasmodium slides were hydrolyzed and stained simultanously for uniform and comparable results. For each stain and staining cycle, one of amoebae and plasmodial slides, pretreated with deoxiribonuclease, acted as a control. These control slides should not stain, demonstrating the specificity of the stain.

The staining technique used was the following:

xylene	10 min
absolute ethanol	10 min
90% ethanol	10 min
70% ethanol	10 min
distilled water	3 min
5 N HCl	45 min
stain (100 ml)	2 hrs
10% K metabisulfite	5 min
10% K metabisulfite	5 min
distilled water	1 min
70% ethanol	3 min
90% ethanol	3 min
absolute ethanol	3 min
xylene	5 min

The stain was fortified with 10% K metabisulfite, to create a 20% solution of Schiff's reagent (Lillie, 1951), just prior to staining (80 ml stain + 20 ml 10% K metabisulfite).

After the initial staining, the remaining Schiff's reagents were covered with aluminum foil and

stored in the refrigerator at 5 degrees C.

Following the initial staining, three consecutive stainings were performed at two week intervals using the same stain and same technique to determine the stability period of the prepared Schiff's reagent

The initial experiment should tell us if the age of the pre-prepared stain has an effect on quantitative determinations. The second, third and fourth stainings should tell us how long, once prepared, the stain is stable.

Microspectrophotometric Method

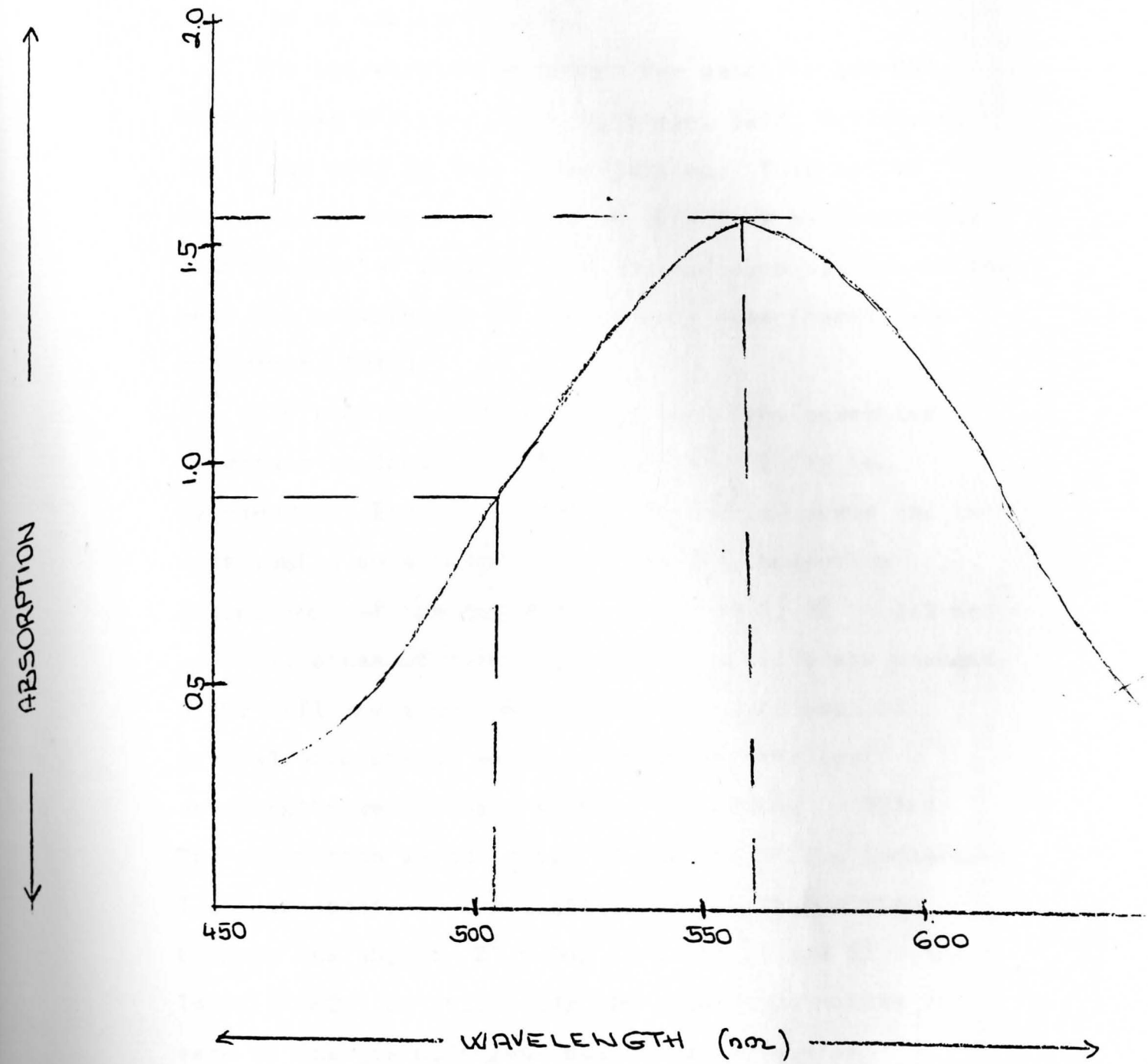
The quantitative basis for the Feulgen reaction is due to the alkyl-sulfonic acid bond between the dye and the deoxyribose sugar which is formed stoichiometrically.

For the microspectrophotometric measurements of the amoebal and plasmodial DNA a Zeiss Universal Type 01 microspectrophotometer was used with a planachromatic 100 X oil immersion objective. To isolate the chosen wavelengths of light, a Zeiss continuous interface-filter monochrometer was used. Alignment and linearity of the photo tube were checked before any measurements were made.

For each stain 100 nuclei of amoeba and 100 nuclei of plasmodium were analyzed for each staining time. The

Figure 3

Combined Feulgen-DNA spectral absorption curve
for both amoeba and plasmodium


ABSORPTION
2.0
1.5
1.0
0.5
450
500
550
600
WAVELENGTH (nm)

DNA measurements of these cells are relative and are reported in arbitrary units.

The two-wavelength method for quantitative DNA measurement (Pateau, 1952; Ornstein, 1952; Mendelson, 1961) was used in this investigation. This method corrects any error that may be caused by heterogeneous distribution of stained material and also eliminates the need for measurement of the nuclear area (Mayall and Mendelson, 1970).

The reliable estimation of the light-absorbing materials is insured by the selection of the two wavelengths. From the spectral absorption curve the two wavelengths were selected so that the respective extinctions of the dye form a ratio of E1:E2 = 1:2 and no local areas of very high optical density are present. After defining a photometric field within each of several homogeneous areas of specimen, the two wavelengths were chosen as $\lambda 1$ = 560 and $\lambda 2$ = 505nm. The extinction is the ratio of the log of the background light intensity to the light intensity transmitted through the object E1 = log Io/Is at $\lambda 1$ and E2 = log Io/IS at $\lambda 2$. In this study the absorption maxima for each of the six different stained materials is represented in a combined spectro absorption curve (Figure 3).

Once the two wavelengths (560 and 505nm) were selected to correspond with the maximum and half-maximum

absorption values of Feulgen DNA, areas with heterogeneous dye distributions were measured. The nucleus was moved to the optical axis and the photometric field was adjusted to completely enclose it.

For all the measurements 100 nuclei (amoeba and 100 plasmodium) were randomly chosen from the slides for each of the six different stain solutions (for all four different staining periods).

Four photometric readings were recorded per nucleus. At each wavelength one reading was taken through the nucleus (Is) and one reading was taken through the background (Io). From the readings the transmissions could be calculated as T_1 = Is/Io at λ_1 and T_2 = Is/Io at λ_2.

The amount of chromophore (absorbing material) within this field of measured area (A) was determined by the equation $M = kAL_1 C$. K is the absorptivity constant required for the determination of absolute values, and was eliminated since only relative values were required in this study of DNA determination. Q is the ratio between L_1 and L_2. $Q = L_2/L_1$ from which the value of C or correction factor for the distributional error can be calculated. $C = (2 - Q)^{-1} \ln(Q - I)^{-1}$ (Swift and Rasch, 1956). Pateau (1952) lists a series of Q functions with the corresponding C values.

Since the extinction ratio at the 2 wavelengths is known as 2:1, Q permits an elimination of the influence

of the unoccupied portion of measured area.

Calculation of content is permitted without reference to the geometry of the structure analyzed since only the area of the aperture is required (Kurnick, 1955). Measurement errors due to erroneous light transmission (caused by dispersion and reflection) are negligible.

All M values were calculated and statistically treated by an IBM computer using Dr. Yemma's program.

Results

Quantitative nuclear DNA measurements made on plasmodia and amoeba are presented in the form of frequency histograms. The histograms represent nuclear DNA frequency distributions of cellular populations and allow for detection of shifts in DNA which may be due to ploidy difference, or increased template activity. The dye concentration presented in arbitrary units appear on the ordinate, while the number of nuclei measured is on the abscissa. As previously stated haploid amoeba, and diploid plasmodium, the result of crossing compatible mating types, were measured for DNA content. The comparison of the mean DNA values for the amoeba and for the plasmodium in all stains used were designated as A,B,C,D,E and F (fig. 10). The difference in their DNA level or concentration (in arbitrary values) is readily observable, the haploid myxamoeba having approximately one-half the DNA value of the diploid plasmodium. The amoebae are considered to be in the 2C category while the plasmodia are in the 4C category - utilizing Swiff's designation of DNA content or ploidy level. This finding is in accord with the results observed by Yemma and Therrien (1972) - that 99% of the time the plasmodia formed from heterothallic isolates of *D. iridis* are in the premitotic replicated diploid state (4C).

These results are analyzed in two categories:

a) the analysis of the first staining (original) of cells in which the pre-prepared or freshly prepared basic fuchsins were utilized, and

b) the analysis of cells stained with the same dyes which were allowed to age continuously (two, four and six weeks).

Analysis of the first staining

(Original staining - 0 week)

Measurements of the mean nuclear DNA in the original staining are shown in figure 4 through figure 9. The histograms presented demonstrate a plot of the quantitative DNA dye binding in relative numbers of a population of nuclei versus the number of nuclei measured.

Analysis of Stain A

A comparison of the mean DNA values for the amoeba and plasmodium clearly demonstrate a ploidy difference for these two stages of the life cycle. Observation of figure 4 (Stain A) demonstrates that the mean DNA content of the myxamoeba is approximately one-half the diploid value: 0.32 and 0.66 respectively. Both

histograms show a characteristic unimodal distribution, with the plasmodium having a wider range of values, due to the diffuse nature of DNA in this case and a high rate of template activity. This is of normal occurance when measurements of the plasmodial nuclear DNA are made. In both cases one can normally expect nuclei to demonstrate DNA synthesis activity, and as a result also demonstrates that cells are largely near G2 or in the G2 phase of the cell cycle. These data are in agreement with values reported in the literature (Yemma and Perry, 1985).

Cells of the organism used in this study, whether amoeba or plasmodium, spend essentially 90% of the cell cycle in the G2 phase or 12 hours prior to mitosis (Rausch, 1969).

It is, however, obvious that in this case the haploid versus the diploid condition is well demonstrated using this stain and this time period.

Analysis of Stain B

Measurements of nuclear DNA using Stain B are shown in figure 5. The mean DNA value of the myxamoeba is 0.32, once again approximately one-half that of the plasmodium (0.57) due to the conditions previously described for stain A. The populations are essentially in G2 of the cell cycle, which explains the unimodal distribution. Most of the myxamoeba appear to be haploid with nuclei in the 2C or replicated DNA

category, and the plasmodium in 4C. Once again as expected the haploid versus the diploid state is well demonstrated.

Analysis of Stain C

The relative mean DNA value of the myxamoeba is 0.30 while the plasmodium's value is 0.56 (figure 6). The data indicates that the nuclei of the plasmodium are in the diploid or 4C category, while the myxamoeba demonstrate, when compared, a haploid nuclear DNA content. The DNA distribution is once again unimodal. The exhibited consistency of measurements can be noted.

Analysis of Stain D

Figure 7 shows a much wider distribution of the mean DNA values than the previous stains, and also demonstrates the highest relative amount of DNA. It is obvious that the kinetics of the cell cycle affect measurements. Nevertheless the consistency of measurements, as well as subtle differences due to elevated template activity and DNA replication, are readily noticeable. It is also evident that this dye provides for a greater depth of staining. The CI 42500 demonstrates a molecular structure with more substituted groups than the other dyes and hence accounts for the greater depth of staining (Conn, 1977; Yemma and Penza, 1987). The mean DNA value of the myxamoeba is 0.49 while the plasmodium has a 0.86 value, approximately double that of the amoeba. The histograms of Stain D

also demonstrate an unimodal distribution and similar cell cycle function, as previously demonstrated.

Analysis of Stain E

The relative DNA values for myxamoeba and plasmodium very closely follow values reported for Stain A, B and C and once again demonstrate remarkable consistency. Note from figure 8 that the relative mean DNA value for the amoeba is 0.32, and the plasmodium's is slightly more than double this number (0.65). The distribution of values is unimodal.

Analysis of Stain F

Figure 9 shows values closer to values obtained using Stain D (figure 7) ; it is important to note that the molecular structure of these dyes is the same. The relative myxamoebal value recorded was 0.43 while the mean plasmodial DNA value is 0.81. The distribution is once again unimodal. When measured nuclear DNA values for the plasmodium are viewed, it can be noted that, although a unimodal distribution is evident, the tendency toward a bimodal distribution is present. These data demonstrate a small G1 population followed by S cells at .3 to .8 arbitrary units. This is followed by a pronounced G2, which exhibits, as expected, greater dye binding. This tends to elevate the mean to 0.81. A CI of 42500 with several substituted groups, as previously explained, also contributes to the depth of staining. However, the consistency of measured values

for this dye is evident when compared with those already discussed.

Upon examination of figures 4 through figure 9, it can be noted that all the myxamoebae as well as plasmodium histograms demonstrate a unimodal distribution and are consistent with values previously reported in the literature for a haploid versus a diploid population (Therrien and Yemma, 1975). Interestingly, the sensitivity of dye binding is also able to reflect subtle differences due to increased template activity, S activity, and cell cycle phase differences. It can be noted that histograms representing the analysis of the first (original), and immediate staining for both amoeba and plasmodium demonstrate that the age of the pre-prepared dye stored properly in its original container has no significant bearing on the intensity of the color developed. It follows that the ability to yield reliable quantitative data in arbitrary units when immediately used (table I) is not affected. The staining ability of the dye manufactured in 1972 (the oldest dye used in this study - manufactured by Baker - figure 4), appears to be approximately the same as the staining ability of the dye manufactured in 1986 (manufactured by Fisher -figure 8). For the amoeba both values were 0.32 relative arbitrary units, and for the plasmodium 0.66 and 0.65 respectively (figure 10 and table I). Although the

manufacturers are different, the CI number, which is 42510 for both of these products, indicates the similarity in their chemical composition (figure 1). All basic fuchsins with CI number 42500 contain triaminotriphenylmethane chloride (pararosaniline) as a basic component. On the other hand CI number 42510 dyes will have magenta I (rosanilin) as a base and also contain magenta II (Conn, 1977; Yemma and Penza, 1985).

A comparison can readily be made by observation of stains C, D, E and F which were prepared from dyes of the same manufacturer, Fisher Scientific. C (1976 - figure 6) and E (1986 - figure 8) have CI numbers 42510; D (1985 - figure 7) and F (1987 - figure 9) have CI number 42500. Comparing the histograms (figures 6,7,8 and 9) and graphs (figure 10) of the original staining with these dyes, it can be observed that the dye concentration measures for stain C and E are approximately the same. Recorded values were 0.30 and 0.32 respectively for amoeba and 0.56 and 0.65 for the plasmodium. These are well within one standard deviation of one another and show a very small standard error, demonstrating no significant differrence (table I). In the case of dyes D and F these values are 0.49 and 0.43 for the amoeba and 0.86 and 0.81 for the plasmodium. The consistency of measurements is evident when comparing mean values for amoeba and plasmodia. This is quite interesting, for in the case of stain C

and E, the time span represented is over 10 years - showing no significant difference in the staining ability. Any slight difference observed can be attributed to the differing rate of DNA synthesis, or the relative diffuse state of DNA as may occur during template activity, as previously explained.

As observed in figure 10 and table I the relative DNA content of the amoeba ranged from 0.30 to 0.49. These values represent approximately half the relative DNA values obtained from plasmodia (0.56 - 0.86). This fact demonstrates that these cellular populations are 2C and 4C values respectively.

In all six stains the plasmodial nuclear DNA distribution shows a greater variability as demonstrated by the standard deviation value. Any variability can be attributed to slight differences in template activity. It is also important to note that division of nuclei is synchronous, in this case (Guttes, Guttes, Rusch 1961), although the synthesis of DNA is not (Therrien and Yemma, 1975).

Analysis of the post-prepared staines

Kasten noted in 1964 that the same batch of Schiff's reagent may produce different staining if used on different occasions. The following data were generated in experiments designed to determine

whether aging of a dye after preparation affects binding in a quantitative manner.

Analysis of staining after two weeks

By comparing figure 4 and figure 11 and table I of Stain A, it can be noted that the relative DNA values of amoeba were 0.32 and 0.34. These values were significantly different regarding dye binding, obviously metabolic difference, i.e., the diffuse condition of DNA, template activity and replication would account for this. However, the mean values are within less than 1 standard deviation of one another, with a standard error of .013 and of .009 respectively. They therefore represent the same population of cells. It is also important to note the extreme sensitivity of dye binding, for an interval in staining from week 0 to week 2 indicates that the stain is capable of quantitative measurements in spite of a time lapse of two weeks. The plasmodium values recorded were 0.66 and 0.58. This change is still within one standard deviation, with a standard error of .02 and .016.

Figure 5, figure 12 and table I, respectively, represent Stain B, where the change in the relative mean DNA value was from 0.32 to 0.29 in the amoeba, and 0.57 to 0.55 in the plasmodium. These values demonstrate differences once again in dye binding, but not in

populations as previously explained.

Comparing the mean values of Stain C (figures 6, 13 and figure I) the recorded values for amoeba were 0.30 and 0.32, and plasmodial values of 0.56 and 0.53 also demonstrate similar cellular populations.

The relative DNA mean values of Stain D (figures 7 and 14 and table I) were 0.49 and 0.52 in the amoebae and 0.86 and 0.75 in the plasmodia. Significant dye binding reflects once again metabolic differences, but represents similar populations, as is reflected in the small variability in these populations and is demonstrated by the insignificant standard deviation of less than one and a very small standard error. This represents a slightly higher value than seen in the previous stains, but because of higher relative values observed in this stain, due to chemical structure and metabolic activity, it represents only a 12% difference and is within 1 S.D.

The relative DNA values using Stain E, as seen in figures 8 and 1, are recorded as 0.32 to 0.33 in the amoeba, and 0.65 to 0.63 in the plasmodium and demonstrate no significant changes (table I).

Figures 9 and 16 show the staining ability of Stain F. The relative mean DNA of the amoeba changed from 0.43 to 0.50, while the plasmodium value went from 0.81 to 0.78. The variability is within one standard

deviation, which can be expected of a growing population of cells. It is important to note that in all cases dye binding in the plasmodia is significantly higher (doubled) than that of the amoeba (table I). This is expected since one population is haploid (amoeba) and one diploid.

All histograms of figures 11 through 16 show unimodal distributions as expected.

Results of the second staining, which were obtained two weeks following the first one, using the same hydrated dyes, show similar results as the original one with no loss of sensitivity in the amoeba. The variations, of course, are due to the difference in the cell population as previously noted. Due to random selection of cells to be measured, in certain cases the relative DNA values obtained may be slighty higher. The slight difference demonstrated in histograms can be attributed to progressive DNA synthesis and the diffuse nature of DNA during template activity. Also, as previously noted, consideration has to be given as to what period the cell may be in regarding the cell cycle.

It can also be assumed that deterioration of certain stains occur earlier than in others and may be more prevalent in stains with low original intensity or sensitivity.

Analysis of staining after four weeks

Comparing figures 4 and 17 (also considering table I) of Stain A, the mean DNA value of amoeba dropped from 0.31 to 0.27. The change in the plasmodial values is greater, dropping from 0.66 to 0.52. The dyes appear to be able to maintain the ability to stain these cells in a quantitative manner since they are haploid; this does not appear to be the case in the plasmodia, however. This may be due to the condition of DNA in the plasmodial 4C diploid nuclei versus that of the haploid amoeba.

Observing Stain B the change is even more obvious. The mean DNA value in the amoeba changed from 0.32 to 0.23, while the plasmodial staining changed from 0.57 to 0.40 (figures 5 and 18, table I). The week 4 value is different from that recorded for the first staining by more than one standard deviation, indicating that deterioration of the stain must be occurring, but remain within two standard deviations.

Figures 6 and 19 (week 4) for stain C show a smaller change in the amoebal DNA value: 0.30 to 0.28. But the change in the plasmodial value is from 0.56 to 0.44.

Values obtained after staining with Stain D show the greatest change up to this point. Comparing figures 7 and 21, the change in the DNA value in the amoeba is

from 0.49 to 0.35, while the change in the plasmodial relative DNA value is from 0.86 to 0.58. This change is greater than one standard deviation and clearly reflects DNA values which indicate stain deterioration.

From figures 8 and 21 the changes obtained using Stain E are as follow: relative DNA value of amoeba changed from 0.32 to 0.29, and the plasmodia had a value of 0.65, and by the end of the fourth week this changed to 0.55. One can readily note that sensitivity to dye binding is greater in the plasmodium than in the amoeba, as already noted. It also appears that stability to some extent may also be related to the stain being used.

A change from 0.43 relative DNA value to 0.35 is observed in the amoeba stained with Stain F. There is a much larger change in the plasmodial staining: from 0.81 to 0.63 (figures 9 and 22). These values have standard deviations slightly greater than one and demonstrate a tendency consistently observed for this time period toward degeneration of the stain.

All histograms of figures 18 through 23 have a unimodal distribution and a diminishing range of DNA values. The standard deviation and standard errors as well as the test for significance (T-test) tend to support this contention. The histograms presented show a diminishing staining ability in all stains, in general (figures 4-22, table I). This is true for both the amoeba and the plasmodium, with respect to time.

DYE	0 wk	2 wks	4 wks
AMOEBA			
A	0.32	0.34 *	0.27 *
B	0.32	0.29 *	0.23 *
C	0.30	0.32 *	0.28 *
D	0.49	0.52 *	0.35 *
E	0.32	0.33	0.29 *
F	0.43	0.50 *	0.35 *
PLASMODIUM			
A	0.66	0.58 *	0.52 *
B	0.57	0.55 *	0.40 *
C	0.56	0.53 *	0.44 *
D	0.86	0.75 *	0.58 *
E	0.65	0.63 *	0.55 *
F	0.81	0.78 *	0.63 *

Table I

Relative mean DNA values obtained using Stains A, B, C, D, E and F

Two sample t-distribution of mean DNA significance regarding dye binding denoted by *

$\alpha = 0.01$

Analyzing data obtained at four weeks following the original staining, it can be observed that values of relative DNA in both amoeba and plasmodium have dropped without exception. This lowering of values is consistent and significant at the 0.01 level, as demonstrated by the T-test. In the amoebal population the change ranges from 6% (Stain C - figures 6 and 19) to 30% (Stain D -figures 7 and 20); in the plasmodial population the change translates into 17% (Stain E - figures 8 and 21) to 33% (Stain D - figures 7 and 20).

It can be observed (table I) that reasonably consistent mean results were obtained from Stains C and E, which are manufactured by Fisher and share CI number 42510. A total of 6% and 7% change is seen in the amoeba, and a 22% and 17% change is observable in the plasmodial population (least change in general).

Analysis of staining after 6 weeks

By the end of the sixth week reading of values became very difficult if not impossible, and when read gave erratic results and many nonstaining of nuclei. Reliable quantitative data could not be obtained, demonstrating that the period at which the usability of the stain regarding a stoichiometric relationship had expired.

Figure 4

Histograms representing Feulgen-DNA values using

STAIN A - 0 WEEK STAINING for:

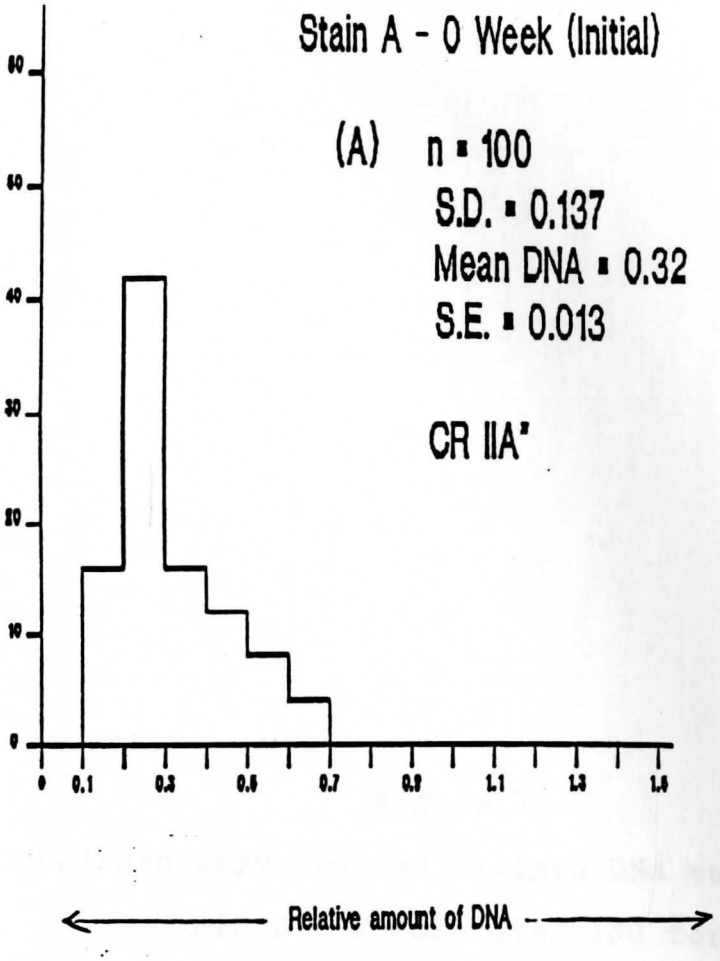
A) Costa Rican - 11A" myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A5

Stain A - 0 Week (Initial)

(A) n = 100
S.D. = 0.137
Mean DNA = 0.32
S.E. = 0.013

CR IIA"



Stain A - 0 Week (Initial)

(B) n = 100
S.D. = 0.227
Mean DNA = 0.66
S.E. = 0.022

CR IIA" x CR 5A

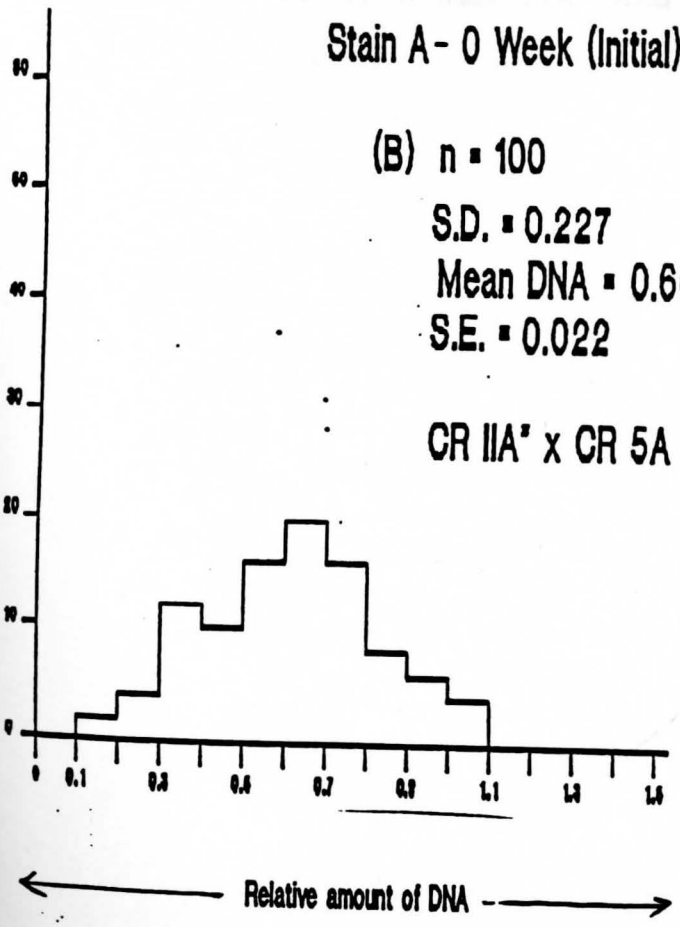


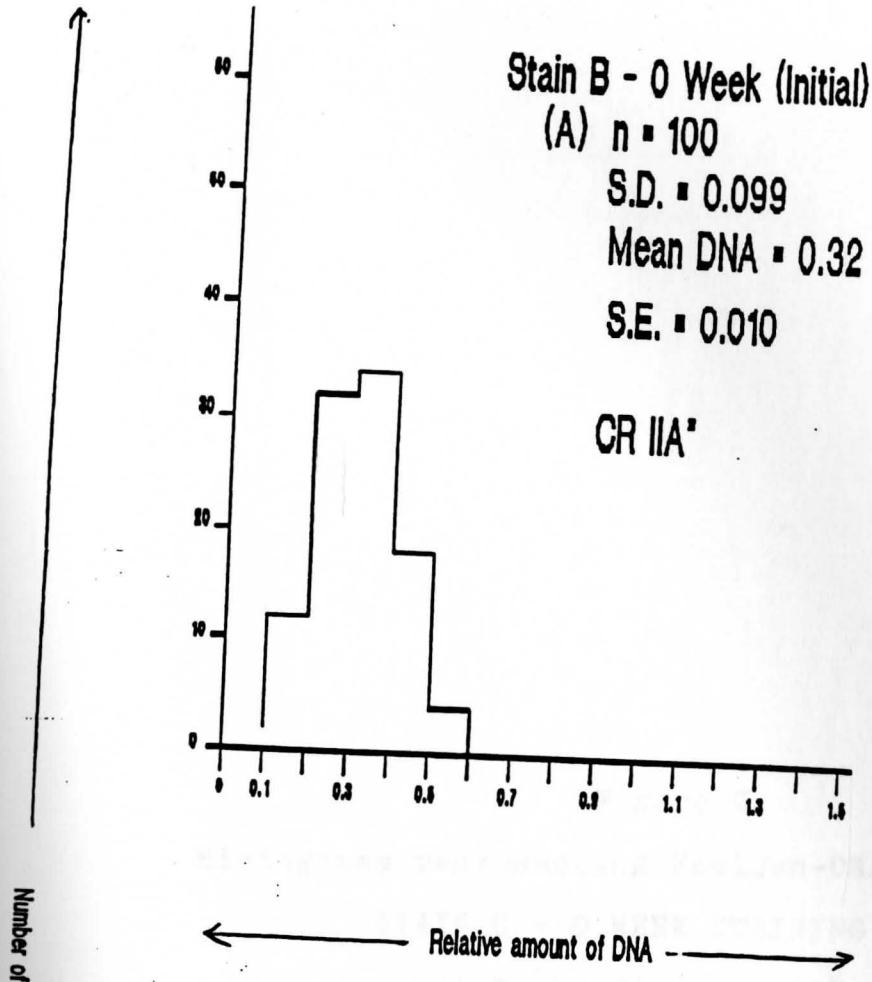
Figure 5

Histograms representing Feulgen-DNA values using

STAIN B - O WEEK STAINING for:

A) Costa Rican - 11A" myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A5


Stain B - 0 Week (Initial)
(A) n = 100
S.D. = 0.099
Mean DNA = 0.32
S.E. = 0.010
CR IIA"
Number of nuclei
Relative amount of DNA

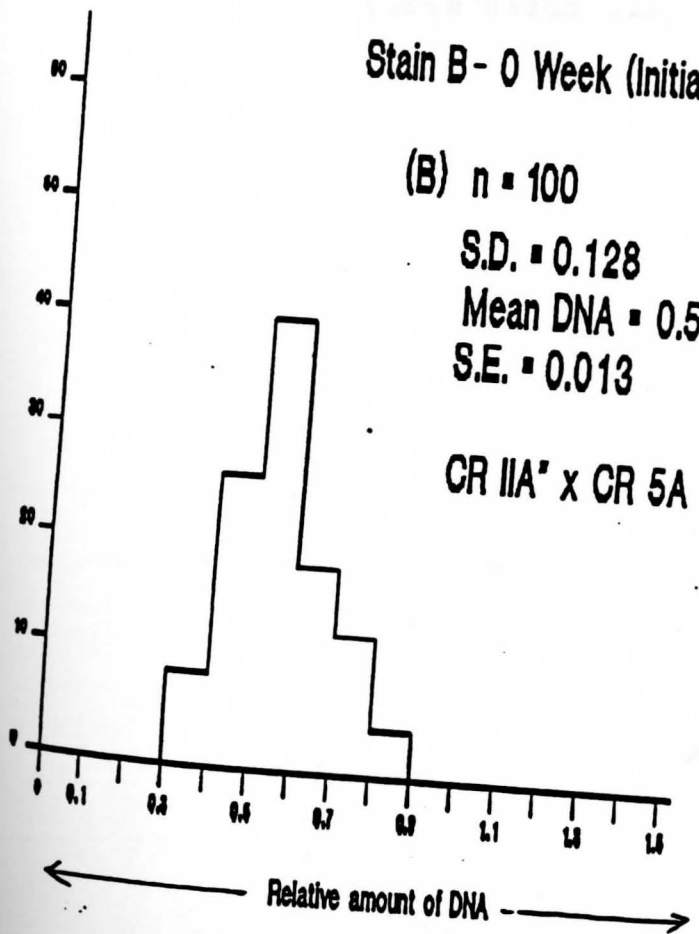

Stain B - 0 Week (Initial)
(B) n = 100
S.D. = 0.128
Mean DNA = 0.57
S.E. = 0.013
CR IIA" x CR 5A
Relative amount of DNA

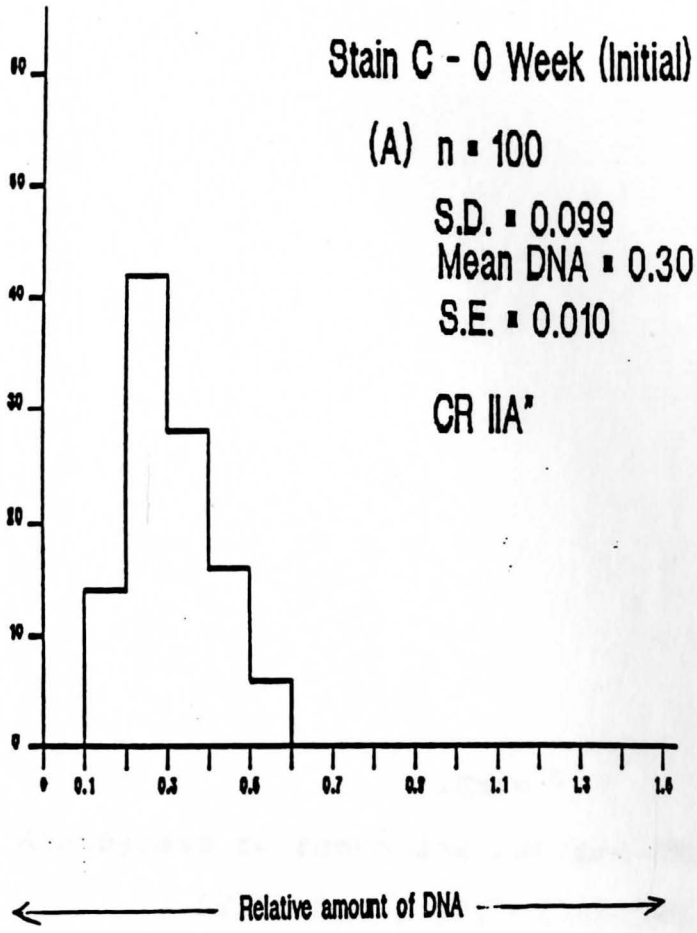
Figure 6

Histograms representing Feulgen-DNA values using

STAIN C - O WEEK STAINING for:

A) Costa Rican - 11A" myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A5


Stain C - 0 Week (Initial)
(A) n = 100
S.D. = 0.099
Mean DNA = 0.30
S.E. = 0.010
CR IIA"
0
10
20
30
40
50
60
0 0.1 0.3 0.5 0.7 0.9 1.1 1.3 1.5
Relative amount of DNA
Number of nuclei

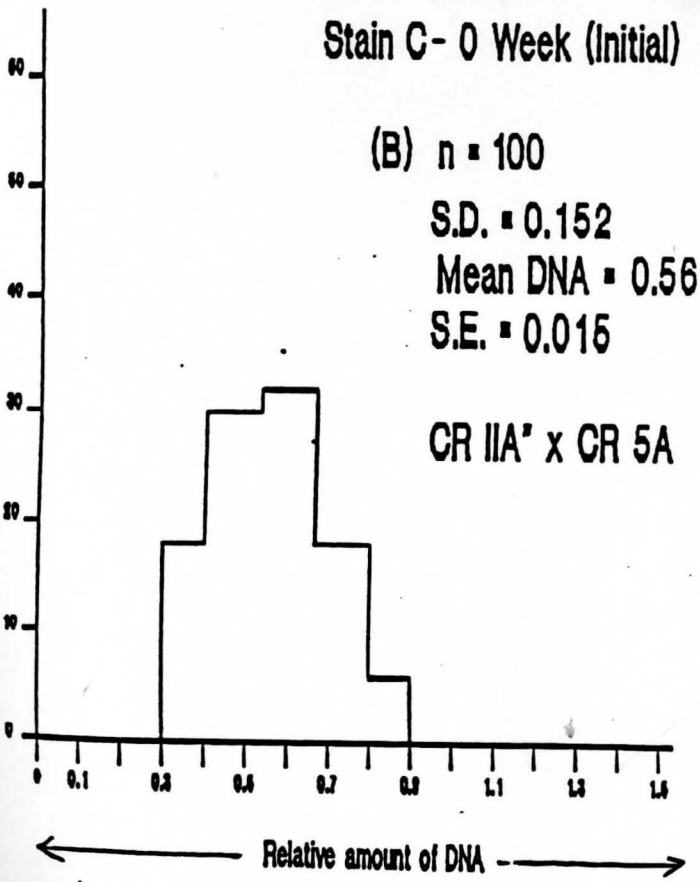
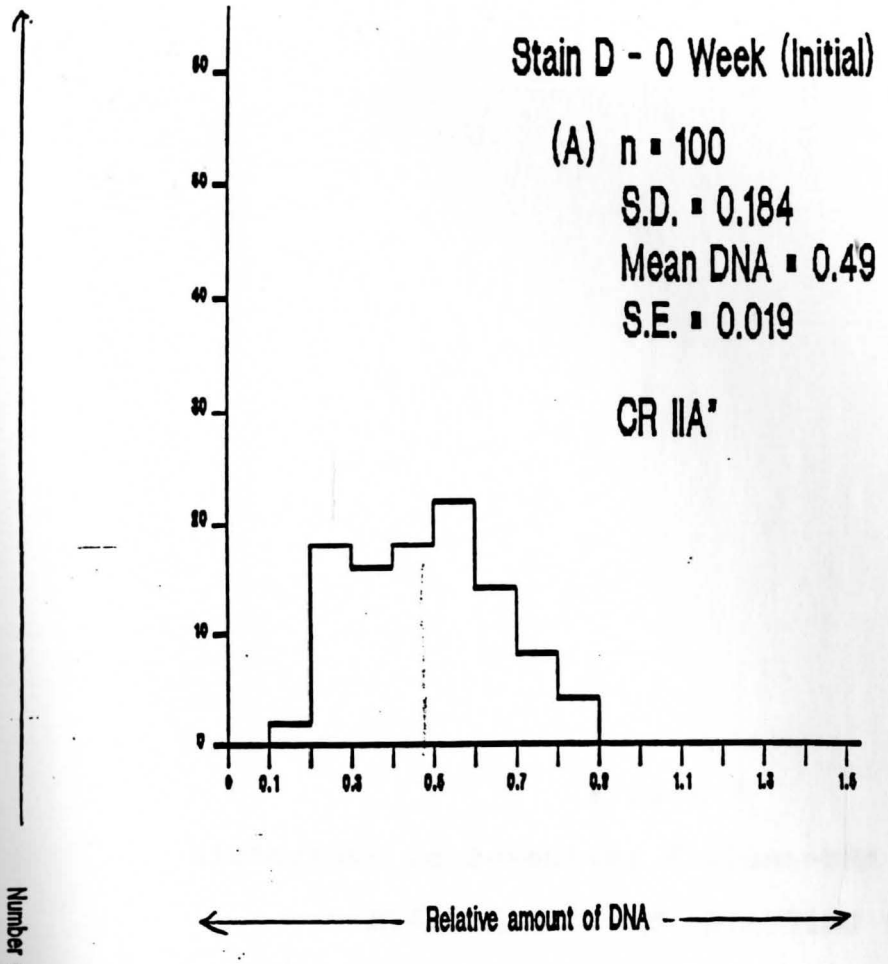

Stain C- 0 Week (Initial)
(B) n = 100
S.D. = 0.152
Mean DNA = 0.56
S.E. = 0.015
CR IIA" x CR 5A
0
10
20
30
40
50
60
0 0.1 0.3 0.5 0.7 0.9 1.1 1.3 1.5
Relative amount of DNA

Figure 7

Histograms representing Feulgen-DNA values using STAIN D - 0 WEEK STAINING for:

A) Costa Rican - 11A[11] myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A[5]


Stain D - 0 Week (Initial)
(A) n = 100
S.D. = 0.184
Mean DNA = 0.49
S.E. = 0.019
CR IIA"
Number of nuclei
Relative amount of DNA

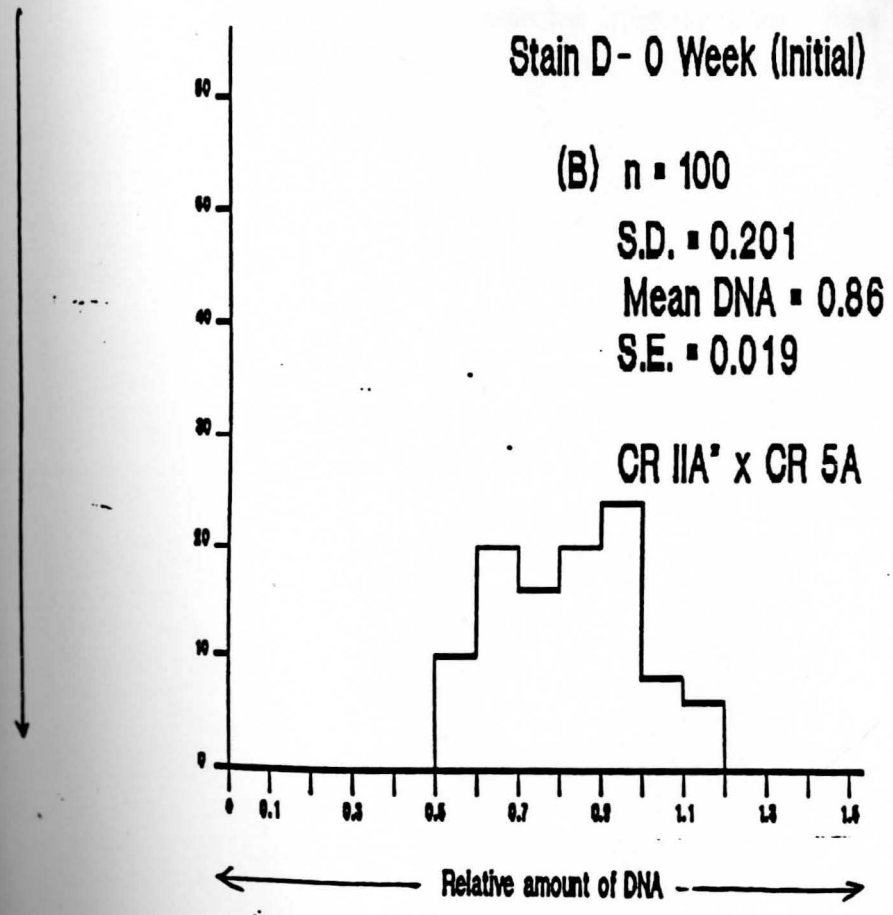

Stain D - 0 Week (Initial)
(B) n = 100
S.D. = 0.201
Mean DNA = 0.86
S.E. = 0.019
CR IIA" x CR 5A
Relative amount of DNA

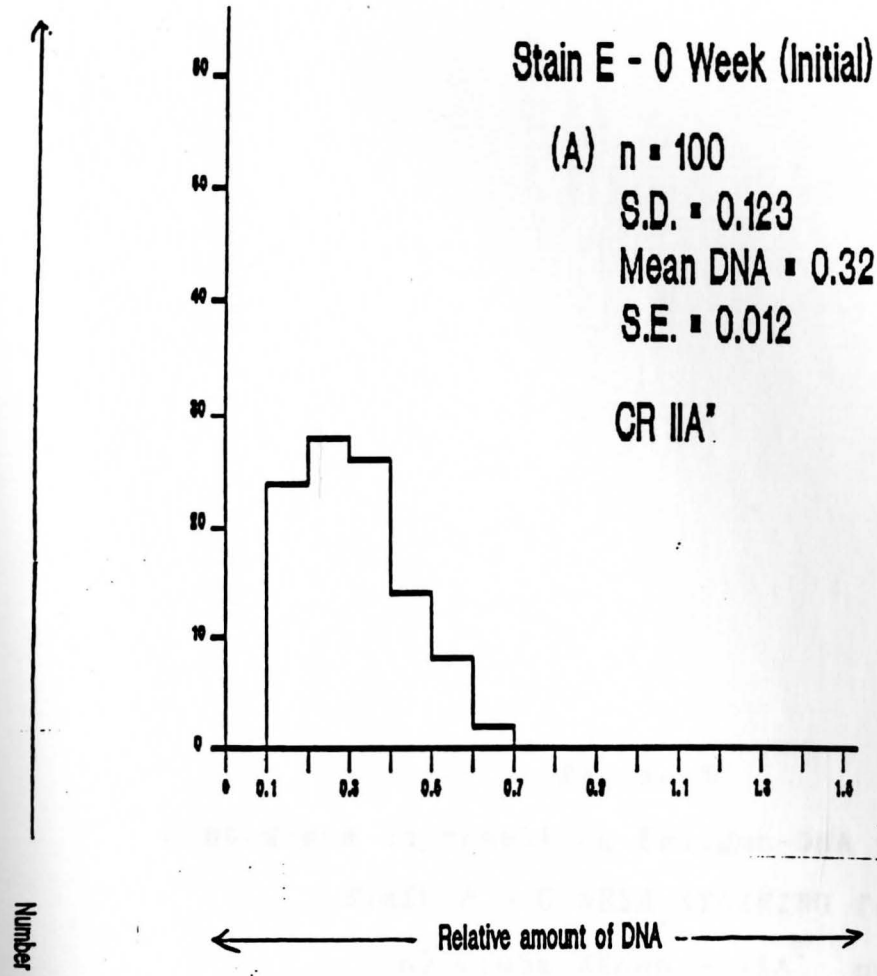
Figure 8

Histograms representing Feulgen-DNA values using

STAIN E - O WEEK STAINING for:

A) Costa Rican - 11A" myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A5


Stain E - 0 Week (Initial)
(A) n = 100
S.D. = 0.123
Mean DNA = 0.32
S.E. = 0.012
CR IIA"
Number of nuclei
Relative amount of DNA

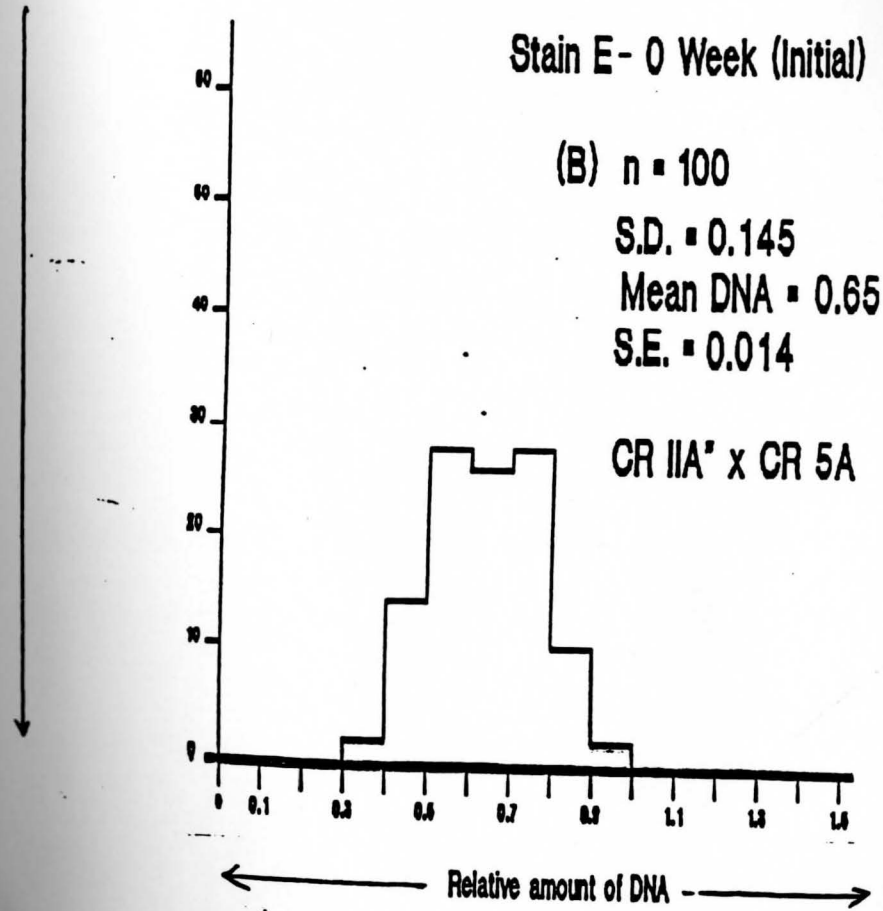

Stain E- 0 Week (Initial)
(B) n = 100
S.D. = 0.145
Mean DNA = 0.65
S.E. = 0.014
CR IIA" x CR 5A
Relative amount of DNA

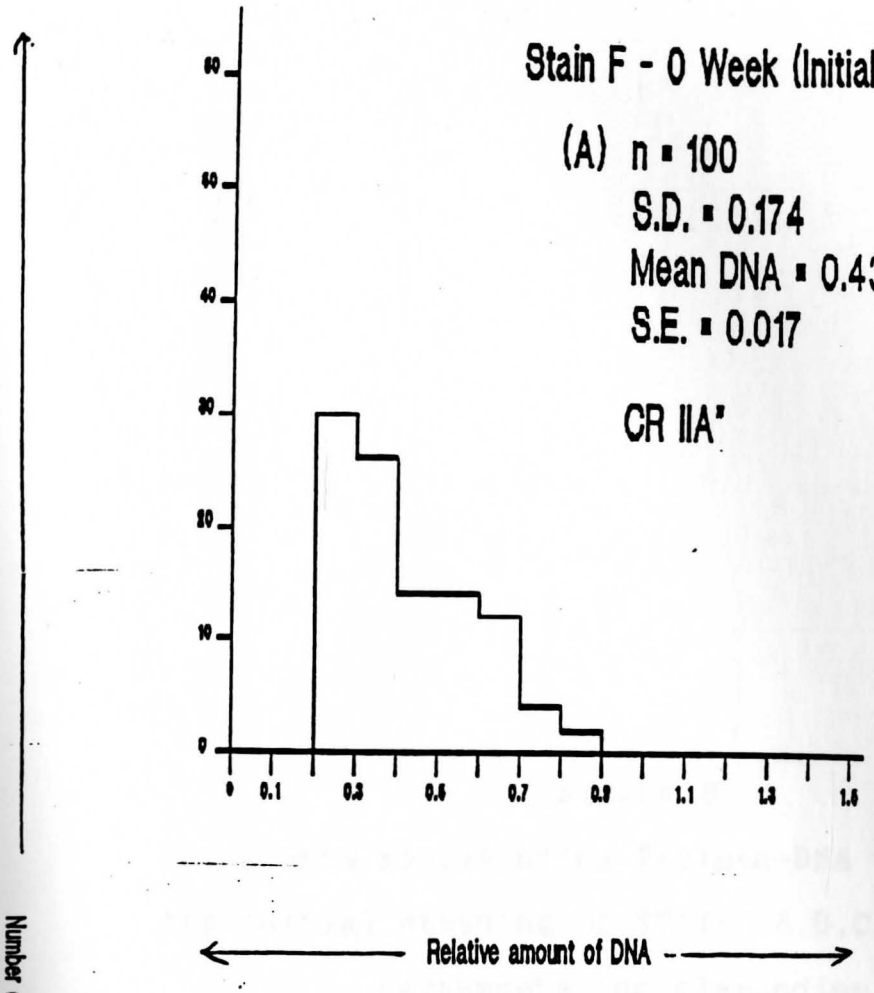
Figure 9

Histograms representing Feulgen-DNA values using

STAIN F - 0 WEEK STAINING for:

A) Costa Rican - 11A^{II} myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A^{5}


Stain F - 0 Week (Initial)
(A) n = 100
S.D. = 0.174
Mean DNA = 0.43
S.E. = 0.017
CR IIA"
Number of nuclei
Relative amount of DNA

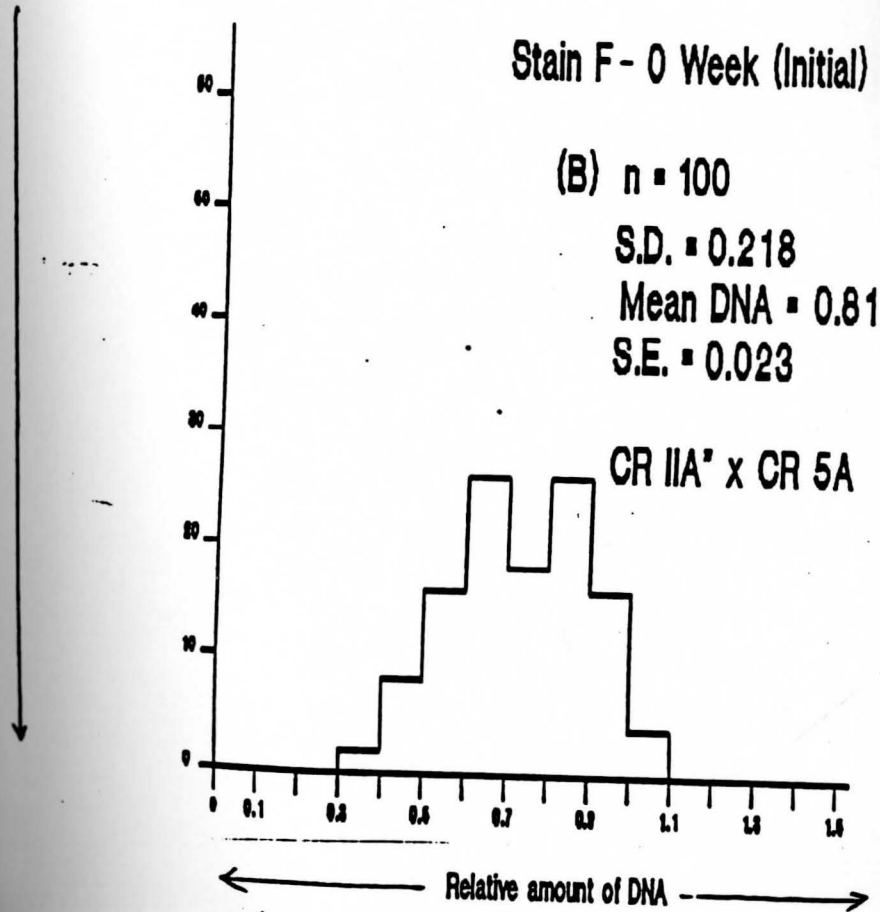
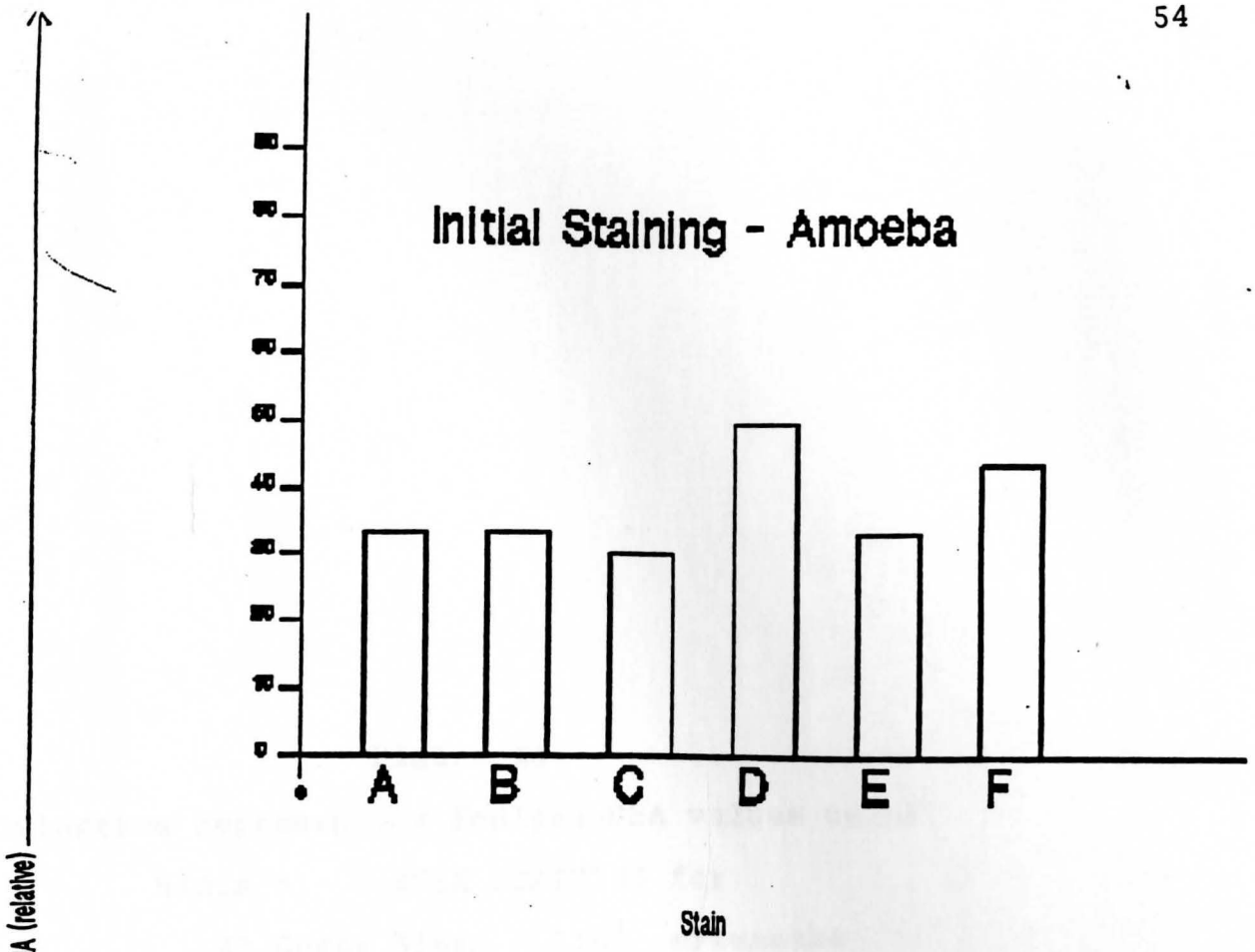

Stain F - 0 Week (Initial)
(B) n = 100
S.D. = 0.218
Mean DNA = 0.81
S.E. = 0.023
CR IIA" x CR 5A
Number of nuclei
Relative amount of DNA

Figure 10

Bar graphs representing Feulgen-DNA values of the initial staining of STAINS A,B,C,D,E and F (myxamoeba and plasmodium)

Initial Staining - Amoeba



Initial Staining - Plasmodium

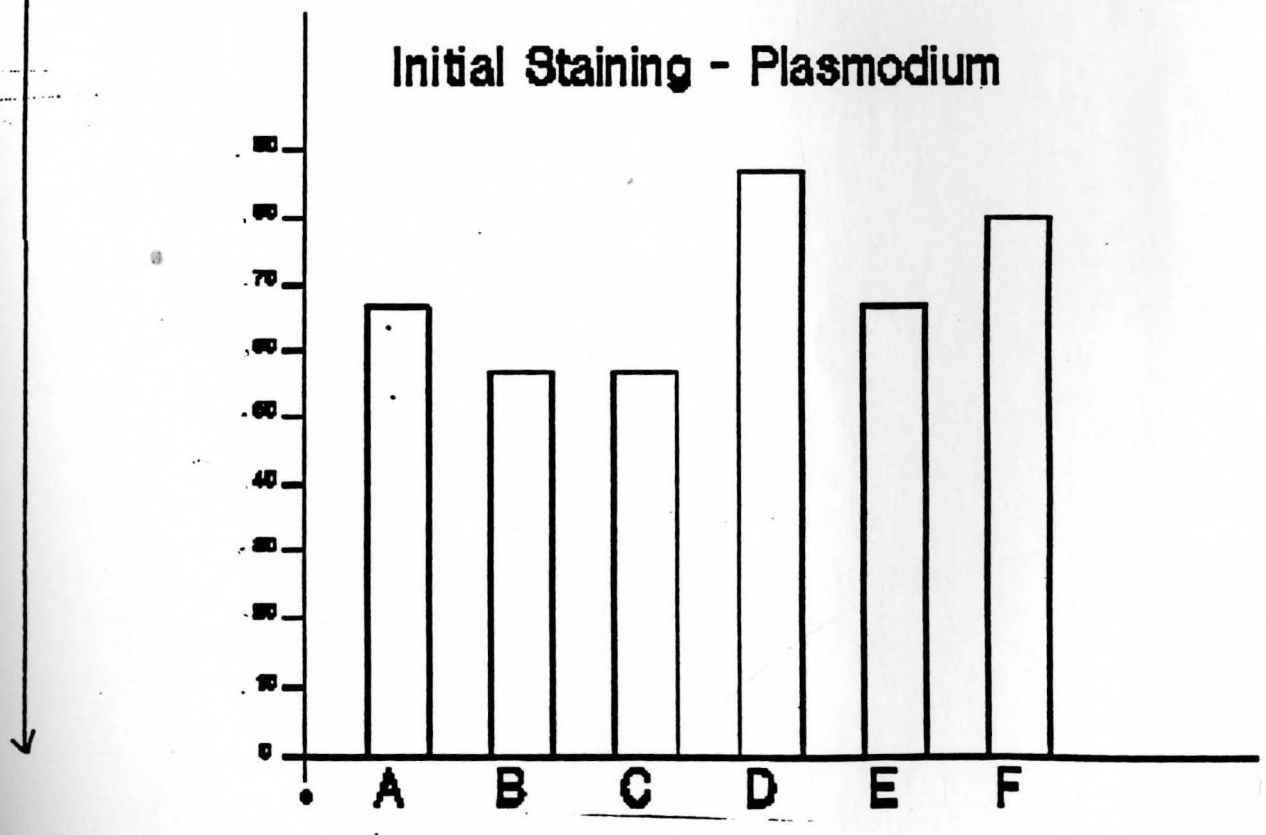


Figure 11

Histograms representing Feulgen-DNA values using

STAIN A - 2 WEEK STAINING for:

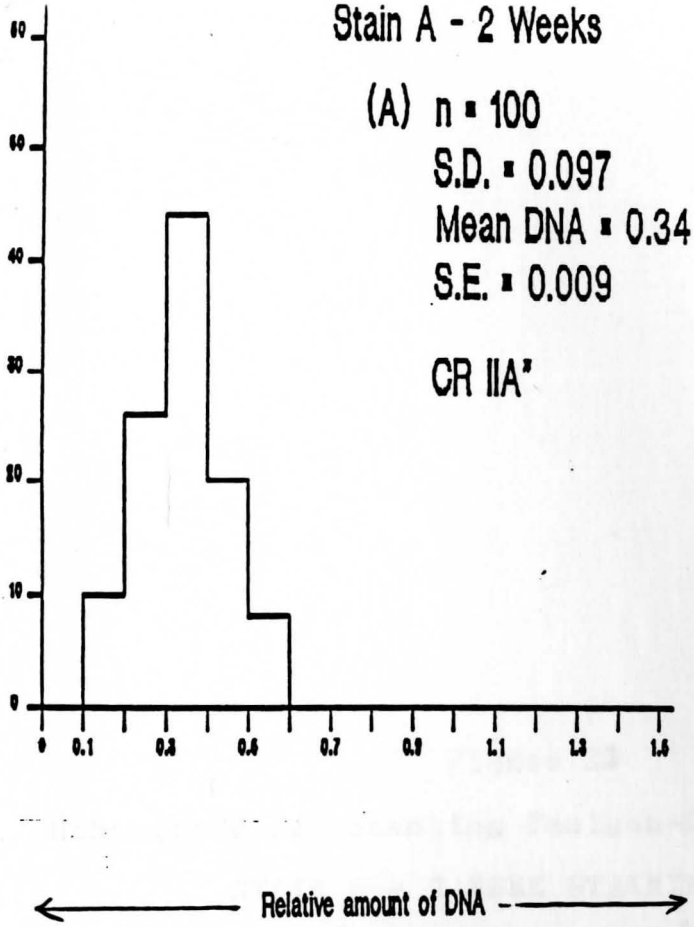
A) Costa Rican - 11A[11] myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A[5]

Stain A - 2 Weeks

(A) n = 100
S.D. = 0.097
Mean DNA = 0.34
S.E. = 0.009

CR IIA"



Stain A - 2 Weeks

(B) n = 100
S.D. = 0.167
Mean DNA = 0.58
S.E. = 0.016

CR IIA" x CR 5A

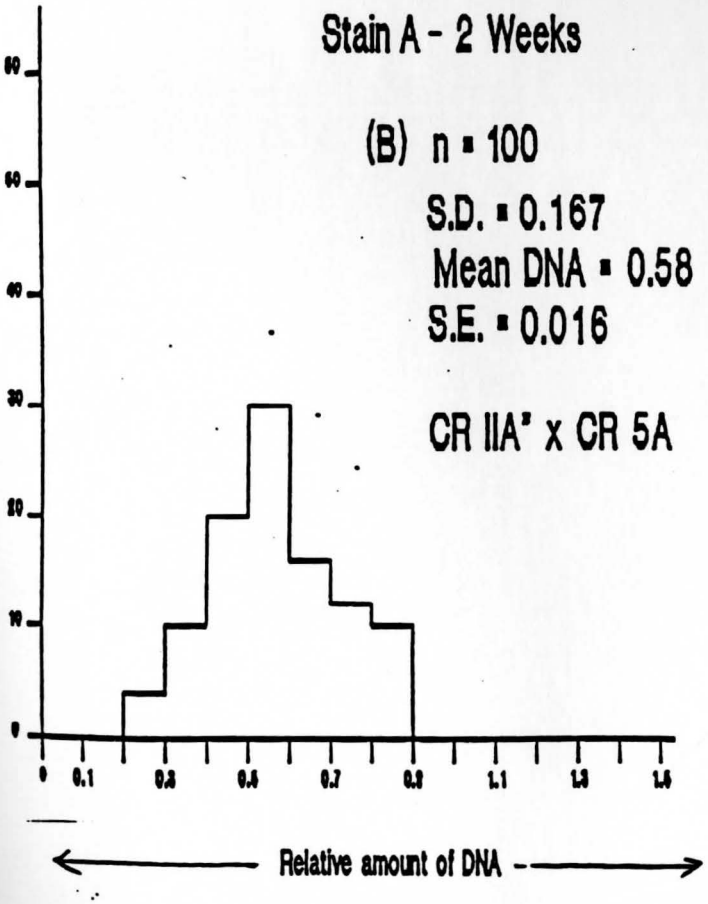


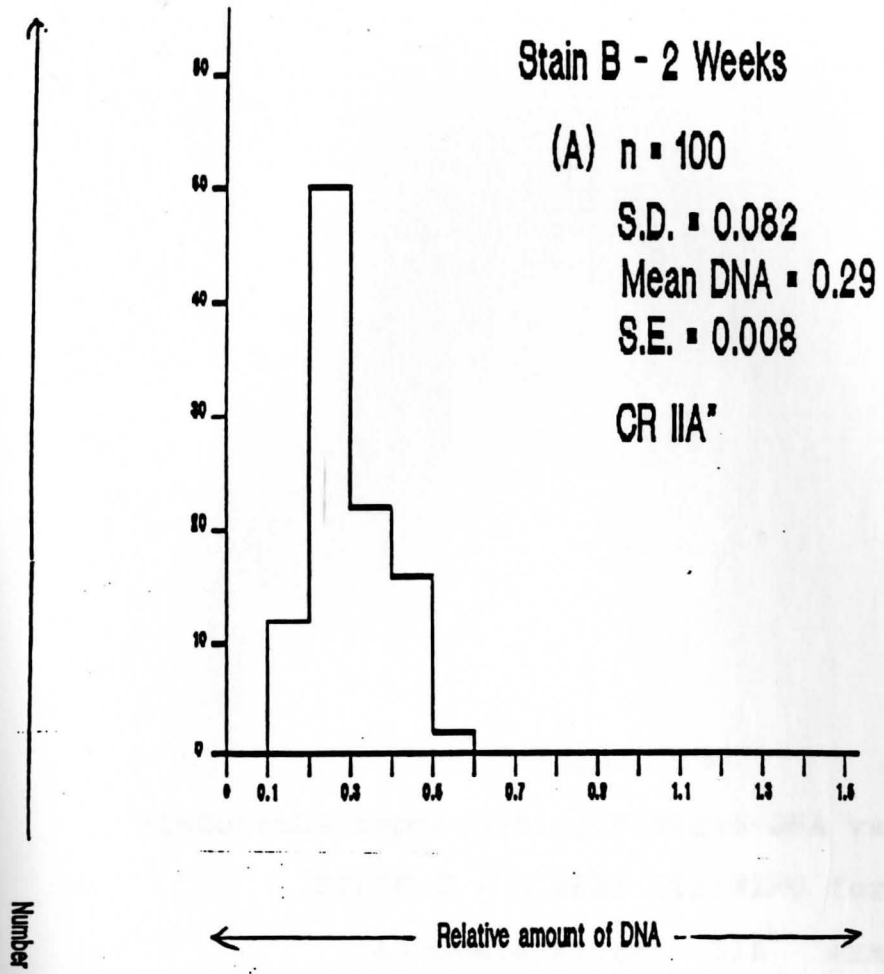
Figure 12

Histograms representing Feulgen-DNA values using

STAIN B - 2 WEEK STAINING for:

A) Costa Rican - 11A[N] myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A[5]


Stain B - 2 Weeks
(A) n = 100
S.D. = 0.082
Mean DNA = 0.29
S.E. = 0.008
CR IIA"
Number of nuclei
Relative amount of DNA

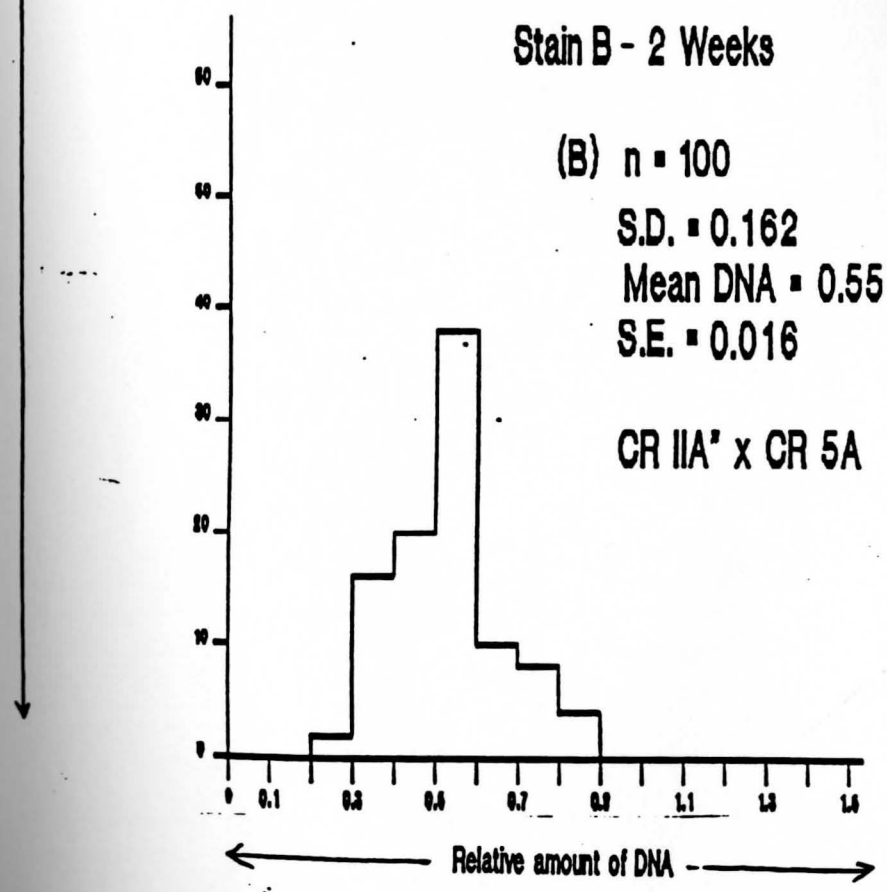

Stain B - 2 Weeks
(B) n = 100
S.D. = 0.162
Mean DNA = 0.55
S.E. = 0.016
CR IIA" x CR 5A
Relative amount of DNA

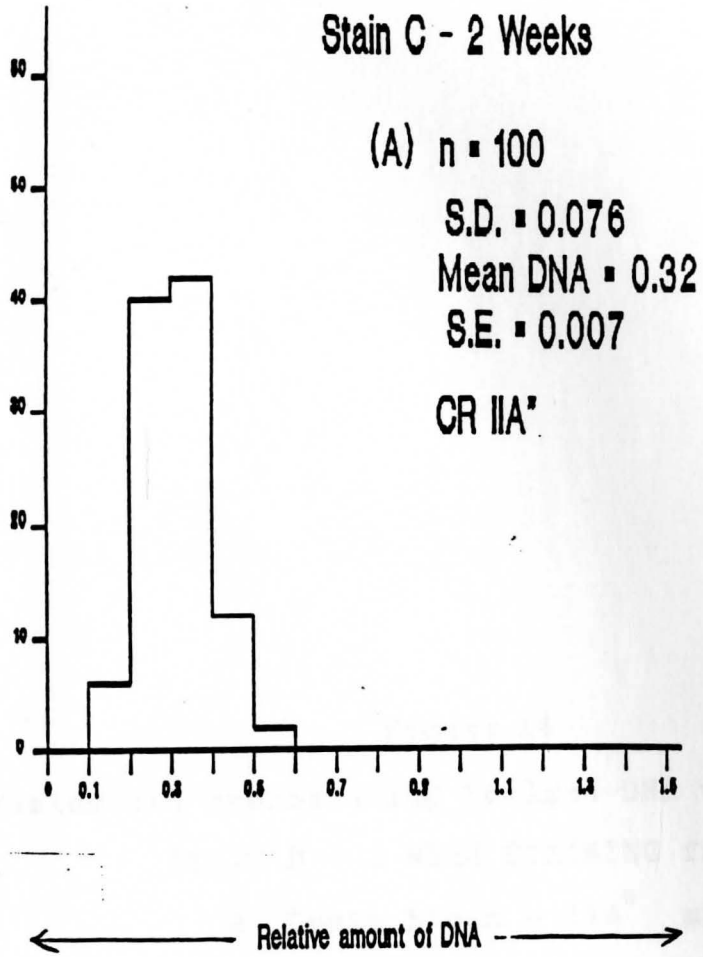
Figure 13

Histograms representing Feulgen-DNA values using

STAIN C - 2 WEEK STAINING for:

A) Costa Rican - 11A" myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A 5


Stain C - 2 Weeks
(A) n = 100
S.D. = 0.076
Mean DNA = 0.32
S.E. = 0.007
CR IIA"
Number of nuclei
Relative amount of DNA

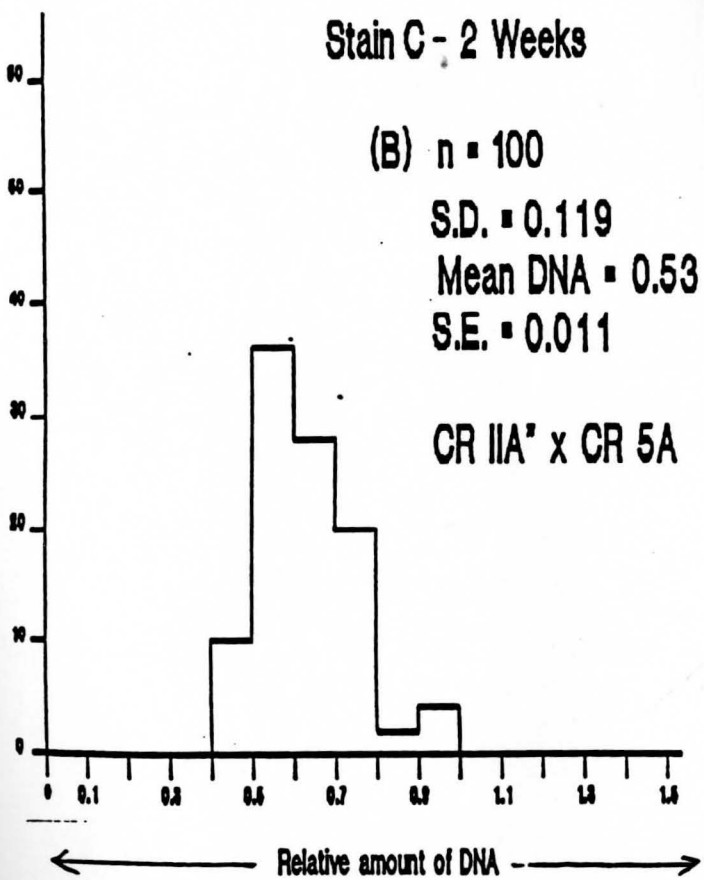

Stain C - 2 Weeks
(B) n = 100
S.D. = 0.119
Mean DNA = 0.53
S.E. = 0.011
CR IIA" x CR 5A
Relative amount of DNA

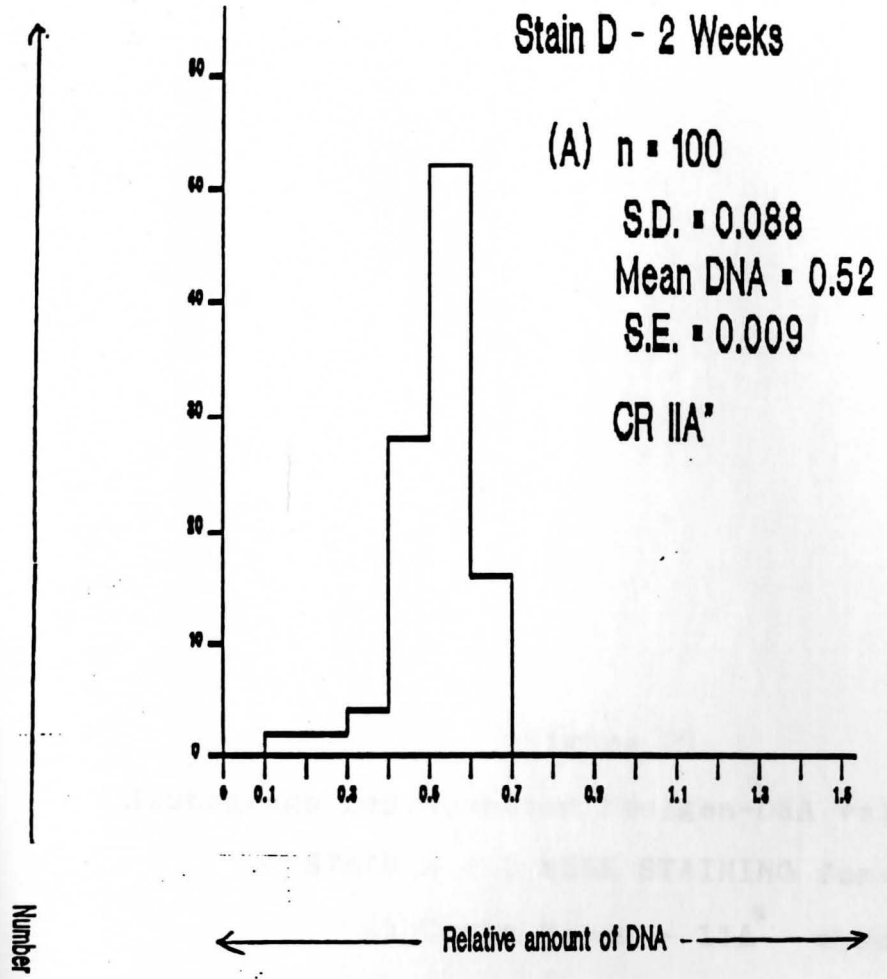
Figure 14

Histograms representing Feulgen-DNA values using

STAIN D - 2 WEEK STAINING for:

A) Costa Rican - 11A" myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A5


Stain D - 2 Weeks
(A) n = 100
S.D. = 0.088
Mean DNA = 0.52
S.E. = 0.009
CR IIA"
Number of nuclei
Relative amount of DNA

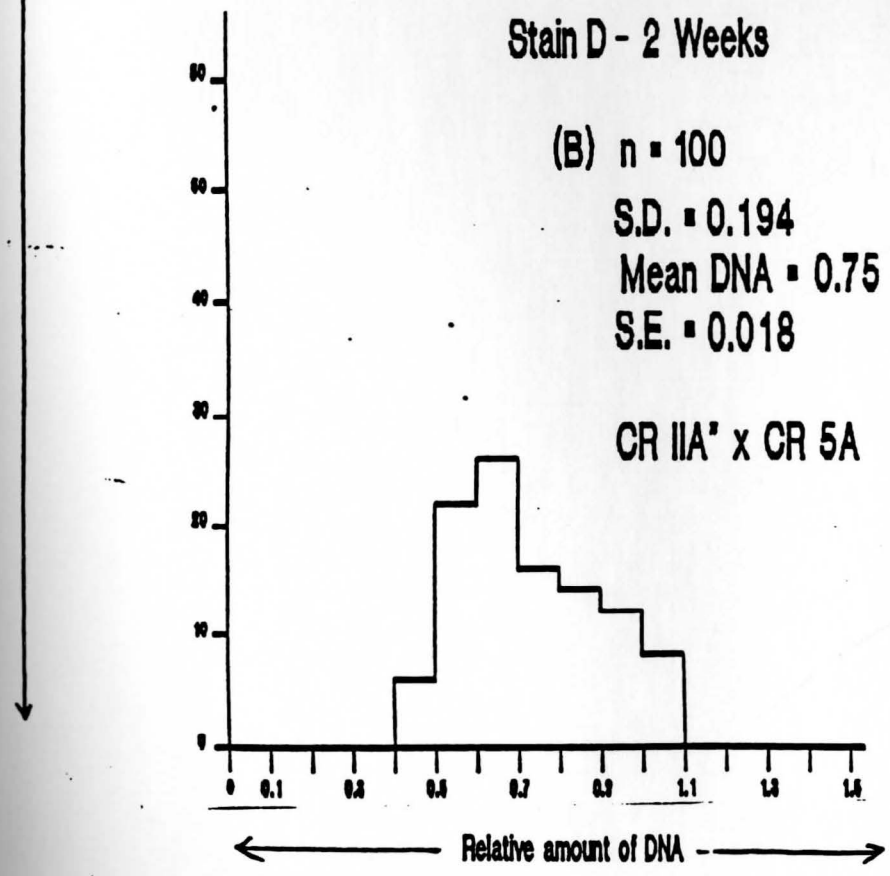

Stain D - 2 Weeks
(B) n = 100
S.D. = 0.194
Mean DNA = 0.75
S.E. = 0.018
CR IIA" x CR 5A
Relative amount of DNA

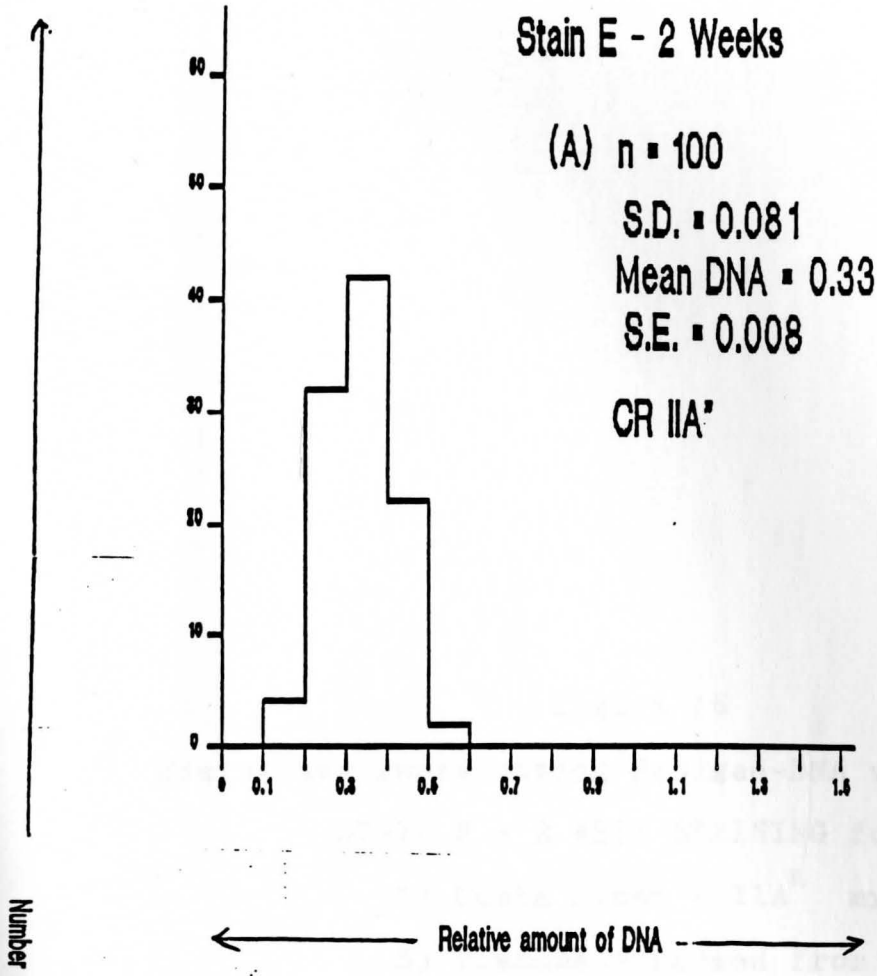
Figure 15

Histograms representing Feulgen-DNA values using

STAIN E - 2 WEEK STAINING for:

A) Costa Rican - 11A[1] myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A[5]


Stain E - 2 Weeks
(A) n = 100
S.D. = 0.081
Mean DNA = 0.33
S.E. = 0.008
CR IIA"
Number of nuclei
Relative amount of DNA

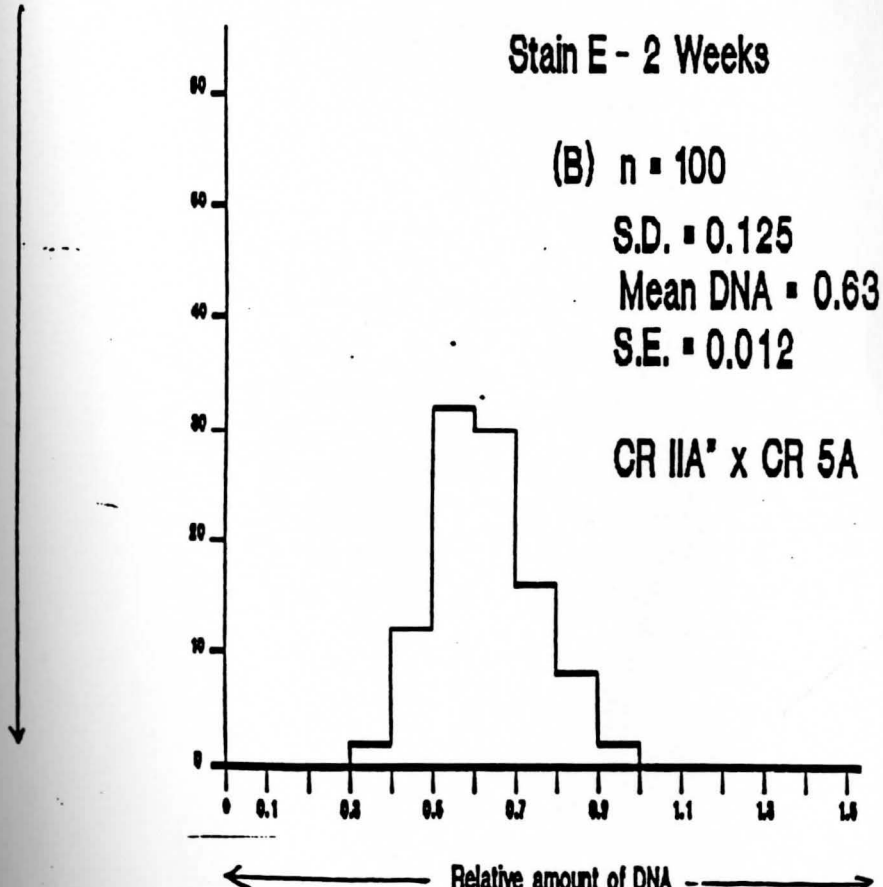

Stain E - 2 Weeks
(B) n = 100
S.D. = 0.125
Mean DNA = 0.63
S.E. = 0.012
CR IIA" x CR 5A
Relative amount of DNA

Figure 16

Histograms representing Feulgen-DNA values using

STAIN F - 2 WEEK STAINING for:

A) Costa Rican - 11A[11] myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A[5]

Stain F - 2 Weeks

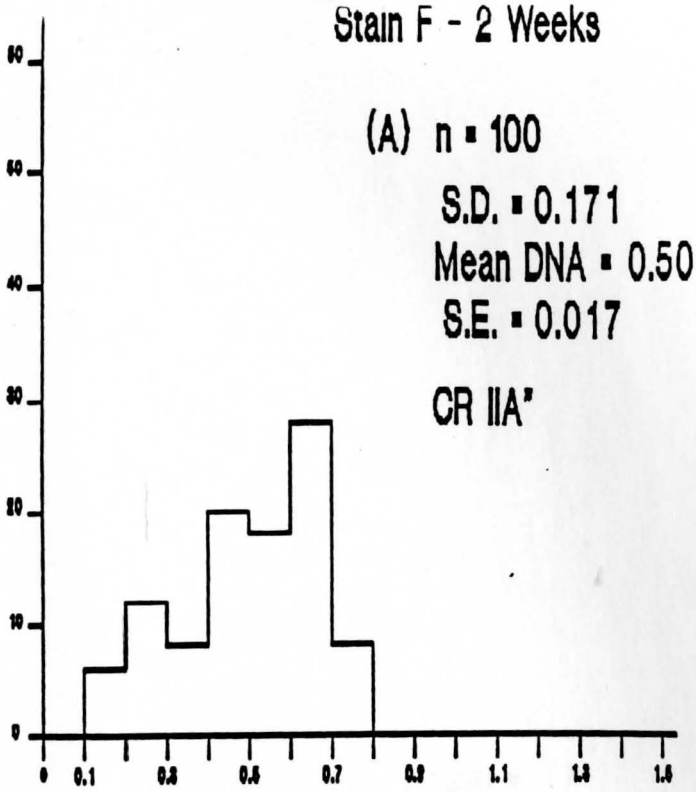
(A) n = 100

S.D. = 0.171

Mean DNA = 0.50

S.E. = 0.017

CR IIA"



Stain F - 2 Weeks

(B) n = 100

S.D. = 0.138

Mean DNA = 0.78

S.E. = 0.013

CR IIA" x CR 5A

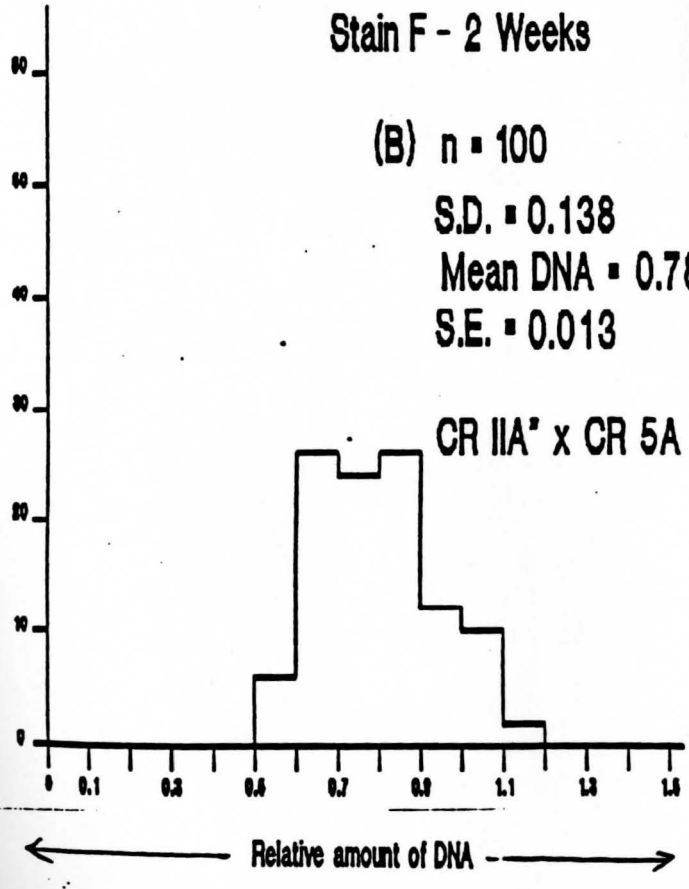


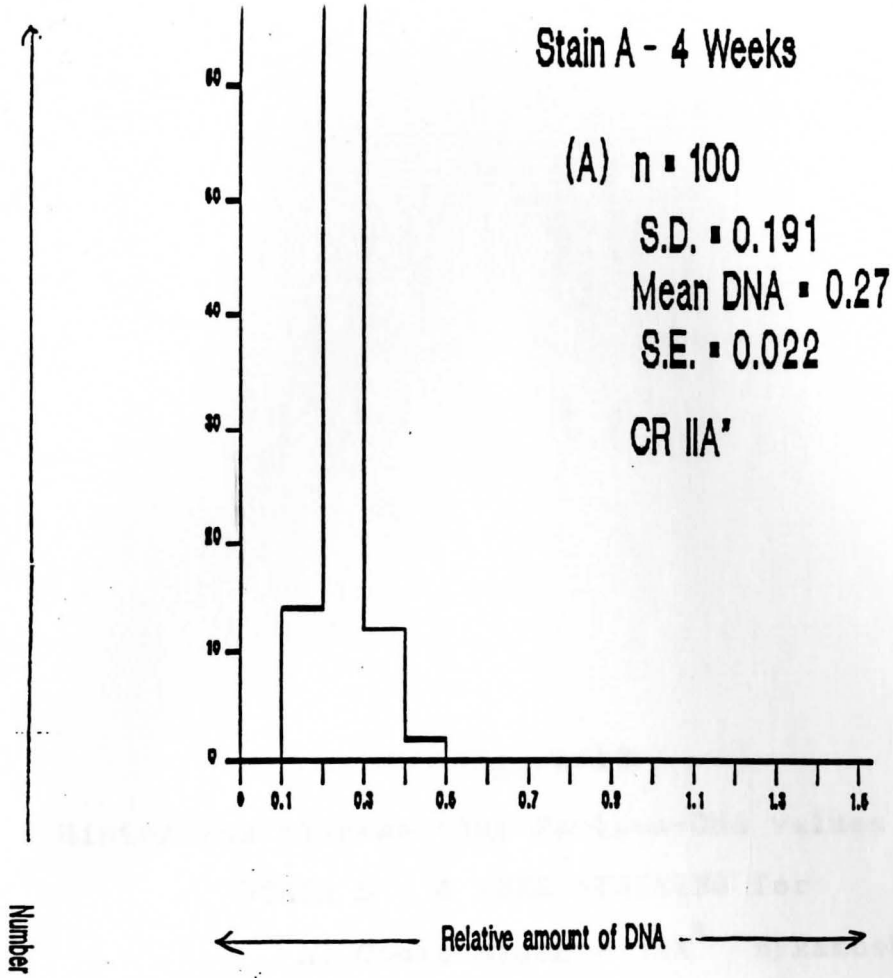
Figure 17

Histograms representing Feulgen-DNA values using

STAIN A - 4 WEEK STAINING for:

A) Costa Rican - 11A" myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A5


Stain A - 4 Weeks
(A) n = 100
S.D. = 0.191
Mean DNA = 0.27
S.E. = 0.022
CR IIA"
Number of nuclei
Relative amount of DNA

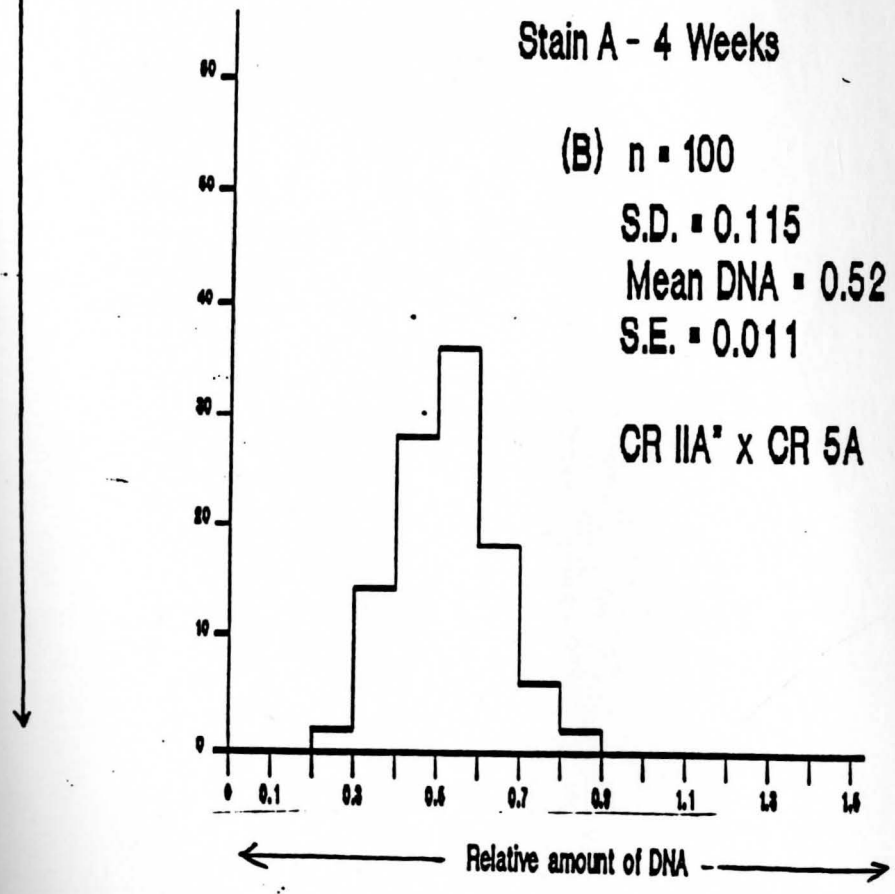

Stain A - 4 Weeks
(B) n = 100
S.D. = 0.115
Mean DNA = 0.52
S.E. = 0.011
CR IIA" x CR 5A
Relative amount of DNA

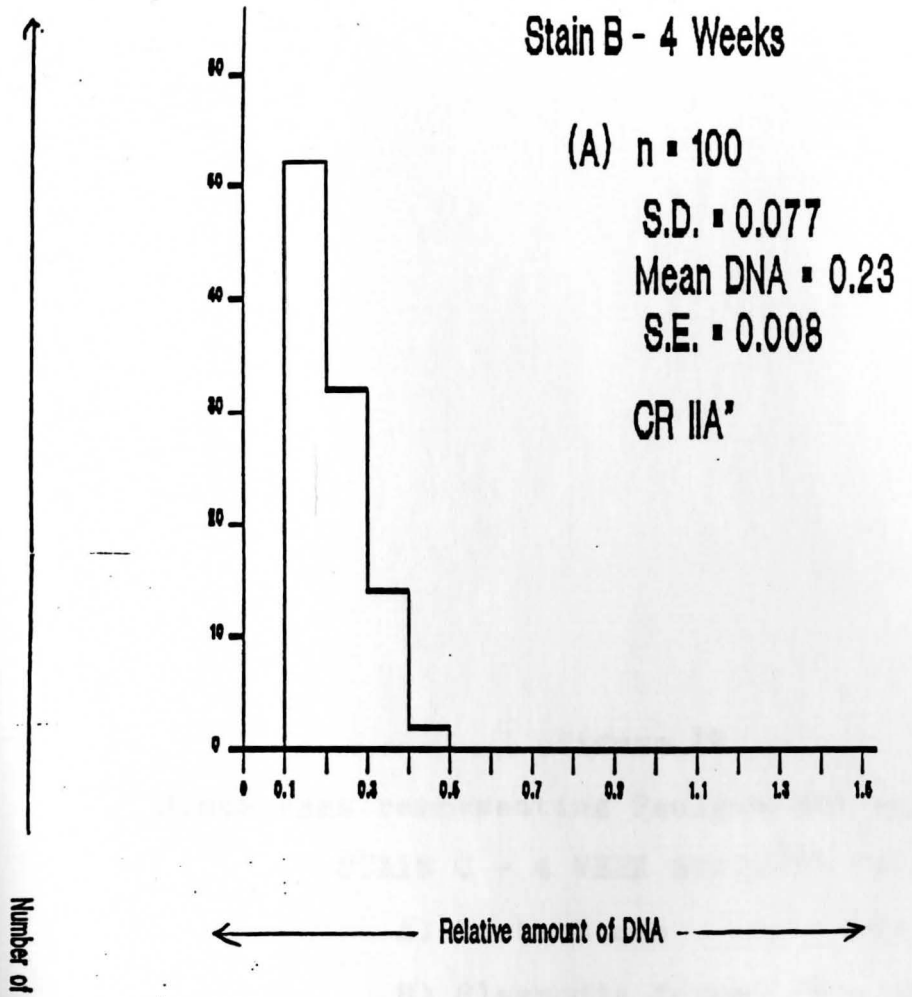
Figure 18

Histograms representing Feulgen-DNA values using

STAIN B - 4 WEEK STAINING for:

A) Costa Rican - 11A[II] myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A[5]


Stain B - 4 Weeks
(A) n = 100
S.D. = 0.077
Mean DNA = 0.23
S.E. = 0.008
CR IIA"
Number of nuclei
0
10
20
30
40
50
60
0 0.1 0.3 0.5 0.7 0.9 1.1 1.3 1.5
Relative amount of DNA

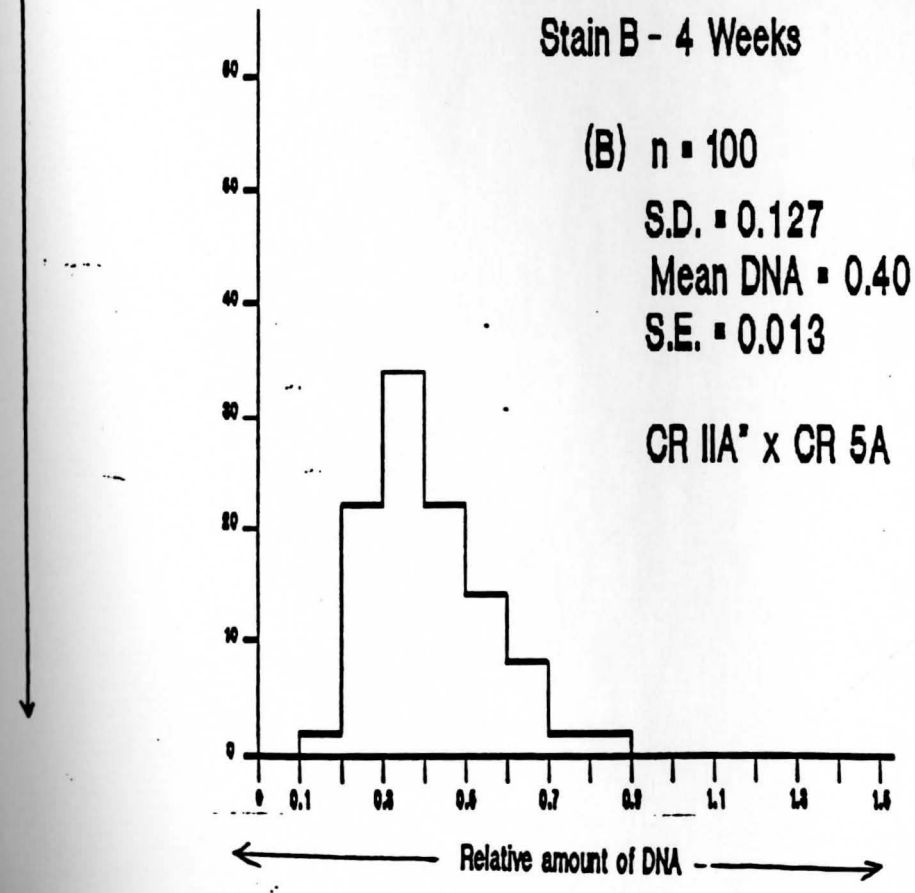

Stain B - 4 Weeks
(B) n = 100
S.D. = 0.127
Mean DNA = 0.40
S.E. = 0.013
CR IIA" x CR 5A
0
10
20
30
40
50
60
0 0.1 0.3 0.5 0.7 0.9 1.1 1.3 1.5
Relative amount of DNA

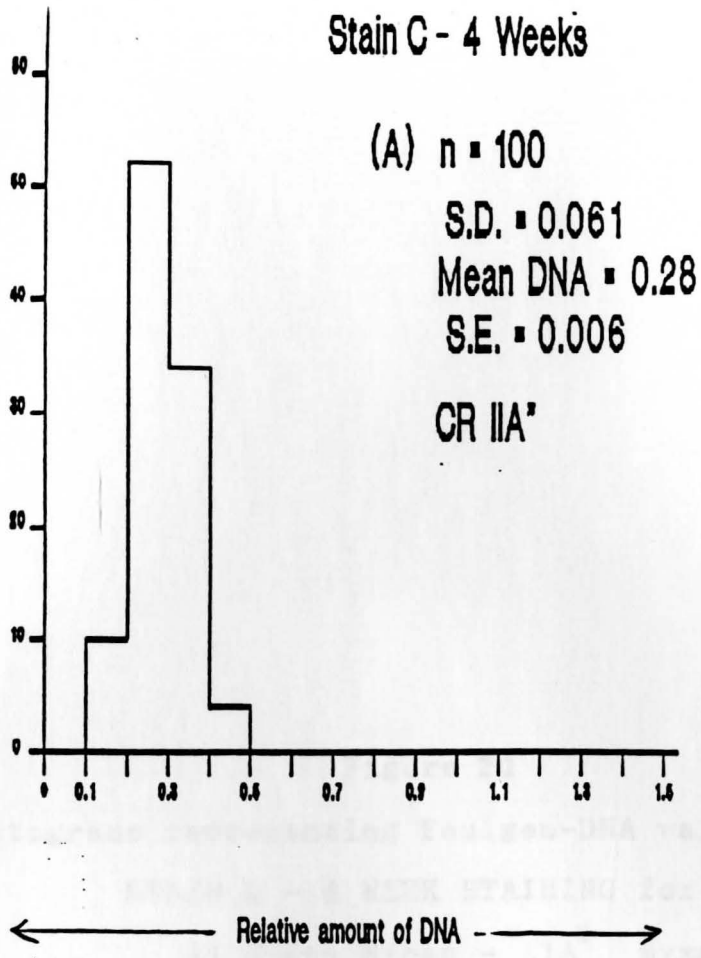
Figure 19

Histograms representing Feulgen-DNA values using

STAIN C - 4 WEEK STAINING for:

A) Costa Rican - 11A" myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A5


Stain C - 4 Weeks
(A) n = 100
S.D. = 0.061
Mean DNA = 0.28
S.E. = 0.006
CR IIA"
0
10
20
30
40
50
60
0 0.1 0.3 0.5 0.7 0.9 1.1 1.3 1.5
Relative amount of DNA
Number of nuclei

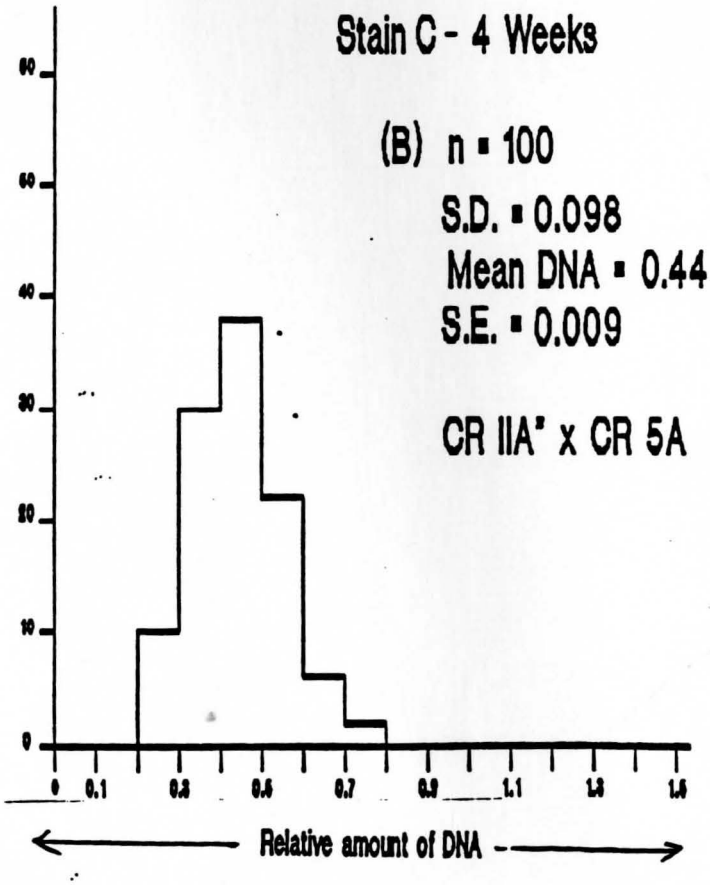

Stain C - 4 Weeks
(B) n = 100
S.D. = 0.098
Mean DNA = 0.44
S.E. = 0.009
CR IIA" x CR 5A
0
10
20
30
40
50
60
0 0.1 0.3 0.5 0.7 0.9 1.1 1.3 1.5
Relative amount of DNA

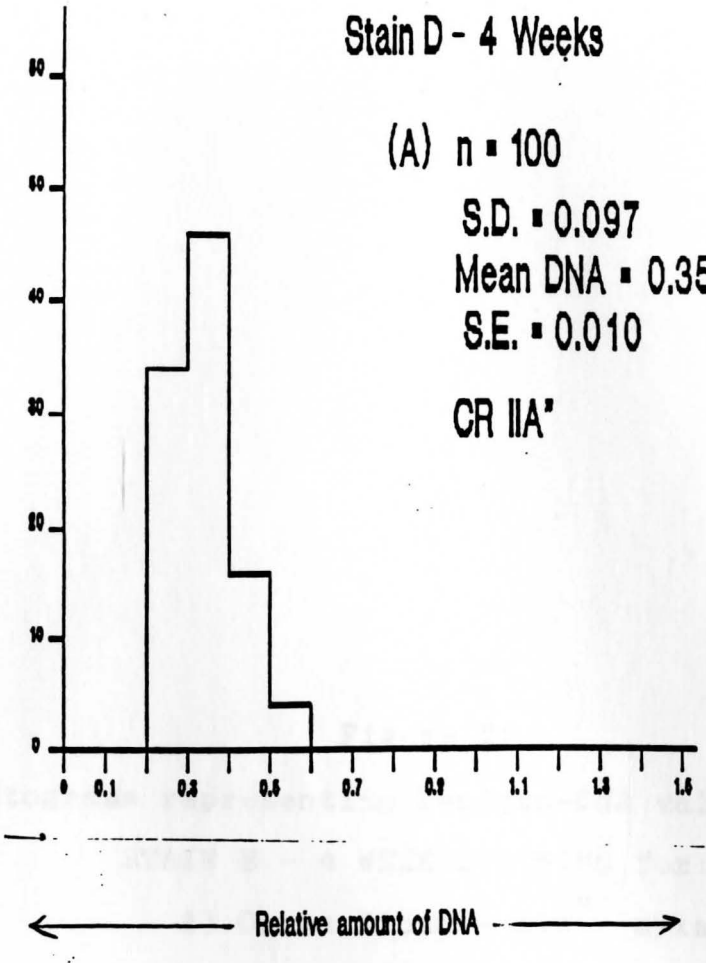
Figure 20

Histograms representing Feulgen-DNA values using

STAIN D - 4 WEEK STAINING for:

A) Costa Rican - 11A[11] myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A[5]


Stain D - 4 Weeks
(A) n = 100
S.D. = 0.097
Mean DNA = 0.35
S.E. = 0.010
CR IIA'
Number of nuclei
Relative amount of DNA

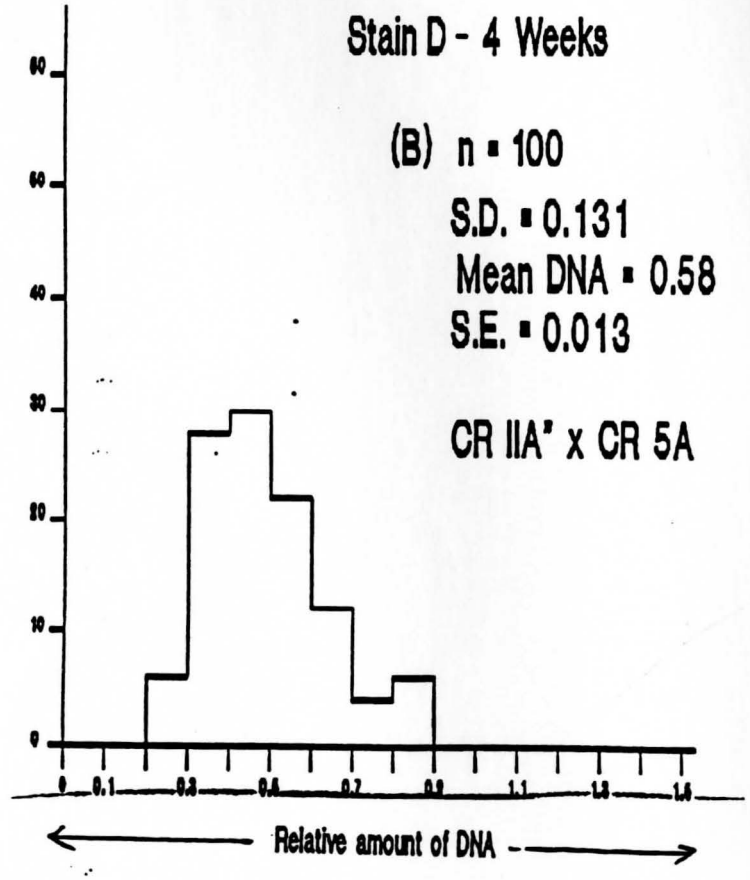

Stain D - 4 Weeks
(B) n = 100
S.D. = 0.131
Mean DNA = 0.58
S.E. = 0.013
CR IIA' x CR 5A
Relative amount of DNA

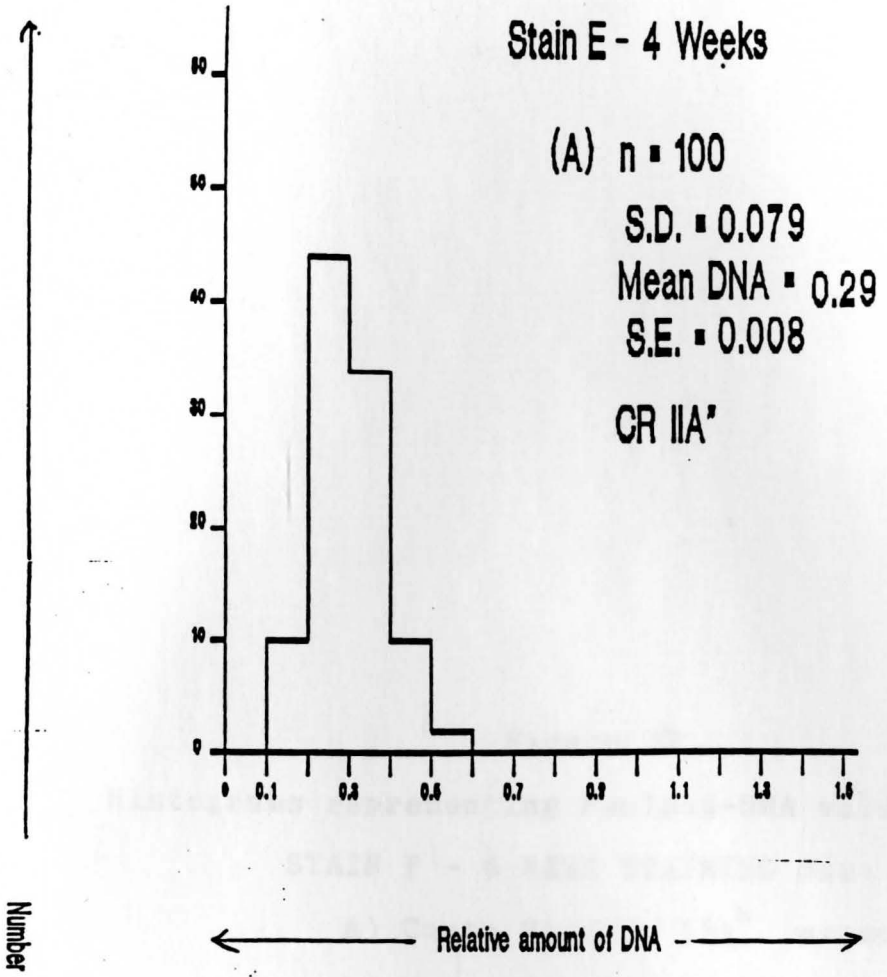
Figure 21

Histograms representing Feulgen-DNA values using

STAIN E - 4 WEEK STAINING for:

A) Costa Rican - 11A" myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A5


Stain E - 4 Weeks
(A) n = 100
S.D. = 0.079
Mean DNA = 0.29
S.E. = 0.008
CR IIA"
Number of nuclei
Relative amount of DNA

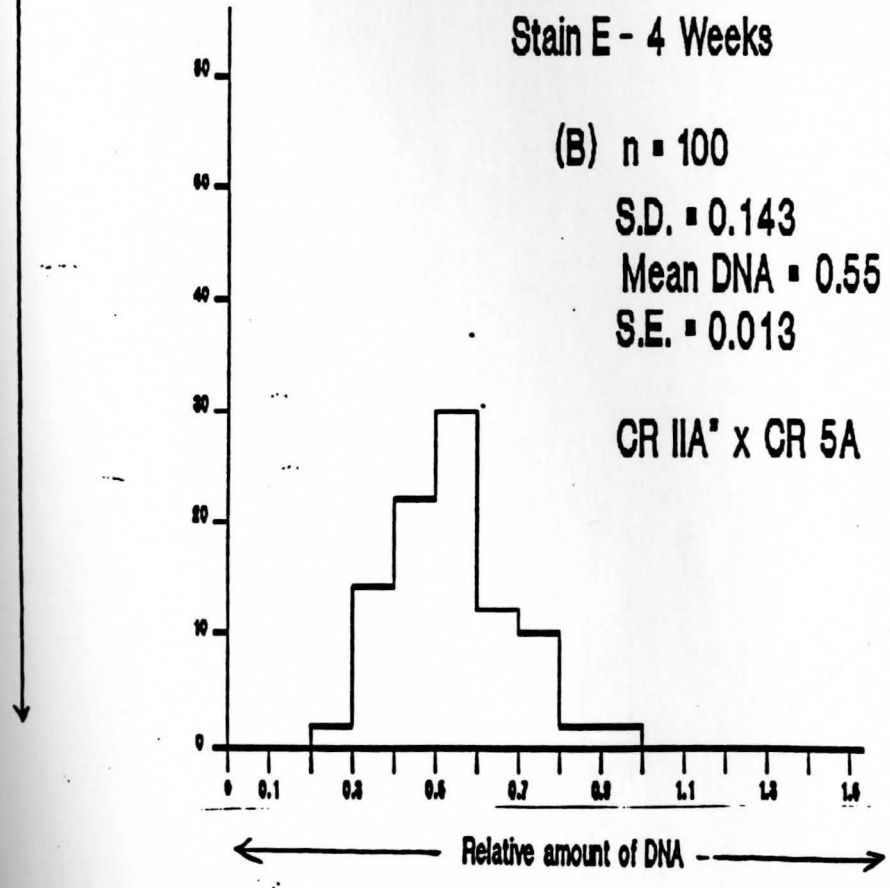

Stain E - 4 Weeks
(B) n = 100
S.D. = 0.143
Mean DNA = 0.55
S.E. = 0.013
CR IIA" x CR 5A
Relative amount of DNA

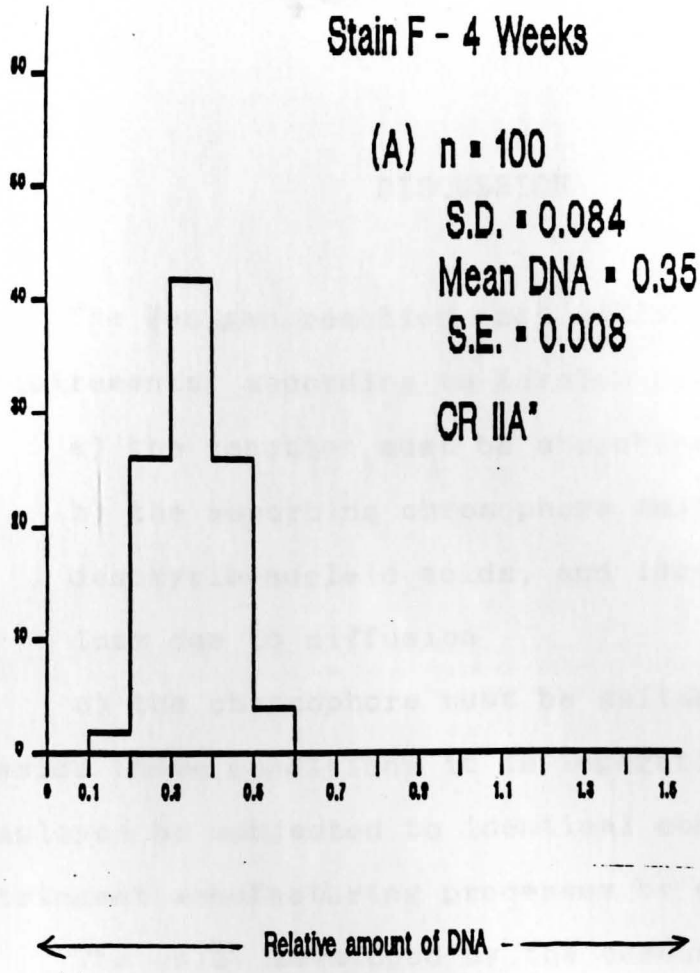
Figure 22

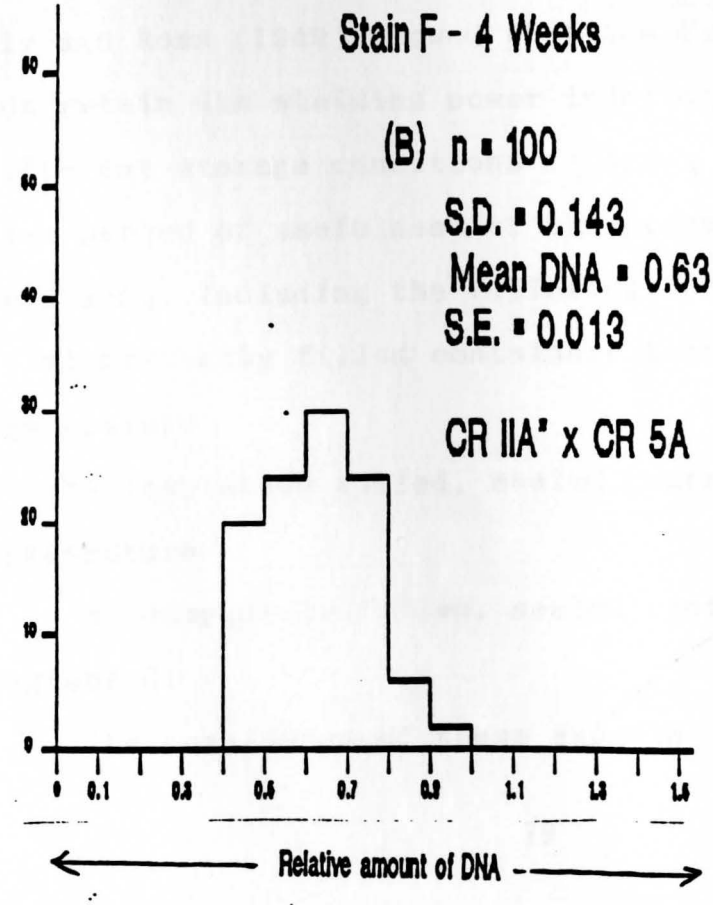
Histograms representing Feulgen-DNA values using

STAIN F - 4 WEEK STAINING for:

A) Costa Rican - 11A^{11} myxamoeba

B) Plasmodia formed from the cross of Costa Rican-11A and Costa Rican 5A^{5}


Stain F - 4 Weeks
(A) n = 100
S.D. = 0.084
Mean DNA = 0.35
S.E. = 0.008
CR IIA"
Number of nuclei
Relative amount of DNA


Stain F - 4 Weeks
(B) n = 100
S.D. = 0.143
Mean DNA = 0.63
S.E. = 0.013
CR IIA" x CR 5A
Relative amount of DNA

DISCUSSION

The Feulgen reaction must satisfy the following requirements, according to Kurnich (1955):

a) the reaction must be stoichiometric

b) the absorbing chromophore must be specific for desoxyribonucleic acids, and localize it without loss due to diffusion

c) the chromophore must be suitable for photometry

Beside these conditions it is imperative that the stains employed be subjected to identical conditions and that stringent manufacturing processes be observed.

The color developed by the combination of hydrolysis products derived from nucleic acid and the Feulgen stain is proportional to the measured DNA value. Ely and Ross (1949) showed that the Feulgen reagent does not retain its staining power indefinitely. They used different storage conditions in order to determine the time period of usefulness of the prepared stain solutions, including the following:

a) partially filled containers kept at room temperature

b) completely filled, sealed containers kept at room temperature

c) completely filled, sealed containers kept at 7 degrees C.

The conclusion of these experiments is that

sections stained well even after two months in reagent that had been kept at 7 degrees C in a completely filled, sealed container, while sections stained with the other two reagents did not stain as well.

In the present investigation any variations in stain intensity observed during the initial staining can be attributed to the diffuse nature of the DNA at a given time during the cell cycle, which affects staining, and is expected within a metabolically active cellular population. Functionally, the intensity of the color appears to be most influenced by the chemical composition of the stain rather than the age of the original dye (Yemma and Penza, 1985), although both bind in a stoichiometric manner (table I). More than likely both are able to measure absolute DNA values upon calculation of the extinction coefficient (Ornstein, 1952), in order to determine absolute DNA values as has become conventional. In this study, however, only arbitrary DNA values are reported. The findings also support the premise that basic fuchsins with CI number of 42500 (pararosanilin -Stains D and F) stain DNA with a greater stain intensity than those having CI number 42510, and give a slightly higher measurable DNA content. This is explained by the differing molecular structure of these dyes (figure 1). But as previously mentioned the arbitrary values recorded upon measurement of DNA demonstrates that they both yield reliable

quantitative datas. It follows that upon calculation of an extinction coefficient, all dyes would yield reliable absolute values. Also the presented data support the fact that these same stains lose their staining ability faster than the stains with CI number 42510, showing a significantly lowered value in case of the plasmodia. In diploid cells having more DNA than those which are haploid - deterioration appears to occur earlier. Interestingly, the stains of CI number 42510 (Stains A, B, C and E) show a longer shelf-life; they also maintain their staining ability for a longer time (without loss of intensity) in both amoeba and plasmodium.

However, the intensity of the color developed is definitely influenced by the age of the post-prepared Feulgen stain, since after the two weeks' staining, a gradual decrease in color intensity is observed in both amoeba and plasmodium. In some of the dyes (A, C, D, E and F) the 2-week staining values for the amoeba show some increase over the first staining values. This can be explained by the fact that the cells differ metabolically and are actively in the S and G2 of the cell cycle. As a result the DNA is in a more diffuse state (Yemma and Perry, 1985); the values are, therefore, in some cases higher than in 0 week. This does not indicate that the cellular population in this case represents differences in nuclear DNA concentration, although dye binding may significantly

differ (figure 4 and 11). However, the means of these populations are less than 1 standard deviation apart and exhibit a very small standard error. This evidence supports the contention that they represent the same population that stains similarly to give similar mean DNA values. By virtue of the dye's ability to bind quantitatively and to demonstrate extreme sensitivity to these changes in DNA, the data strongly support the contention that the dye binds stoichiometrically at this time. Whereas a drop (significant) in week 4 staining, for example, demonstrates a loss of stoichiometric value.

Plasmodia, are in G2 for 90% of the total cell cycle time and will, therefore, be normally in this phase when measurements are made. Dye binding normally will not show an increase here because 0 Week and 2 weeks show G2 cells and will similarly stoichiometrically bind dye (table I, figures 4,5,6,7,8, 9,11,12,13,14,15 and 16). However if we observe stains A and D (figures 4,7,11 and 14) they represent an exception in that dye binding is reduced in week 2 when compared with 0 week but not significantly so. Evidently although 0 week represents G2 cells, differences in means among them are not significant, and well within 1 S.D. and as well show a very small standard error. The 2 weeks data shows a drop in measurable DNA and shows these cells to be in the G1 DNA

category, having undergone a synchronous mitosis (figures 4 and 11) and, as previously pointed out, are well within one standard deviation of one another and exhibit a very small standard error, and therefore represent the same population and a similar valid measurement of DNA concentration. Week 4 (figures 17 through 22) shows a significant drop in dye binding in all cases when compared to week 0; histograms for these time periods support these observations. DNA is not measurable at the 6-week time period.

According to data presented in this study, post-prepared dye-reagent can be used with confidence for at least two weeks. This period of time gives optimal results in staining. After this period, the staining intensity of the nuclei produced by the dye-reagent is gradually diminished, however, the stain is still capable of stoichiometric DNA measurement where the dye is gradually oxidized, but haploid-diploid relationships are maintained. The observed means for all stains used through week four are within statistically acceptable ranges. This time period is definitely shorter than the one Ely and Ross determined in their 1949 study (two months), but it is longer than the period suggested by Dutt in 1980 (one week). DeTomasi (1936) also suggests a shelf-life of one to two months.

Increasing the shelf-life of the post-prepared solutions preoccupied many scientists. Dutt (1981)

describes several variations to the preparation of Schiff's reagent for use in Feulgen procedures. He replaced N HCl by 5N H SO - obtaining a highly sensitive Schiff's reagent which requires only 4-5 minutes for nuclear staining and produces perfect Feulgen staining up to six months after preparation. Dutt used this method with different quantities than used by Hormann et al, (1958) to prepare this perfect stain. However, he did not attempt to determine a quantitative specificity at that time, but dealt only with staining characteristics.

Dutt (1981) also introduced another substitution by using propionic acid instead of HCl (which stabilizes the pH of Schiff's reagent). The shelf-life of these reagents is also increased it was contended, due to their less acidic nature.

Bloch and Godman (1955) suggested the use of organic acids like trichloroacetic (TCA) in the preparation of Schiff's reagent. This substitution following Feulgen hydrolysis gave weak nuclear staining intensity and the shelf-life was very short. This method was modified by Dutt (1979) by increasing the pH to increase the shelf-life of this preparation.

These modifications of the Schiff's reagent, it can be concluded, form the basis of the longer shelf-life. Increased staining ability of the modified Schiff's reagents is requiring increased pH values when prepared,

compared with that of the conventional Schiff's reagent. It is important to note that increasing shelf life by preparation in a more basic environment does not apply to the quantitative measurement of DNA. In fact, it is now generally accepted that high acidity in preparation of Schiff's reagent by sulfonation enhances staining, but lowers shelf-life of the stain.

In conclusion, experiments conducted indicated that a shelf-life of about two weeks for post-prepared Schiff's reagent using the conventional method of preparation and 5N HCl hydrolysis. It is reasonable to assume by observation of the results of these values in arbitrary units, that absolute values could also be obtained. Ornstein (1952) indicates that if one calculates an extinction coefficient for each dye, from similar artirarily obtained data, they would yield quantitative absolute values for DNA. One major source of error, fading, was considered during microspectrophotometric measurements in this study. Fading was first reported by Hillary (1939). In 1955 Kurnick reported fading of the stain upon exposure of the slide containing tissue upon which DNA measurements were to be made, was affected by the intense illumination required in microspectrophotometry, but he also offered a solution by reducing and filtering the light source during the examination and focusing process, which will reduce the fading effect. According

to Kasten et al. (1962), fading involves in situ formation of leuco-base of the dye in the presence of light, with a change in the absorption spectrum of the dye complex and reduction in the color intensity. To minimize the fading, slides were read as soon as was possible after mounting, and measurements were completed rapidly to reduce fading by monochromatic light on the absorption peak of the stain, while the readings were actually being made. Slides also were stored in the dark.

Besides fading, stain impurities were reported to interfere with accurate readings, by causing artifacts of color in the cytoplasm of cells (Hillary, 1939, Kasten, 1964). However, once again these conclusions were speculative.

It can be concluded from the data presented that:

a) the age of the undissolved stain does not affect its staining ability as long as the dyes are properly stored and handled.

This contention is clearly demonstrated by the data presented as histograms detailed in the "Analysis of the first staining". Regardless whether the results obtained were from the amoeba or the plasmodium, a haploid and diploid cell line, the oldest dye (1972 - Baker) Stain A has the same staining ability as the Stain E (1986 - Fisher) comparing the obtained DNA values: 0.32 for both amoebas and 0.66 and 0.65

respectively for the plasmodium (table I, figures 4,8 and 10). Stain A is compared to Stain E rather than to Stain F (1987 - Fisher) because Stain F has a different C.I. number (C.I. 42500), thus eliminating the introduction of a new factor into our comparison. The results obtained by using Stains A,B,C and E (figure 10) speak for themselves. All these dyes have C.I. numbers 42510. In the amoeba the variation in the mean DNA value is minimal, all having a value of 0.32 except Stain C with 0.31. The plasmodial staining shows slightly higher variation (0.66, 0.57, 0.56 and 0.65) due to higher relative DNA content of the 4C state and differing rate of DNA synthesis. There is no downward trend observed when comparing mean DNA values obtained by using dyes dating from 1986 to 1972.

Comparing Stains D and F both having C.I. numbers 42510, manufactured by Fisher and having the same dye content 97%, the only variable in this case is the age of the dye. This represents only a 2-year time period compared to the 10 years represented by the previous group of dyes (Stains A,B,C and E) but still sufficient to prove a point. There is no increase of staining ability with the newer dye (Stain F - 1987), Stain D (1985) giving slightly, but insignificantly higher values than Stain F: 0.49 for amoeba Stain D and 0.43 for amoeba Stain F, respectively 0.86 for plasmodia Stain D and 0.81 plasmodia Stain F. The presented data

clearly demonstrate that the age of the preprepared Basic Fuchsin has no effect on the quantitative cytophotometric properties of the Feulgen nucleal reaction.

b) the time period (duration) for stability of postprepared Schiff's reagents was established as two weeks for optimal results in staining. After this time period the stain is still capable of stoichiometric DNA measurement but a decrease in overall stain intensity occurs, due to some fading.

By comparing results of the second week staining to the original staining results no loss of sensitivity is observed. Tests of significance were performed on data obtained from the first and second staining, and the results showed no significant differences. By comparing the histograms obtained from initial staining data (figures 4 through 9) to histograms of the second staining (figures 11 through 16) there is only very slight variation observed. This variation is due to the difference in the cell population analyzed and due to the diffuse nature of DNA during template activity. By comparing the original staining results (figures 4 through 9) to the four week results (figures 17 through 22), the difference in results is more obvious. The significant decrease in values is demonstrated by the T-test also. The stability of postprepared stains was established as 2 weeks under controlled temperatures and

circumstances. This determination of stability of the post-prepared dye is important as part of a reaction parameter best suited to producing optimal Feulgen staining.

By analyzing table I it appears that haploid cells do not show or reflect aging of prepared stain as easily as 4C or diploid cells due to a greater amount of DNA, and complexity, a greater content of diffuse DNA. Also, it can be concluded that stain stability varies according to molecular type of the dye. The deterioration of certain stains may in fact occur earlier, as seen from Table I data. But one can safely stain tissue with the intent of studying nuclear DNA content within a population of cells by use of quantitative cytophotometric methods, using Schiff's reagent of almost any shelf life or storage period, and regardless of manufacturer. But, upon hydration of preparation, use in quantitative measurements is limited. It is best that any prepared dye be used within two weeks.

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