CYTOPHOTOMETRIC ANALYSIS OF HYDROLYSIS TIMES AND FIXATION EFFECTS ON QUANTITATION OF THE FEULGEN REACTION

by

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ABSTRACT

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Four fixatives, ten percent buffered formalin, twenty percent buffered formalin, bicarbonate formaldehyde, and calcium-acetate-formalin, were used to study the fixation and acid hydrolysis effects on the Feulgen nucleal reaction used in quantitative cytochemical determinations for deoxyribonucleic acid. The two-wavelength method of microspectrophotometry was used to analyze maximal DNA staining values at various hydrolysis times for two distinctly diverse tissue types. With the use of hydrolysis curves generated in this study, relative DNA measurements of myxamoebal and liver nuclei were compared to determine the fixative and hydrolysis time best suited to produce maximal Feulgen staining.

It was determined that the two tissue types differed with respect to fixatives employed to produce maximal staining. Optimum staining intensity was obtained when liver nuclei were fixed in bicarbonate formaldehyde, while fixation in calcium-acetate-formalin yielded the best maximal intensity for myxamoebal nuclei. Similar maximal stain intensity values were obtained for liver fixed in ten percent buffered formalin, twenty percent buffered formalin, and calcium-acetate-formalin. However hydrolysis curves for liver fixed with ten percent buffered formalin, twenty percent buffered formalin, and calcium-acetate-formalin exhibited a significant decline in maximum staining intensity

with continued hydrolysis. For liver nuclei, the maximum Feulgen intensity was achieved at similar time periods for ten percent buffered formalin, bicarbonate formaldehyde, and calcium-acetate-formalin (at 50 minutes). On the other hand, maximal Feulgen intensity for liver nuclei fixed in twenty percent buffered formalin reached a plateau significantly later (at 90 minutes).

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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_s	Transmitted flux	
k	Specific absorptivity constant of the chromophore at a defined wavelength	
L	Parameter equivalent to one minus the transmission (1-T) at a defined wavelength	
M	Chromophore mass in the measured field	
Q	Ratio of L_2/L_1	
T	Transmission of the field	
λ	Wavelength	nm
α	Confidence interval	

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CHAPTER I

INTRODUCTION

Cellular investigations involving cytochemical methods have in recent years become increasingly important as evidenced by the numerous scientific publications that have appeared regarding these studies. These investigations often center on the subcellular organelles, such as the nucleus. Cytochemical research complements and extends those investigations which formerly focused on nuclear morphology, by employing biochemical methods. Recent advances regarding cellular metabolism have accorded a central position for nucleic acids. The biochemical changes which occur are reflected and accompanied by changes in nuclear deoxyribonucleic acid (Yemma and Therrein, 1972). Early quantitative studies regarding DNA were made on the basis of tissue weight (Swift, 1953). In 1948, Boivin, Vendrely, amd Vendrely were the first to determine the amount of DNA per nucleus. Today, nucleic acids (both RNA and DNA) are recognized by the properties imparted by their purine, pyrimidine, or sugar moieties. The absorption of specific wavelengths of ultraviolet light distinguishes purine and pyrimidine, but this will not distinguish between RNA and DNA (Ely and Ross, 1949). However the literature of the past as is true of the present is replete with quantitative methods by which one can study DNA and its changes. Paramount among these, and one which has received widespread use is the Feulgen reaction (Feulgen and Rossenbeck, 1924), a cytochemical procedure which under proper conditions can lead to a quantitative measurement of DNA changes that occur during the cell cycle (Andersson and Kjellstrand, 1971, 1972, and 1975; DeCosse and Aiello, 1966). Since its development in 1923, the Feulgen

reaction had an immediate and tremendous impact on all fields of biological and medical sciences that persists today. To illustrate this point, only ten years after the discovery of the Feulgen reaction, thousands of scientific papers had been published on the use of this technique (Kasten, 1964).

Nevertheless, the application of the Feulgen reaction has been challenged even though much progress has been made since Baker (1944) stated the "Feulgen's test has been much used but less understood." Some of the problems that have been associated with Feulgen reaction include the following: 1) Stain impurities which cause artifacts of color in the cytoplasm of cells (Hillary, 1939, and Kasten, 1964), 2) Irregularities in staining from one batch of tissue to another (Kasten, 1964), 3) Irregular results obtained in staining at different times (Kasten, 1964), 4) Nuclei on the edge of a tissue block give significantly higher values than those in the central portion (Swift, 1966), 5) Inconsistencies in the proportion of DNA bound by Feulgen dye (Patau and Swift, 1953), 6) Hydrolysis allows for the loss of some stainable material (Stedman and Stedman, 1943 and 1947; Ely and Ross, 1949), 7) Varying shapes of hydrolysis curves and indiscriminate use of fixatives resulting in different plateau regions of maximum dye concentration for the same fixatives (Mayall, 1970), 8) Optical errors (Patau, 1952), and 9) Fading of the stain upon exposure of the slide to the intense illumination required in microspectrophotometry (Kurnick, 1955).

Although there initially appear to be many problems associated with the Feulgen reaction, each has been either disproven or a method developed which overcomes the difficulty (Haselkorn and Doty, 1961). The staining artifacts following fixation are not commonly observed provided the tissues are thoroughly washed following fixation. It has been found that by filtering the Feulgen solution with activated charcoal, impurities are removed

which results in a colorless Leuco basic fuchsin dye (Kasten, 1960). The irregularities in staining exhibited from one batch of tissue to another and at different times may be prevented by the use of consistent handling procedures. Thus the materials (tissues) to be studied should be fixed, mounted, and stained simultaneously. However, it has been found that valid data is consistently obtained even when different slides are stained together (Kasten, 1964). In order to obtain Feulgen-DNA values, tissues may be homogenized or fixed in blocks (Swift, 1953) and allowed adequate time in fixative to ensure penetration to the center. While some investigators may argue that cells containing identical amounts of DNA per nucleus may not bind identical amounts of dye in the Feulgen reaction, cytophotometric and biochemical analyses performed by Ris and Mirsky (1949) and Leuchtenberger (1952) have shown agreement in DNA amounts using both methods. Therefore the Feulgen reaction gives a constant amount of dye bound per nucleus, in spite of the variations in nucleic acid concentrations (Swift, 1950). Studies performed by Greenwood and Berlyn (1968), have demonstrated that spectral absorption is proportional to DNA content per nucleus. Although some of the stainable material may be lost during acid hydrolysis, Swift (1966) felt that since there would be an equal loss of DNA following hydrolysis procedures, the resultant DNA measurements could be treated as arbitrary rather than absolute values. In addition, using in vitro studies, Caspersson (1936) showed that the Feulgen reaction would give a valid estimate of the amount of DNA within a variation of about two percent. In response to varying plateaus of hydrolysis curves, Mayall (1970) contended that errors due to this factor may be decreased or eliminated by hydrolysis in 5 Normal hydrochloric acid at room temperature. Swift (1966) added that ideally the curves that are to be compared should have had similar chemical treatment. The optical errors

can be reduced by choosing a wavelength for cytophotometric studies at which the absorption by the dye is less than maximal (Patau, 1952) as first used by Swift (1950). In his investigations Swift felt that "measurements made at only one wavelength . . . seem inadequate in studying the kinetics of Feulgen hydrolysis." The fading effect caused by illumination can be minimized by reducing and filtering the light source during the searching and focusing processes (Kurnick, 1955). In addition several researchers (Stowell, 1945; Ris and Mirsky, 1949) found that Feulgen stained nuclei are not decolorized by exposure to light.

When one considers the above arguments, it is quite evident why most of the investigators who are familiar with the technique accept the Feulgen reaction as a valid and specific histochemical test for DNA (Ely and Ross, 1949). Today there is widespread agreement that under appropriate conditions the Feulgen reaction is applicable to quantitation of DNA (Lison, 1960 and Thomas, 1950). Perhaps the Feulgen reaction is best summarized by Kasten (1964), "in spite of . . . difficulties, the (Feulgen) reaction has allowed important contributions to be made about DNA and is still regarded today as the most important and quantitative reaction in histo- and cytochemistry."

Because of its widespread use, many studies have centered around the various factors which may affect the intensity of and interpretation of results obtained from the use of the Feulgen reaction. The result has been a wide spectrum of uses for this reaction in cellular research. In this study several of these factors have been investigated including hydrolysis conditions and procedures, fixative employed, and tissue type.

In 1933, Bauer realized the maximum staining in the Feulgen reaction required an optimal hydrolysis time. This was supported by Patau (1952), Whitaker (1939), Hillary (1939) and other investigators. Acid hydrolysis is an

essential part of the Feulgen reaction since unhydrolyzed tissues do not stain (Kasten, 1960). Hydrolysis is a necessary feature of the Feulgen reaction since it cleaves the purine-sugar linkages of DNA to expose aldehyde sites which react with Schiff's reagent (Kasten, 1960). However the optimum hydrolysis time is affected by several factors such as acid concentration, temperature, and fixation (Kasten, 1960; Pöppe, Pellicciari, and Bachmann, 1979; Scherini, 1982; Magakyan, Karalova, Khachikyan, and Avetissyan, 1980; and Karalova, Khachikyan, Avetissyan, and Magakyan, 1980). Ely and Ross (1949) also suggested that the optimal hydrolysis time may also vary with tissues. Pöppe, Pellicciari, and Bachmann (1979) concurred and added that a comparison of tissues from different taxonomic categories would prove to be a promising field of application of Feulgen hydrolysis. Although it is generally agreed upon that various fixatives affect the Feulgen reaction (Mayall, 1970; Bauer, 1933; Hillary, 1939; and Kotelnikov and Litinskaya, 1981), Hillary (1939) stated that "the duration of fixation has no effect on the intensity of the stain." The Feulgen reaction is also affected by the dye solution (Patau, 1952), as the Feulgen reagent does not retain its staining power as normally stored in the lab (Ely and Ross, 1949). Also, Kasten (1964) stated that the same batch of Schiff's reagent may produce differences if used on different occasions. Patau (1952) indicated that the Feulgen reaction may vary between different kinds of nuclei and different tissues. But since different nucleic acids contain different ratios of purine to pyrimidine the amount of aldehyde exposed for the reaction would also vary (Kurnick, 1955). Because of all the factors known or thought to affect the Feulgen reaction, cytological studies of the Feulgen reaction including hydrolysis have been performed (Bauer, 1933; DiStefano, 1948; Ris and Mirsky, 1949; Ely and Ross, 1949; and Taylor, 1958).

In this study an attempt has been made to demonstrate the effects of hydrolysis times and fixatives on two widely diverse tissue types: rat liver tissue, considered a highly developed multicellular life form and slime mold myxamoeba, a lower organism. This investigation expands upon an initial study performed by Deitch et al. (1967). To achieve the goals of this study, absorption microspectrophotometry, using the two-wavelength method of Patau (1952), was employed to analyze the DNA content of Feulgen-stained tissues using four separate fixatives: 1) ten percent buffered formalin, 2) twenty percent buffered formalin, 3) bicarboante formaldehyde, and 4) calcium-acetate-formlain. The purpose of this investigation was to determine the optimum hydrolysis interval for each fixative that can produce and maintain maximum Feulgen stain intensities, and to determine whether the use of diverse tissue types produces similar or different hydrolysis characteristics when employing the methods of quantitative cytophotometry.

CHAPTER II

MATERIALS AND METHODS

Tissue Sources

The cultures of Didymium iridis used in this study were originally obtained from Dr. O.R. Collins, Department of Botany, University of Califronia, Berkley. The specific isolate is designated Honduran 1-2A'. Mammalian liver tissue was obtained from the albino rat, Rattus rattus, supplied by the Holtzman Laboratory Animal Supply Company, Wisconsin. However both are currently maintained in the Youngstown State University laboratories.

Cultivation of D. iridis

Myxamoebal clones of D. iridis are maintained on slants of half strength Difco corn meal agar for future use. The bacterium Escherichia coli was used as a food source on these slants. When needed, the isolate used in this investigation was transferred from slants to separate plates of sterile media which had been previously inoculated with 2ml of E. coli suspension.

The culture media was prepared by dissolving 8.5 grams of Difco Corn Meal Agar and 8.0 grams of Difco Agar in 1000ml of distilled water. This mixture was then covered and autoclaved for one hour (Collins, 1963; Yemma et al., 1974). The autoclaved media was then poured into sterile plastic Petri dishes and refrigerated in an inverted position until needed.

Originally four agar plates were inoculated with E. coli suspension.

This suspension was prepared by washing the bacterium from an agar slant with 10ml of sterile distilled water. Another final bacterial suspension was prepared by adding 2ml of the bacteria-water suspension to 5ml of sterile distilled water. A 2ml quantity of this diluted suspension was spread on the agar media surface with a sterile glass rod. These four plates were inverted at room temperature to develop into a bacterial lawn. After seven days the bacterial growth was sufficient to facilitate the inoculation and subsequent cultivation of the myxamoeba. Following inoculation, the plates were inverted and incubated at 21 degrees Centigrade for ten days (Yemma and Therrien, 1972; Yemma et al., 1974).

Once achieving log phase of growth, these four myxamoebal isolate transfers were used to inoculate 200 additional plates which were prepared in the same manner described previously. All of the myxamoebal subcultures were again inverted and incubated.

To prevent contamination of the cultures, sterile technique was utilized throughout all the transferring and inoculating procedures. Although contamination was not evident in this study, had any of the culture plates shown foreign or bacterial or fungal growth, they would have been discarded.

Preparation of Material for Cytochemical Studies

Myxamoeba

Fixation of Cells

All of the myxamoebal cultures were fixed in the same manner while still on the culture medium. The fixatives used in this investigation were prepared as follows:

(1) Ten Percent Buffered Formalin: 1000ml of 10% formalin, 4.0 grams

of sodium acid phosphate ($NaH_2PO_4 \cdot H_2O$), and 6.5 grams of anhydrous disodium phosphate (Na_2HPO_4).

(2) Twenty Percent Buffered Formalin: 100ml of 40% formaldehyde, 450ml distilled water, 8.0 grams of sodium acid phosphate, and 13.0 grams of anhydrous disodium phosphate.

(3) Bicarbonate Formaldehyde: 100ml of 40% formaldehyde solution, 1000ml of distilled water, and add 12.5 grams of sodium bicarboante to 1000ml of the above solution.

(4) Calcium-Acetate-Formalin: 100ml of 40% formaldehyde solution, 900ml distilled water, and 20 grams of calcium acetate monohydrate.

Initially 50 plates per fixative containing myxamoeba were used. The plates were flooded with each fixative and allowed to stand at room temperature for 24 hours before harvesting.

Harvest of Cells

The method for harvesting the fixed myxamoebal cells was the same for each of the four fixatives. The cells were "washed" from the media surface by drawing up the fixative on the culture plate into a sterile pipette and then with moderate force ejecting back onto the plate. This was repeated several times for each plate. The washings were collected in a sterile flask and spun down in 20ml aliquots in 50ml conical centrifuge tubes at 1000rpm for 20 minutes in an Adams Dynac swinging bucket centrifuge (Yemma, 1971). The supernatant, consisting of fixative and bacteria, was removed using vacuum suction and then discarded. The resultant plugs were washed by first covering it with 70% ethanol then agitated and centrifuged. The centrifuging process was performed several times until a plug of myxamoebal cells free from bacterial cells was obtained. These then were allowed to

post fix in 70% ethanol for 12 hours.

Finally, 24 slides containing cells isolated from each fixative were prepared. Slides were previously albumenized to insure proper adhesion of the cells to the slides. Subsequently, three drops of the myxamoebae were smeared onto each slide and allowed to dry overnight on a slide warmer set at 40 degrees Centigrade.

Liver

Preparation of Tissues

The experimental rat was sacrificed. The liver was immediately cut into $1cm^3$ cubes. The tissue blocks of liver were divided into four groups with six cubes per fixative type.

Fixation of Tissues

The fixation, dehydration, and paraffin imbedding techniques employed for each of the four fixatives were the same. In each case, the procedure was as follows:

(1) The liver blocks were placed into large volumes of each fixative at room temperature for 24 hours.

(2) The fixed liver tissues were washed in 70% ethanol for 12 hours and passed through a graded ascending concentration ethanol series to xylene and paraffin imbedded.

(3) The liver was then sectioned at 8 microns on an American Optical microtome.

(4) The liver tissue sections were affixed to previously albumenized slides and allowed to dry overnight on a slide warmer set at 30 degrees Centigrade.

(5) Immediately prior to staining, the liver tissue slides were cleared in xylene for one half hour and rehydrated by passing through a graded descending concentration of ethanol series to water.

Cytochemical Methods

The Feulgen reaction was employed in order to quantitatively localize the nuclear DNA for both myxamoebal cells and liver tissue (Feulgen and Rossenbeck, 1924; as modified by Therrien, 1966; and Bryant and Howard, 1969). A primary requirement of the Feulgen reaction in order to ensure that accurate and quantitative measurement be made is proper acid hydrolysis of tissues.

Hydrolysis and Staining Technique

Both liver tissue and myxamoebal cells were placed in distilled water for one hour prior to hydrolysis. The liver section and myxamoebal slides, along with control slides previously treated with deoxyribonuclease, were simultaneously hydrolyzed in 5 Normal hydrochloric acid (5N HCl) at room temperature at the the intervals ranging from 15 to 90 minutes. An additional time period of 120 minutes was added for both tissue types for twenty percent buffered formalin. 5N HCl hydrolysis rather than 1N HCl at 60 degrees Centigrade was used for it is superior and has been found to produce optimum staining in tissues (DeCosse and Aiello, 1966; Magakyan, Karalova, Khachikyan and Avetissyan, 1980). After rinsing in distilled water, all the slides were stained in the same manner in order to insure accurate and comparable results for one hour in Schiff's reagent (Lillie, 1951) freshly fortified by adding 1 part 10% potassium metabisulfite to 4 parts Schiff's reagent. These reagents were prepared as follows:

(1) HCl-Schiff's - Add 1 gram basic fuchsin and 2.2 grams of potassium meta-bisulfite to 100ml of 1N HCl. Shake mechanically for 2 hours and let stand overnight in a dark place. Mix activated charcoal and filter until water clear. Store in a dark bottle in refrigerator.

(2) 10% Potassium-metabisulfite - Dissolve 10 grams of potassium-metabisulfite in 100ml of distilled water.

Following the staining procedure, the slides were rinsed for 5 minutes in freshly prepared 10% potassium meta-bisulfite. This was followed by a distilled water rinse and dehydration in ethanol (70% ethanol for 10 minutes, 95% ethanol for 10 minutes, and absolute ethanol for 30 minutes). Lastly the slides were cleared in xylene for one half hour, mounted in permount, and allowed to dry.

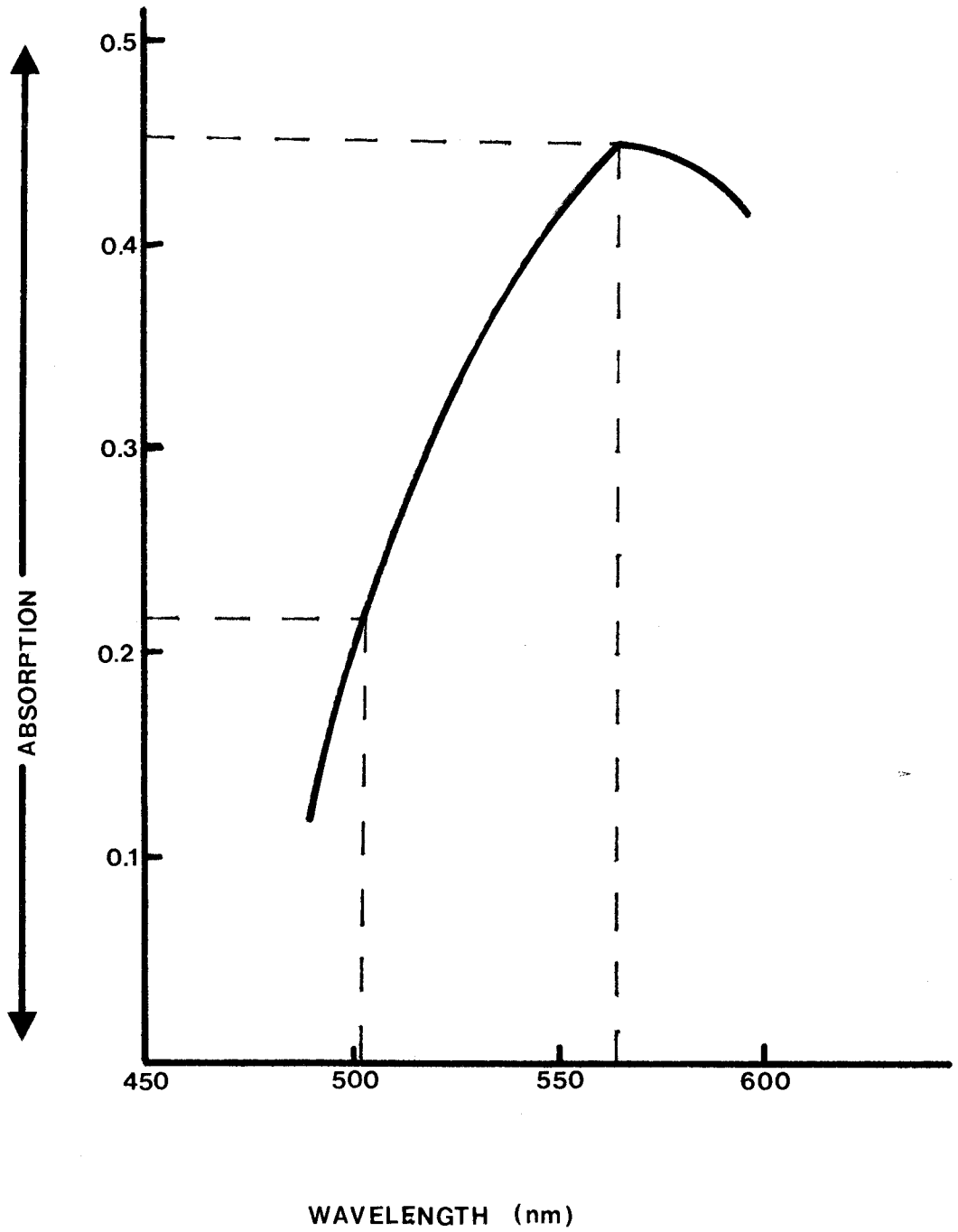
Microspectrophotometric Methods

The two-wavelength method of microspectrophotometry requires the establishment of absorption curves for each chromophore-molecular complex being studied. In this study, the absorption maxima for each of the four fixatives is represented in a combined spectral absorption curve (Figure 1). Since both liver tissue and myxamoebal cells for each fixative were treated under the same conditions, one absorption curve is sufficient to represent both tissue types. The wavelength representing the absorption maximum and half maximum were 565 nanometers (nm) and 505 nm, respectively. Control slides treated with deoxyribonuclease for the removal of DNA did not stain, indicating the absence of DNA and the specificity of the Feulgen stain for DNA.

Microspectrophotometric measurements were performed on a Zeiss Type 01 microspectrophotometer using a Planachromat oil immersion objective,

Figure 1

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


0.5
0.4
0.3
0.2
0.1
ABSORPTION
450
500
550
600
WAVELENGTH (nm)

N.A. 1.30 x 100. Before each use the instrument was aligned and the light source was centered. In order to isolate the chosen wavelength of light, a Zeiss continuous interference-filter monochrometer was used.

This study employed the two-wavelength method of microspectrophotometry described by Patau (1952), Ornstein (1952), and Mendelsohn (1961) for both myxamoebal cells and liver tissue. The use of this method corrects any errors that may be caused by heterogeneous distribution of stained material. Also, the measurement of the nuclear area is not necessary (Mayall and Mendelsohn, 1970). In order to eliminate errors produced by shifts in absorption curves and stain intensity, all of the slides used in this study were hydrolyzed and stained in the same manner (Swift and Rasch, 1956.)

The two wavelengths (λ_1 and λ_2) chosen enable accurate estimation of the absorbing material. Selection of the two wavelength were made so that the respective extinctions of the dye form a ratio of $E_1 : E_2 = 1 : 2$. The extinction is the ratio of the log of the background light intensity to the light intensity transmitted through the object or $E = \log Io/Is$. Thus $E_1 = \log Io/Is$ at λ_1 and $E_2 = \log Io/Is$ at λ_2. An absorption curve for all the stained material was made using several slides. The absorption curve is the same for all the tissue types since all the tissues were stained in the same manner (Figure 1).

Since the two-wavelength method corrects for errors caused by heterogeneous stain distribution, the linearity between absorption and DNA concentration could be measured. For all the measurements, seventy-five nuclei were randomly chosen from the slides for each of the corresponding hydrolysis time periods of each fixative and tissue type. Each of the nuclei was centered on the optical axis and the optimum photometric field was

chosen to surround the nucleus with the least amount of unoccupied space (light) appearing around the borders of the nucleus for each tissue type. The aperature chosen for the myxamoebal cells was 4 microns while that for the liver was 7 microns.

This method requires four photometric readings to be recorded per nucleus. At each wavelength (λ_1 and λ_2) one reading is taken through the nucleus (Is) and one reading is taken through the background (Io). These result in the transmissions where $T_1 = Io/Is$ at λ_1 and $T_2 = Is/Io$ at λ_2. Furthermore, the absorbances may be calculated: $L_1 = 1 - T_1$ and $L_2 = 1 - T_2$. The ratio between L_1 and L_2 is equal to Q, where $Q = L2/L_1$ and represents a value that may be used to determine the correction factor (C) between two wavelengths. This corrects for the unoccupied space that surrounds the nucleus. The correction factor for the distributional error may be determined by: $C = (2 - Q)^{-1} \ln(Q - 1)^{-1}$ (Swift and Rasch, 1956; and Leuchtenberger, 1958). However the actual computation of the correction factor is unnecessary since a table formulated by Patau (1952) lists a series of Q functions with the corresponding C values.

The amount of absorbing molecules in the measured field can be determined by the following equation: $M = kAL_1C$. In this equation M represents the amount of dye bound in the nucleus (chromophore), k is the extinction coefficient of the dye at the particular wavelength, A represents the measured area in the field, L is the absorbance, and C is the correction factor. The extinction coefficient (k) was eliminated in the calculations for this study since only relative values of DNA content were necessary. These relative values are presented as arbitrary units of DNA content in this investigation.

For the sake of accuracy and to save time, all the arbitrary DNA

values obtained from the microspectrophotometric data and statistical analysis were performed using an IBM 470 Model B-5 computer.

CHAPTER III

RESULTS

The data collected in this investigation are presented in tabular form in Tables I and II. The Feulgen-DNA measurements for both liver and myxamoebal nuclei are graphically presented as hydrolysis curves and histograms in Figures 2 through 13. The hydrolysis curves serve two functions in this study: 1) to determine the hydrolysis time required for maximum Feulgen stain intensity to occur and 2) to illustrate the duration of hydrolysis which permits peak staining to occur. The histograms represent cellular populations at particular hydrolysis time periods for each fixative and tissue type. By observing the means of these populations of cells the variation in Feulgen-DNA content can be determined.

Microspectrophotometric Analysis of Nuclear DNA

The amount of nuclear DNA, measured in arbitrary units, along with the standard deviation and standard error for each of the four fixatives is reported in Tables I and II. At each of the hydrolysis time periods, 75 nuclei were measured and recorded. However each of the recorded values for the 75 nuclei is actually a population mean for that number of cells pulsed 280 times per one tenth of a second which would yield a population size of 350,000 nuclei and thus represents maximum randomization of the population sample. The mean DNA content for each time period was used to plot Feulgen hydrolysis curves for each fixative and tissue type. On each curve the abscissa represents the hydrolysis time in minutes while the ordinate indicates the relative amount of dye binding

Table I

Standard deviations, standard errors, and two sample t-distribution of mean DNA values for myxamoebal tissue hydrolysis curves.

n=75 per time period	TEN PERCENT BUFFERED FORMALIN				TWENTY PERCENT BUFFERED FORMALIN				BICARBONATE FORMALDEHYDE				CALCIUM-ACETATE FORMALIN			
Hydrolysis Time(min.)	Mean DNA	S.D.	S.E.	t	Mean DNA	S.D.	S.E.	t	Mean DNA	S.D.	S.E.	t	Mean DNA	S.D.	S.E.	t
15	3.67	0.84	0.10		2.64	0.99	0.11		3.18	0.62	0.08		2.76	1.44	0.17	
20	3.66	0.68	0.08		2.90	1.14	0.14		3.00	0.87	0.10		2.15	0.91	0.11	
30	3.98	0.69	0.08		3.43	1.31	0.15		3.28	0.72	0.09		4.25	0.81	0.10	
40	3.86	0.75	0.09		2.66	0.87	0.10		3.42	0.76	0.10		4.52	0.86	0.10	
				1.80												
45	3.86	0.65	0.08		3.11	1.13	0.13		3.51	0.69	0.08		3.98	0.71	0.08	
50	3.78	0.64	0.07		3.30	1.05	0.12		3.65	0.70	0.08		4.22	0.85	0.10	0.73
												1.08				
75	3.70	0.76	0.09		4.08	1.39	0.16		3.52	0.72	0.08		4.41	0.78	0.09	
								0.55								
90	3.99	0.70	0.08		4.19	1.08	0.12		3.62	0.72	0.08		4.41	0.92	0.11	
120					2.77	1.39	0.19									

df = 148 α = 0.05 * = significant

Table II

Standard deviations, standard errors, and two sample t-distribution of mean DNA values for liver tissue hydrolysis curves.

n=75 per time period	TEN PERCENT BUFFERED FORMALIN				TWENTY PERCENT BUFFERED FORMALIN				BICARBONATE FORMALDEHYDE				CALCIUM-ACETATE FORMALIN			
Hydrolysis Time	Mean DNA	S.D.	S.E.	t	Mean DNA	S.D.	S.E.	t	Mean DNA	S.D.	S.E.	t	Mean DNA	S.D.	S.E.	t
15	24.00	3.92	0.45		27.37	7.73	0.90		23.74	2.25	0.26		28.70	2.80	0.32	
20	29.62	3.14	0.36		30.67	5.83	0.68		31.25	4.31	0.50		28.96	2.22	0.26	
30	32.48	2.71	0.31		28.92	6.94	0.80		35.07	1.68	0.19		33.11	3.19	0.37	
40	33.85	2.22	0.26		24.33	5.20	0.60		37.12	1.96	0.23		37.73	3.19	0.37	
45	33.76	2.35	0.27		28.47	5.86	0.68		37,83	1.86	0.21		39.26	5.95	0.69	
50	40.60	3.88	0.45	4.44*	32.35	8.22	0.96		43.60	2.30	0.26	1.55	39.92	3.19	0.37	3.62*
75	37.06	5.02	0.58		36.34	6.17	0.71		43.01	2.42	0.28		37.57	4.64	0.54	
90	37.17	4.18	0.48		39.86	7.82	0.90	3.06*	44.02	3.96	0.46		35.70	2.40	0.28	
120					21.50	5.71	0.66									

df = 148 α = 0.05 * = significant

or DNA for each of the time periods.

Analysis of F-DNA

Ten Percent Buffered Formalin

Myxamoeba

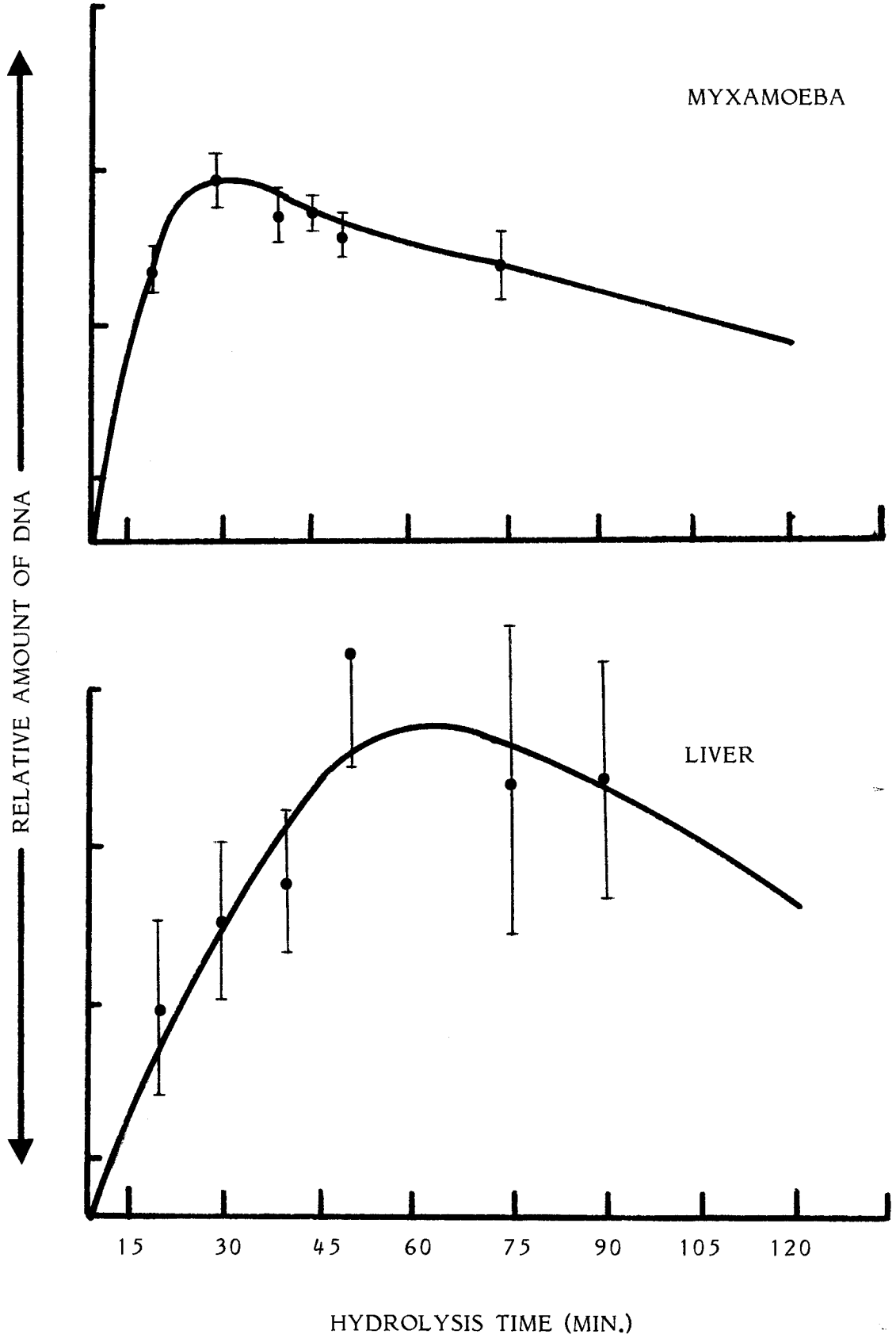
Hydrolysis curves for myxamoebal cells fixed in ten percent buffered formalin are presented in Figure 2. The curve for these cells demonstrates a rapid increase in dye binding capacity and therefore stain intensity. The maximum value is 3.98 ± 0.08 arbitrary units at 30 minutes. The plateau continues, then decreases significantly at 75 minutes. Notice that the maximum stain intensity is reached sooner for myxamoebal tissue than for liver tissue with this fixative (at 30 minutes versus 50 minutes). This also represents the quickest attainment of maximum Feulgen stain intensity for any of the fixatives or tissue types.

Liver

Liver tissue treated with ten percent buffered formalin produced a relatively steep ascending slope (Figure 2). The maximum stain intensity plateau of 40.60 ± 3.88 was achieved at 50 minutes, followed by a decrease in stain intensity at the 75 minute hydrolysis time period. For this fixative both myxamoebal and liver tissue reach significant decreases in stain intensity after 75 minutes of hydrolysis. However the maximum stain intensity achieved for liver is considerably greater than that for myxamoeba (40.60±3.88 compared to 3.98 ± 0.08 arbitrary units).

Figure 2

Hydrolysis curves representing Feulgen stained liver nuclei and myxamoebal nuclei fixed in ten percent buffered formalin.


MYXAMOEBA
LIVER
RELATIVE AMOUNT OF DNA
15
30
45
60
75
90
105
120
HYDROLYSIS TIME (MIN.)

Twenty Percent Buffered Formalin

Myxamoeba

The ascending slope of the hydrolysis curve for myxamoebal cells fixed in twenty percent buffered formalin rises rather gradually (Figure 3). The maximum staining intensity of 4.19 ± 0.12 is not achieved until 90 minutes. This is considerably longer than the other three fixatives and persists for 15 minutes (75 - 90 minutes).

Liver

Liver cells fixed in twenty percent buffered formalin (Figure 3) display a steep ascending slope until the twenty minute hydrolysis time period. However at this point the curve very gradually rises to the maximum of 39.86 ± 7.82 achieved at 90 minutes as was also the case for myxamoebal cells. Once again this is also considerably longer than the time to reach the maximum for the other three fixatives. This maximum decreases significantly at 120 minutes. Once again the maximum stain intensity of 39.86 ± 7.82 arbitrary units is significantly greater than that achieved with this fixaitve for myxamoeba (4.19 ± 0.12).

Bicarbonate Formaldehyde

Myxamoeba

The ascending slope of the hydrolysis curve (Figure 4) for bicarbonate formaldehyde is not as steep as that of ten percent buffered formalin or calcium-acetate-formalin. Also the maximum stain intensity of 3.65 ± 0.08 achieved at 50 minutes is not as great as any of the other three fixatives and decreases significantly at the next hydrolysis time period of 75 minutes.

Figure 3

Hydrolysis curves representing Feulgen stained myxamoebal nuclei and liver nuclei fixed in twenty percent buffered formalin.

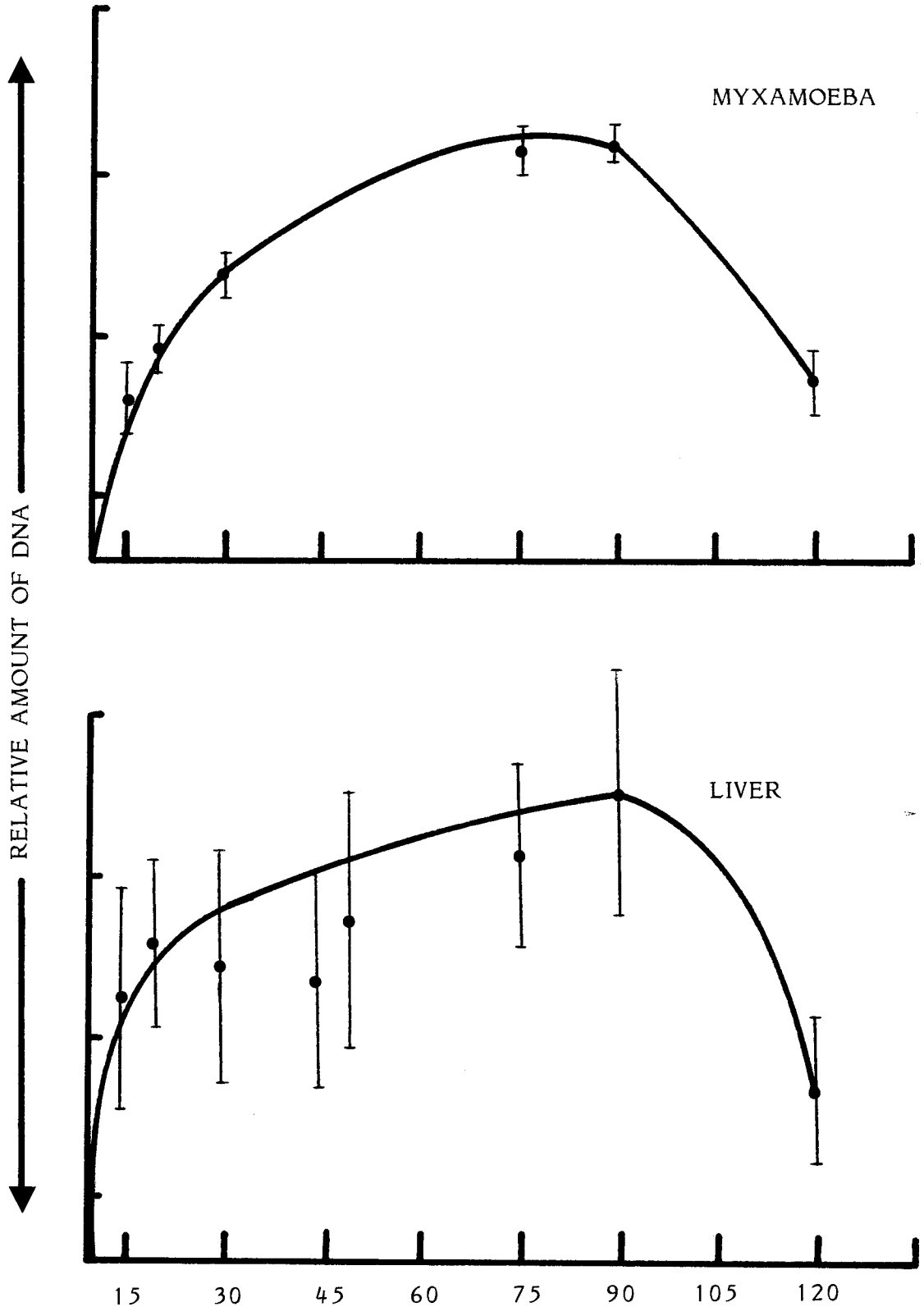
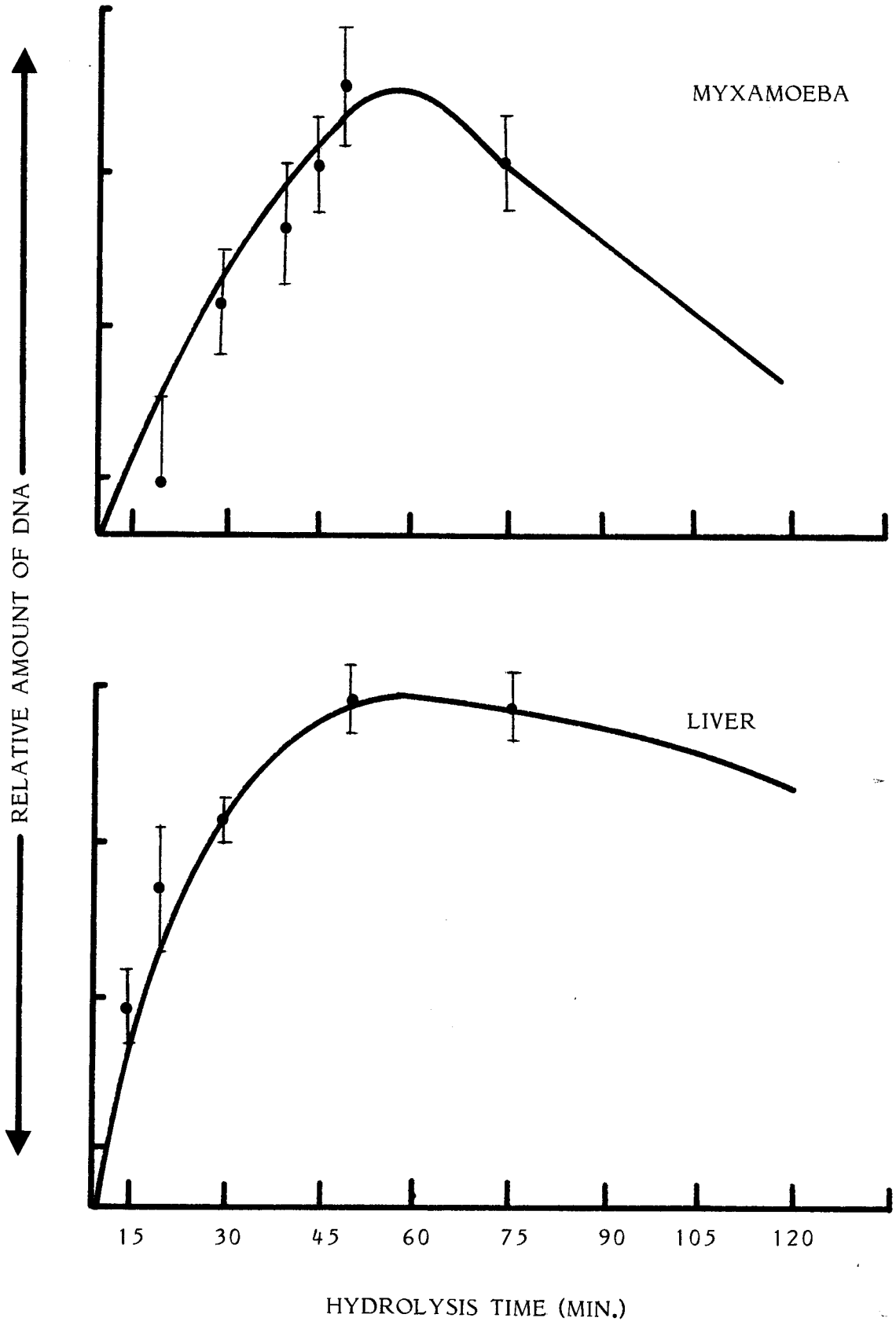

MYXAMOEBA
LIVER
RELATIVE AMOUNT OF DNA
15
30
45
60
75
90
105
120
HYDROLYSIS TIME (MIN.)

Figure 4

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


MYXAMOEBA
LIVER
RELATIVE AMOUNT OF DNA
15
30
45
60
75
90
105
120
HYDROLYSIS TIME (MIN.)

Liver

Liver cells fixed in bicarbonate formaldehyde stain maximally relatively quickly and demonstrate the steepest ascending slope of the four fixatives (Figure 4). The maximum stain intensity of 43.60 ± 1.86 is achieved at 50 minutes as was the case for myxamoebal cells. This maximum is considerably greater and persists longer than either that attained with the other three fixatives. The plateau of the bicarbonate formaldehyde fixative lasts for 25 minutes, ranging from the 50 to 75 hydrolysis time interval until a significant decrease at the 90 minute hydrolysis time period. Once again the maximum stain intensity for liver is greater than that for myxamoeba (43.60 ± 1.86 versus 3.65 ± 0.08 arbitrary units).

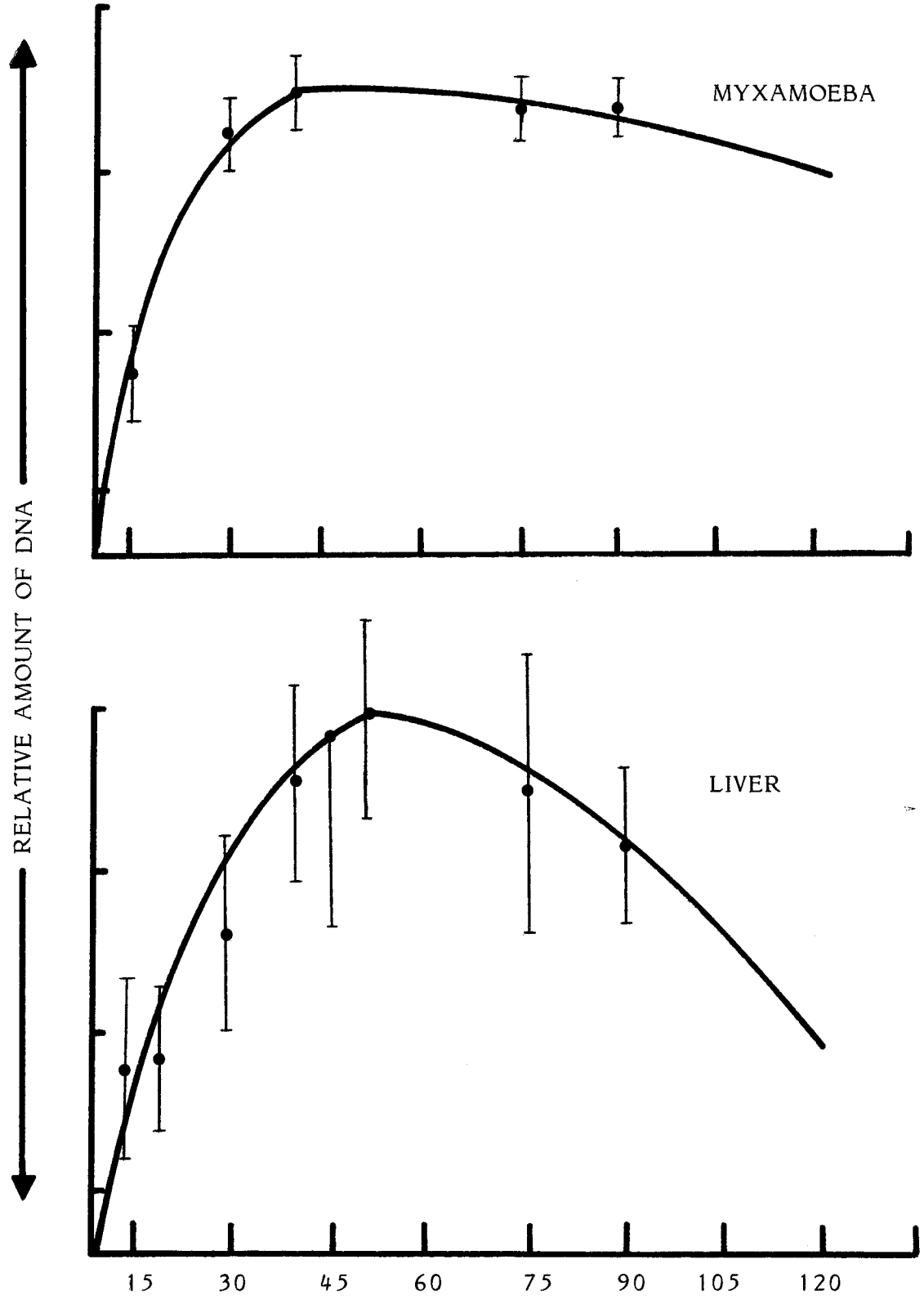
Calcium-Acetate-Formalin

Myxamoeba

The ascending slope of the hydrolysis curve (Figure 5) for the myxamoeba fixed in calcium-acetate-formalin rises rapidly, similar to the increase shown by ten percent buffered formalin. The maximum stain intensity, which occurred at 40 minutes, is 4.52 ± 0.10. This represents a much greater maximum than that achieved by the other three fixatives. There is also a slow decline in the staining intensity once the maximum is reached over a long period of time. Thus the plateau persists from the 40 to 90 minute hydrolysis times, longer than ten percent buffered formalin, twenty percent buffered formalin, and bicarbonate formaldehyde. Notice that for this fixative the maximum stain intensity is reached more quickly than for liver (at 40 rather than 50 minutes).

Figure 5

Hydrolysis curves representing Feulgen stained myxamoebal nuclei and liver nuclei fixed in calcium-acetate-formalin.


MYXAMOEBA
LIVER
RELATIVE AMOUNT OF DNA
15
30
45
60
75
90
105
120
HYDROLYSIS TIME (MIN.)

Liver

For the fourth fixative, calcium-acetate-formalin, the moderately steep ascending slope continues to a maximum stain intensity of 39.92 ± 3.19 that is reached at the 50 minute interval (Figure 5). This is followed by a sharp decrease in the hydrolysis curve and a significant decrease in stain intensity reached at the next time period of 75 minutes. However, as is the case for all the other three fixatives, the maximum reached for myxamoeba (4.52 ± 0.10) is not as great as that achieved for liver tissue (39.92 ± 3.19).

Statistical Analysis of Mean DNA Values

Tables I, II, III, and IV contain statistical data for both liver and myxamoebal nuclei. Inasmuch as one of the purposes of this study was to determine the optimum time for acid hydrolysis, it was necessary to determine the plateau periods by comparing the hydrolysis time of maximum stain intensity to that of the other hydrolysis times. For each tissue type and fixative, the two sample t-distribution analysis for detecting differences between means was used to signal significant differences between the mean DNA values plotted on the hydrolysis curves. Thus the time of peak stain intensity (the beginning of the plateau) and the period at the end of the plateau could be determined. These plateau periods indicate the hydrolysis times necessary to attain and maintain the maximum stain intensity. The data for these maximum stain intervals are presented in Table III.

The frequency distribution histograms of Figures 6 through 13 show significant differences between the initial period and that of the mean DNA values along with that at the end of the plateau. Although statistical analysis was not performed on the initial periods, this was not necessary for it is quite obvious that there were significant differences between the initial periods and

Table III

Maximum stain interval and maximum stain value for each fixative and tissue.

TISSUE TYPES	FIXATIVE	MAXIMUM STAIN INTERVAL	MAXIMUM STAIN VALUE
MYXAMOEBA	Ten Percent Buffered Formalin	30 - 50 minutes	3.98
	Twenty Percent Buffered Formalin	75 - 90 minutes	4.19
	Bicarbonate Formaldehyde	50 minutes	3.65
	Calcium-Acetate Formalin	40 - 90 minutes	4.52
LIVER	Ten Percent Buffered Formalin	50 minutes	40.60
	Twenty Percent Buffered Formalin	90 minutes	39.86
	Bicarbonate Formaldehyde	50 - 75 minutes	43.60
	Calcium-Acetate Formalin	50 minutes	39.92

that of the maximal periods.

Myxamoebal Nuclei

Statistical analysis of mean DNA values of myxamoeba fixed in ten percent buffered formalin show a 9 percent difference between the initial and peak hydrolysis times. However once the peak of 3.98 ± 0.69 is reached the maximum staining continues to the 50 minute interval since there is no significant difference between the peak time (at 30 minutes) and at 50 minutes as shown in Table I. Thus hydrolysis for maximum staining may occur within this 29 minute time lapse. The histograms of these time periods (Figure 6) also show only very slight shifts which serve to support the statistical findings.

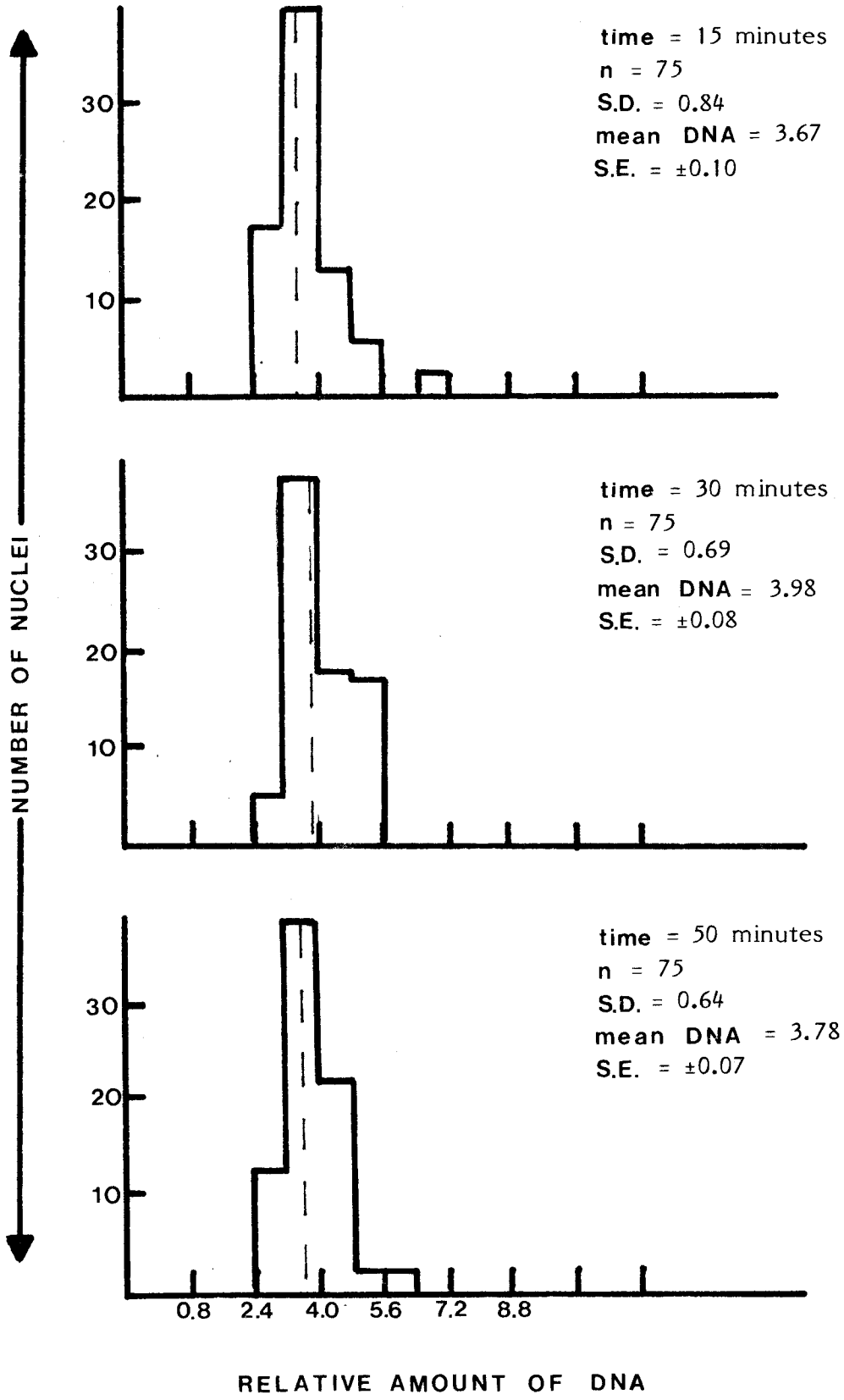
The histograms of mean DNA values of myxamoebal cells fixed in twenty percent buffered formalin are shown in Figure 7. The maximum value reached at 90 minutes is shifted to the right of the histogram of the initial hydrolysis time of 15 minutes. Since there is no significant difference between the maximum value reached at 90 minutes and the value attained at 75 minutes, maximum Feulgen staining may occur between these time periods.

The shift in the histogram of the 50 minute time interval is definitely to the right of the 15 minute hydrolysis time for myxamoeba fixed in bicarbonate formaldehyde. Statistical analysis (Table II) show that there is no significant difference between the maximum value (at 50 minutes) and that at the next interval of 75 minutes. Therefore hydrolysis for maximum Feulgen staining may continue through this 25 minute time interval. The histograms substantiating these findings are presented in Figure 8.

Histograms of mean DNA values for myxamoeba fixed in calcium-acetate-formalin are illustrated in Figure 9. The histograms of the maximum (at 40 minutes) is once again shifted to the right of the initial hydrolysis time

Figure 6

Histograms representing Feulgen-DNA values for myxamoebal nuclei fixed in ten percent buffered formalin.


time = 15 minutes
n = 75
S.D. = 0.84
mean DNA = 3.67
S.E. = ±0.10
time = 30 minutes
n = 75
S.D. = 0.69
mean DNA = 3.98
S.E. = ±0.08
time = 50 minutes
n = 75
S.D. = 0.64
mean DNA = 3.78
S.E. = ±0.07
NUMBER OF NUCLEI
30
20
10
0.8
2.4
4.0
5.6
7.2
8.8
RELATIVE AMOUNT OF DNA

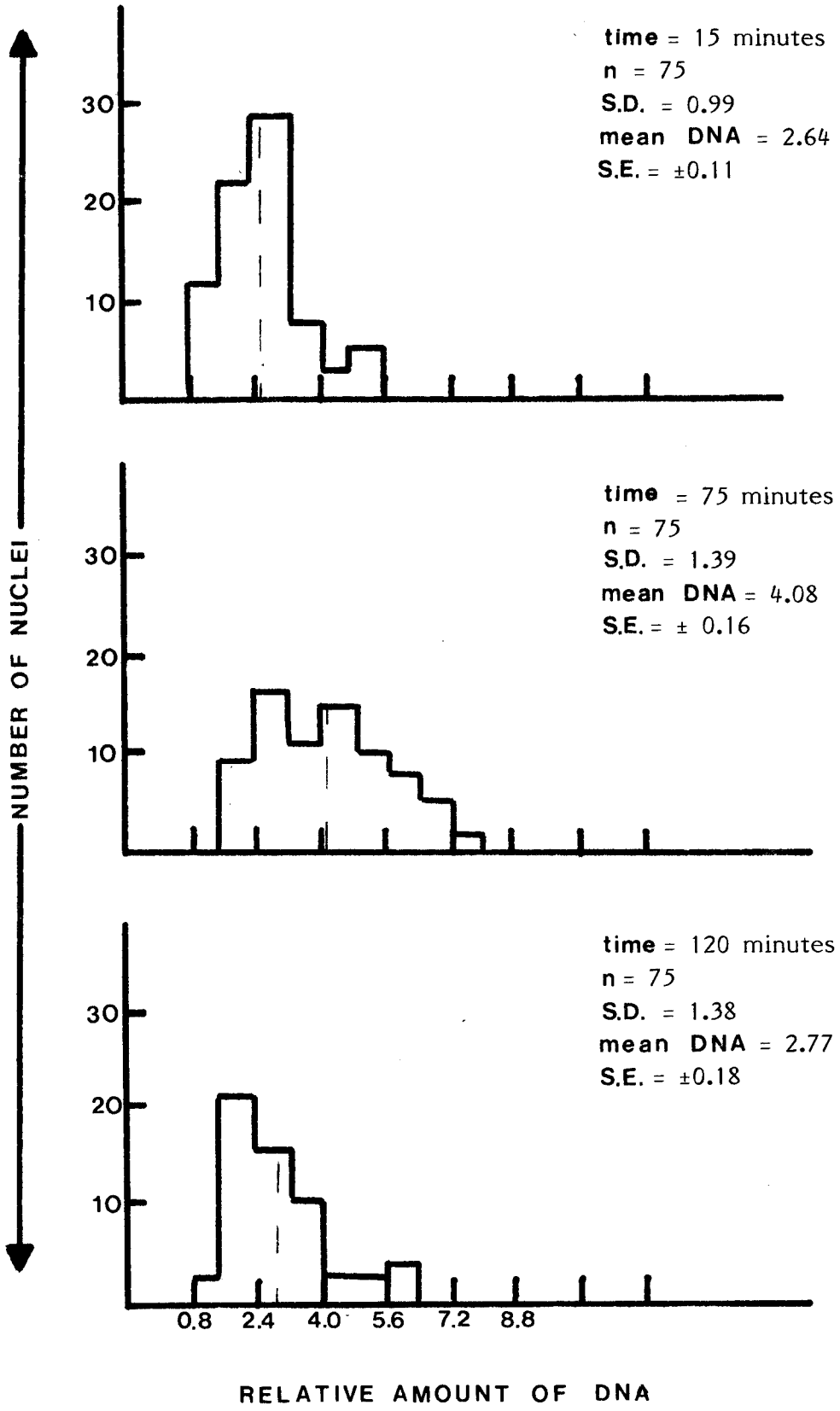

time = 15 minutes
n = 75
S.D. = 0.99
mean DNA = 2.64
S.E. = ±0.11
time = 75 minutes
n = 75
S.D. = 1.39
mean DNA = 4.08
S.E. = ± 0.16
time = 120 minutes
n = 75
S.D. = 1.38
mean DNA = 2.77
S.E. = ±0.18
NUMBER OF NUCLEI
30
20
10
0.8
2.4
4.0
5.6
7.2
8.8
RELATIVE AMOUNT OF DNA

Figure 8

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

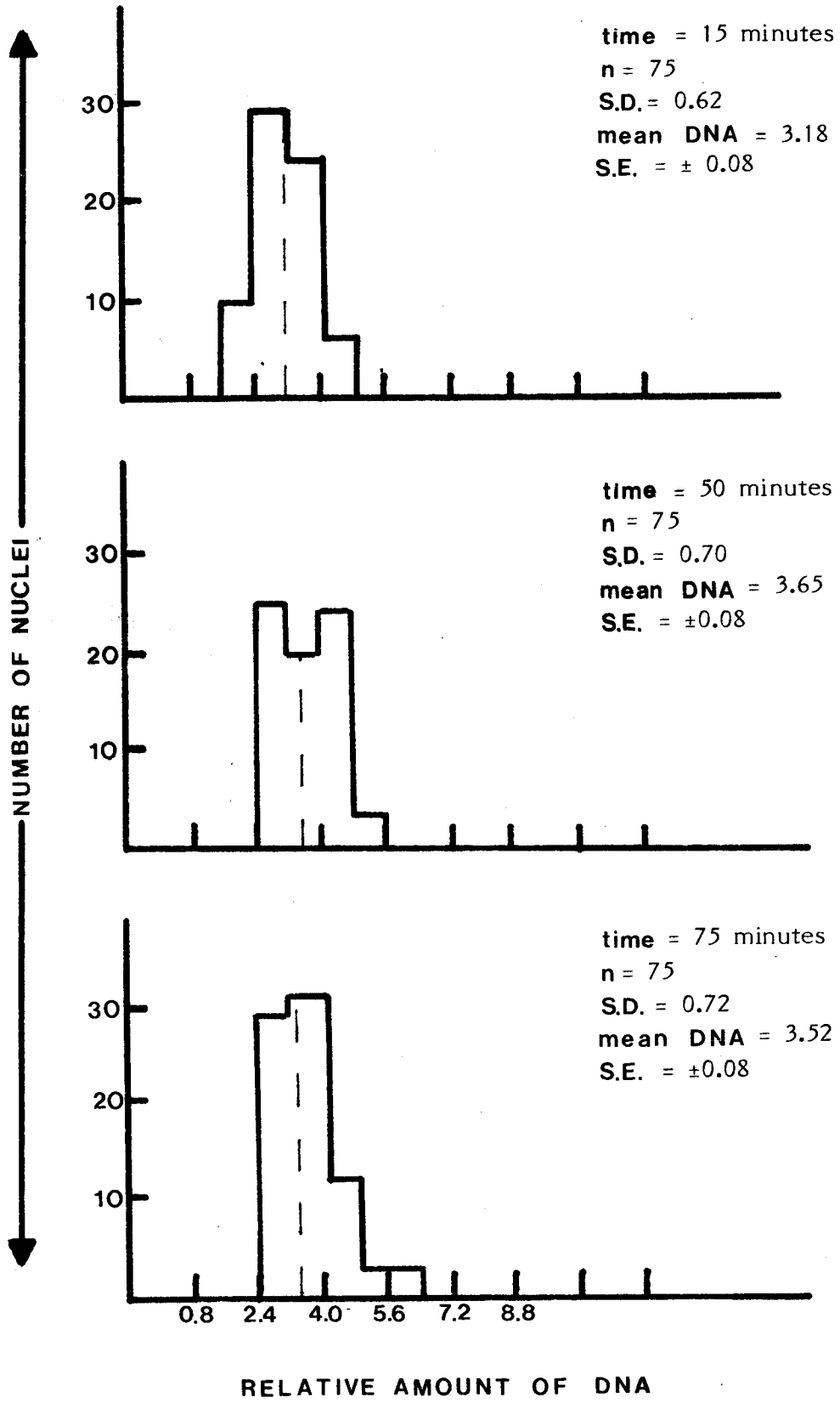
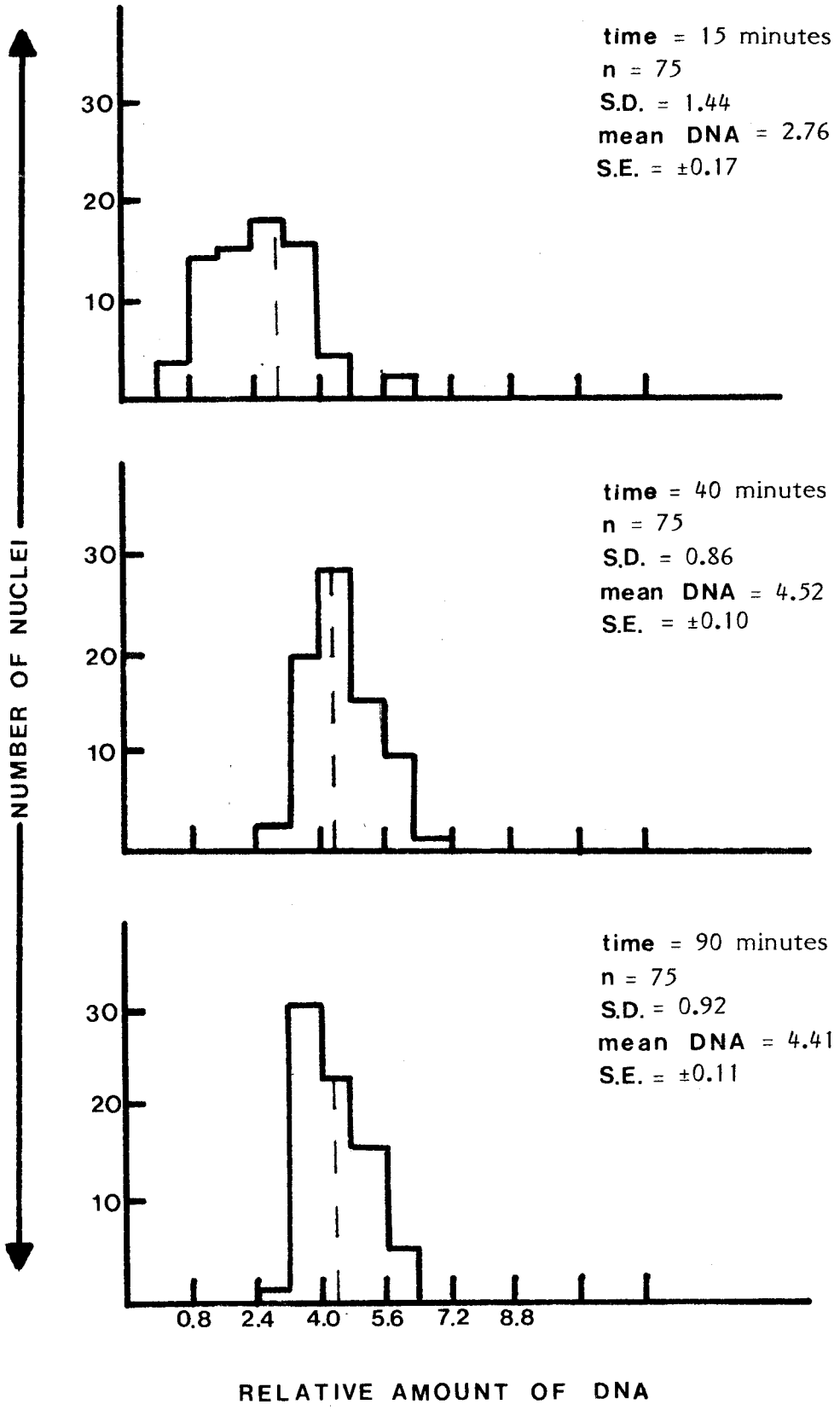

time = 15 minutes
n = 75
S.D. = 0.62
mean DNA = 3.18
S.E. = ± 0.08
time = 50 minutes
n = 75
S.D. = 0.70
mean DNA = 3.65
S.E. = ±0.08
time = 75 minutes
n = 75
S.D. = 0.72
mean DNA = 3.52
S.E. = ±0.08
NUMBER OF NUCLEI
30
20
10
0.8
2.4
4.0
5.6
7.2
8.8
RELATIVE AMOUNT OF DNA

Figure 9

Histograms representing Feulgen-DNA values for myxamoebal nuclei fixed in calcium-acetate-formalin.


time = 15 minutes
n = 75
S.D. = 1.44
mean DNA = 2.76
S.E. = ±0.17
time = 40 minutes
n = 75
S.D. = 0.86
mean DNA = 4.52
S.E. = ±0.10
time = 90 minutes
n = 75
S.D. = 0.92
mean DNA = 4.41
S.E. = ±0.11
NUMBER OF NUCLEI
30
20
10
0.8
2.4
4.0
5.6
7.2
8.8
RELATIVE AMOUNT OF DNA

of 15 minutes. Statistical data in Table I show no significant difference between the maximum, plateau or final hydrolysis values (at 90 minutes) which indicates a rather extended time period available for maximum stain binding.

Liver Nuclei

The Feulgen-DNA values for liver cells fixed in ten percent buffered formalin are graphically shown in Figure 10. The difference between the 15 minute hydrolysis time period and the remaining time periods are significant as shown by the pronounced shift of the 15 minute histogram from those of the remaining time periods. It should be remembered that the histograms demonstrate a plot of the relative amounts of DNA dye binding of a population of cells versus the number of nuclei representing these amounts. The Feulgen value at the maximum (50 minutes) is approximately 69 percent higher than that at 15 minutes which substantiates the shift. There is a 10 percent difference between the maximal value and the end of the plateau and is statistically significant. This indicates that the optimum hydrolysis time for maximum staining utilizing this fixative is rather precise.

Liver cells fixed in twenty percent buffered formalin do not exhibit a plateau until the 90 minute hydrolysis time (Figure 11). Once again, the difference in values recorded at the beginning of hydrolysis and the plateau are evident in the shifts of these histograms.

Treatment of liver cells with bicarbonate formaldehyde show an extended plateau with only a 1 percent difference between the maximum and plateau level. However, there is a very significant 84 percent difference between the plateau and initial hydrolysis times. The histograms of the maximum and plateau values are shifted greatly to the right of the 15 minute time

Figure 10

Histograms representing Feulgen-DNA values for liver nuclei fixed in ten percent buffered formalin.

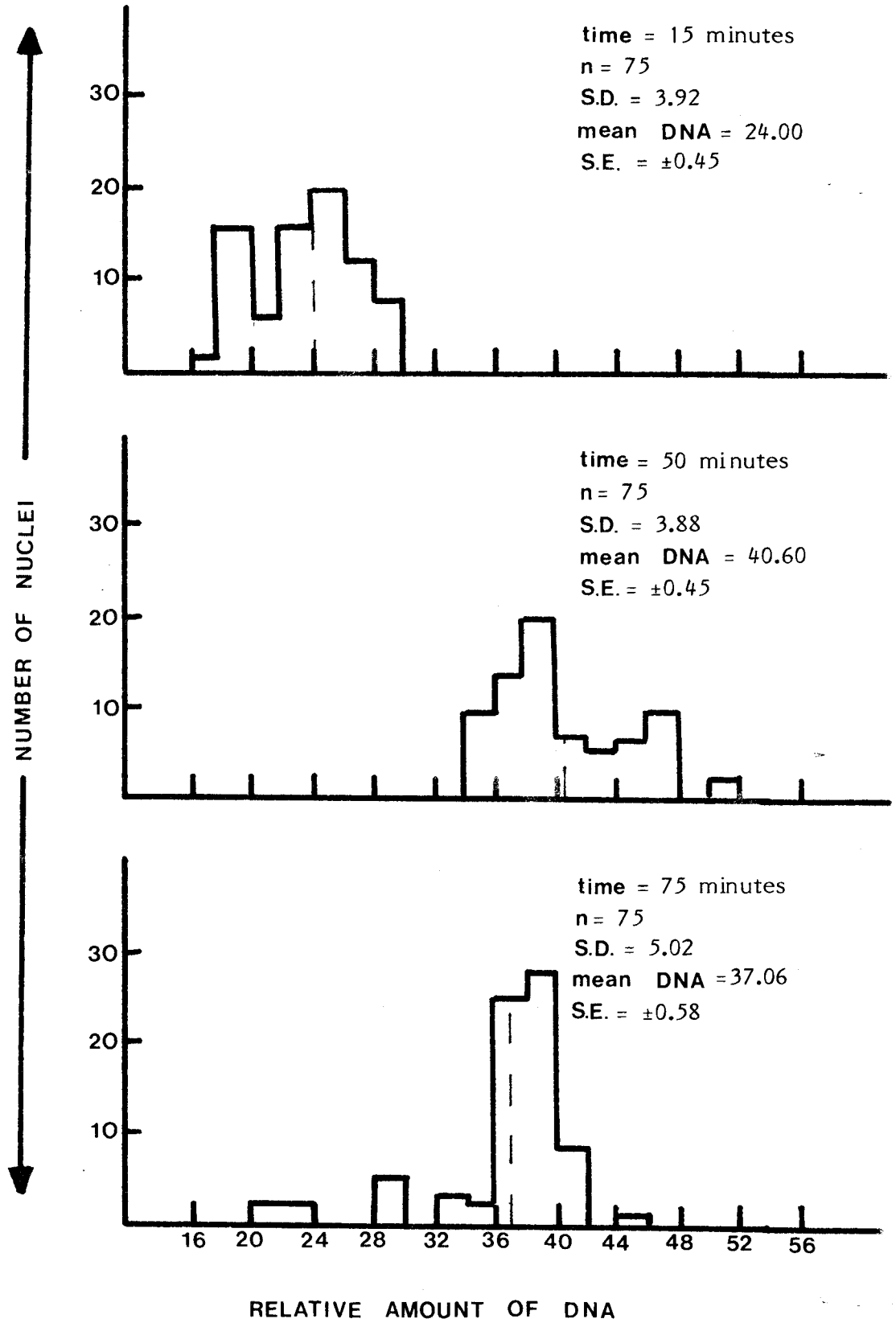
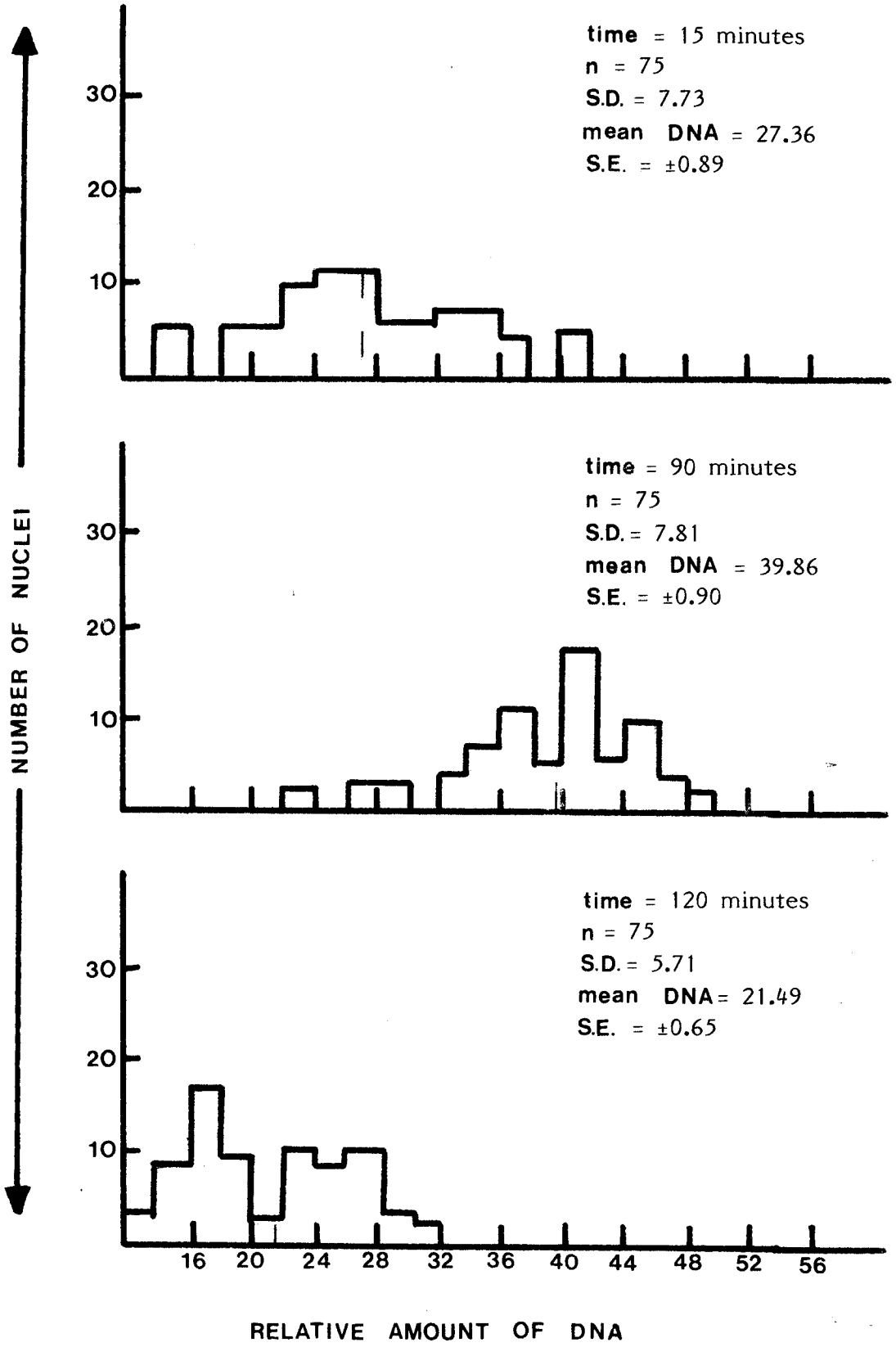

time = 15 minutes
n = 75
S.D. = 3.92
mean DNA = 24.00
S.E. = ±0.45
time = 50 minutes
n = 75
S.D. = 3.88
mean DNA = 40.60
S.E. = ±0.45
time = 75 minutes
n = 75
S.D. = 5.02
mean DNA = 37.06
S.E. = ±0.58
NUMBER OF NUCLEI
30
20
10
16 20 24 28 32 36 40 44 48 52 56
RELATIVE AMOUNT OF DNA

Figure 11

Histograms representing Feulgen-DNA values for liver nuclei fixed in twenty percent buffered formalin.


time = 15 minutes
n = 75
S.D. = 7.73
mean DNA = 27.36
S.E. = ±0.89
time = 90 minutes
n = 75
S.D. = 7.81
mean DNA = 39.86
S.E. = ±0.90
time = 120 minutes
n = 75
S.D. = 5.71
mean DNA = 21.49
S.E. = ±0.65
NUMBER OF NUCLEI
30
20
10
16 20 24 28 32 36 40 44 48 52 56
RELATIVE AMOUNT OF DNA

period (Figure 12). This shift represents approximately 20 relative mean DNA units between the initial and the maximum value.

Finally, liver cells treated with calcium-acetate-formalin show a similarity to those treated with formalin in that the period available for maximum stain intensity is relatively short. The maximum achieved at 50 minutes shows a statistically significant difference from that at the next interval of 75 minutes. This is equivalent to a 6 percent decrease in the relative amount of DNA dye binding. Once again these differences are apparent when comparing the histograms for this fixative (Figure 13).

Chi-square Analysis of Mean DNA Values

By performing a chi-square analysis it is possible to determine differences existing between the mean DNA dye binding values of maximal stain intensity of each fixative for each of the two different tissue types. According to the results shown in Table IV, for each case the observed frequencies in its chi-square vlaue, show that each fixative represents a comparable stoichiometric relationship to the chromatin despite differences in maximum staining intensity. Therefore, one may conclude that the choice of any of the four fixatives will accurately represent the relative dye binding for either liver or myxamoebal nuclei.

Figure 12

Histograms representing Feulgen-DNA values for liver nuclei fixed in bicarbonate formaldehyde.

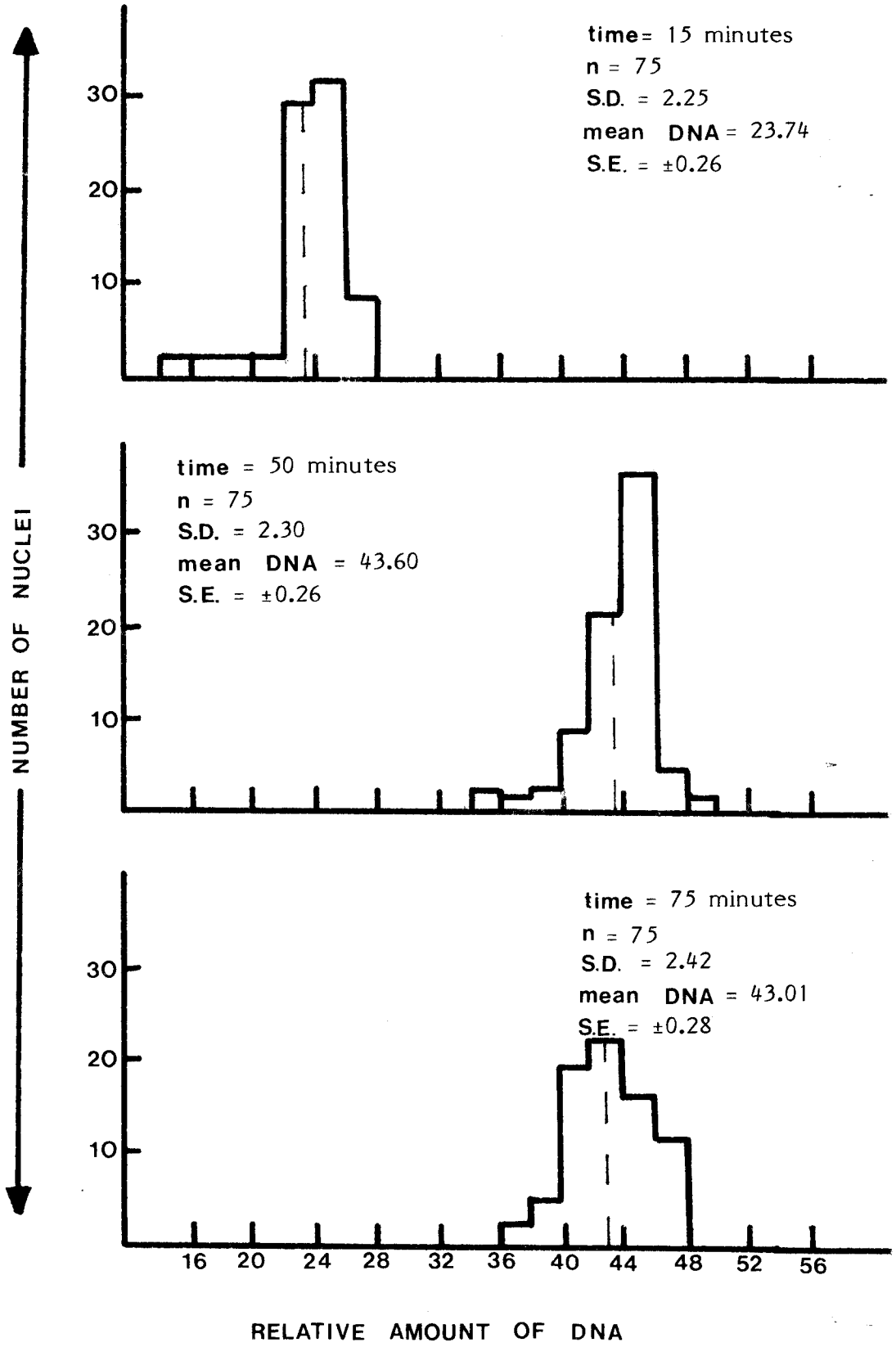

time = 15 minutes
n = 75
S.D. = 2.25
mean DNA = 23.74
S.E. = ±0.26
time = 50 minutes
n = 75
S.D. = 2.30
mean DNA = 43.60
S.E. = ±0.26
time = 75 minutes
n = 75
S.D. = 2.42
mean DNA = 43.01
S.E. = ±0.28
NUMBER OF NUCLEI
30
20
10
16 20 24 28 32 36 40 44 48 52 56
RELATIVE AMOUNT OF DNA

Figure 13

Histograms representing Feulgen-DNA values for liver nuclei fixed in calcium-acetate-formalin.

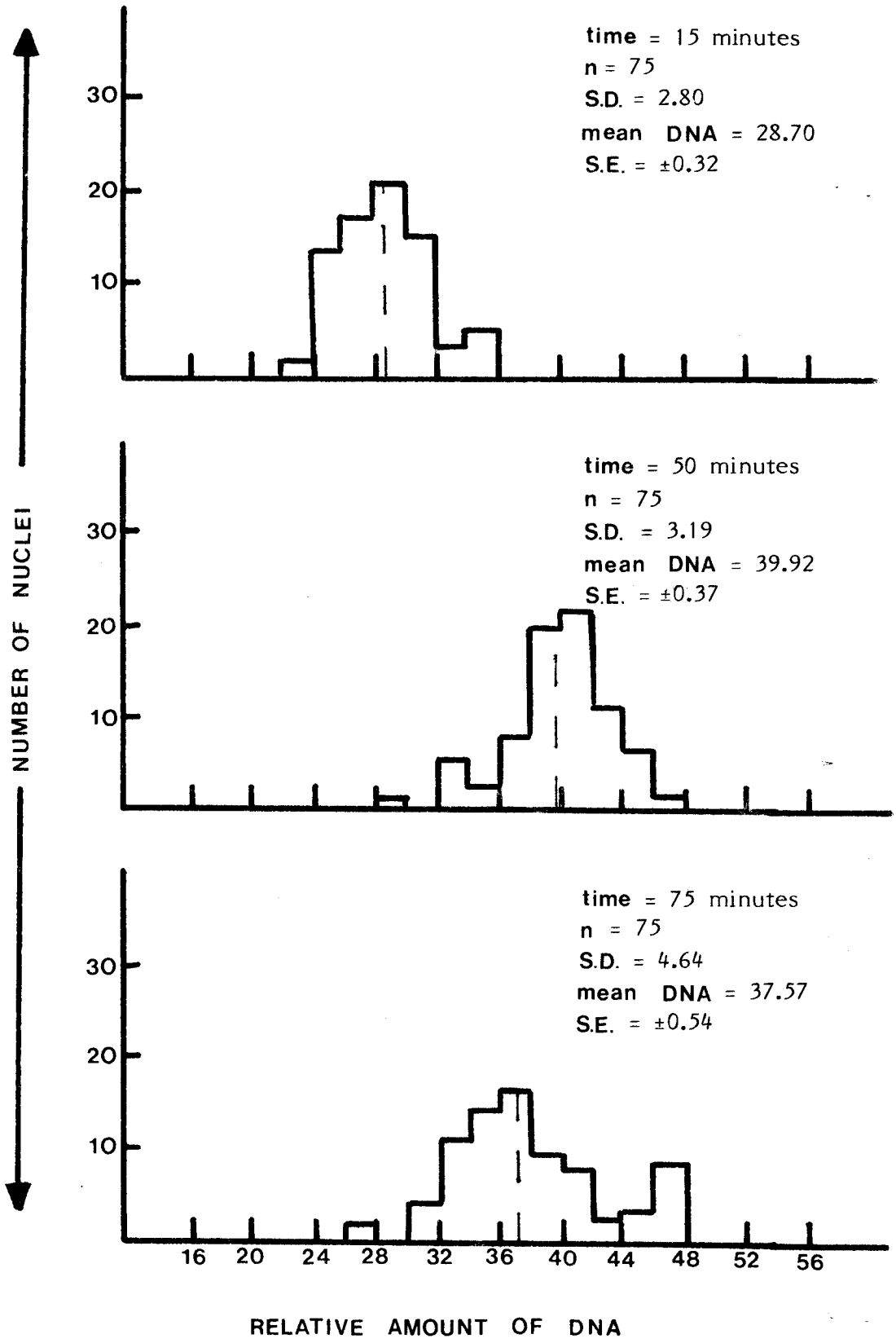

time = 15 minutes
n = 75
S.D. = 2.80
mean DNA = 28.70
S.E. = ±0.32
time = 50 minutes
n = 75
S.D. = 3.19
mean DNA = 39.92
S.E. = ±0.37
time = 75 minutes
n = 75
S.D. = 4.64
mean DNA = 37.57
S.E. = ±0.54
NUMBER OF NUCLEI
30
20
10
16 20 24 28 32 36 40 44 48 52 56
RELATIVE AMOUNT OF DNA

Table IV

Chi-square analysis of myxamoebal and liver mean DNA values at maximal stain intensities for four fixatives.

	Observed Frequency	Expected Frequency	Deviation	$\frac{\text{Deviation}^2}{\text{Expected}}$
MYXAMOEBA				
10% Buffered Formalin	3.98	4.085	-0.105	0.0027
20% Buffered Formalin	4.19	4.085	0.105	0.0027
Bicarbonate Formaldehyde	3.65	4.085	-0.435	0.0463
Calcium-Acetate-Formalin	4.52	4.085	0.435	0.0463
TOTALS	16.34	16.34	0	0.0980
LIVER				
10% Buffered Formalin	40.60	40.995	-0.395	0.0038
20% Buffered Formalin	39.86	40.995	-1.135	0.0314
Bicarbonate Formaldehyde	43.60	40.995	2.605	0.1655
Calcium-Acetate-Formalin	39.92	40.995	-1.075	0.0282
TOTALS	163.98	163.98	0	0.2289

df = 3 $\alpha = 0.05$

CHAPTER IV

DISCUSSION

Quantitative cytophotometric studies employing the Feulgen reaction have greatly improved since Mainland in 1933 measured the amount of dye bound per nucleus using a photometer consisting of five cards painted with a square of water color paint, by matching the color of the nuclei to the painted square. Later, quantitative determinations of cellular DNA were made on the basis of tissue weight (Swift, 1966). Since then, increasing importance has been placed on the microspectrophotometric evaluation of cytochemical color reactions (Patau, 1952). The development of ultraviolet microscopic spectrophotometric methods by Caspersson (1936) made it possible to analyze individual cells and cell parts for their chemical components (Leuchtenberger, 1952). Thus cytophotometric measurement of Feulgen-DNA in nuclei has provided a new approach to the intracellular level of quantitative analyses of DNA changes (Kasten, 1964) as evidenced in studies by Swift (1950, 1950, 1953), Swift and Rasch (1956), Swift, Rebhun, Rasch and Woodard (1956), Leuchtenberger (1958), Ely and Ross (1949), Lison (1960), and Pasteels and Lison (1950, 1951). Cytophotometry was employed in this study as an invaluable tool when coupled with the Feulgen reaction which meets the following requirements detremined by Kurnick (1955) for quantitation: 1) the absorbing material be specific for nucleic acids and localize it without diffusion, 2) the reaction be stoichiometric, and 3) the material be suitable for photometry.

Studies done in the past have indicated that the maximum stain intensity and therefore maximum absorption of the Feulgen stain, results

in a stoichiometric relationship with the DNA of the cell. This then permits the quantitative measurement of the chromophore by cytophotometric means. Quantitation is affected by several factors, among these are: 1) the length of hydrolysis (Ely, 1949; Patau, 1952; Hillary, 1939: Bauer, 1933; DiStefano, 1948; Tamm et al., 1952; Sibatani and Fukuda, 1953; Jordanov, 1963; and DeCosse and Aiello, 1966), and 2) the choice of fixative (Kotelnikov and Litinskaya, 1981; Swift, 1966; Deitch, 1967; Ely, 1949; Patau, 1952; Kurnick, 1955; Artvinli, 1975; Greenwood and Berlyn, 1968; Hillary, 1939; Hopwood, 1967; and Sibatani and Fukuda, 1953). The results of this investigation substantiate these observations and are confirmed by hydrolysis curves, histograms, and statistical analyses presented for both Feulgen stained liver and myxamoebal nuclei using four different fixatives, ten percent buffered formalin, twenty percent buffered formalin, bicarbonate formaldehyde, and calcium-acetate-formalin. Another factor, determined by this study to greatly influence the intensity of the Feulgen reaction is that of tissue differences. Although frequently mentioned in the literature (Ely and Ross, 1949; Patau, 1952; and Pöppe, Pellicciari, and Bachmann, 1979), this investigation employed Feulgen-DNA measurements for two distinct tissue types using quantitative cytophotometry for analysis in order to determine through the examination of hydrolysis curves generated if a given fixative can generally be used with any tissue type or whether a specific fixative (best fixative) must be used to produce a maximum stain intensity in different tissue types.

Observation of the hydrolysis curves generated (Figures 2 - 5) demonstrate in the case of each fixative an increase in the Feulgen-DNA values with increased time, followed by a plateau region of varying duration, dependent on fixative and tissue, and a subsequent decrease in Feulgen intensity with prolonged hydrolysis. The Feulgen reaction necessitates mild hydrolysis of DNA

with hydrochloric acid in order to facilitate dye binding. This procedure results in purine liberation from the DNA molecule (depurinization) by cleaving the glycosidic linkage between the sugars and purines (Feulgen and Rossenbeck, 1924). The result is the subsequent exposure of free aldehyde groups on the deoxyribose moiety. Consequently the DNA is converted into apurinic acid (Kasten, 1964). The overall shape of the hydrolysis curve depends on the fixative and preparative procedures (Deitch, 1967). The ascending slope of the hydrolysis curve represents a period of minimal hydrolysis where a part of the DNA remains untransformed into apurinic acid and therefore few aldehyde groups are available for dye binding (Jordanov, 1963). The absorption peak occurs during that period of hydrolysis when the number of exposed aldehyde groups is at a maximum (Greenwood, 1968). The maximum absorption plateau region was found by Deitch (1967) to be generally in the hydrolysis range between 30 - 60 minutes using different fixatives and tissues than that used in this study. Leveling of the curve at maximum stain intensity represents the points at which depurinization of DNA may proceed without decomposition and subsequent loss from tissue. DeCosse (1966), using one fixative and tissue type indicated the interval of 30 - 120 minutes to be the end of depurinization and the beginning of further degredation of the residual polymeric DNA complex. This study is in agreement with the hydrolysis times proposed by DeCosse and Deitch. Deitch (1967) found that maximum DNA values began to diminish to 75-90% of the maximum at 120 minutes. Andersson (1972) used labelling experiments to determine depurinization and depolymerization processes based on the measurement of dye molecules bound to DNA in accordance with Feulgen procedures. His contention was that the less steep downslope of the hydrolysis curve represented a slower depolymerization of DNA. Prolonged hydrolysis destroyed the Feulgen positive material

in the nucleus in his study and caused a decrease in optical density which approached zero when DNA was completely removed or inactivated; this data is in agreement with the work of Greenwood (1968). The change of the aldehyde groups to acid groups by long continued hydrolysis (Ely, 1949) would also account for a decrease in stain intensity as hydrolysis continues. In addition, Ely and Ross (1949) found that the diminution as hydrolysis proceeds was due to hydrolysis not only removing purines (necessary in order to expose aldehyde groups to Feulgen dye) but also the splitting off of deoxyribose which passes into solution. This study demonstrated that hydrolysis time is an important factor one must consider in order to ensure attainment of maximum Feulgen intensity. The time period for hydrolysis most conducive to maximum staining for both tissue types varied with tissue and fixative and centered around a 50 minute interval.

This present study is in agreement with other investigations indicating that the choice of fixative affects the Feulgen reaction. The results of liver fixation demonstrate that the best maximal stain intensity is observed when fixation by bicarbonate formaldehyde is carried out. In the case of myxamoebal nuclei, calcium-acetate-formalin produced the best results in the attainment and maintenance of maximal values when compared to the other fixatives. This clearly indicates a tissue specific difference with regard to fixatives. The maximal stain intensity (optimal staining) attained reflects the high stability of DNA using these fixatives under conditions of Feulgen hydrolysis procedures. Any nucleic acid conformational changes are minimal (Andersson and Kjellstrand, 1971, 1972, and 1975). Therefore the chromatin stability is dependent on the type of fixative employed prior to the staining procedure.

While myxamoebal cells fixed in ten percent buffered formalin do not demonstrate the highest maximum stain binding achieved, the maximum

staining is reached at an earlier time period (at 30 minutes) than for the other three fixatives (Table III). In addition a plateau conducive to maximum staining from the 30 through 50 minute hydrolysis time periods represents an interval comparable to that of the other three fixatives (Figure 2). Thus ten percent buffered formalin exhibits the ability to stabilize DNA and maintains maximum stain intensity over extended hydrolysis time. This is supported by Deitch, et al. (1967) who achieved longest hydrolysis plateaus with HeLa cells fixed for 18 - 24 hours. An extended plateau period of maximum Feulgen intensity is due to slower extraction of DNA depolymerization products by formalin (Andersson and Kjellstrand, 1975). Liver tissue fixed in ten percent buffered formalin achieved maximum staining at 50 minutes (Figure 10) which is also the case using bicarbonate formaldehyde (Figure 12) and calcium-acetate-formalin (Figure 13). The maximum stain value of 40.60 ± 3.88 (Table III) is not significantly different from that attained by twenty percent buffered formalin or calcium-acetate-formalin. Ten percent buffered formalin is recommended by most authors in studies involving the Feulgen reaction and quantitative cytophotometric studies because it penetrates tissues rapidly, is chemically stable, and is economical (Chambers, 1968). However, Greenwood and Berlyn (1968) argued that the use of formalin fixation in quantitative Feulgen-cytochemistry appears to have some pitfalls concerning the effect of formaldehyde on the resistance of DNA to hydrolysis. It is also important to note that paramount among these is the need to guard against overfixation which can cause the loss of detectable amounts of proteins and nucleic acids (Sibatani and Fukuda, 1953; Baker and McCrae, 1966; and Chambers, 1968) if carried on for extended periods of time. A survey of pertinent literature indicates a variety of optimum time periods of fixation ranging from 2 - 6 hours (Abramczuk, 1971) to a maximum of 18 - 24 hours is acceptable

by Naora, Matsuda and Sibatani (in Sibatani and Fukuda, 1953) in which more intense Feulgen staining was observed employing 20% formaldehyde than that after using 50% formaldehyde. Romakov (1965) reported an increase in formalin concentration from 1% to 4% resulted in an increased amount of bound formaldehyde. Possible explanations for achieving the maximum later using 20% buffered formalin in this study are probably due to the fact that formalin penetrates tissues rapidly but appears to fix slowly. Ericsson's work (1967) supports this contention. Also, Jordanov (1963) found that formaol-containing fixatives preserve a maximal Feulgen reaction for up to four hours of hydrolysis.

Although bicarbonate formaldehyde produced maximal staining (43.60 ± 2.30) and a prolonged plateau (for 30 minutes) for liver tissue, the results for myxamoebal tissue demonstrate the lowest maximum stain value of 3.65 ± 0.08 and the shortest plateau of the four fixatives (Figure 4) strongly supporting the hypothesis that tissue differences strongly influence the entire staining process. The favorable results attained in this study using bicarbonate formaldehyde for liver were also observed and explained by several other investigators. Bicarbonate formaldehyde is a suitable fixative because it only slightly denatures proteins (Hopwood, 1967; Grillo et al., 1971; and Hopwood, 1971), shrinkage is minimal (Artvinli, 1975) and the solubility of tissue proteins is decreased. Experiments on protein fixation reveal bicarbonate formaldehyde has a high fixation rate (Cantarow and Shepartz, 1967) due to bicarbonate ions penetrating cells readily and acting as a buffer during the liberation of hydrogen ions resulting from an interaction between formaldehyde and the free amino groups of proteins. Artvinli (1975) noted two advantages of bicarbonate formaldehyde: 1) formalin-protein interaction becomes irreversible, and 2) the number of formalin molecules decreases continually

(Deitch, 1967). In this study a fixation period of 24 hours was employed and demonstrated consistently acceptable results, with no appreciable loss of DNA.

Both myxamoebal and liver cells fixed in twenty percent buffered formalin exhibit similar hydrolysis curves (Figure 3) in that each achieves maximum absorbance considerably later than that for the other three fixatives (at 75 minutes for myxamoeba and 90 minutes for liver). The maximum value for myxamoeba of 4.19 ± 0.12 (Table III) using this fixative is greater than that achieved with either ten percent buffered formalin or bicarbonate formaldehyde. However liver cells fixed in twenty percent buffered formalin achieved the lowest maximum of the four fixatives (Table III). Once again this indicates a tissue specific difference concerning the use of fixatives. Baker (1966) found that the fixation results were similar whether the formalin concentration was 1%, 4%, or 16%. While the results of this investigation showed similar Feulgen-DNA values for cells fixed in both concentrations of buffered formalin, the cells fixed in the higher concentration (20%) reached the maximum value much later, 50 versus 90 minutes for liver and 30 versus 75 minutes for myxamoeba (Figures 2 and 3). Deitch (1967) using lymphocytes, found that hydrolysis was not attained until 90 minutes when fixed in formalin. On the other hand, Dutt (1970) reported higher Feulgen-DNA values in rat liver fixed in 40% formaldehyde than in ten percent formaldehyde whereas the present study demonstrated a slightly lower Feulgen-DNA value for liver and a slightly higher Feulgen-DNA value for myxamoeba fixed in the increased concentration of formalin. Dutt explained these findings citing the possibility that greater denaturation of DNA molecules takes place when fixation is carried out using 40% buffered formalin than by 10% buffered formalin and more polymerized DNA-protein complex retains a larger proportion of aldehyde, giving rise to enhanced staining. However, Dutt's findings contradicted the results obtained

through binding of the molecule to the protein that maintains the difference between concentration of formalin in the tissue and fixative solution thus preventing a deceleration of the diffusion rate.

Myxamoebal nuclei fixed in calcium-acetate-formalin produced the best results in the attainment and maintenance of maximal values when compared to the other fixatives. The maximal stain intensity (optimal staining) attained reflects the high stability of DNA using this fixative under conditions of Feulgen-hydrolysis procedures. The maximum stain value of 4.52 ± 0.86 arbitrary units persisted the longest for a total of 50 minutes (Figure 5). In the case of liver fixed in calcium-acetate-formalin the maximum stain value of 39.92 ± 3.19 is less than that of bicarbonate formaldehyde but similar to that of 10% buffered formalin and 20% buffered formalin. However the plateau reached at 50 minutes does not persist, and provides a very critical peak period. The success achieved using calcium-acetate-formalin on myxamoeba can be explained by the ability of calcium salts to decrease the solubility of tissue proteins during acid hydrolysis (Artvinli, 1975). Fixatives similar to calcium-acetate-formalin have been shown to decrease or inhibit the dissolution of DNA during acid hydrolysis (Sibatani and Fukuda, 1953). In addition to the ability of this fixative to preserve the integrity of DNA it has also been shown to be a good fixative for preserving enzyme activity (Eapen, 1960) with the exceptions of alkaline phosphatase and aminopeptidase (Baker, 1944).

One of the main areas of emphasis in this study was to determine the effect of fixatives used on widely diverse tissue types in order to determine whether a given fixative can generally be used with any tissue type or whether a specific fixative must be used to produce maximum stain intensity for distinctly different tissue types. The findings of this study indicate that

different fixatives appear to be most compatible regarding different tissues. For example, myxamoebal cells fixed in calcium-acetate-formalin produced the highest maximal stain value and the longest plateau while liver cells fixed in calcium-acetate-formalin produced one of the lowest maximal stain values and plateau periods. In addition bicarbonate formaldehyde produced the maximum stain intensity and longest plateau for liver cells but the lowest maximum stain value and shortest plateau for myxamoebal cells. Variations in the ratio of purine to pyrimidine in different nucleic acids would cause differences in the amount of deoxyribose uncovered for reaction with leuco-basic fuchsin (Kurnick, 1955). Therefore, one would not expect similar Feulgen intensities from two widely diverse cell types when using the same fixative because of differences in the type and amount of DNA along with the manner in which the particular nucleic acid interacts with the fixative in producing a characteristic stearically altered molecular configuration for dye bonding. By examining the hydrolysis curves for liver tissue and myxamoebal tissue for each particular fixative, the differences are illustrated by comparison of the shapes and height of the curves (Figures 2 through 5).

Evidence presented in this study indicates that in order to achieve optimal conditions which will permit quantitative cytophotometric analysis of Feulgen or nuclear DNA, several important criteria must be met: the fixative used, length of fixation, length of hydrolysis time, and persistence of the critical maximum staining plateau regarding the tissue type under investigation. This study reports the effects of four fixatives on Feulgen staining intensity of two distinctly different cell types and implies similar results for any other tissue type using these fixatives, a problem that has not been adequately investigated in the past. Although the fixatives in this study differ in the maximal staining intensities obtained, they all may be

used to quantify relative amounts of nuclear DNA present. Haselkorn and Doty suggested this in 1961. Evidence presented in this paper has adequately demonstrated this. However if intense staining coupled with a prolonged hydrolysis plateau is desired, the results indicate that the choice for mammalian liver tissue would be bicarbonate formaldehyde while calcium-acetate-formalin for myxamoebal nuclei would be appropriate. Finally one must also take into account the cellular systems used in quantitation because of the fixative effects on the Feulgen nuclear reaction in order to obtain accurate results.

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