# MICROSPECTROPHOTOMETRIC ANALYSIS OF THE EFFECTS OF VARIOUS HYDROLYSIS TIMES AND FIXATIVES ON THE INTENSITY OF THE FEULGEN REACTION ON RAT LIVER AND SLIME MOLD MYXAMOEBAE

by

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ABSTRACT

MICROSPECTROPHOTOMETRIC ANALYSIS OF THE EFFECTS
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The two wavelength method of microspectrophotometry was used to analyze the effects of various hydrolysis times for the four different fixatives (Calcium-acetate-formalin, Acetic-alcohol-formalin, Neutral buffered formaldehyde and Fixcel - a new fixative); to determine the best hydrolysis time for each fixation procedure that will give maximal intensity and quantification of the Feulgen reaction for both tissue types (liver and myxamoebae), and therefore most types of tissues.

Optimum Feulgen staining intensity for both tissue types was obtained with Calcium-acetate-formalin (CAF) as a fixative when compared to the other three fixatives. Liver and myxamoebae tissue fixation with Acetic-alcohol-formalin (AAF) and fixcel attained maximum Feulgen intensity rapidly. Liver tissue fixation with Neutral buffered formalin exhibited the longest plateau period of maximal Feulgen intensity, and in myxamoebal tissue fixation with AAF exhibited the longest extended plateau period of maximal Feulgen intensity reflecting tissue differences.

The hydrolysis curve for liver tissue fixed in CAF exhibited an abrupt decline in maximal Feulgen staining intensity following a 5 minute plateau period. Such an abrupt decline was not evidenced in the

descending slope of the hydrolysis curve for myxamoebal nuclei fixed in CAF, nor in comparison with other fixatives. The results of this investigation indicate that fixcel is a very poor fixative for liver tissue, but myxamoebal tissue fixed in fixcel suggests that it may be used in certain tissue types for quantification of DNA; furthermore, similar Feulgen intensities should not be expected to occur between two cell types when using the same fixatives.

# ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. John J. Yemma whose guidance and support made the realization of this thesis possible. A special thanks goes to my friend, Dr. Henry Musoke Sendaula, for his aid and moral support in this study.

Finally, this thesis is dedicated to my parents, Mr. and Mrs. Samuel Onyechere Ibezim, and family for their continued support and encouragement throughout this study.

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# LIST OF SYMBOLS

| SYMBOL | DEFINITION | UNITS OF REFERENCE |
|---|---|---|
| A | Area | $\mu$ |
| 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 | |
| $\lambda$ | Wavelength | nm |
| $\alpha$ | Confidence interval | |

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# INTRODUCTION

The determination of the chemical composition of various cellular constituents has been of profound importance to the cell and molecular biologist. This is often true at both the gross and cytochemical levels as demonstrated by Pollister et al., (1949). The gross method is utilized often by the analytical biochemist who isolates cellular structures in masses and subjects them to a variety of analytical procedures. In the cytochemical method, the cell biologist studies single cells with various tools and is often restricted to methods which are based upon light absorption for the analysis of these structures. Gross and cytochemical methods are complementary in many respects, for example, in evaluating changes which might have been brought about by normal and abnormal cellular process as well as the isolation fixation and staining procedures which may be used in analysis. Analytical methods have gained in importance in recent years and can readily be employed in assessing nuclear activity including changes in DNA content (Yemma and Therrien, 1972).

In view of this Swift (1956) and Caspersson (1936) proposed a method in which relative amounts of desoxyribonucleic acid (DNA) per single cell nucleus could be determined spectrophotometrically. This method proposed would compensate for the factors which may influence staining intensity and thus spectrophotometric analysis providing the following conditions were met: 1) results must be reproducible,

2) measured extinctions in photometric determinations must be directly proportional to the number of dye molecules present throughout the range of the concentration studied, and 3) there must be a constant relationship between the number of dye molecules and the absolute amount of desoxyribonucleic acid. To prove the validity of the quantitative cytophotometric method Swift measured the DNA content of murine somatic nuclei and murine spermatid nuclei and showed them to have a ratio of 2:1 respectively. This ratio was in agreement with gross analysis of nucleic acid and protein in isolated nuclei and chromosomes made later by several investigators working independently (Pollister et al, 1949 and Moore, 1951). Publication on suspension count analysis of nuclear suspensions of several beef tissues done by Boivin, Vendrely and Vendrely (1948) and Vendrely and Vendrely (1948, 1949) also support the above ratio. To verify further the accuracy of quantitative microspectrophotometry, Ris and Mirsky (1949), used several different species of animals and obtained two sets of nuclear DNA values for each specific group which when compared are within ten percent of one another when the nuclei are measured by both spectrophotometric and biochemical methods. Patau (1952), Ornstein (1952) and Mendelsohn (1961) working independently formulated the two-wavelength method of microspectrophotometry. This method eliminates the need for direct measurements of nuclear area (Mayall and Mendelsohn, 1970) and corrects for errors caused by heterogenous distributions of stained material.

In recent years most cell biologists have embarked on studies designed to evaluate procedures that will minimize errors in studies involving the cell nucleus. The Feulgen nuclear reaction has long been considered a reliable tool for the quantitative cytochemical

determination of desoxyribonucleic acid. The method was developed by Feulgen and Rossenbeck (1924) as an application of Feulgen's discovery of a positive Schiff test for thymonucleic acid after acid hydrolysis of thymus nucleic acid. The Feulgen reaction has since then been used as a histochemical test for desoxyribonucleic acid. Most investigators who are familiar with the technique accept it as a specific test for desoxyribonucleic acid (Deitch et al., 1967; and Jordanov, 1963) while some like Stedman and Stedman (1943, 1947) and Carr (1945) question such specificity. But recently, most cytochemists using more refined techniques (Andersson and Kjellstrand, 1971, 1972 and 1975; and DeCosse and Aiello, 1966) agree that under proper conditions with appropriate controls the reliability of the Feulgen reaction for localizing DNA within the cell nuclei can be accepted universally as a valid procedure. Questions about the right procedure and conditions that should be utilized for Feulgen staining have arisen, for example, the intensity of the Feulgen reaction can be influenced considerably by factors involved in the reaction procedure; Deitch et al., (1967), Andersson and Kjellstrand (1972), DeCosse and Aiello (1966), and Jordanov (1963). Jordanov (1963) indicated that one argument against the quantitative value of the Feulgen reaction is that a solubilization of apurinic acid may occur during the standard acid hydrolysis at $60^{o}$ C. Ely and Ross (1949) concurred and said that Feulgen reaction technique is satisfactory for qualitative work but quantitatively there is loss of Feulgen reacting substances from the cell because of the conditions of hydrolysis. Caspersson (1936) and Swift (1956) indicated that since there is an equal amount of DNA in the cells of a species, that if the cells are subjected to the same Feulgen

reaction procedure there will be an equal loss of the stainable material (Feulgen reacting substances or DNA) in the cells. In view of this Swift (1956) said that the amount of DNA remaining in the cells after the Feulgen reaction procedure would represent in comparable relative values and not absolute values. In Swift's work establishing the validity of quantitative microspectrophotometry, he confirmed the observation of other investigators which indicated that some steps taken in the cytochemical procedure (fixation and hydrolysis) influenced the staining intensity of the Feulgen reaction. Recently acid hydrolysis carried on at room temperature gives satisfactory results (Andersson and Kjellstrand, 1971, 1972). Swift also indicated that changes were observed in the Feulgen reaction when the hydrolysis time and/or fixation procedure is varied. Ely and Ross (1949) also concurred and pointed out that, "Those who are familiar with the Feulgen technique recognize that the staining results with sections may vary greatly, because the technique involves a series of steps which are not so clearly understood and a change in any one of the reactions might have considerable influence on the final results."

Previous experiments and discussions show that there are various factors possible for a negative Feulgen nucleal reaction. These factors can be grouped under five headings according to Hillary (1939): 1) unsuitable general methods; that is wrong fixative, insufficient fixation and preparation or unsuitable object (presence of thick cell walls or heavy cuticle or fats which would hinder observations of the nucleus). 2) Incompleteness in the methods employed in the nucleal reaction; that is wrong period of hydrolysis as well as improper concentration of acid and temperature of hydrochloric acid (HCl),

3) Presence of materials which hinder the normal process of the reaction; that is, neutralization or binding of HCl, binding of the liberated aldehyde groups, disturbances of the penetration of reagents by resins. 4) Strong dispersion of a small amount of chromatin in a large nuclear volume. 5) Chemical alteration of thymonucleic acid. Hillary (1939) also indicated that various experiments have shown that Feulgen nucleal stain is possible with all kinds of plants, and he further said that the intensity of this stain and thereby its usefulness is dependent on the interrelation of two factors (fixation and hydrolysis). Whitaker (1939) remarked that the time allowed for hydrolysis is one of the critical points of the Feulgen technique, but then makes the statement: "In general, it is governed by the lengths of the period of fixation, that is, if fixation lasts 30 minutes, hydrolysis should extend over the same period." After comparing series of experiments, Hillary (1939) generalized that: 1) Fixatives containing chromic acid have optimum $60^{o}$ C. hydrolysis period for maximum stain of 5-30 minutes; 2) while with fixatives lacking chromic acid, it is 4-8 minutes. He further indicated that in cases where no stain is obtained in the normal hydrolysis range, a stain can often be obtained by increasing the time of hydrolysis. Other investigators like Kurnick (1955), Deitch et al., (1967) and Sibatani and Fukuda (1953) agreed with the above. And Deitch et al, (1967) remarked that it should be noted that the choice of fixatives and hydrolysis procedures is not the only procedural details affecting the intensity of the Feulgen reaction, the pH and $SO_4$ content of the Schiff reagent, the presence of dye impurities and even the bleach, wash and dehydration steps all affect the stain intensity; this is confirmed by Kasten (1960 and 1964), Schiff (1955) and Hillary (1939).

Where information on localization and quantitation of cellular constituents is desired, it is mandatory that the cell be unaltered by the techniques of preparation and examination, and this ideal is often not attained in the physical world let alone in the biological world. Very few procedures can be performed on living cells in a physiological environment. Even there, the measuring procedures may not be innocuous as in the ultraviolet examination of protozoa (Kurnick, 1955), for most procedures, fixation will therefore be necessary. Tissue fixation is a process in which the proteins become less soluble and catabolic reactions stop; and without such denaturation of tissue proteins components would be dissolved during further procedures. In most cases, fixation involves some distribution and loss of cellular components. In the case of nucleic acids, organic solvents, formalin and most fixatives do not cause significant losses from the cell (Kurnick, 1955). Several investigators have further shown in their studies that fixatives during the fixation processes interact with polynucleotides and nucleic acid proper (Haselkorn and Doly, 1961; and Romakov, 1965), therefore, redistribution is a significant problem since most fixatives produce some shrinkage. Pearse (1968) concurred with this and gave several examples of effects of different fixation procedures. Changes in the physical state of nucleic acids (for example polymerization) occurs with any method of fixation (Kurnick, 1955). Therefore if good fixation is to be achieved alteration in tissue proteins must be kept to a minimum. Considering these, good nuclear fixatives for quantification studies should be those which alter the nucleic acid conformation slightly and produces maximum chromatin stability that is needed for optimum staining.

The mechanism of DNA dissolution caused by acid hydrolysis during Feulgen reaction is not fully understood, Sibatani and Fukuda (1953), but the extent of the dissolution is partially conditioned by the behavior of the protein bound to DNA during acid hydrolysis, for example formalin fixation renders histones less soluble in acid medium so that DNA resists dissolution more strongly. Deitch et al., (1967), Hillary (1939), Sibatani and Fukuda (1953), DeCosse and Aiello (1966), Jordanov (1963), Kasten (1959) and Itikawa et al., (1953) point out that improper acid hydrolysis procedures involved in Feulgen staining can contribute significantly to the loss of Feulgen intensity through depolymerization of DNA, and that the extent of acid degradation of DNA and subsequent loss of the material is dependent upon the concentration of the acid, the temperature involved, and the stability of the chromatin in the DNA. These investigators noted that 5 N hydrochloric acid hydrolysis at room temperature is preferable over the conventional Feulgen hydrolysis at $60^{o}$ C. for two reasons: 1) the performance of the Feulgen reaction is less critically dependent on time and temperature when hydrolyzed at room temperature and 2) the maximal Feulgen values obtained may be from 5 to 30% higher than those found for similar cells after conventional hydrolysis.

These conditions being considered, good fixatives for Feulgen staining should render high chromatin stability to acid hydrolysis, and allow for maximum Feulgen intensity, and the maximum values should persist over extended period of hydrolysis. The need for such parameter has been confirmed by Andersson and Kjellstrand (1971, 1972, and 1975). Sibatani et al. (1967), Kurnick (1955), Swift (1966) and Ruch (1966) recommend formalin fixation and Sibatani et al. (1967) indicates

that DNA dissolution can be inhibited by treating tissue with salt of a metallic element in formalin (for example Calcium acetate formalin or Lanthanum acetate formalin) or slightly acid-alcohol formalin (like glacial acetic-acid and ethyl alcohol formalin). Formalin fixation has been used in most studies involving Feulgen cytophotometry, fixation with formalin involves the formation of a network between the proteins of the cell nuclei and this stabilizes the protein molecules (DNA molecule) and makes it to be resistant to acid hydrolysis, Greenwood and Berlyn (1968).

Since the intensity of the Feulgen reaction and thus the quantitative aspects is influenced by the fixative and hydrolysis time, microspectrophotometry is used in this investigation to analyze the Feulgen-stained tissues from rat liver and slime mold myxamoeba fixed with four different fixatives: 1) Calcium-acetate-formalin, 2) Acetic-alcohol-formalin, 3) Neutral buffered formaldehyde, and 4) Fixcel (a new commercial quick spray fixative used by hospital pathology laboratories); and subjected to 5 N hydrochloric acid hydrolysis at room temperature. The aim being to determine the best hydrolysis time for each fixative that will provide maximal intensity and allow precise quantitation of the Feulgen reaction for both tissue types, and thus most types of tissues.

# MATERIALS AND METHODS

## Tissue Preparation

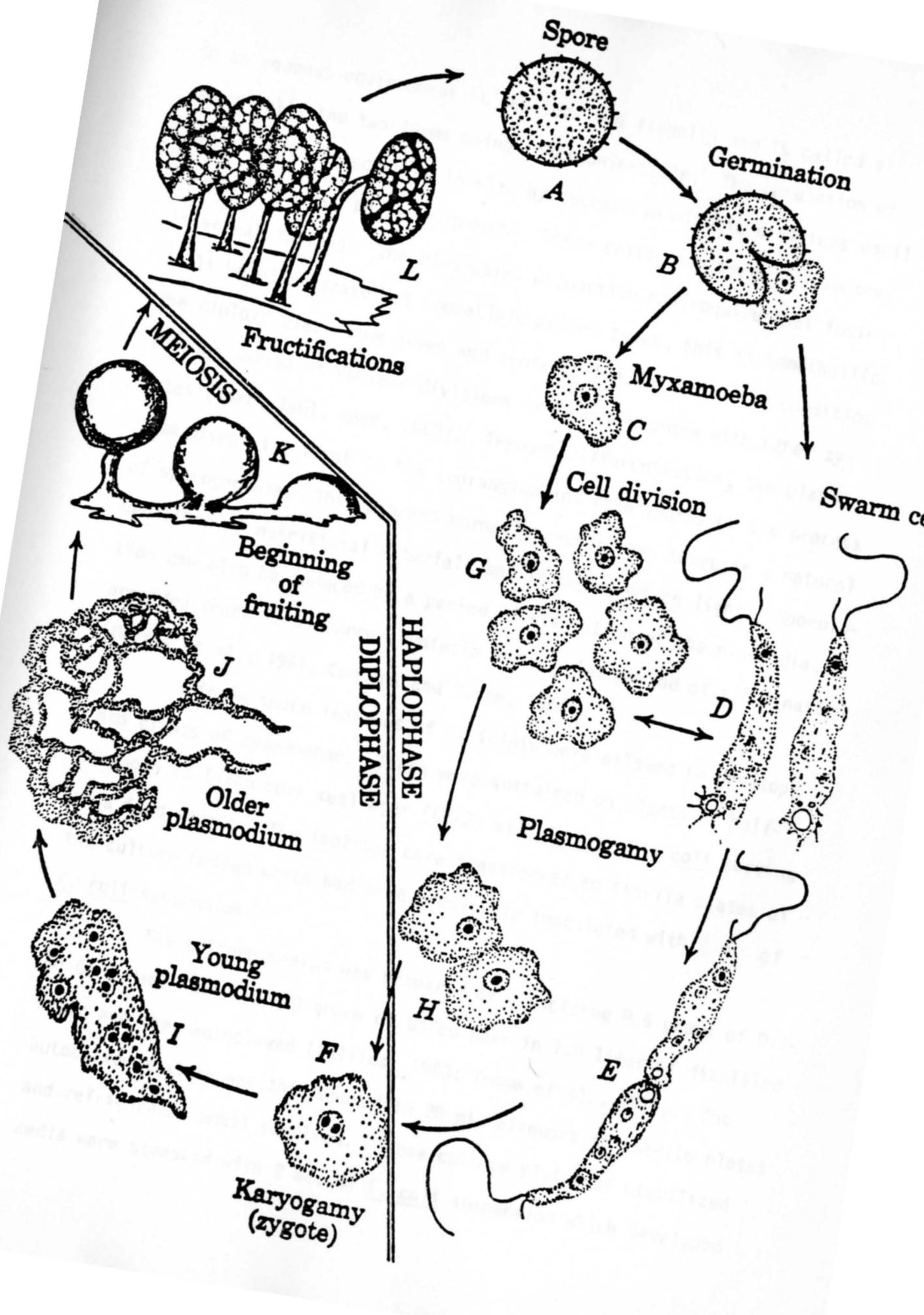
The S-2 isolates of Didymium iridis used during this investigation were originally obtained from Dr. O. R. Collins, Department of Botany, University of California, Berkeley. Mammalian liver tissue used was excised from the albino rat Rattus rattus, supplied by the Holtzman Laboratory Animal Supply Company, Wisconsin.

## Cultivation of D. iridis

A brief review of the life cycle of D. iridis would serve to clarify the mode of development and the nuclear content of the amoeboid stage that was used in this study. D. iridis is a myxomycete or true acellular slime mold. The synthrony of DNA synthesis and mitosis that occur in some parts of the life cycle permit the analyses of a macroscopic cell. The characteristically plant and animal stages of the life cycle offer varied opportunities for biological research primarily because of the ease by which study materials may be obtained. The stages in the life cycle of the myxomycetes have been well described by Gray and Alexopoulos (1968). The life cycle comprise of a unicellular myxamoeboid stage, a multinucleate plasmodial stage and a sporulation stage (see plate 1). The myxamoeboid stage or haplophase results from the germination of a spore. Upon spore germination, a single protoplast is released (Collins, 1961) and assumes the myxamoeboid form or

PLATE 1

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


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

in an aqueous environment it develops two flagella and is called a swarm cell, the two forms being interconvertible. The population of the cells then increases in size by successive mitotic divisions until reaching the log phase of growth. These cells then serve as gametes in sexual fusions. The plasmodial production may require that fusing cells be of separate but compatible mating types, this is homothallic. The diploid plasmodium grows and achieves the multinucleate condition through series of nuclear divisions or by coalescence with other zygotes (Kerr, 1961, Ross, 1967). Through differentiation, the plasmodium gives rise first to the sporangium and then spores by the process of sporogenesis. This process normally ensues when there is a natural depletion of nutritional materials and is dependent on light. Sporulation can also be induced by a period of starvation of the plasmodia, provided there is a source of niacin and a brief period of illumination (Guttas et al., 1961, Cummins and Rusch, 1968).

Single spore isolates of D. iridis were allowed to develop into clones of myxamoebae. These were sustained on slants of half-strength 2% Difco corn meal agar (CM/2) with Escherichia coli serving as a food source. The isolates were transferred to sterile plates of the culture medium which had been previously inoculated with 2 ml. of E. coli suspension.

The culture medium was prepared by dissolving 8.5 grams of Difco Corn Meal Agar and 8.0 grams of Difco Agar in 1.0 liter of distilled water and then autoclaved (Collins, 1963; Yemma et al., 1974). The autoclaved mixture was then poured in 20 ml. aliquots to sterile plates and refrigerated until required. These culture plates of sterilized media were streaked with 2 ml. of E. coli suspension which developed

into an even bacterial lawn. These plates were used for subculturing of the stock clones. Subcultures from each clone to fresh media with bacterium suspension were made once the log phase of growth had been achieved, and transfers were made weekly. All inoculated plates were incubated in an inverted position in an incubator at 21 degrees C. (Yemma and Therrien, 1972; Yemma et al., 1974). All inoculated plates were examined periodically under the microscope for bacterial or fungal contamination, and any contaminated plates were discarded.

The E. coli suspension was initially prepared by washing bacterium from an E. coli nutrient agar slant culture with 15 ml. of sterile distilled water. The 2 ml. E. coli suspension was then formed by adding 2 ml. of the bacteria-water suspension to a culture tube containing 5 ml. of sterile distilled water. Drops of this E. coli suspension was spread over the culture media plates using sterile cotton swab (Q-tips). These E. coli inoculated culture media plates were then incubated in an inverted position in an incubator at 21 degrees C. and are inoculated with myxamoebae once the log phase of growth had been achieved.

Sterile technique was used throughout all inoculation procedure to prevent bacterial or fungal contamination. In five days myxamoebal growth can be seen and by the eight (8th) day all the plates had sizable number of myxamoebal cells.

## Preparation and Treatment of Materials for Cytochemical Studies

### Harvest of Myxamoebal Cells

The method used in collecting log phase myxamoebal cells used in this study were the same for the four different fixatives (Calcium acetate-Formalin, Acetic-alcohol-Formalin, Neutral buffered formaldehyde, and Fixcel).

(1) Plates containing myxamoebal cells in log phase were flooded with sterile 0.25 Molar Sucrose Phosphate buffer (SPB) at pH 6.5, prior to harvesting. Isotonic 0.25 Molar Phosphate Buffer used in this study was prepared by dissolving 3.0 grams of potassium chloride, 9.04 grams of dibasic sodium phosphate, and 18.4 grams of monobasic potassium phosphate, in 4 liters of distilled water. This preparation gives a buffer of pH 6.5. These plates were allowed to soak for 45 to 60 minutes, then the myxamoebal cells were washed from the media surface with additional SPB buffer.

(2) The cell suspensions were transferred to 50 ml. conical centrifuge tubes using sterile pasteur pipettes, and spun down at 70X gravity for 20 minutes in an Adams Dynac swinging bucket centrifuge (Yemma, 1971). The bacterial laden supernatant was discarded using vacuum suction and the myxamoebal cell pellets were resuspended in SPB buffer and re-centrifuged. The centrifuging process was done several times until a

final packed volume of myxamoebal cells free from bacterial cells were obtained.

Myxamoebal Fixation

The fixation procedure employed for fixing myxamoebal cells with Calcium acetate-Formalin, Acetic-alochol-Formalin and Neutral buffered formaldehyde was the same. The fixatives used in this study were prepared in the following manner:

Lillies Calcium Acetate-Formalin: 100 ml. of 37% to 40% of formaldehyde solution, 900 ml. of distilled water and and 20 grams of calcium acetate monohydrate.

Lillies Acetic-alcohol-Formalin: 5 ml. formaldehyde, 5 ml. glacial acetic acid, and 100 ml. ethyl alcohol.

Lillies Neutral buffered formaldehyde: 100 ml. of 37% to 40% formaldehyde solution, 900 ml. of distilled water, 4 grams of monohydrate acid sodium phosphate ($NaH_2PO_4H_2O$), and 6.5 grams of anhydrous disodium phosphate ($Na_2HPO_4$).

The myxamoebal fixation procedure using the three above-mentioned fixatives was as follows:

(1) The pellets of myxamoebae cells were re-suspended in 10 ml. of fixative and allowed to fix for 12 hours. The myxamoebae cells in fixative suspension was then centrifuged at 70X gravity for 20 minutes and the supernatant was discarded.

(2) Fixed myxamoebae cells were then washed with three changes of 10 ml. of 70% ethyl alcohol and allowed to post-fix in another 10 ml. of 70% ethyl alcohol for 12 hours. The myxamoebae cells in ethyl alcohol

suspension were then centrifuged at 70X gravity for 20 minutes and the supernatant was discarded.

(3) Finally, the myxamoebae cells were smeared on previously albuminized slides and allowed to dry overnight on a slide warmer set at 40 degrees centigrade.

The fixation procedure employed for fixing myxamoebal cells with fixcel was as follows:

(1) The myxamoebae cells fixed with fixcel were washed three times in 10 ml. of 70% ethyl alcohol after harvest, and allowed to post-fix in another 10 ml. of 70% ethyl alcohol for 12 hours. The myxamoebae cells were then centrifuged after each washing time at 70X gravity for 20 minutes and the supernatant discarded.

(2) Finally, the myxamoebae cells were smeared on previously albuminized slides and allowed to dry overnight on a slide warmer set at 40 degrees centigrade.

(3) The slides with myxamoebae smears were then fixed with fixcel spray fixative and then left overnight on a slide warmer set at 40 degrees centigrade for complete drying. Fixcel is a commercial quick spray fixative used by hospital pathology laboratories. It is a product of Fixcel Incorporated, Boardman, Ohio. It consists mainly of Ethyl alcohol and Methyl alcohol in the ratio of 20:1. In every 500 gallons of the above ratio 5 cc of Isopropyl glycol and green food coloring was added.

Liver Tissue Preparation and Fixation

The albino rat, Rattus rattus, was sacrificed in chamber and the liver was excised immediately and washed in cold Hanks Salt solution then cut into small cubes of approximately 0.8 $cm^3$. The liver tissue fixed with fixcel was smeared on already albuminized slides and allowed to dry on a slide warmer set at 40 degrees centigrade. After the slides with smeared liver cells were dried, they were then fixed with fixcel spray fixative by spraying the whole slide. The slides were dried overnight on a slide warmer set at 40 degrees centigrade for complete drying.

The liver tissues that were to be fixed with the other three fixatives (Calcium-acetate-Formalin, Acetic-alcohol-Formalin, and Neutral buffered formaldehyde) were divided into three groups. The fixation, dehydration and paraffin imbedding techniques employed for these three fixatives were the same in each case. The fixation procedure was as follows:

(1) The approximately 0.8 $cm^3$ freshly excised liver tissues were immediately placed in large volumes of each of the already mentioned fixatives and allowed to fix for 12 hours.

(2) Fixed liver tissues were then placed in 70% ethyl alcohol and held here prior to the subsequent dehydration in 95% ethanol for 2 hours and then dehydrated in absolute ethanol for 2 hours.

(3) The fixed tissues were then passed through two series of xylene for an hour each time, followed by hot

paraffin for an hour, and finally the tissues were imbedded in a second change of paraffin.

## Cytochemical Methods

### Nuclear Desoxyribonucleic Acid Analysis

The Feulgen nucleal reaction (Feulgen and Rossenbeck, 1924; as modified by Therrein, 1966; and Bryant and Howard, 1969) was employed for the specific localization and quantitation of nuclear desoxyribonucleic acid (DNA) in both liver tissue and myxamoebal cells. Feulgen quantitation relies on the stable bond formed in a stoichiometric manner between the dye molecules and the polyaldehyde formed when DNA is hydrolyzed (Nauman, West et al., 1960). The mechanism of staining includes mold acid hydrolysis, in 5N hydrochloric acid at room temperature which unmasks aldehyde groups in the DNA and also removes the purine rings. This then exposes the aldehyde groups to the Schiff reagent which creates a bisulfite bond. The result of this is that the nuclei will be stained deep magenta (reddish) color. One dye molecule binds per base pair of DNA molecule (Andersson and Kjellstrand 1971 and 1975).

Prior to staining, all tissue sections were cut at 8 microns, and then affixed to previously albuminized slides. Paraffin sections of liver tissue were cleared twice in xylene for 2 minutes, and hydrated through a graded series of ethyl alcohol of descending concentration then to distilled water. The myxamoebae slides were placed in distilled water for one hour prior to staining. All slides both liver tissue slides and myxamoebae cell slides were hydrolyzed and stained simultaneously for uniform and comparable results. The staining technique employed is as follows:

(1) Liver section slides and the myxamoebae cell slides were hydrolyzed in 5 Normal Hydrochloric Acid (5N HCl) at room temperature at predetermined time intervals from 15 minutes to 80 minutes.

(2) The slides were rinsed in distilled water after hydrolysis and stained for one hour in Schiff's reagent (Lillie, 1951) which was fortified by adding 10 parts of ten percent aqueous solution of potassium metabisulfite to 40 parts of Schiff's reagent.

(3) This was followed by two 5 minute rinses in freshly prepared bisulfite rinse. The bisulfite rinse was prepared by mixing 5 ml. of 10 percent potassium meta-bisulfite and 5 ml. of 1N hydrochloric acid in 100 ml. of distilled water.

(4) This was followed by rinsing the slides in distilled water, and then dehydration in a graded series of ethyl alcohol of ascending concentration. Finally, the slides were cleared in xylene, and mounted in permount.

## Microspectrophotometric Methods

A Zeiss Type 01 microspectrophotometer using a Planachromat oil immersion objection, N.A. 1.30 X 100 at an optovar setting of 1.25X was used for all cytophotometric measurements. The alignment of the instrument and the linearity of the photo-tube were checked each time the instrument was used. A Zeiss continuous interference-filter monochrometer was used to isolate the chosen wavelengths of light.

Relative amounts of Feulgen stained Deoxyribonucleic Acid (DNA) for both myxamoebal cells and liver tissue were made photometrically (Ris and Mirsky, 1949). The two wavelength method of microspectrophotometry (Patau, 1952; Ornstein, 1952; and Mendelsohn, 1961) was utilized in the photometric determinations. This method eliminates the need for direct measurement of nuclear area (Mayall and Mendelsohn, 1970) and the optical density measurements made by this method minimizes the distributional error caused by heterogeneity of staining within the specimen. This method permits calculation of content without reference to the geometry of the structure analyzed since only the area of the aperture is required, (Kurnick, 1955). In this instance, measurement errors due to erroneous light transmission due to dispersion, refraction, and reflection are considered noncontributory. Hydrolysis and staining of experimental slides of each comparison fixation procedure were done simultaneously to obtain a uniformity in staining intensity and absorption curves for the chromophore-molecular complex that are identical (Swift and Rasch, 1956).

The selection of the two wavelengths is critical for correct estimation of the absorbing material and they should give specific absorptivities in the ratio of 2:1 for the chromophore when uniformly stained material is in the field. This means that the ratios of the molar extinctions of the stained material at the two wavelengths should be 2:1 and that no local areas of very high optical density be present. The estimation of the absorbing materials depends on the difference between the transmissions at the two wavelengths (1 and 2). Wavelength 2 ($L_2$) corresponds to the wavelength at maximum extinction ($E_2$) of the

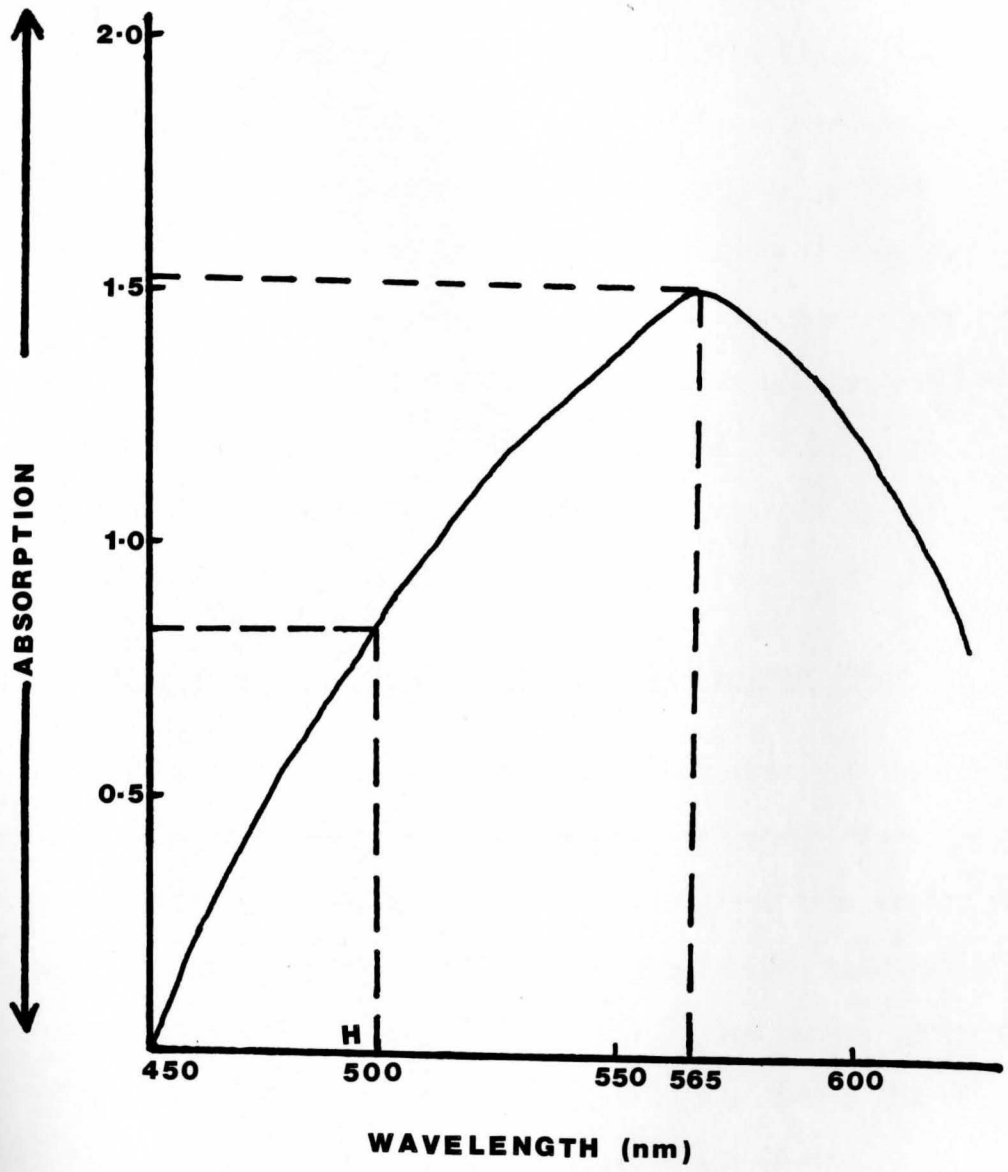
spectral absorption curve and wavelength 1($L_1$) is the wavelength of half maximum extinction ($E_1$), that is $2E_1 = E_2$. A homogenous area of the specimen was chosen for this measurement. The extinction, (E), is equal to the log Io/Is; Io is the intensity of the background light and Is is the intensity of light after passing through the specimen. Therefore $E_2$ = log Io/Is at $_2$ and $e_1$ = log Io/Is at $_1$. Several absorption curves for the Feulgen stained specimens were made. Since all tissues were stained simultaneously (under the same condition), a combined spectral absorption curve for both tissue types (liver tissue and myxamoebae cells) fixed with the four fixation procedure is shown in Figure 1.

Once the two wavelengths (565 nm and 500 nm) were selected to correspond with the maximum and half maximum absorption values of Feulgen DNA, areas with heterogeneous dye distributions were measured. Measured nuclei were selected at random from slides representing each hydrolysis time. In making the measurements, the photometric field was adjusted to completely circumscribe the nucleus in such a way that a minimal amount of light (unoccupied space) past the borders of the nuclei. The amount of chromophore or absorbing material (M) within this field of measured area (A) was determined by the equation $M = KAL_1Q$. K = the absorptivity constant is required for the determination of absolute values, it was eliminated since only relative values were required in this study of relative DNA determinations. $L_1$ and $L_2$ were calculated from transmissions ($T_1$ and $T_2$) taken at wavelengths 1 and 2 for each nucleus; i.e. $L_1 = 1 - T_1$ and $L_2 = 1 - T_2$.

Q corresponds to $L_2/L_1$ and can be used to determine the correction factor (C) for the distribution error (Swift and Rasch, 1956;

FIGURE 1

A combined Feulgen-DNA spectral absorption curve for both tissue types fixed with the four fixation procedure.


2·0
1·5
1·0
0·5
ABSORPTION
H
450
500
550
565
600
WAVELENGTH (nm)

Leuchtenberger, 1958). The C value that corresponds to a particular Q ratio can be found in a table formulated by Patau (1952).

Calculations of all relative DNA values and the statistical analysis were performed using an IBM 370 Model 145 computer.

# RESULTS

The results of Feulgen-DNA measurements of myxamoebal nuclei and liver nuclei are presented graphically as hydrolysis curves and histograms. The hydrolysis curves represent the maximal Feulgen intensity attained for a specific hydrolysis time and the duration of peak dye binding for cells treated at subsequent time periods. The histograms represent nuclear DNA frequency distributions of cellular populations and provide a means for detection of any shifts in the DNA content. Measurements were limited to interphase nuclei and were reported as relative amounts of DNA in arbitrary units.

## Microspectrophotometric Analysis of Nuclear DNA

Relative measurements of the nuclear DNA content of liver nuclei and myxamoebal nuclei fixed in each of the four different fixatives were made. In each case, one hundred nuclei per hydrolysis time period were analyzed and the ones relevant in generating the Feulgen hydrolysis curves were plotted as mean DNA values. The abscissa of the curve represents the hydrolysis time in minutes, and the ordinate represents the relative amount of dye binding (or DNA stained) for each time period.

## Analysis of F-DNA

### Neutral Buffered Formaldehyde

The hydrolysis curve for the liver tissue fixed in Neutral buffered formaldehyde (Figure 2) shows a gradual rise to a maximum of 81.31 ± 19.24 at 45 minutes followed by a plateau of maximal Feulgen intensity for the next 15 minutes, and, then a subsequent marked decline in intensity. The hydrolysis curve for myxamoebae cells fixed in Neutral buffered formaldehyde (Figure 2) shows a gradual rise to a maximum of 7.59 ± 3.22 at 40 minutes followed by a plateau of maximal Feulgen intensity for the next 20 minutes, and, then a subsequent marked decline in intensity. The maximum Feulgen intensities for both tissue types fixed with the same fixative (Neutral buffered formaldehyde) are significant and there are considerable differences in the shapes of the hydrolysis curves. In myxamoebae cells the plateau of maximal Feulgen intensity is achieved earlier at 40 minutes and persists longer than it does for liver tissue.

### Acetic-alcohol-Formalin

The hydrolysis curve for the liver tissue fixed with Acetic-alcohol-Formalin (Figure 3) shows a typical rapid rise to a maximum of 90.14 ± 14.59 at 20 minutes followed by a plateau of maximal Feulgen intensity for the next 10 minutes, and, then a gradual decline in average intensity. The hydrolysis curve for myxamoebae cells fixed in Acetic-alcohol-Formalin (Figure 3) shows a gradual rise to a maximum of 6.25 ± 2.40 at 30 minutes followed by a plateau of maximal Feulgen intensity for the next 30 minutes, and, then a subsequent gradual decline in Feulgen

Figure 2

Hydrolysis curves representing Feulgen stained liver and myxamoebal nuclei fixed in Neutral buffered formaldehyde.

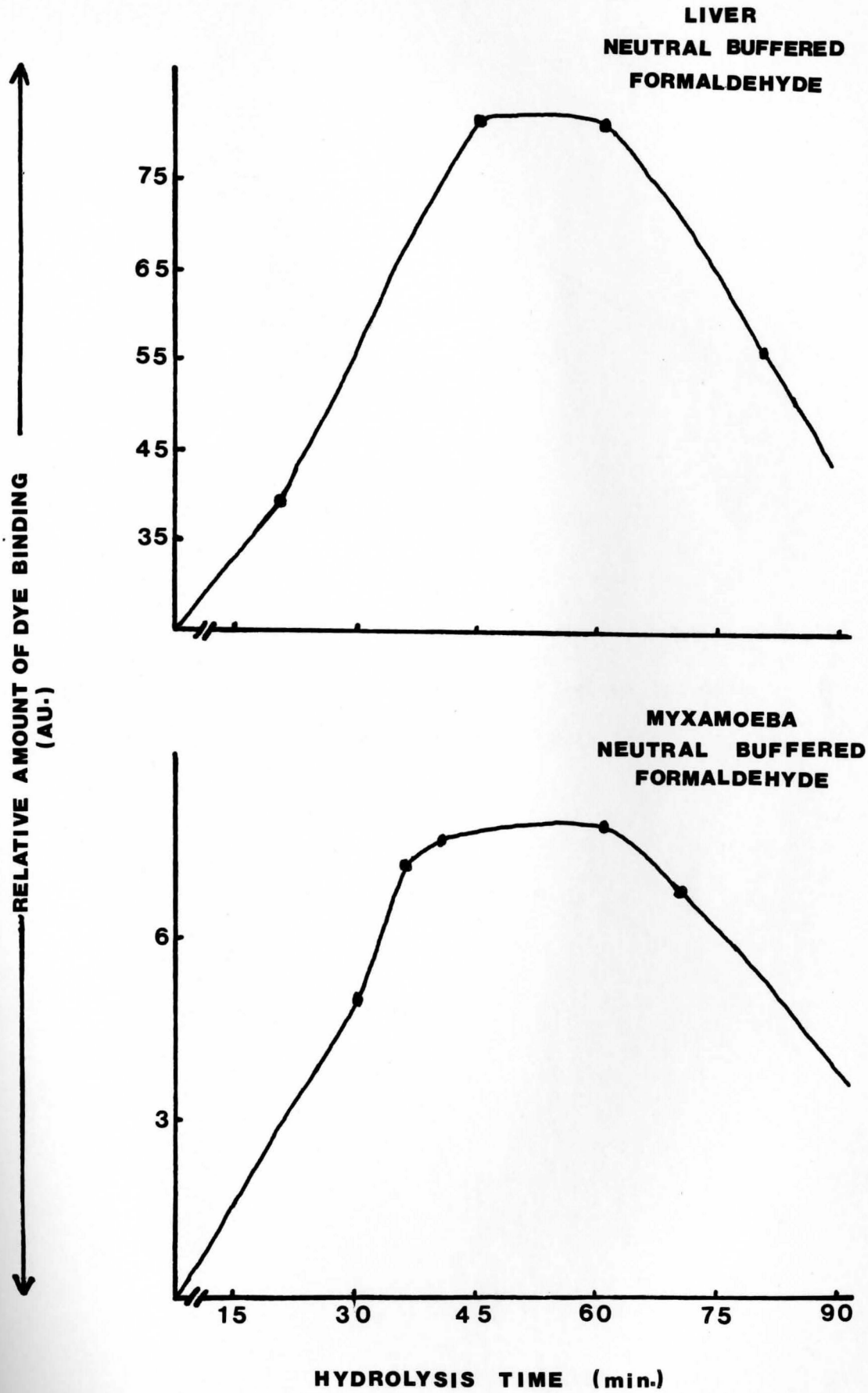
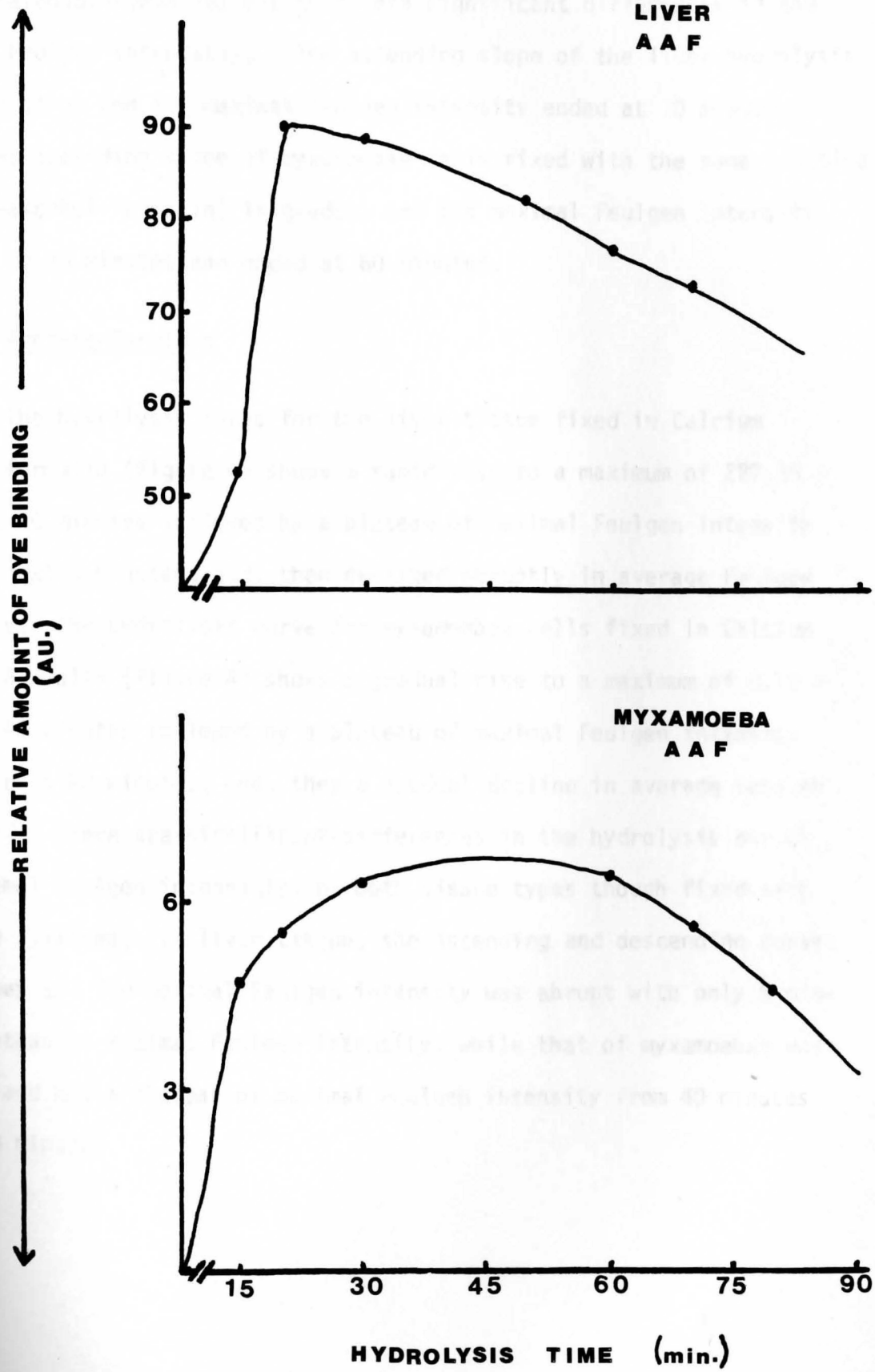

LIVER
NEUTRAL BUFFERED
FORMALDEHYDE
75
65
55
45
35
MYXAMOEBA
NEUTRAL BUFFERED
FORMALDEHYDE
6
3
15
30
45
60
75
90
RELATIVE AMOUNT OF DYE BINDING
(AU.)
HYDROLYSIS TIME (min.)

Figure 3

Hydrolysis curves representing Feulgen stained liver and myxamoebal nuclei fixed in Acetic-alcohol-Formalin (AAF).


LIVER
A A F
90
80
70
60
50
MYXAMOEBA
A A F
6
3
RELATIVE AMOUNT OF DYE BINDING (A.U.)
15
30
45
60
75
90
HYDROLYSIS TIME (min.)

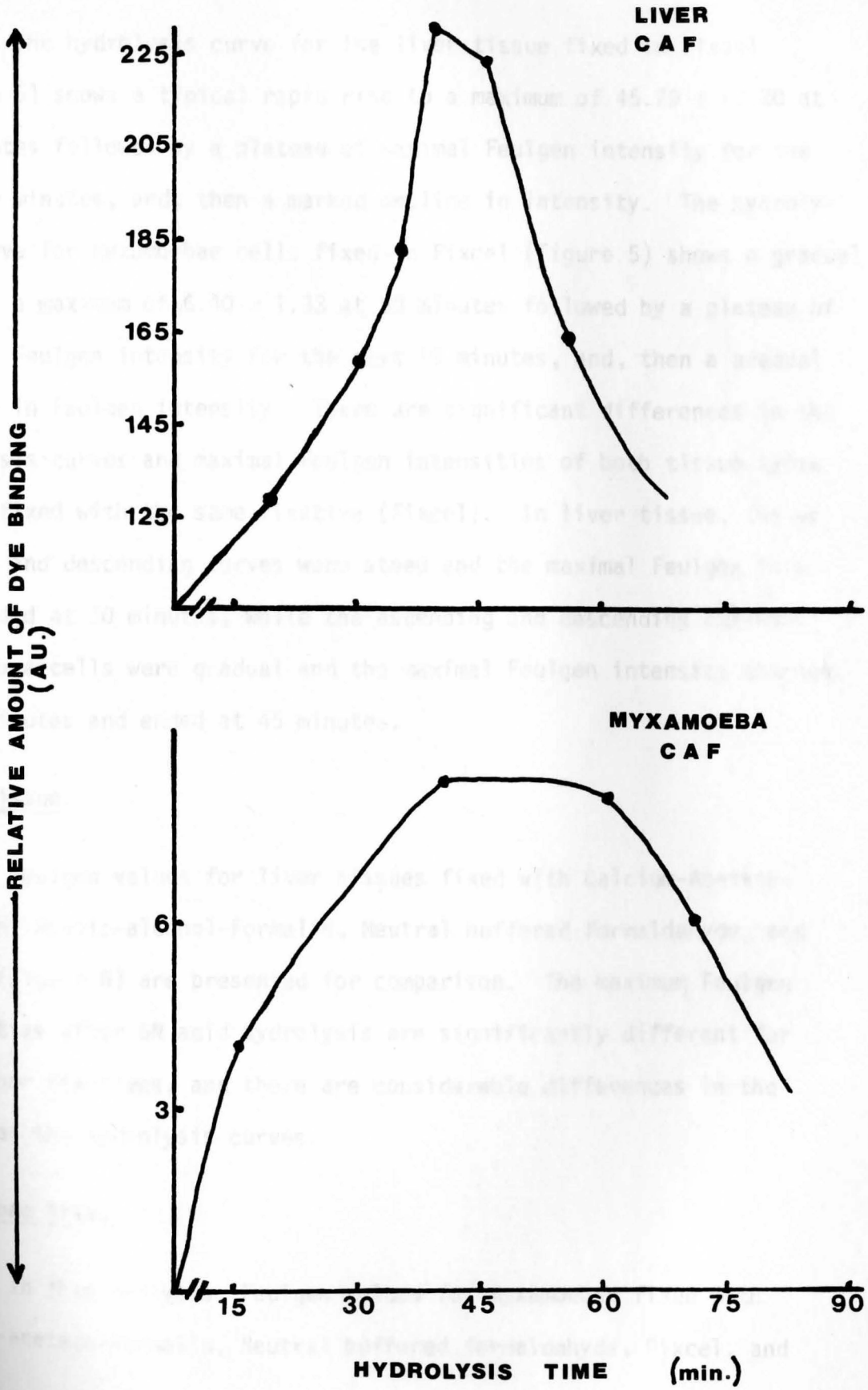
intensity. Though both tissue types were fixed with the same fixative (Acetic-alcohol-Formalin) but there are significant differences in the maximum Feulgen intensities. The ascending slope of the liver hydrolysis curve is steep and its maximal Feulgen intensity ended at 30 minutes; while the ascending slope of myxamoebae cells fixed with the same fixative (Acetic-alcohol-Formalin) is gradual and its maximal Feulgen intensity started at 30 minutes and ended at 60 minutes.

Calcium Acetate-Formalin

The hydrolysis curve for the liver tissue fixed in Calcium acetate-Formalin (Figure 4) shows a rapid rise to a maximum of 227.35 ± 49.91 at 40 minutes followed by a plateau of maximal Feulgen intensity for the next 5 minutes, and, then declined abruptly in average Feulgen intensity. The hydrolysis curve for myxamoebae cells fixed in Calcium Acetate-Formalin (Figure 4) shows a gradual rise to a maximum of 8.19 ± 3.86 at 40 minutes followed by a plateau of maximal Feulgen intensity for the next 20 minutes, and, then a gradual decline in average Feulgen intensity. There are significant differences in the hydrolysis curves and maximal Feulgen intensities of both tissue types though fixed with the same fixative. In liver tissue, the ascending and descending curves were steep and the maximal Feulgen intensity was abrupt with only 5 minutes plateau of maximal Feulgen intensity, while that of myxamoebae was gradual and had a plateau of maximal Feulgen intensity from 40 minutes until 60 minutes.

Figure 4

Hydrolysis curves representing Feulgen stained liver and myxamoebal nuclei fixed in Calcium-Acetate-Formalin (CAF).


LIVER
CAF
225
205
185
165
145
125
MYXAMOEBA
CAF
6
3
RELATIVE AMOUNT OF DYE BINDING
(A.U.)
15
30
45
60
75
90
HYDROLYSIS TIME (min.)

Fixcel

The hydrolysis curve for the liver tissue fixed in Fixcel (Figure 5) shows a typical rapid rise to a maximum of 45.79 ± 17.20 at 20 minutes followed by a plateau of maximal Feulgen intensity for the next 10 minutes, and, then a marked decline in intensity. The hydrolysis curve for myxamoebae cells fixed in Fixcel (Figure 5) shows a gradual rise to a maximum of 6.40 ± 1.33 at 30 minutes followed by a plateau of maximal Feulgen intensity for the next 15 minutes, and, then a gradual decline in Feulgen intensity. There are significant differences in the hydrolysis curves and maximal Feulgen intensities of both tissue types though fixed with the same fixative (Fixcel). In liver tissue, the ascending and descending curves were steep and the maximal Feulgen intensity ended at 30 minutes, while the ascending and descending curves of myxamoebae cells were gradual and the maximal Feulgen intensity started at 30 minutes and ended at 45 minutes.

Liver Tissue

Feulgen values for liver tissues fixed with Calcium-Acetate-Formalin, Acetic-alcohol-Formalin, Neutral buffered formaldehyde, and Fixcel (Figure 6) are presented for comparison. The maximum Feulgen intensities after 5N acid hydrolysis are significantly different for these four fixatives, and there are considerable differences in the shapes of the hydrolysis curves.

Myxamoebae Tissue

In this analysis, Feulgen values for myxamoebae fixed with Calcium-acetate-Formalin, Neutral buffered formaldehyde, Fixcel, and

Figure 5

Hydrolysis curves representing Feulgen stained liver and myxamoebal nuclei fixed in Fixcel.

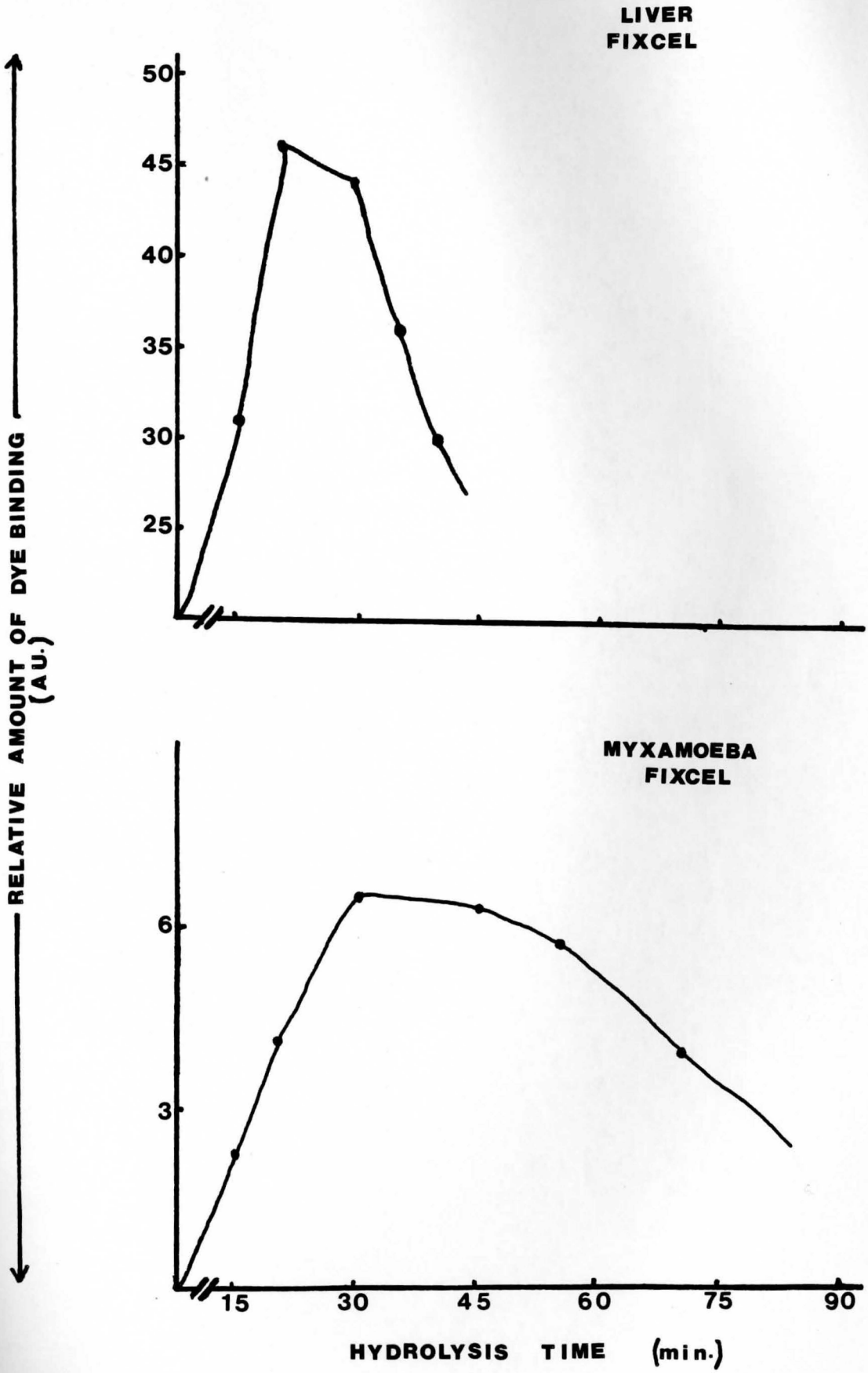
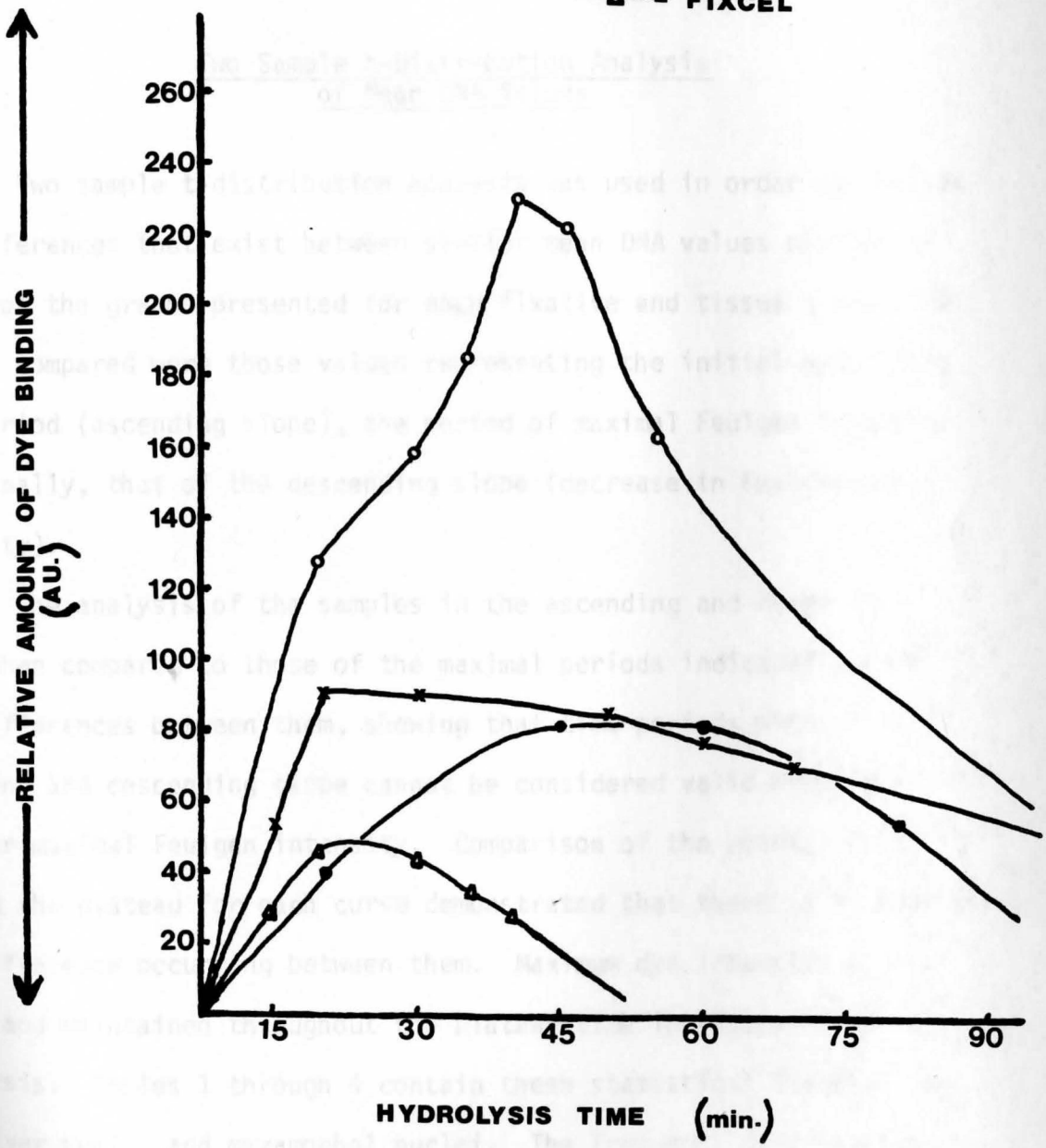

LIVER
FIXCEL
50
45
40
35
30
25
MYXAMOEBA
FIXCEL
6
3
RELATIVE AMOUNT OF DYE BINDING
(A.U.)
15
30
45
60
75
90
HYDROLYSIS TIME (min.)

Figure 6

Hydrolysis curves representing Feulgen stained liver tissue nuclei fixed in Neutral buffered formaldehye, Acetic-alcohol-Formalin (AAF), Calcium-acetate-Formalin (CAF) and Fixcel.


NEUTRAL BUFFERED FORMALDEHYDE
A A F
C A F
FIXCEL
RELATIVE AMOUNT OF DYE BINDING (A.U.)
260
240
220
200
180
160
140
120
100
80
60
40
20
15
30
45
60
75
90
HYDROLYSIS TIME (min.)

Acetic-alcohol-Formalin (Figure 7) are presented for comparison. The maximum Feulgen intensities after 5N acid hydrolysis are significantly different for these four fixatives, and there are considerable differences in the shapes of the hydrolysis curves as previously noted for liver tissues.

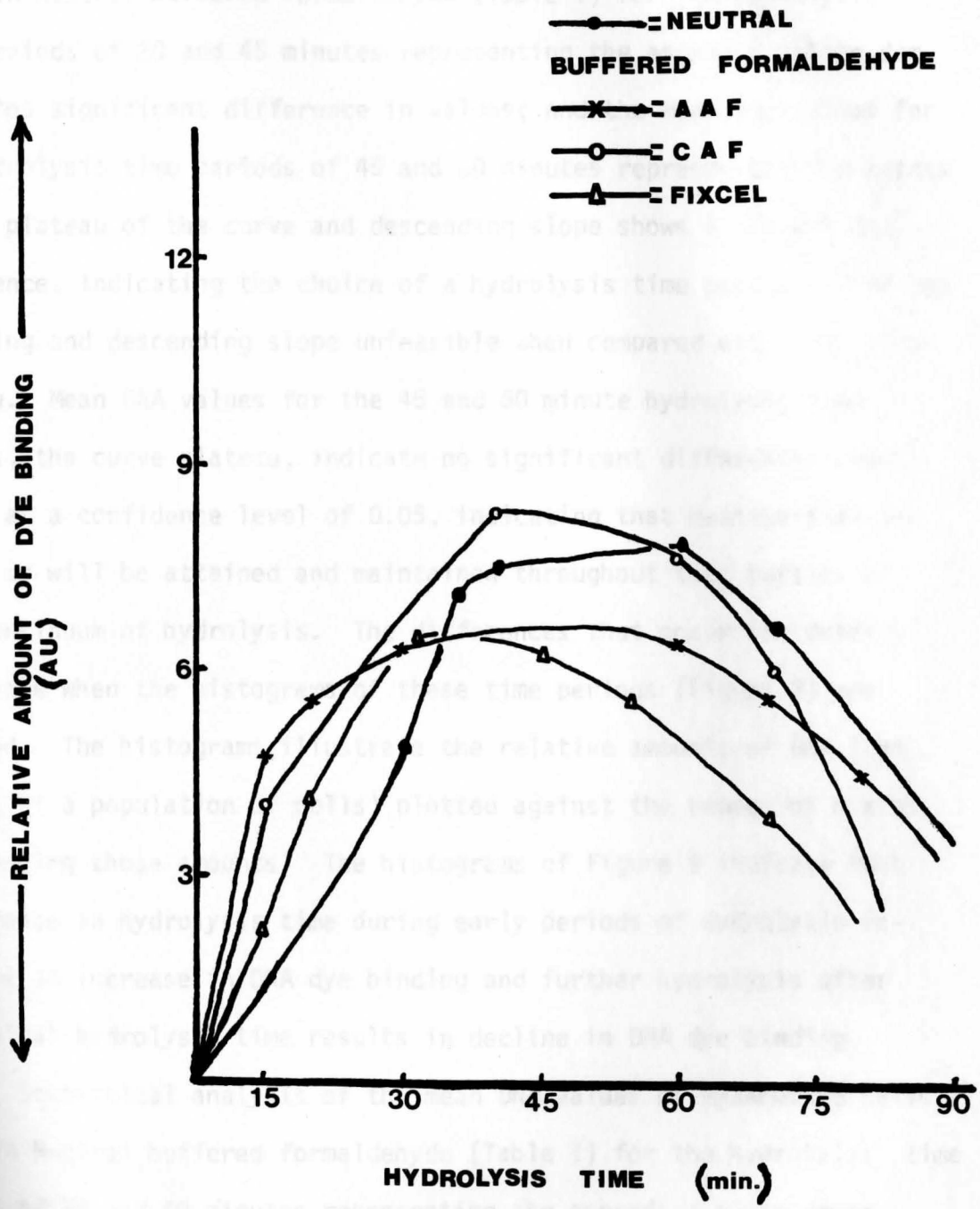
## Two Sample t-Distribution Analysis of Mean DNA Values

Two sample t-distribution analysis was used in order to analyze for differences that exist between similar mean DNA values plotted as points on the graphs presented for each fixative and tissue type. The samples compared were those values representing the initial hydrolysis time period (ascending slope), the period of maximal Feulgen intensity, and, finally, that of the descending slope (decrease in Feulgen stain intensity).

The analysis of the samples in the ascending and descending slope when compared to those of the maximal periods indicated significant differences between them, showing that time periods within the ascending and descending slope cannot be considered valid hydrolysis time for maximal Feulgen intensity. Comparison of the points representing the plateau for each curve demonstrated that there is no significant difference occurring between them. Maximum dye intensity is attained and maintained throughout the plateau time indicated for each hydrolysis. Tables 1 through 4 contain these statistical findings for both liver nuclei and myxamoebal nuclei. The frequency distribution histograms of Figures 8 through 15 also show these significant differences.

Figure 7

Hydrolysis curves representing Feulgen stained myxamoebal nuclei fixed in Neutral buffered formaldehye, Acetic-Alcohol-Formalin (AAF), Calcium-Acetate-Formalin (CAF) and Fixcel.


NEUTRAL
BUFFERED FORMALDEHYDE
A A F
C A F
FIXCEL
RELATIVE AMOUNT OF DYE BINDING (AU.)
12
9
6
3
15
30
45
60
75
90
HYDROLYSIS TIME (min.)

Neutral Buffered Formaldehyde

Statistical analysis of the mean DNA values of liver tissue fixed in Neutral buffered formaldehyde (Table 1) for the hydrolysis time periods of 20 and 45 minutes representing the ascending slope demonstrates significant difference in values; and the mean DNA values for the hydrolysis time periods of 45 and 80 minutes representing the points of the plateau of the curve and descending slope shows a significant difference, indicating the choice of a hydrolysis time period within the ascending and descending slope unfeasible when compared with the curve plateau. Mean DNA values for the 45 and 60 minute hydrolysis time periods, the curve plateau, indicate no significant differences among points at a confidence level of 0.05, indicating that maximum staining intensity will be attained and maintained throughout that particular time continuum of hydrolysis. The differences that occur are readily noticeable when the histograms of these time periods (Figure 8) are compared. The histograms illustrate the relative amounts of DNA (dye binding of a population of cells) plotted against the number of nuclei representing those amounts. The histograms of Figure 8 indicate that an increase in hydrolysis time during early periods of hydrolysis results in an increase in DNA dye binding and further hydrolysis after the maximal hydrolysis time results in decline in DNA dye binding.

Statistical analysis of the mean DNA values of myxamoebae cells fixed in Neutral buffered formaldehyde (Table 1) for the hydrolysis time periods of 30 and 40 minutes representing the ascending slope demonstrates a significant difference in values; and the mean DNA values for the hydrolysis time periods of 60 and 70 minutes representing the points

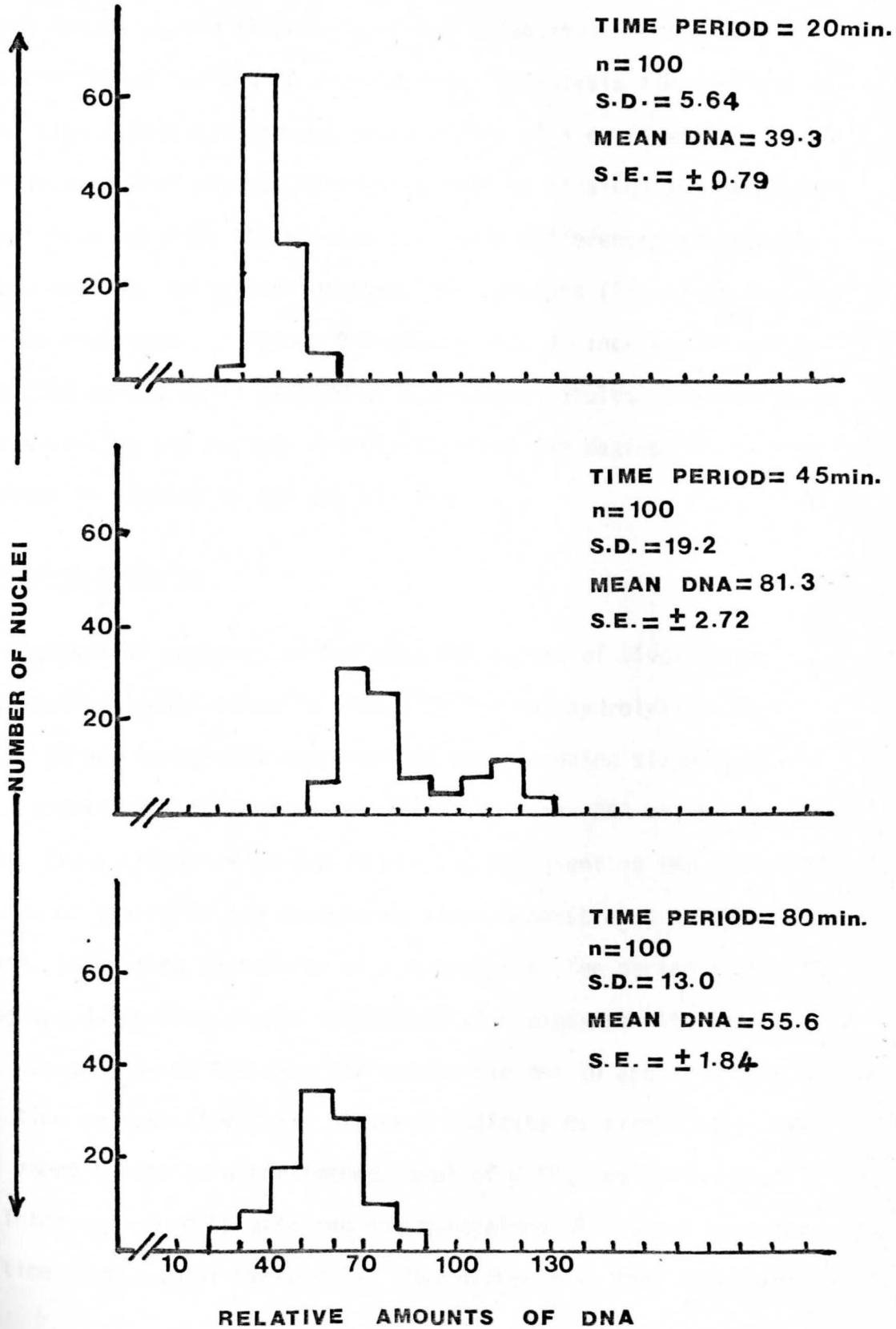
TABLE 1 - TWO SAMPLE t-DISTRIBUTION COMPARISON OF MEAN DNA VALUES FOR LIVER AND MYXAMOEBAE TISSUE FIXED IN NEUTRAL BUFFERED FORMALDEHYDE

| Tissue Type | Hydrolysis Periods Compared (Min) | Mean | Standard Deviation | t | (+) = No Difference (-) = Significant Difference Exists |
|---|---|---|---|---|---|
| Liver | 20 | 39.39900 | 5.64253 | -14.63151 | - |
| | 45 | 81.30678 | 19.23912 | | |
| | 45 | 81.30678 | 19.23912 | 0.0044976 | + |
| | 60 | 81.28931 | 19.01207 | | |
| | 45 | 81.30678 | 19.23912 | 7.7351233 | - |
| Myxamoebae | 30 | 4.81549 | 1.17198 | -5.62505 | - |
| | 40 | 7.59146 | 3.22107 | | |
| | 40 | 7.59146 | 3.22107 | -0.6359366 | + |
| | 60 | 7.97179 | 2.61713 | | |
| | 60 | 7.97179 | 2.61713 | 2.6463367 | - |
| | 70 | 6.73894 | 1.93927 | | |

df = 198
$\alpha$ = 0.05

Figure 8

Histograms representing Feulgen-DNA values for liver tissue nuclei fixed in Neutral buffered formaldehyde.


TIME PERIOD = 20min.
n = 100
S.D. = 5.64
MEAN DNA = 39.3
S.E. = ± 0.79
TIME PERIOD = 45min.
n = 100
S.D. = 19.2
MEAN DNA = 81.3
S.E. = ± 2.72
TIME PERIOD = 80min.
n = 100
S.D. = 13.0
MEAN DNA = 55.6
S.E. = ± 1.84
NUMBER OF NUCLEI
60
40
20
10
40
70
100
130
RELATIVE AMOUNTS OF DNA

of the plateau of the curve and descending slope demonstrates a significant difference, indicating the choice of a hydrolysis time period within the ascending and descending slopes unfeasible. Comparison of the mean DNA values for the 40 and 60 minute hydrolysis time periods indicate no significant differences among points at a confidence level of 0.05, indicating that maximum intensity could be attained and maintained throughout this interval of hydrolysis. These differences are readily noticeable when the histograms of these time periods (Figure 9) are compared. The histograms of Figure 9 indicate that an increase in the hydrolysis time during early periods of hydrolysis results in an increase in DNA dye binding and further hydrolysis after the maximal hydrolysis time results in decline in DNA dye binding.

Acetic-Alcohol-Formalin

Statistical analysis of the mean DNA values of liver tissue fixed in Acetic-Alcohol-Formalin (Table 2) for the hydrolysis time periods of 15 and 20 minutes representing the ascending slope demonstrates a significant difference in values; and mean DNA values for the hydrolysis time periods of 20 and 70 minutes representing the points of the plateau of the curve and descending slope demonstrates a significant difference, indicating the choice of a hydrolysis time period within the ascending and descending slopes unfeasible when compared with the curve plateau. Comparison of the mean DNA values for the 20 and 30 minute hydrolysis time periods (the curve plateau) indicate no significant differences among points at a confidence level of 0.05, indicating that maximum intensity could be attained and maintained throughout that particular time continuum of hydrolysis. The differences that occur are

Figure 9

Histograms representing Feulgen-DNA values for myxamoebal nuclei in Neutral buffered formaldehyde.

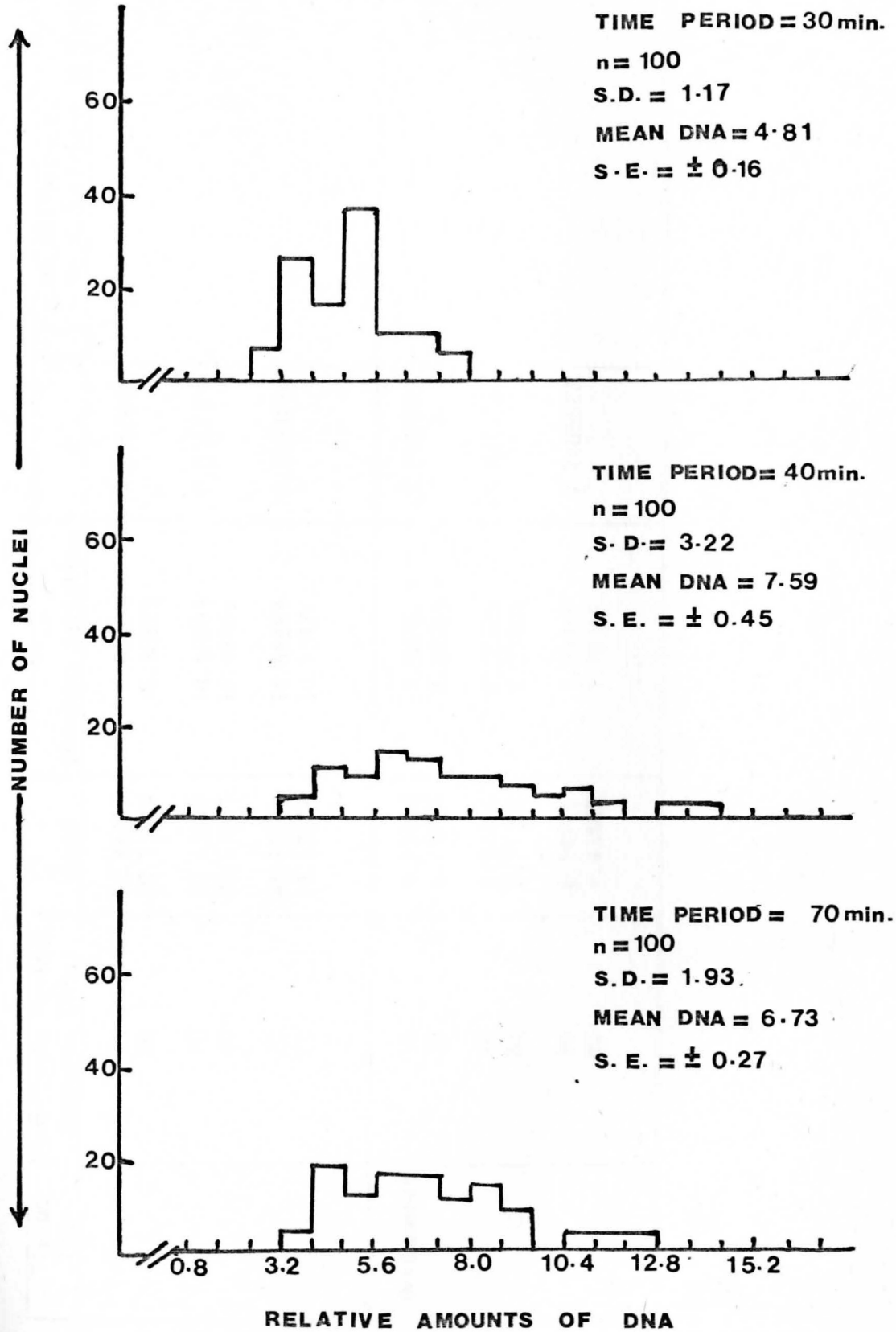

TIME PERIOD = 30 min.
n = 100
S.D. = 1·17
MEAN DNA = 4·81
S·E· = ± 0·16
TIME PERIOD = 40 min.
n = 100
S· D·= 3·22
MEAN DNA = 7·59
S. E. = ± 0·45
TIME PERIOD = 70 min.
n = 100
S.D. = 1·93
MEAN DNA = 6·73
S. E. = ± 0·27
NUMBER OF NUCLEI
60
40
20
0·8
3·2
5·6
8·0
10·4
12·8
15·2
RELATIVE AMOUNTS OF DNA

TABLE 2 - TWO SAMPLE t-DISTRIBUTION COMPARISON OF MEAN DNA VALUES FOR LIVER AND MYXAMOEBAE TISSUE FIXED IN ACETIC-ALCOHOL-FORMALIN

| Tissue Type | Hydrolysis Periods Compared (Min) | Mean | Standard Deviation | t | (+) = No Difference (-) = No Significant Difference Exists |
|---|---|---|---|---|---|
| Liver | 15 | 53.24815 | 8.99198 | -19.096269 | - |
| | 20 | 90.13649 | 14.58583 | | |
| | 20 | 90.13649 | 14.58583 | 0.0741329 | + |
| | 30 | 89.91023 | 16.06451 | | |
| | 20 | 90.13649 | 14.58583 | 6.5039224 | - |
| | 70 | 73.63562 | 10.11477 | | |
| Myxamoebae | 15 | 4.76954 | 1.92867 | -3.2988025 | - |
| | 30 | 6.24726 | 2.39785 | | |
| | 30 | 6.24726 | 2.39785 | -0.052176 | + |
| | 60 | 6.27310 | 2.37804 | | |
| | 30 | 6.24726 | 2.39785 | 4.1405627 | - |
| | 80 | 4.43547 | 1.78316 | | |

df = 198
$\alpha$ = 0.05

readily noticeable when the histograms of these time periods (Figure 10) are compared. The histograms of Figure 10 indicate that an increase in hydrolysis time during the early periods of hydrolysis results in an increase in DNA dye binding and further hydrolysis after the maximal hydrolysis time results in decline in DNA dye binding.

Statistical analysis of the mean DNA values of myxamoebae cells fixed in Acetic-alcohol-Formalin (Table 2) for the hydrolysis time periods of 15 and 30 minutes representing the ascending slope demonstrates a significant difference in values; and mean DNA values for the hydrolysis time periods of 30 and 80 minutes representing the points of the plateau of the curve and descending slope demonstrates a significant difference, indicating the choice of a hydrolysis time period within the ascending and descending slope unfeasible, when compared with the curve plateau. Comparison of the mean DNA values for the 30 and 60 minute hydrolysis time periods (the plateau) indicate no significant differences among points at a confidence level of 0.05, indicating that maximum intensity could be attained and maintained throughout that particular time continuum of hydrolysis. The differences that occur are readily noticeable when the histograms of these time periods (Figure 11) are compared. The histograms of Figure 11 indicate that an increase in hydrolysis time during early periods of hydrolysis results in an increase in DNA dye binding and further hydrolysis after the maximal hydrolysis time results in decline in DNA dye binding.

Figure 10

Histograms representing Feulgen-DNA values for liver tissue nuclei fixed in Acetic-Alcohol-Formalin.

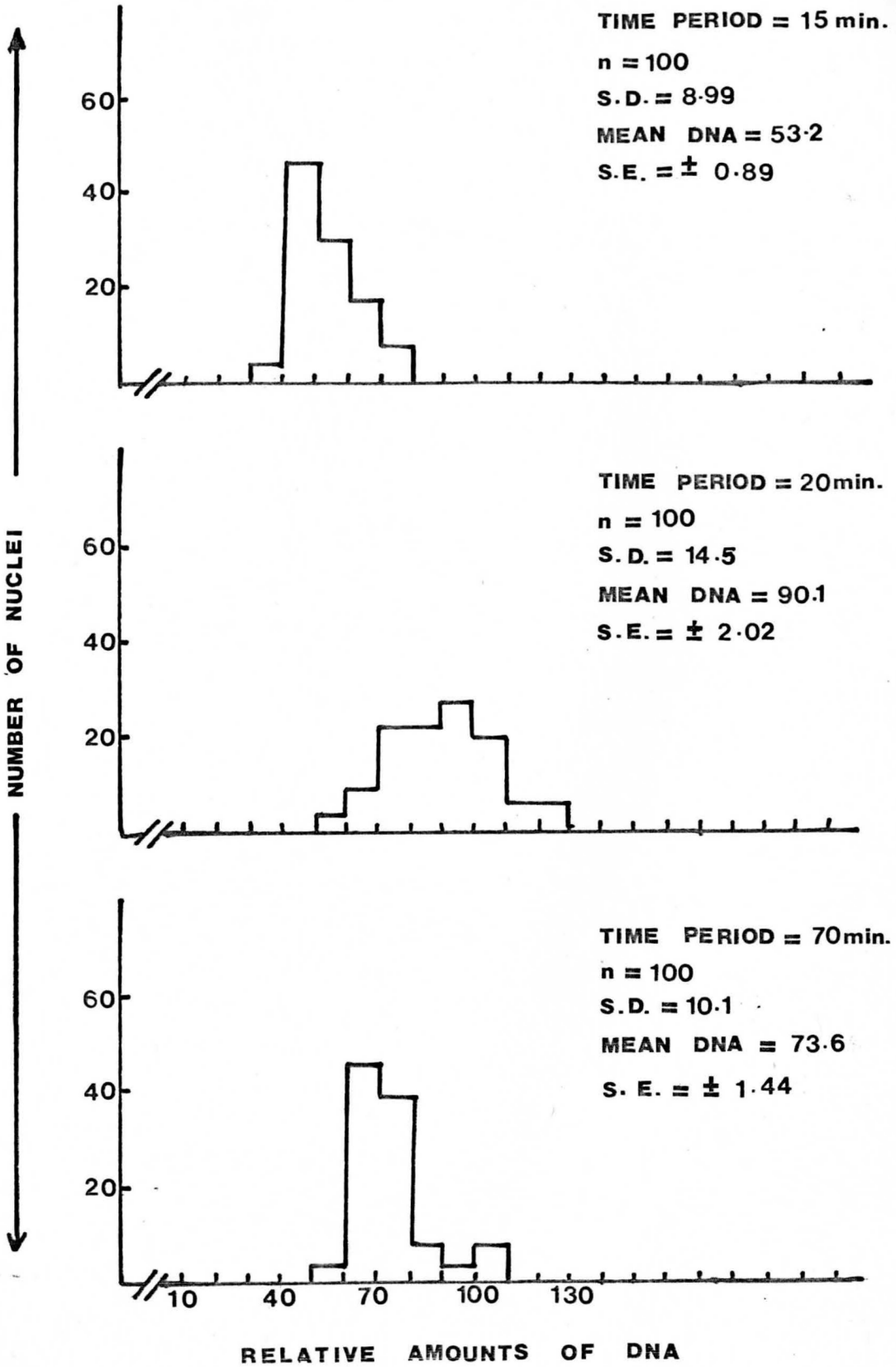
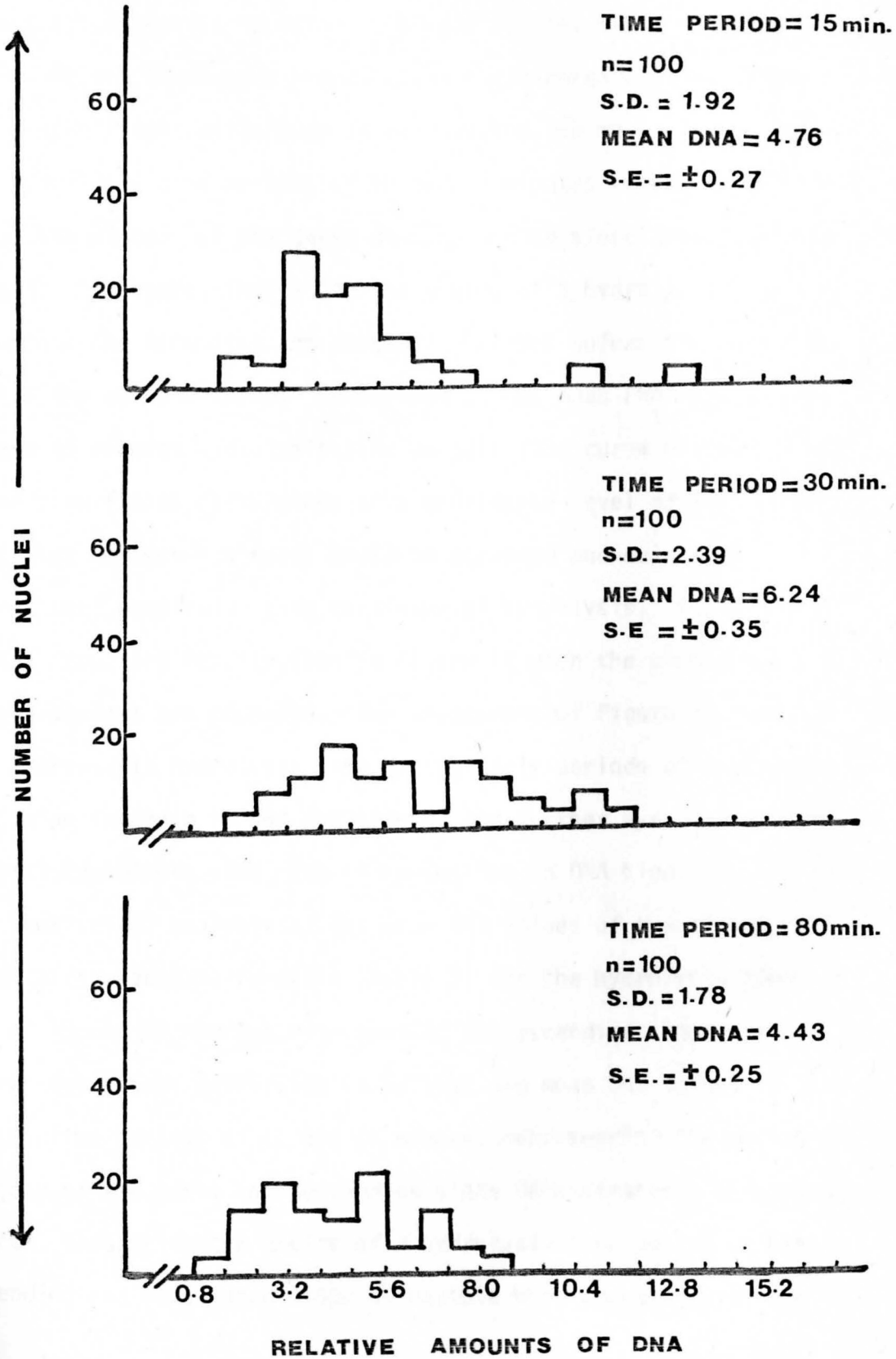

TIME PERIOD = 15 min.
n = 100
S.D. = 8·99
MEAN DNA = 53·2
S.E. = ± 0·89
TIME PERIOD = 20min.
n = 100
S.D. = 14·5
MEAN DNA = 90·1
S.E. = ± 2·02
TIME PERIOD = 70min.
n = 100
S.D. = 10·1
MEAN DNA = 73·6
S.E. = ± 1·44
NUMBER OF NUCLEI
60
40
20
10
40
70
100
130
RELATIVE AMOUNTS OF DNA

Figure 11

Histograms representing Feulgen-DNA values for myxamoebal nuclei fixed in Acetic-alcohol-Formalin.


TIME PERIOD = 15 min.
n = 100
S.D. = 1.92
MEAN DNA = 4.76
S.E. = ±0.27
TIME PERIOD = 30 min.
n = 100
S.D. = 2.39
MEAN DNA = 6.24
S.E = ±0.35
TIME PERIOD = 80 min.
n = 100
S.D. = 1.78
MEAN DNA = 4.43
S.E. = ±0.25
NUMBER OF NUCLEI
60
40
20
0·8
3·2
5·6
8·0
10·4
12·8
15·2
RELATIVE AMOUNTS OF DNA

Calcium-Acetate-Formalin

Statistical analysis of the mean DNA values of liver tissue fixed in Calcium-acetate-Formalin (Table 3) for the hydrolysis time periods of 20 and 40 minutes representing the ascending slope demonstrates a significant difference in values; and the mean DNA values for the hydrolysis time periods of 40 and 55 minutes representing the points of the plateau of the curve and descending slope demonstrates a significant difference, indicating the choice of a hydrolysis time period within the ascending and descending slopes unfeasible when compared with the curve plateau. Comparison of the mean DNA values for the 40 and 45 minutes hydrolysis time periods (the curve plateau) indicate no significant differences at a confidence level of 0.05, indicating that maximum intensity could be attained and maintained throughout that particular time continuum of hydrolysis. The differences that occur are readily seen in Figure 12 when the histograms of these time periods are compared. The histograms of Figure 12 indicate that an increase in hydrolysis time during early periods of hydrolysis results in an increase in DNA dye binding and further hydrolysis after the maximal hydrolysis time results in decline in DNA binding.

Statistical analysis of the mean DNA values of myxamoebae cells fixed in Calcium-acetate-Formalin (Table 3) for the hydrolysis time periods of 15 and 40 minutes representing the ascending slope demonstrates a significant difference in values; and mean DNA values for the hydrolysis time periods of 40 and 70 minutes representing the points of the plateau of the curve and descending slope demonstrates a significant difference, indicating the choice of a hydrolysis time period within the ascending and descending slope unfeasible when compared with the

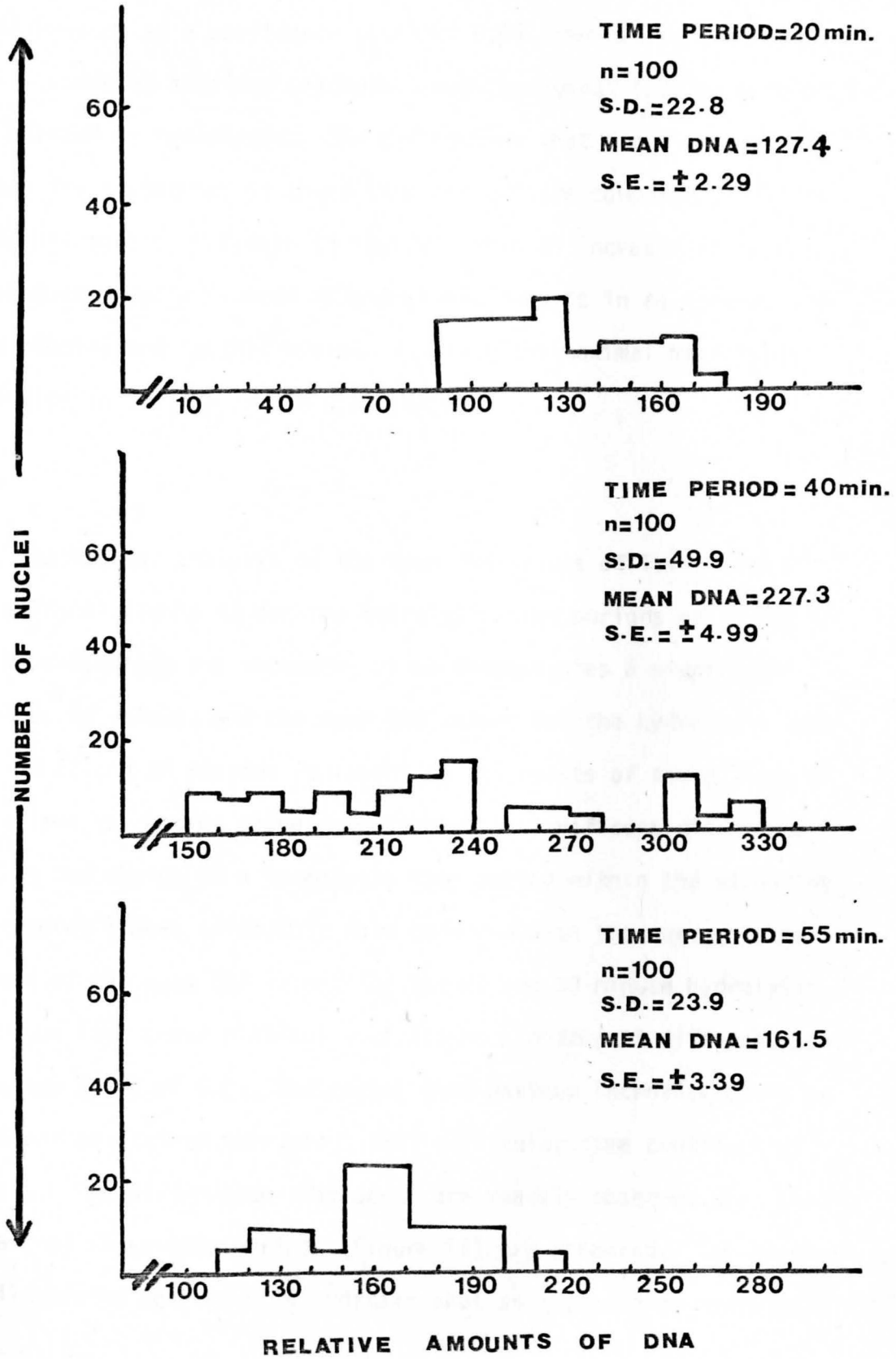
TABLE 3 - TWO SAMPLE t-DISTRIBUTION COMPARISON OF MEAN DNA VALUES FOR LIVER AND MYXAMOEBAE TISSUE FIXED IN CALCIUM-ACETATE-FORMALIN

| Tissue Type | Hydrolysis Periods Compared (Min) | Mean | Standard Deviation | t | (+) = No Difference (-) = Significant Difference Exists |
|---|---|---|---|---|---|
| Liver | 20 | 127.49324 | 22.89563 | -18.094638 | - |
| | 40 | 227.34683 | 49.90611 | | |
| | 40 | 227.34683 | 49.90611 | 0.983596 | + |
| | 45 | 220.15335 | 17.20755 | | |
| | 40 | 227.34683 | 49.90611 | 8.7661411 | - |
| | 55 | 161.56702 | 23.96790 | | |
| Myxamoebae | 15 | 3.87678 | 1.02222 | -7.4750507 | - |
| | 40 | 8.18755 | 3.86243 | | |
| | 40 | 8.18755 | 3.86243 | 0.62694279 | + |
| | 60 | 7.78982 | 2.09753 | | |
| | 40 | 8.18755 | 3.86243 | 3.4381869 | - |
| | 70 | 5.90836 | 2.48493 | | |

df = 198
$\alpha$ = 0.05

Figure 12

Histograms representing Feulgen-DNA values for liver tissue nuclei fixed in Calcium-Acetate-Formalin.


TIME PERIOD=20 min.
n=100
S.D.=22.8
MEAN DNA=127.4
S.E.=±2.29
60
40
20
10
40
70
100
130
160
190
TIME PERIOD=40 min.
n=100
S.D.=49.9
MEAN DNA=227.3
S.E.=±4.99
60
40
20
150
180
210
240
270
300
330
TIME PERIOD=55 min.
n=100
S.D.=23.9
MEAN DNA=161.5
S.E.=±3.39
60
40
20
100
130
160
190
220
250
280
NUMBER OF NUCLEI
RELATIVE AMOUNTS OF DNA

curve plateau. Comparison of the mean DNA values for the 40 and 60 minute hydrolysis time periods (the curve plateau) indicate no significant differences at a confidence level of 0.05, indicating that maximum intensity could be attained and maintained throughout that particular time continuum of hydrolysis. The differences that occur are readily seen when the histograms of these time periods are compared in Figure 13. The histograms of Figure 13 indicate that an increase in hydrolysis time during early periods of hydrolysis results in an increase in DNA dye binding and further hydrolysis after the maximal hydrolysis time results in decline in DNA dye binding.

Fixcel

Statistical analysis of the mean DNA values of liver tissue fixed in Fixcel (Table 4) for the hydrolysis time periods of 15 and 20 minutes representing the ascending slope demonstrates a significant differences in values; and the mean DNA values for the hydrolysis time periods of 20 and 35 minutes representing the points of the plateau of the curve and descending slope demonstrates a significant difference indicating the choice of a hydrolysis time period within the ascending and descending slopes unfeasible when compared with the curve plateau. Comparison of the mean DNA values for the 20 and 30 minute hydrolysis time periods (the curve plateau) indicate no significant differences at a confidence level of 0.05, indicating that maximum intensity could be attained and maintained throughout that particular time continuum of hydrolysis. The differences that occur are readily observed when the histograms of these time periods (Figure 14) are compared. The histograms illustrated in Figure 14 indicate that an increase in hydrolysis

Figure 13

Histograms representing Feulgen-DNA values for myxamoebal nuclei fixed in Calcium-Acetate-Formalin.

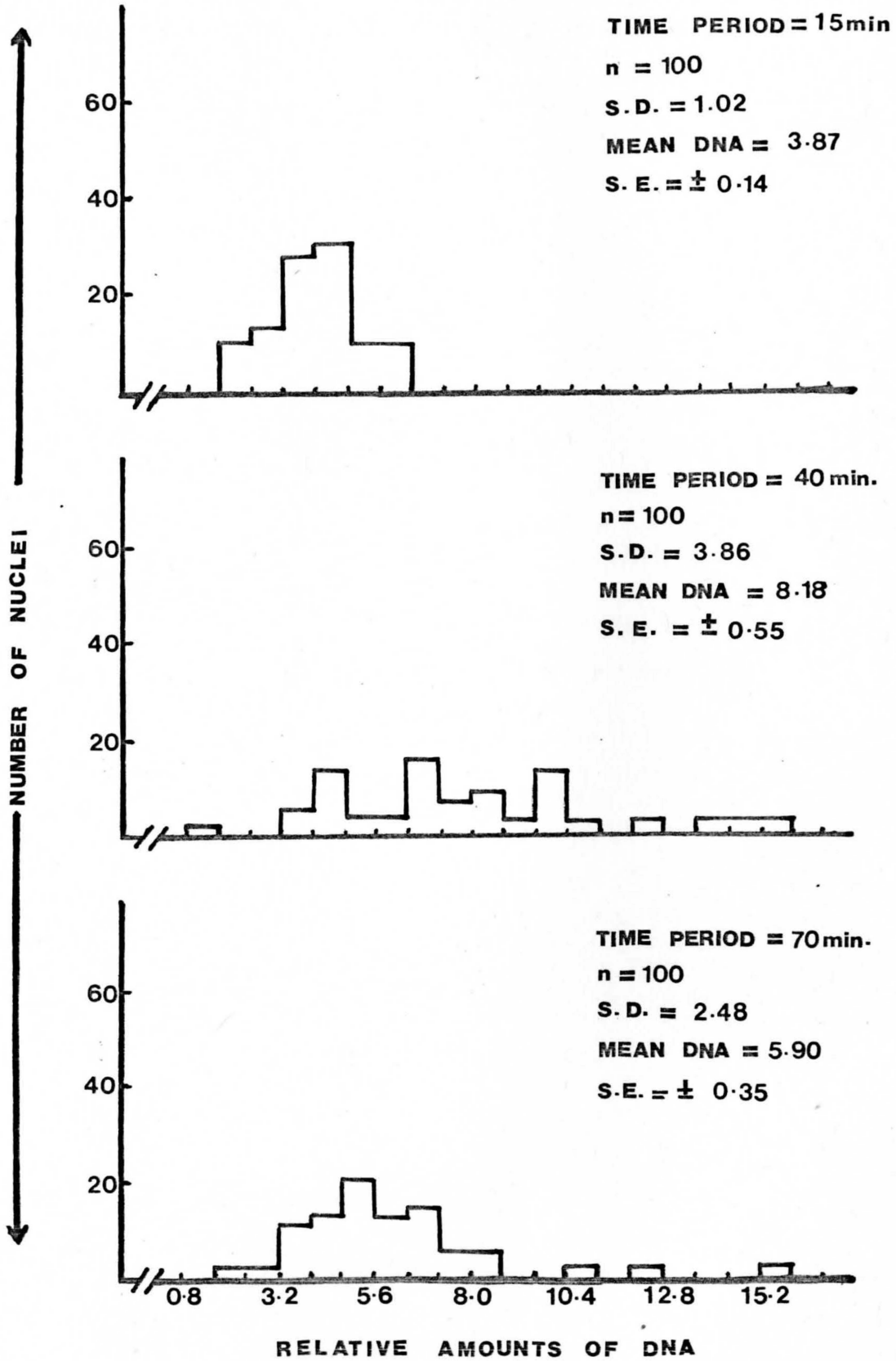

TIME PERIOD = 15min
n = 100
S.D. = 1.02
MEAN DNA = 3·87
S. E. = ± 0·14
TIME PERIOD = 40 min.
n = 100
S.D. = 3·86
MEAN DNA = 8·18
S. E. = ± 0·55
TIME PERIOD = 70 min.
n = 100
S. D. = 2.48
MEAN DNA = 5·90
S.E. = ± 0·35
NUMBER OF NUCLEI
60
40
20
0·8
3·2
5·6
8·0
10·4
12·8
15·2
RELATIVE AMOUNTS OF DNA

Figure 14

Histograms representing Feulgen-DNA values for liver tissue nuclei fixed in Fixcel.

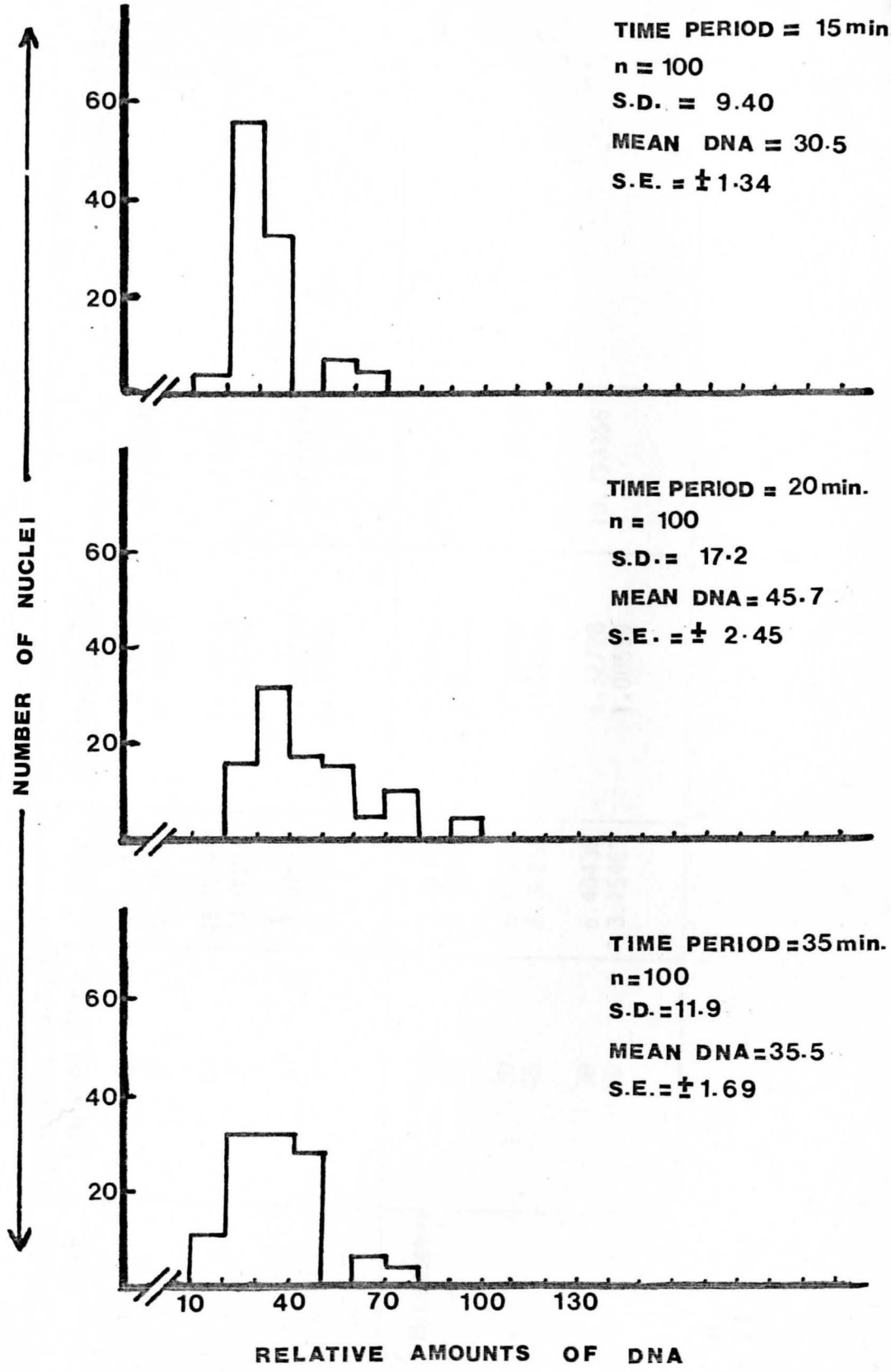

TIME PERIOD = 15 min.
n = 100
S.D. = 9.40
MEAN DNA = 30.5
S.E. = ± 1.34
TIME PERIOD = 20 min.
n = 100
S.D. = 17.2
MEAN DNA = 45.7
S.E. = ± 2.45
TIME PERIOD = 35 min.
n = 100
S.D. = 11.9
MEAN DNA = 35.5
S.E. = ± 1.69
NUMBER OF NUCLEI
60
40
20
10
40
70
100
130
RELATIVE AMOUNTS OF DNA

TABLE 4 - TWO SAMPLE t-DISTRIBUTION COMPARISON OF MEAN DNA VALUES FOR LIVER AND MYXAMOEBAE TISSUE FIXED IN FIXCEL

| Tissue Type | Hydrolysis Periods Compared (Min) | Mean | Standard Deviation | t | (+) = No Difference (-) = Significant Difference Exists |
|---|---|---|---|---|---|
| Liver | 15 | 30.51765 | 9.40379 | -5.396785 | - |
| | 20 | 45.78906 | 17.20235 | | |
| | 20 | 45.78906 | 17.20235 | 0.4856932 | + |
| | 30 | 44.08505 | 17.34792 | | |
| | 20 | 45.78906 | 17.20235 | 3.409682 | - |
| | 35 | 35.54420 | 11.97604 | | |
| Myxamoebae | 15 | 2.22779 | 0.59745 | -19.599599 | - |
| | 30 | 6.40430 | 1.32728 | | |
| | 30 | 6.40430 | 1.32728 | 0.4104409 | + |
| | 45 | 6.30250 | 1.09645 | | |
| | 30 | 6.40430 | 1.32728 | 10.793355 | - |
| | 70 | 3.75463 | 1.08559 | | |

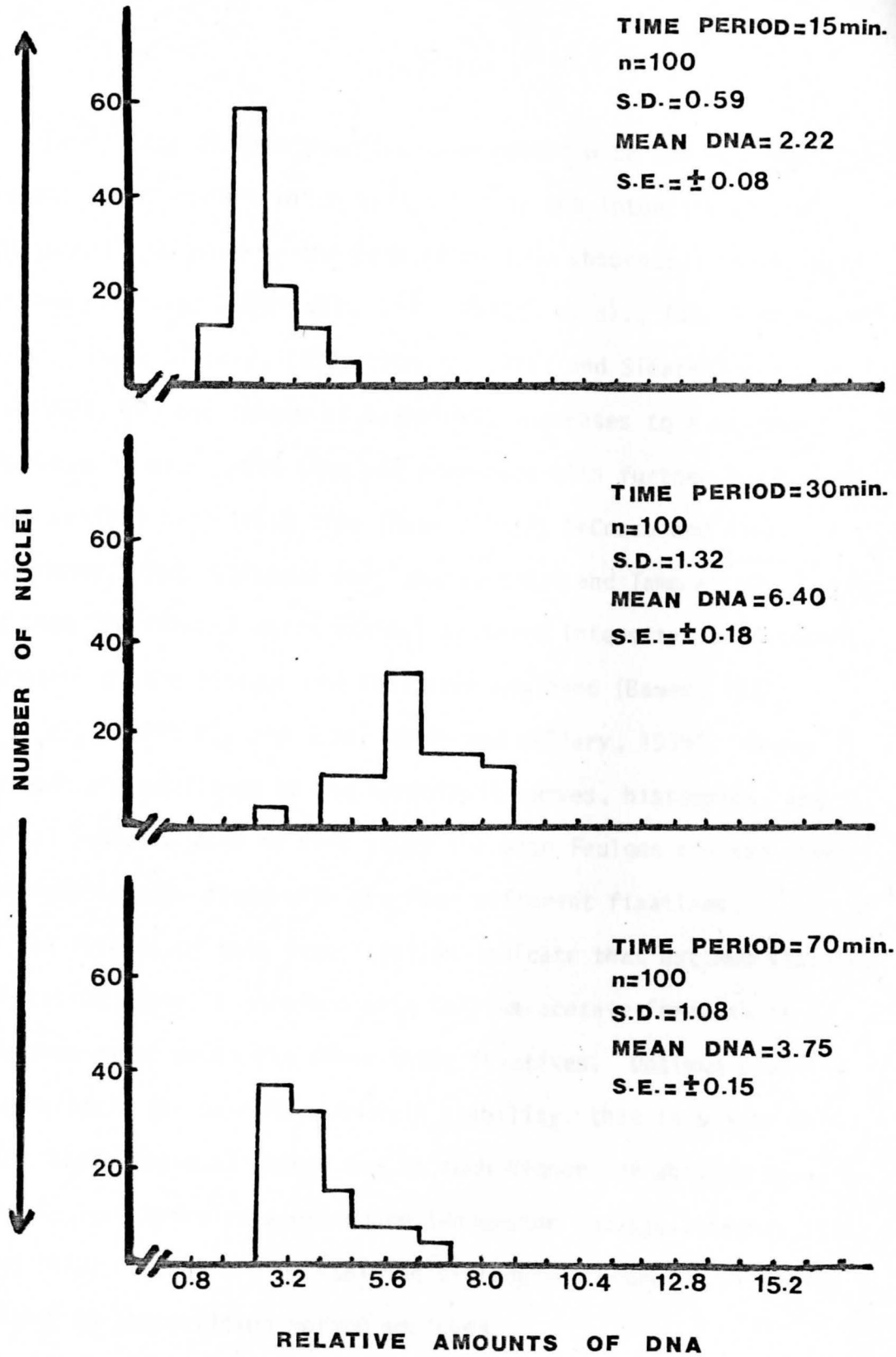
df = 198
$\alpha$ = 0.05

time during early periods of hydrolysis results in an increase in DNA dye binding and further hydrolysis after the maximal hydrolysis time results in decline in DNA dye binding.

Statistical analysis of the mean DNA values of myxamoebae cells fixed in Fixcel (Table 4) for the hydrolysis time periods of 15 and 30 minutes representing the ascending slope demonstrates a significant difference in values; and the mean DNA values for the hydrolysis time periods of 30 and 70 minutes representing the points of the plateau of the curve and descending slope demonstrates a significant difference, indicating the choice of a hydrolysis time period within the ascending and descending slopes unfeasible when compared with the curve plateau. Comparison of the 30 and 45 minute hydrolysis time periods (the curve plateau) indicate no significant differences at a confidence level of 0.05, indicating that maximum intensity could be attained and maintained throughout that particular time continuum of hydrolysis. The differences that occur are readily noticeable when the histograms of these time periods (Figure 15) are compared. The histograms of Figure 15 indicate that an increase in the hydrolysis time during the early periods of hydrolysis result in an increase in DNA dye binding and further hydrolysis after the maximal hydrolysis time results in decline in DNA dye binding.

Figure 15

Histograms representing Feulgen-DNA values for myxamoebal nuclei fixed in Fixcel.


TIME PERIOD=15min.
n=100
S.D.=0.59
MEAN DNA=2.22
S.E.=±0.08
TIME PERIOD=30min.
n=100
S.D.=1.32
MEAN DNA=6.40
S.E.=±0.18
TIME PERIOD=70min.
n=100
S.D.=1.08
MEAN DNA=3.75
S.E.=±0.15
NUMBER OF NUCLEI
60
40
20
0.8
3.2
5.6
8.0
10.4
12.8
15.2
RELATIVE AMOUNTS OF DNA

# DISCUSSION

The results of this investigation concur with the observations of previous investigators which indicate that the intensity of the Feulgen nucleal reaction or the peak of maximum absorption varies with (1) various fixatives (Artvinli, 1975; Deitch et al., 1967; Greenwood and Berlyn, 1968; Hillary, 1939; Hopwood, 1967; and Sibatani and Fukuda, 1953); (2) the length of hydrolysis increases to a maximum with increase in hydrolysis time and decreases with further hydrolysis after the maximal hydrolysis time (Bauer, 1932; DeCosse and Aiello, 1966; Jordanov, 1963; Sibatani and Fukuda, 1953; and Tamm et al., 1952): (3) and that the time at which maximal staining intensity is achieved is a function of the tissues and fixatives employed (Bauer, 1932; Deitch et al., 1964; Ely and Ross, 1949; and Hillary, 1939). These observations are confirmed by the hydrolysis curves, histograms, and statistical analyses done in this study for both Feulgen stained liver and myxamoebal tissue fixed with the four different fixatives.

The results of this investigation indicate that optimum staining for both tissue types is obtained with Calcium-acetate-Formalin as a fixative when compared to the other three fixatives. Optimum staining achieved reflects the maximum chromatin stability, that is slight nucleic acid conformational change and as such higher DNA ability to withstand the Feulgen hydrolysis procedure (Andersson and Kjellstrand, 1971, 1972, and 1973). Therefore in staining procedure, chromatin stability is dependent on the fixation method employed.

In liver tissue experiments, the shape of the hydrolysis curves and the times at which the maximal Feulgen intensity were attained differed among Neutral buffered formaldehyde, Calcium-acetate-Formalin, Acetic-alcohol-Formalin and Fixcel, with Acetic-alcohol-Formalin and Fixcel being similar. The time of maximal Feulgen intensity in liver tissue experiments was attained first in fixation with both Acetic-alcohol-Formalin and Fixcel at 20 minutes followed by that for Calcium-acetate-Formalin at 40 minutes and Neutral buffered formaldehyde at 45 minutes. The maximum Feulgen intensity persisted longer in liver tissue fixed with Neutral buffered formaldehyde (for 15 minutes) followed by those of liver tissues fixed with both Acetic-alcohol-Formalin and Fixcel (for 10 minutes); and Calcium-acetate-Formalin fixed liver tissue had the shortest (5 minutes) degree of persistence of maximal Feulgen intensity, though it gave the highest maximum Feulgen intensity.

In myxamoebae cells experiments, the shape of the hydrolysis curves and the times at which maximum Feulgen intensity were attained did not differ significantly among the different fixatives. The time of maximal Feulgen intensity in myxamoebal cells experiments was attained first in fixation with both Acetic-alcohol-Formalin and Fixcel at 30 minutes, and last for cells fixed with both Neutral buffered formaldehyde and Calcium-acetate-Formalin at 40 minutes. The maximum Feulgen intensity persisted longest for myxamoebal cells fixed with Acetic-alcohol-Formalin (for 30 minutes) followed by those of myxamoebal cells fixed with both Neutral buffered formaldehyde and Calcium-acetate-Formalin (for 20 minutes), and Fixcel-fixed myxamoebal cells had the shortest (15 minutes) degree of persistence of maximal

Feulgen intensity, with progressive decline in intensity until 55 minutes.

The ability of Calcium-acetate-Formalin (CAF) to decrease the solubility of tissue proteins during acid hydrolysis is a reasonable explanation for the attainment of high maximum Feulgen intensity values in both liver and myxamoebae tissues fixed in this fixative. Artvinli (1975) concurred with this result and indicated that Calcium salts is suitable in decreasing the solubility of tissue proteins provided that the fixation time is kept short. Sibatani and Fukuda (1953) also indicated that DNA dissolution during acid hydrolysis can be decreased to a minimum or inhibited by the addition of Lanthanum acetate to formalin, a similar fixative to Calcium-acetate-Formalin. Eapen (1960) supported the above authors and indicated that Calcium formalin is a good fixative for enzyme activity; Humason (1979) agreed partly and indicated that Calcium formalin is a good enzyme fixative but it is not good in the preservation of alkaline phosphatase or aminopeptidase. Humason (1979) also indicated that the problem with this fixative is that Calcium deposits may be heavily concentrated in the tissue that they may interfere with sectioning and result in torn sections and nicks on the knife edge. This observation was confirmed in this study while sectioning liver tissue blocks fixed in Calcium-acetate-Formalin.

Though the mechanism of DNA dissolution caused by acid hydrolysis during Feulgen reaction is not fully understood, Sibatani and Fukuda (1953). But the extent of the dissolution is partially conditioned by the behavior of the protein bound to the DNA during the hydrolysis. Deitch et al. (1967), Hillary (1939), Kasten (1959),

Jordanov (1963), DeCosse and Aiello (1966) and Itikawa et al. (1954) found that in view of the fact that acid hydrolysis involved in Feulgen reaction contributed to the loss of Feulgen intensity through depolymerization of DNA. But the extent of acid degradation of DNA and subsequent loss of the material is dependent upon the stability of the chromatin in the DNA, the concentration of the acid, and the temperature involved. Since all experimental slides were hydrolyzed together under the same temperature and with the same acid concentration, then the high Feulgen intensity observed in liver and myxamoebal tissues fixed in Calcium-acetate-Formalin was due to the ability of the fixative to stabilize the chromatin and reduce the loss of Feulgen stainable material. Analysis of the hydrolysis curve for liver tissue fixed in Calcium-acetate-Formalin supports the above, and reflects an abrupt decline in maximal staining intensity following 5 minutes hydrolysis plateau period. Such an abrupt decline is not evidenced in the descending slope of the hydrolysis curve for myxamoebal nuclei fixed in Calcium-acetate-Formalin, nor, in comparison with the other fixatives; so this poses a question whether Calcium-acetate-Formalin in all cases produce the best maximal stain intensity, as is demonstrated in its use with liver tissue in this study.

Neutral buffered formaldehyde's ability to stabilize DNA adequately and maintain maximum stain intensity over extended hydrolysis time is portrayed by the liver and myxamoebae nuclei hydrolysis curve. This was supported by Deitch et al. (1967) who also got the longest hydrolysis plateau with Hela cells fixed for 18-24 hours in 10 percent neutral formalin. Andersson and Kjellstrand (1975) observed that the alteration of the hydrolysis pattern of chromatin fixed in formalin was

due to slower extraction of DNA polymerization, and this is a reasonable explanation for the extended plateau period of maximum Feulgen intensity. Neutral buffered formaldehyde adequately stabilize DNA in such a way that the extraction of DNA or depolymerization is very slow and as such extended plateau period of maximum Feulgen intensity is obtained as was observed in this investigation. The low maximum Feulgen intensity obtained with 10 percent Neutral buffered formaldehyde fixation of rat liver, when compared to the maximum Feulgen intensity obtained with Calcium-acetate-Formalin and Acetic-alcohol-Formalin, was supported by the observations of Dutt (1971). In Dutt's experiment, following fixation of rat liver in either 40 percent or 10 percent formalin solution, Feulgen staining was greater in tissues fixed with 40 percent formalin. And his explanation was that polymerized DNA molecule retains a much larger proportion of aldehyde and hence enhanced staining reaction. The aldehyde residues might be fixed by direct chemical bonding to the insoluble DNA-protein complex and hence do not suffer loss during hydrolysis and rinsing operations, and this supports fully why both Calcium-acetate-Formalin and Acetic-alcohol-Formalin fixed liver tissues had higher maximum Feulgen intensity than liver tissue fixed in ordinary buffered formaldehyde; Belanger and Bois (1964) concurred.

The results of myxamoebae cells fixed with Acetic-alcohol-Formalin (AAF) suggest that it does not give a high maximal Feulgen intensity for this tissue. The choice of this fixative (AAF) for fixing myxamoebae cells depends on the investigator's particular research problem because it has lesser hydrolysis time and longest plateau of maximal Feulgen intensity for myxamoebae cells compared to the two previous fixatives (CAF and 10 percent neutral buffered

formaldehyde). On the other hand, the results of liver tissue fixed in AAF indicates that the fixative may offer an alternative to Calcium-Acetate-Formalin (CAF), in that, the maximum stain intensity is readily defined, achieved early, and persists for a longer period of time than CAF. Jordanov (1963) supports the results obtained with AAF for liver tissue in this study, in which maximum staining was reached after 20 minutes hydrolysis, and then the reaction remained unchanged up to 30 minutes before it declined. Belanger and Bois (1964) also indicated that AAF retains a larger stainable material and as such higher Feulgen intensity for rat tissue than Neutral buffered formaldehyde. Hillary (1939), Greenwood and Berlyn (1968), and Sibatani and Fukuda (1953) supported the above mentioned investigators by indicating that their clearest and sharpest preparations were fixed in Carnoy's fluid which contains 25 percent acetic acid (similar to Acetic-alcohol-Formalin); and further they indicated that Carnoy's fluid is superior to buffered formaldehyde in that it facilitated vigorous staining. Though there would appear to be no perfect fixative, some workers have claimed that fixatives with low acetic acid content are best with the Feulgen reaction. Semmens and Bhaduri (1939), for their work on differential staining of nucleoli and chromosomes, suggested fixatives with acetic acid cut down to a minimum. Why, they do not say. Whitaker (1939), working with various plant materials, states that in his experience it is quite important that a fixative be employed which contains a minimum of acetic acid for Feulgen staining. Thus all the evidence indicate that acetic acid has no deleterious effect on the Feulgen nucleal stain.

The results of this investigation indicate that Fixcel (mainly 20:1 ratio of ethanol and methanol) is a very poor fixative for liver

tissue. Ethanol hardens tissue but causes serious shrinkage. It is a strong cytoplasmic coagulant but does not fix chromatin. When alcohol is used alone, nucleic acid is transformed into a soluble precipitate and is lost in subsequent solutions and during staining, Humason (1979). This supports the low maximum Feulgen intensity obtained for liver tissue fixed with Fixcel fixative. Myxamoebae cells fixed in Fixcel suggest that it may be used in certain tissue types for quantification of DNA, since the maximal Feulgen stain intensity here is slightly higher than that of Acetic-alcohol-Formalin (AAF) fixed myxamoebal cells. Fixcel and AAF fixed liver tissue attained their respective maximal Feulgen intensity at the same time and the plateau of maximal Feulgen intensity terminated at the same time. Myxamoebae cells fixed with Fixcel and Acetic-alcohol-Formalin (AAF) attained their respective maximal Feulgen intensity, which was not significantly different, at the same time, but the plateau of maximal Feulgen intensity for AAF extended longer. The possible explanation for these similarities between AAF and Fixcel fixed tissues may be due to the ethanol in them.

Though formalin fixation is recommended by most investigators in quantitative studies involving proteins and the Feulgen reaction; it seems that the type of tissue being fixed is also an important consideration. Literature surveys indicate a variety of formalin fixation periods for various tissues, including those of minimum times of 2 to 6 hours (Abramczuk, 1971) and maximum times of 18 to 24 hours (Deitch et al., 1967; and Sibatani and Fukuda, 1953) have been employed to obtain desirable results. This study has shown that 12 hours fixation period is good for liver tissue but results in the

overfixation of myxamoebal cells, rendering the nuclear material more susceptible to hydrolytic dissolution, and hence decreased staining intensity. Hydrolysis periods between 30 minutes to 60 minutes for myxamoebae nuclei, and between 20 minutes to 60 minutes for liver tissue, is recommended to ensure attainment of maximal Feulgen intensity. Hydrolysis periods outside this range represent depurination (some Feulgen stainable materials unmasked) and depolymerization (loss of Feulgen stainable material) and are not recommended as valid periods to represent maximal stain intensity. Depurination and depolymerization is proposed respectively for the ascending and descending slopes of the hydrolysis curves prior to and after the maximum dye binding plateau. The histograms also exhibit a bi-modal distribution which is characteristic of a population of cells in which some cells are in the process of DNA replication (s-phase), some cells are not replicating in $G_1$-phase, and some cells that have already replicated in $G_2$-phase (Swift, 1950).

Considering the results of this investigation, similar Feulgen intensities should not be expected to occur between two cell types when using the same fixative, because of the differences in the type and amount of DNA and the way the fixative and the particular nucleic acid interact in producing a stable configuration for dye-binding. Also, there may or may not be similarities in the maximal stain intensity in using different fixatives. Even though stain intensities may differ, the choice of using any of these fixatives in representing the maximal stain intensity for a specific tissue type is possible, depending on the particular research problem of the investigator. For example, if the investigator wants intense staining, Calcium-acetate-Formalin may

appeal to him; otherwise time factors like length of fixation, length of hydrolysis before maximal stain intensity is attained, and length of plateau persistence may influence his choice. The results of this investigation represent the best hydrolysis time for each of the four different fixatives that will give maximal intensity and quantitation of the Feulgen reaction for both tissue types and thus most types of tissues. Thus formaldehyde fixatives, especially Calcium-acetate-Formalin, may prove to be very suitable for tissue preparative techniques in all microscopic studies, provided that the fixation time is kept short.

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