THE EFFECTS OF VARIOUS HYDROLYSIS TIMES AND FIXATION ON THE INTENSITY OF THE FEULGEN REACTION IN STUDIES INVOLVING QUANTITATIVE MICROSPECTROPHOTOMETRY

by

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ABSTRACT

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The effect of three different fixatives, ten percent buffered formalin, Lillies alcohol-acetic acid-formalin, and a new fixative, bicarbonate formaldehyde, on the staining intensity of the Feulgen nucleal reaction for both liver and myxamoebal nuclei was analyzed by means of the two wavelength method of microspectrophotometry. Comparisons of the relative DNA measurements of each procedure were made to determine the reaction parameters best suited to each tissue type in providing maximal staining intensity values.

Fixation of liver nuclei in bicarbonate formaldehyde provided the best maximal staining intensity of all three fixatives, while fixation in ten percent buffered formalin yielded the best maximal intensity for myxamoebal nuclei. Identical maximal stain intensity values were obtained for amoebal nuclei fixed in both Lillies AAF and bicarbonate formaldehyde with the hydrolysis curves for each exhibiting extended plateau periods as opposed to a significant decline of plateau in fixation with ten percent buffered formalin. An extended period of hydrolysis before attainment of maximal stain intensity characterized

the effect of Lillies AAF on liver nuclei, while fixation in ten percent buffered formalin resulted in an extremely prolonged plateau period and in early attainment of maximal intensity, as was the case with bicarbonate formaldehyde fixation. The maximal stain intensity values for fixation of liver nuclei in ten percent buffered formalin and Lillies AAF were closely related and fell well below the value obtained with fixation in bicarbonate formaldehyde. In the case of both liver and myxamoebal nuclei, fixation with Lillies AAF resulted in extreme nuclear and cytoplasmic shrinkage and should be regarded as a relatively harsh fixation procedure on tissue preparations.

Statistical analysis of the mean relative DNA values representing the periods of maximal stain intensity of the three fixatives for each cell type indicated that no significant differences exist between these values. Therefore, each procedure can provide both a stoichiometric relationship and valid comparable values of relative amounts of DNA in view of the specific effect each fixative has in determining the resultant configuration of the nucleic acid for dye-binding.

It is evident from these experiments that the fixatives and hydrolysis times selected for the performance of a valid and quantitative Feulgen DNA determination must be chosen properly for each specific tissue type since differences in staining intensity may occur.

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LIST OF SYMBOLS

SYMBOL	DEFINITION	UNITS OR 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 the 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	
X	Mean DNA values	
λ	Wavelength	nm
Σ	Summation of the mathematical terms that follow	
σ	Standard deviation	
α	Confidence interval	

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INTRODUCTION

In recent years research dealing with the cell cycle has become increasingly important to the cell biologist, and, hence, to the biological world as a whole. Such studies have often been concerned with the elucidation and characterization of biochemical changes which occur during the development and differentiation of cells. These changes, of course, invariably are accompanied by nuclear changes most often reflected by changes in DNA. In this respect, widespread use of the Feulgen nucleal reaction has become increasingly important, and, thus, a substantial amount of literature has accumulated concerning the necessary conditions for valid analysis of nuclear changes in relation to nuclear DNA. These conditions include the effects of preparative procedures, i.e., fixation, and the measures employed in the staining procedure to provide a quantitative index of cellular deoxyribonucleic acid (DNA) content.

The specificity of the Feulgen reaction for DNA has been either supported by or challenged by investigators in numerous studies. At present, most cytochemists contend that under proper conditions with appropriate controls the reliability of the reaction for localizing DNA within the cell can be universally accepted as a valid procedure. However, skepticism as to the justification of the quantitative phase of the stain-

ing methodology has arisen since the intensity of the reaction has been shown to be influenced considerably by many factors involved in the reaction procedure. Ely and Ross (1949) argued that computations of the total or absolute amount of DNA within a nucleus cannot be made as hydrolysis allows for the loss of a certain amount of stainable material. Swift (1956) contended that in view of the constancy of DNA in the cells of a species, an equal loss of stainable material would occur in all cells examined if simultaneously subjected to the same reaction procedure. DNA measurements of these cells would not represent absolute values, but, rather, comparable relative values with respect to the constant amount of material remaining after loss. In addition, most early quantitative work utilized inadequate analytical techniques (gross analysis of nuclear suspensions) which only provided average amounts of DNA for large numbers of nuclei, thereby, limiting the nature and adequacy of cytochemical investigation. In compensating for the inherent factors which may influence staining intensity, and by adhering to the requirements for valid photometric measurements (Caspersson, 1936), Swift (1956) postulated an alternative method (to inadequate gross analysis) in which relative amounts of DNA per single nucleus could be spectrophotometrically determined. Such analysis could be accomplished if the following conditions were met: 1) results must be reproducible, 2) measured extinctions in photometric determinations are directly proportional to the number of dye molecules present throughout the range of concentration studied, and, 3) that there be a

constant relationship between the number of dye molecules and the absolute amount of DNA. As evidence of the validity of the quantitative cytophotometric method employed, Swift, in measuring the DNA content of mice and other animal nuclei, showed a 2:1 ratio of nuclear DNA content between murine somatic nuclei and murine spermatid nuclei, respectively. This ratio was in complete agreement with the estimation by suspension count and computation of the amount of DNA per nucleus by Boivin, Vendrely, and Vendrely (1948) and Vendrely and Vendrely (1948, 1949) in their analysis of bovine somatic and spermatid nuclei. To further substantiate the contention of accuracy in quantitative microspectrophotometry, Ris and Mirsky (1949), using several different species of animals, obtained two sets of nuclear DNA values for each specific group, which, when compared, fell within ten percent of one another when the nuclei were measured by both biochemical and spectrophotometric methods. These and other microspectrophotometric analyses of the Feulgen nucleal reaction (Pollister et al., 1949; Moore, 1951) have overcome the skepticism of the quantitative capability of the staining procedure by indicating that the Feulgen reaction can actually serve as a measure of the relative DNA content of cell nuclei. In independent research, Patau (1952) and Ornstein (1952) established the validity of the two wavelength method of microspectrophotometry which was utilized exclusively in this study.

In his work establishing the validity of quantitative microspectrophotometry, Swift (1956) concurred with the obser-

vations of other investigators which indicated that inherent factors of the cytochemical procedure may influence the staining intensity of the reaction. He pointed out that, "Indications were noted of the variability of the reaction with slight changes in the staining procedure, and, ...different fixatives influenced the intensity of the regenerated fuschin obtained". The validity of the means of obtaining quantitative measurements were no longer in doubt, but, rather, the methodology used to obtain optimal stain intensity to accurately represent the total DNA content of cell nuclei.

Fixatives and hydrolysis procedures are two factors known to influence the staining intensity of the Feulgen nucleal reaction by their direct interaction with nuclear DNA. Fraenkel-Conrat (1954) and Sauerbier (1960), in their work with the formalin-induced inactivation of the RNA tobacco mosaic virus and the DNA phage T_1, respectively, indicated that inactivation resulted as a function of fixative binding to the amino groups of the nucleic acid bases. Other investigators have been able to show in both in vitro and in vivo studies that fixatives in their fixation processes do interact with polynucleotides and nucleic acids proper (Haselkorn and Doty, 1961; Romakov, 1965). Changes in the physical state of nucleic acids occur using any method of fixation (Pearse, 1968). In view of this, the choice of nuclear fixatives for quantification studies should center on those which only slightly alter the nucleic acid's conformation in producing maximal chromatin stability.

Acid hydrolysis procedures have been shown to contribute to the loss of Feulgen intensity through depolymerization of DNA in the latter part of these procedures. The extent of acid degradation of chromatin and the subsequent loss of the material is dependent upon the concentration of acid, the temperature involved and the stability of the chromatin. Itikawa and Ogura (1953), Jordanov (1963), and DeCosse and Aiello (1966), noted that 5N hydrochloric acid hydrolysis at room temperature as a substitute for the more conventional "hot" hydrolysis in 1N hydrochloric acid at 60 degrees C prolonged the period of maximal Feulgen intensity. In their studies of acid-induced depolymerization of DNA, Andersson and Kjellstrand (1971, 1972, 1975) reported that the exposure of purine bases and the elimination of the aldehydes (stainable groups) through DNA depolymerization (two factors responsible for hydrolysis curves) are dependent to a considerable degree on chromatin stability.

In view of this, the best fixatives for Feulgen reaction procedures would be required to render high chromatin stability toward acid hydrolysis, and, thus, allow for the maximal Feulgen intensity possible and the persistence of maximal values over extended periods of hydrolysis. In Feulgen microspectrophotometry, formalin fixation of tissue has been recommended by several authors to accomplish such objectives (Kurnick, 1955; Ruch, 1966; Swift, 1966). Formalin fixation involves the formation of a net-work between fibrous proteins of the cell nuclei, and, as previously mentioned, the binding of the fixative direct-

ly to amino groups of DNA bases, thereby, stabilizing the molecule to a greater degree than most fixatives. The ability of formalin to stabilize chromatin was evidenced in the works of Greenwood and Berlyn (1968) in which formalin treated DNA greatly resisted DNase activity and extraction by mineral acid. It was suggested that such resistance may be due to the denaturation of DNA molecules and the maintenance of the denatured state by formalin fixation. Such stability would, therefore, allow for slower DNA depolymerization resulting in more DNA available for dye combination and extended hydrolysis periods giving optimal Feulgen intensities. As a result of these findings, studies were undertaken to determine the ability of formalin and other nuclear fixatives to effect such occurrences.

Estimations of acid depolymerization of DNA and the loss of the latter from tissue as the resultant effect of various concentrations of formaldehyde (Sibatani and Fukuda, 1953; Baker and McCrae, 1966; Dutt, 1971) have produced directly opposing views in regard to the best concentration of fixative for Feulgen staining procedures. Other studies have contrasted ten percent neutral buffered formalin with a variety of commonly used fixatives considering factors of penetration rates and modes of fixation (Hillary, 1940; Ericsson and Beberfeld, 1967; Hopwood, 1967; Artvinli, 1972). Deitch et al. in 1967 made a comprehensive examination of the collective effects of different fixatives, hydrolysis times, temperatures and acid concentrations on several distinct cell types to

determine the reaction parameters best suited to producing optimal Feulgen intensities.

In view of this, the objectives of this investigation were twofold: 1) to determine the best combination of fixation procedure and hydrolysis periods employed in order to obtain optimal staining intensity, and, 2) to determine the effects of these parameters on different tissue types. To accomplish these objectives, absorption microspectrophotometry was employed to measure nuclear deoxyribonucleic acid content of both myxamoebae and liver tissue groups fixed in 1) ten percent buffered formalin, 2) Lillies alcohol-acetic acid-formalin, and, a new fixative, 3) bicarbonate formaldehyde.

MATERIALS AND METHODS

Tissue Culture

The Honduran a-2A[2] isolate of Didymium iridis used in this investigation was supplied by Dr. O. R. Collins, Department of Botany, University of California, Berkeley. Mammalian liver tissue utilized was excised from the albino rat, Rattus rattus, supplied by the Holtzmann Laboratory Animal Supply Company, Wisconsin.

Growth of D. iridis on Solid Media

The myxamoebal clone of Didymium iridis was maintained on the bacterium Escherichia coli, on slants of half strength Difco corn meal agar (CM/2). The clone used throughout this study was originally propagated from a single spore isolate.

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 dispensed in 15 ml aliquots to sterile plates and refrigerated until required.

Portions of the myxamoebal isolate were transferred from the stock culture slants to several plates of CM/2

which had been previously inoculated with an E. coli suspension. The bacterial suspension was initially prepared by washing bacterium from a 48 hour old E. coli nutrient agar slant culture with 10 ml of sterile distilled water. A final bacterial inoculum was then formed by adding 2 ml of the bacteria-water suspension to a culture tube containing 5 ml of sterile distilled water. Three drops of this dilute inoculum was spread over the entire media surface by means of a sterile glass rod. The inoculated media plates were then incubated in an inverted position in a 21 degree C incubator for 24 hours to ensure bacterial lawn growth and were subsequently innoculated with the myxamoebae.

The first of the myxamoebal isolate transfers were allowed to attain log phase growth and were then transferred to several other CM/2 plates which had been prepared in the manner previously described. Subcultures of the clone to fresh media were made every 3-5 days post-transfer. All amoebal cultures were maintained in an incubator at 21 degrees C (Yemma and Therrien, 1972; Yemma et al., 1974). Sterile technique was implemented throughout all transferring procedures to prevent contamination of the cultures by other micro-organisms. Culture plates were examined microscopically for contamination, and, if present, the plates were discarded.

Preparation of Material for Cytochemical Study

Myxamoebae

Harvest of Cells

Although three different fixatives were employed in this study, (ten percent buffered formalin, Lillies alcohol-acetic acid-formalin, and bicarbonate formaldehyde), the method of collecting or "harvesting" the log phase amoebal cells for fixation remained the same in each case.

Ten ml of sterile room temperature 0.25M Sucrose Phosphate Buffer (PBS) was added to each of the approximately 75 culture plates used per preparation. Cells were then "washed" from the media surface with the PBS buffer. Forceful washing was avoided since it not only resulted in the removal of the media-associated cells, but, also in the removal of unwanted bacterial plaques. The washings were collected in a sterile flask and were spun down as 20 ml aliquots in 50 ml conical centrifuge tubes at 70X gravity for 20 minutes in an Adams Dynac swinging bucket centrifuge (Yemma, 1971). The supernatant, consisting mainly of bacteria, was discarded. The remaining pellets were then resuspended in additional buffer-cell solution and re-centrifuged. The process was repeated until a final packed volume of 0.5 to 1 ml of amoebal cells was attained. The final pellet was washed several times using additional 0.25M PBS.

The phosphate buffer employed in this study was prepared by dissolving 3.0 grams of potassium chloride, 2.32 grams of sodium chloride, 9.04 grams sodium phosphate, dibasic and 18.4 grams potassium phosphate, monobasic in 4 liters of distilled water. This preparation resulted in a 50 mM phosphate buffer at pH 6.5. Occasionally, the pH of the buffer fell below pH 6.5 and was adjusted to the appropriate level using 0.1N NaOH.

Microscopic observation of the final pellet indicated that most, but not all, of the bacteria was discarded in the supernatant. In order to remove the remaining pellet-associated bacteria, the pellet was re-suspended in 2 ml of 10 percent PBS (w/v) and layered over 2 ml of 30 percent PBS (w/v) in 15 ml conical centrifuge tubes, and was centrifuged at 42X gravity for 25 minutes. The bacteria appeared at the gradient interface in the 10 percent layer as a white band and the amoebae as a dark yellow pellet. The procedure was repeated until the white band of bacteria was no longer visible. The pellet was then washed once again with 0.25M PBS and centrifuged at 70X gravity for 20 minutes to give a relatively bacteria-free preparation of amoebal cells. Centrifugation procedures appeared to have caused any swarmer cells present to assume the amoeboid condition so that the majority of cells present in the final pellet were myxamoebae. Cells of the final clean pellet were used for cytochemical analysis.

Liver

Tissue Preparation

The experimental animal was killed by placing it in a closed chloroformed chamber. The liver was immediately excised, rinsed twice in cold Hanks Salt Solution, placed into a beaker containing additional cold salt solution, and then cut into tissue blocks approximately 0.8 to 1.0 cm^3 and 0.5 cm thick. The tissue blocks were divided into three groups (approximately 10 blocks per fixative type) and readied for fixation.

Tissue Fixation

Myxamoebae

Ten Percent Buffered Formalin

(1) The pellet of myxamoebae was re-suspended in 5 ml of fixative and allowed to fix for a 12 hour period.

(2) Fixed myxamoebae were then washed with two changes of 70 percent ethanol, and allowed to post-fix in another change of 70 percent ethanol for 12 hours.

(3) The myxamoebal suspension was then centrifuged at 70X gravity for 20 minutes, the supernatant discarded and the pellet re-suspended in a small amount of 70 percent

ethanol.

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

Lillies Alcohol-Acetic Acid Formalin

(1) The myxamoebal pellet was re-suspended in a small amount of 0.25M PBS.

(2) The myxamoebae were then smeared onto previously albuminized slides and were allowed to air dry for several minutes.

(3) The dried smears were then placed into a staining dish containing the fixative. The smears were allowed to fix at 4 degrees C for a 24 hour period.

(4) Fixed smears were rinsed in 70 percent ethanol and then post-fixed in 70 percent ethanol for 12 hours.

(5) Finally, the slides were removed from the 70 percent ethanol and dried overnight on a slide warmer set at 40 degrees C.

Bicarbonate Formaldehyde

Treatment was similar to that of fixation in Lillies alcohol-acetic acid-formalin,

differing only in that fixation with bicarbonate formaldehyde was carried out at room temperature for 30 minutes.

Liver

Fixation in ten percent buffered formalin at room temperature and in Lillies alcohol-acetic acid-formalin at 4 degrees C was carried out for a 24 hour period. Liver tissue fixed in bicarbonate formaldehyde occurred at room temperature for 30 minutes. Dehydration and parafin imbedding techniques employed were similar in each case. The fixation procedure is as follows:

(1) Freshly excised liver tissue was immediately placed in large volumes (approximately 10 times the volume of the tissue) of the aforementioned fixatives and allowed to fix for the required time periods as indicated.

(2) Fixed liver tissue was then placed in a running water rinse for 1 hour and subsequently dehydrated in a graded ethanol series.

(3) The tissue was then passed through a graded benzene series, followed by several changes in parafin, and, finally, was parafin imbedded.

(4) All sections were cut at 8 microns, then affixed to previously albuminized slides.

Cytochemical Methods

The Feulgen nucleal reaction (Feulgen and Rossenbeck, 1924; as modified by Bryant and Howard, 1969) was employed for the localization of nuclear deoxyribonucleic acid (DNA) in both the myxamoebal cells and the liver tissue.

Prior to staining, parafin sections of liver tissue were cleared in xylene, and hydrated through a graded ethanol series to distilled water. The myxamoebal smears were placed in distilled water 3 hours prior to staining. Control slides, hydrolyzed for 15 minutes at 90 degrees C in 5 percent trichloroacetic acid (TCA) in order to remove the DNA, were run through the staining procedure along with the tissue to be stained in order to ensure that the stain was always DNA specific. The staining technique employed is as follows:

(1) Liver section slides and the myxamoebal smears, along with control slides, were simultaneously hydrolyzed in 5N HCl at room temperature at predetermined time intervals over a 90 minute time period (5 minute hydrolysis time intervals for the 15 to 35 and the 50 to 90 minute time periods, and 2 to 3 minute intervals for the 38 to 50 minute period).

(2) The slides were stained for two hours in Schiff's reagent (Lillie, 1951) which was fortified by adding 1 part of a 10 percent aqueous solution of potassium meta-bisulfite to 4 parts Schiff's reagent. The basic fuschin used to prepare the dye was obtained from Fisher Scientific Company (C.I. # 42500).

(3) Two 5 minute rinses in freshly prepared bisulfite rinse (5 ml of 1.ON HCl and 5 ml of 10 percent potassium meta-bisulfite added to 100 ml of distilled water) was followed by a distilled water rinse and then dehydration in a graded ethanol series.

(4) Finally, the specimens were cleared in xylene, and mounted in permount.

Cytophotometric Methods

All cytophotometric measurements were made with a Zeiss Type 01 Microspectrophotometer using a Planachromat oil immersion objective, N.A. 1.30, X 100. Instrument alignment and linearity of the phototube were checked before the operation of the instrument. A Zeiss continuous interference-filter monochrometer (Zeiss #47 43 10) was used in the isolation of chosen wavelengths of light.

The two wavelength method of microspectrophotometry (Patau, 1952 and Ornstein, 1952; Mendelsohn, 1961) was employed in the photometric determination of relative Feulgen-DNA measurements (Ris and Mirsky, 1949) for both the liver tissue and amoebal cell preparations. A reliable estimation of the absorbing material is critically dependent upon the accuracy of selection of the two wavelengths, since the difference between transmissions at the two wavelengths will determine that estimate. Error due to heterogeneous distribution of stained material, and the need for direct measurement of the nuclear area are eliminated using this method of optical density measurement (Mayall and Mendelsohn, 1970). Hydrolysis and staining of all comparable materials should be carried out at the same time to avoid differences in staining intensity and to produce identical absorption curves for the chromophore-molecular complex (Swift and Rasch, 1956).

In the selection of the appropriate wavelengths (λ_1 and λ_2), homogeneous areas of the specimen nuclei are measured such that the extinction (E_2) at λ_2 is in a 2 to 1 ratio with the extinction (E_1) at λ_1, i.e. $2E_1 = E_2$. The extinction, (E), is equal to the log I_o/I_s, (where I_o is the background light and I_s is the light intensity after passing through the specimen), so that $E_1 = \log I_o/I_s$ at λ_1 and $E_2 = \log I_o/I_s$ at λ_2. Several absorption curves for the Feulgen stained specimens representing the three fixatives utilized were made. Spectral absorption curves of each fixative for the Feulgen stained liver and amoebae nuclei are

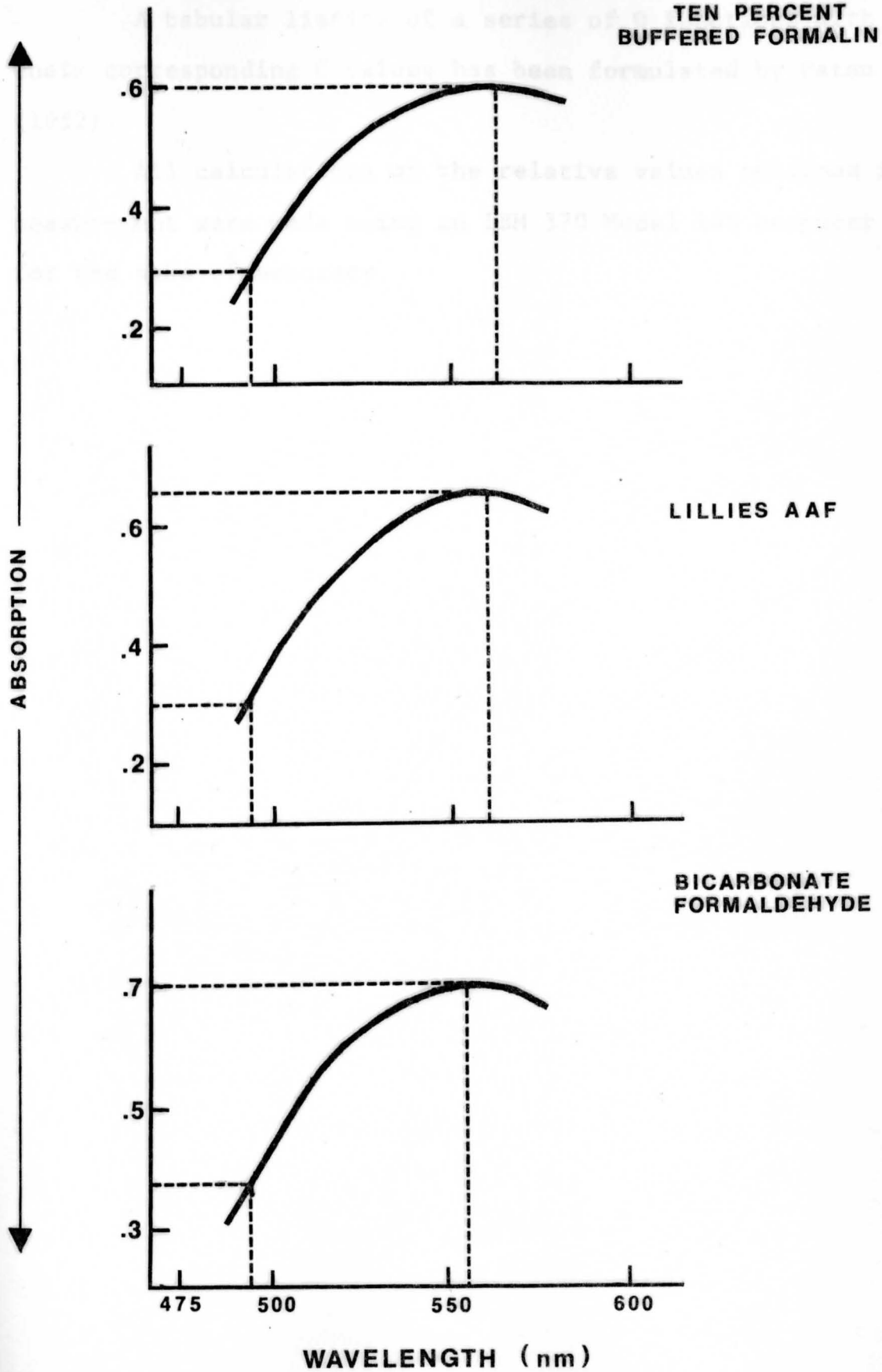
shown in Figure 1.

Areas of heterogeneous dye distribution can be measured once the two wavelengths have been selected. In making the measurements, all nuclei were selected at random from several slides representing specific time periods. For each measurement, the nuclei were centered on the optical axis, and the photometric field was adjusted (by means of an aperature) to surround the object to be measured. The aperature chosen allowed only a minimal amount of light (unoccupied space) past the borders of the nuclei.

The amount of absorbing material (chromophore), (M), within the measured area (A) is determined from the following equation:

$$M = kAL_1C \qquad (1)$$

K, the absorptivity constant, was omitted from the equation in this study, since only relative values were required. For each nucleus, transmission reading (T) were taken, where $T_1 = {}^{I}s/_{I_o}$ at λ_1 and $T_2 = {}^{I}s/_{I_o}$ at λ_2. From these transmission values, L_1 and L_2 were calculated such that $L_1 = (1 - T_1)$ and $L_2 = (1 - T_2)$. With the extinction of the two wavelengths being a 2 to 1 ratio, Q, the ratio between L_1 and L_2 ($Q = {}^{L_2}/_{L_1}$), is used in the determination of the correction factor (C) for the distributional error, where $C = (2 - Q)^{-1} \ln (Q - 1)^{-1}$. The Q value allows for the elimination of the influence of unoccupied portions of the measured area.


TEN PERCENT
BUFFERED FORMALIN
.6
.4
.2
LILLIES AAF
.6
.4
.2
BICARBONATE
FORMALDEHYDE
.7
.5
.3
ABSORPTION
475
500
550
600
WAVELENGTH (nm)

A tabular listing of a series of Q functions with their corresponding C values has been formulated by Patau (1952).

All calculations of the relative values obtained in measurement were made using an IBM 370 Model 145 computer for the sake of accuracy.

RESULTS

The results of Feulgen-DNA measurements of liver nuclei and of myxamoebal nuclei are presented in tabular form and graphically as histograms and hydrolysis curves. The histograms represent nuclear DNA frequency distributions of cellular populations and provide a means for detection of any shifts in the DNA content, while the hydrolysis curves illustrate both the maximal Feulgen intensity attained for a specific hydrolysis time period and the degree of its persistence for a specific treatment of cells. Measurements were limited to interphase nuclei and were reported in arbitrary units as relative amounts of DNA.

Spectral Absorption Curves for Feulgen-DNA Stain

In the utilization of the two-wavelength method of microspectrophotometry, it is essential to establish absorption curves for each particular chromophore-molecular complex being studied. In this investigation, Feulgen stained liver cell nuclei were used in the determination of absorption maxima for each of the fixatives employed (Figure 1). Since both liver tissue sections and myxamoebal smears for each fixative type were subjected to the same reaction procedure, the maximal absorption peaks determined

from the stained liver nuclei representing each fixative were also used in DNA measurements for the myxamoebal nuclei of each specific treatment case, since all tissue was stained at the same time and under the same conditions. The maximal absorption for the Feulgen-DNA complex of tissue fixed in ten percent buffered formalin and in Lillies alcohol-acetic acid-formalin was at a wavelength of 560 nm, with the half-maximal value at a wavelength of 495 nm. The wavelengths representing the absorption maximum and the half-maximum for the tissue fixed in bicarbonate formaldehyde were 555 nm and 495 nm, respectively. Liver tissue control slides hydrolyzed in 5 percent tri-chloroacetic acid, in order to remove DNA, did not stain, indicating the absence of DNA, and, hence, the strict specificity of the stain employed.

Microspectrophotometric Analysis of Nuclear DNA

Relative measurements of the nuclear DNA content of myxamoebal nuclei and of liver nuclei for each fixative employed are presented in Tables I and II. In each case, 25 nuclei per hydrolysis time period were analyzed and were reported, collectively, as mean DNA values; each value accompanied by its standard deviation and standard error, with certain mean values plotted to generate Feulgen hydrolysis curves for both myxamoebal and liver nuclei. The abscissa of the curve represents the hydrolysis period in minutes and the ordinate, the relative mean amount of DNA or dye binding for each period.

TABLE I

STANDARD DEVIATIONS AND STANDARD ERRORS OF MEAN DNA VALUES FOR LIVER TISSUE HYDROLYSIS CURVES

n = 25/time period	Ten Percent Buffered Formalin			Lillies AAF			Bicarbonate Formaldehyde		
Hydrolysis Time (Min.)	Mean DNA	Standard Deviation	Standard Error	Mean DNA	Standard Deviation	Standard Error	Mean DNA	Standard Deviation	Standard Error
15	42.87	1.45	0.29	38.37	2.23	0.45	50.30	2.72	0.54
20	42.87	2.38	0.48	43.42	2.81	0.56	53.40	2.23	0.45
25	47.57	1.84	0.37	41.05	2.09	0.42	55.44	1.97	0.40
30	47.63	1.54	0.31	42.62	1.75	0.35	55.75	2.49	0.50
35	48.66	2.74	0.55	44.89	1.84	0.37	58.84	2.23	0.45
38	44.61	1.69	0.34	44.36	1.63	0.33	57.03	2.00	0.40
40	43.04	2.14	0.43	42.31	2.41	0.48	55.71	1.66	0.33
42	47.42	1.83	0.37	43.45	1.54	0.31	57.25	1.79	0.36
45	46.72	3.39	0.68	43.97	1.33	0.27	58.55	1.94	0.39
48	46.31	1.59	0.32	43.37	2.53	0.51	57.63	2.18	0.44
50	47.11	1.59	0.32	46.68	1.36	0.27	57.73	2.62	0.52
55	43.94	3.01	0.62	47.32	1.13	0.23	58.93	2.94	0.59
60	46.80	1.71	0.34	46.02	1.67	0.33	57.38	1.84	0.37
65	45.95	1.61	0.32	47.88	1.18	0.24	57.83	2.84	0.57
70	46.37	2.57	0.51	45.95	1.77	0.35	56.12	2.14	0.43
75	47.70	3.06	0.61	44.62	1.67	0.33	56.90	2.71	0.54
80	49.48	1.21	0.24	44.53	1.88	0.38	55.69	1.77	0.35
85	51.94	4.29	0.86	44.25	1.71	0.34	56.08	2.98	0.60
90	51.29	2.25	0.45	46.61	1.79	0.36	54.84	1.71	0.34

TABLE II

STANDARD DEVIATIONS AND STANDARD ERRORS OF MEAN DNA VALUES FOR AMOEBAL TISSUE HYDROLYSIS CURVES

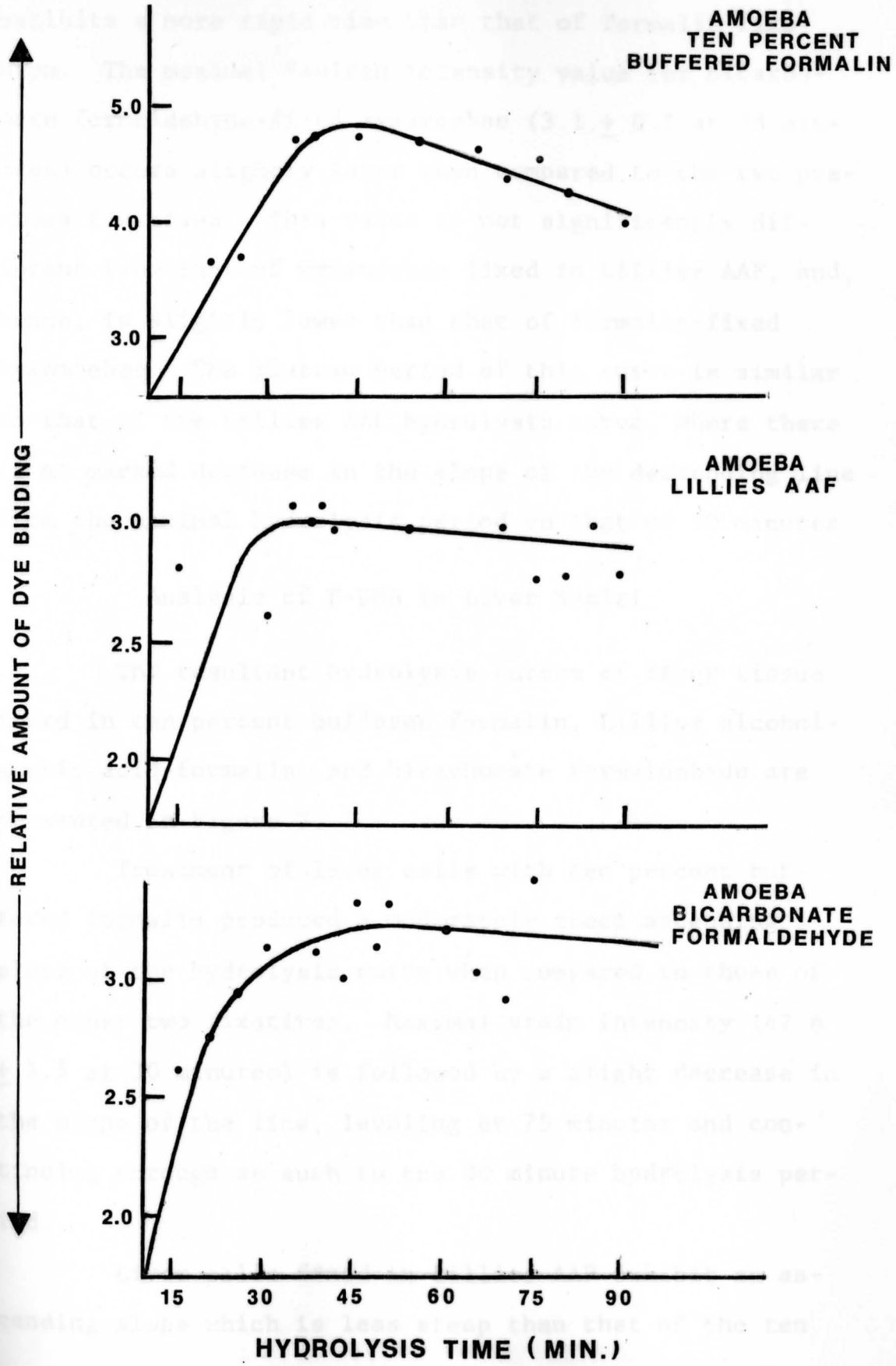
n = 25/time period	Ten Percent Buffered Formalin			Lillies AAF			Bicarbonate Formaldehyde		
Hydrolysis Time (Min.)	Mean DNA	Standard Deviation	Standard Error	Mean DNA	Standard Deviation	Standard Error	Mean DNA	Standard Deviation	Standard Error
15	3.80	0.83	0.17	2.84	0.71	0.14	2.61	0.41	0.08
20	3.65	0.45	0.09	3.34	0.75	0.15	2.69	0.55	0.11
25	3.69	0.77	0.15	3.11	0.66	0.13	2.90	0.42	0.08
30	3.57	0.68	0.14	2.55	0.46	0.92	3.06	0.44	0.09
35	4.66	0.64	0.13	3.07	0.58	0.12	2.66	0.38	0.08
38	4.68	0.82	0.16	2.95	0.71	0.14	3.10	0.32	0.07
40	5.90	1.12	0.22	3.07	0.73	0.15	2.95	0.42	0.08
42	4.47	0.93	0.19	2.93	0.68	0.14	3.04	0.34	0.07
45	4.73	0.69	0.14	2.39	0.41	0.08	3.34	0.57	0.11
48	4.44	0.84	0.17	3.57	0.82	0.17	3.09	0.45	0.09
50	5.49	0.89	0.18	2.53	0.54	0.11	3.26	0.59	0.12
55	4.70	0.68	0.14	2.96	0.51	0.10	2.89	0.54	0.11
60	5.05	1.08	0.22	2.45	0.44	0.09	3.17	0.53	0.11
65	4.57	1.14	0.23	2.48	0.56	0.11	3.04	0.46	0.09
70	4.39	0.78	0.16	2.98	0.55	0.11	2.86	0.34	0.07
75	4.53	0.89	0.18	2.76	0.45	0.09	3.46	0.50	0.10
80	4.23	0.65	0.13	2.77	0.50	0.10	3.53	0.46	0.09
85	3.86	0.63	0.13	2.99	0.49	0.10	3.49	0.30	0.06
90	3.96	0.71	0.14	2.84	0.45	0.09	3.54	0.39	0.08

Analysis of F-DNA in Myxamoebal Nuclei

The hydrolysis curve for myxamoebae fixed in ten percent buffered formalin (Figure 2) shows a gradual rise to a maximum (4.6 ± 0.6 at 35 minutes) followed by a plateau of maximal Feulgen intensity for the next 15 minutes, and, then, a subsequent marked decline in intensity.

In the second experiment, representing myxamoebae fixed in Lillies alcohol-acetic acid-formalin (Figure 2), the rise of the ascending slope is slightly more rapid than that of the formalin-fixed myxamoebae, reaching a slightly lower maximum intensity value at the same time period (3.0 ± 0.5 at 35 minutes). There is a difference in the shapes of the hydrolysis curves of these two fixatives following the attainment of maximal staining intensity. Whereas, in the case of formalin-fixed myxamoebae, where a short plateau period followed by a progressive decline in maximum intensity was noted, the plateau of myxamoebae fixed in Lillies AAF is shown to persist for a longer period of time. This is exemplified by no abrupt decrease in the slope of the line between the maximal intensity hydrolysis time period of 35 minutes to the final hydrolysis time period of 90 minutes.

The ascending slope of the hydrolysis curve for myxamoebae fixed in bicarbonate formaldehyde (Figure 2) approximates that of the Lillies AAF curve, in that, it


AMOEBA
TEN PERCENT
BUFFERED FORMALIN
5.0
4.0
3.0
AMOEBA
LILLIES AAF
3.0
2.5
2.0
AMOEBA
BICARBONATE
FORMALDEHYDE
3.0
2.5
2.0
15
30
45
60
75
90
HYDROLYSIS TIME (MIN.)
RELATIVE AMOUNT OF DYE BINDING

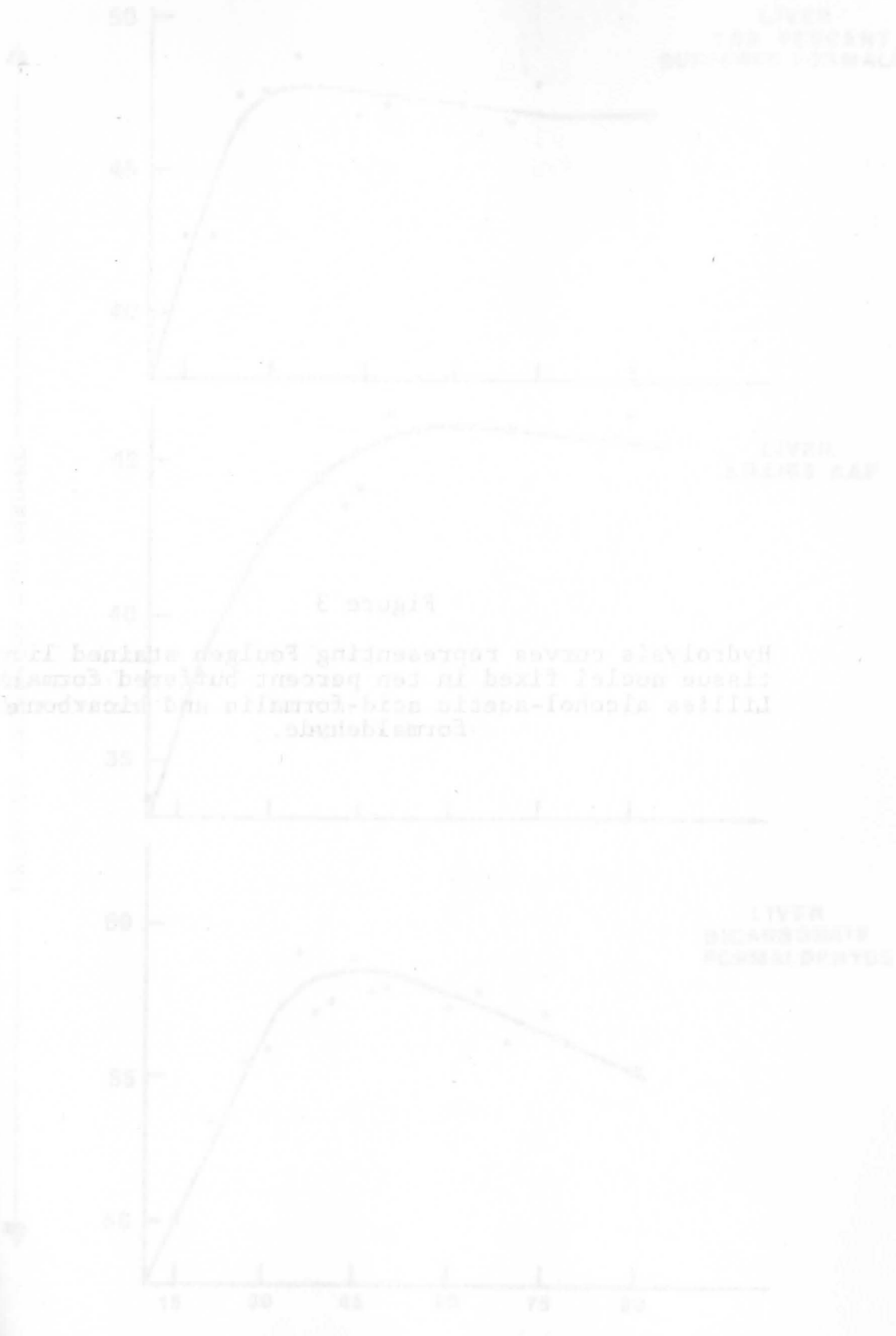
exhibits a more rapid rise than that of formalin fixation. The maximal Feulgen intensity value for bicarbonate formaldehyde-fixed myxamoebae (3.1 $\pm$ 0.3 at 38 minutes) occurs slightly later when compared to the two previous fixatives. This value is not significantly different from that of myxamoebae fixed in Lillies AAF, and, hence, is slightly lower than that of formalin-fixed myxamoebae. The plateau period of this curve is similar to that of the Lillies AAF hydrolysis curve, where there is no marked decrease in the slope of the descending line from the maximal hydrolysis period to that of 90 minutes.

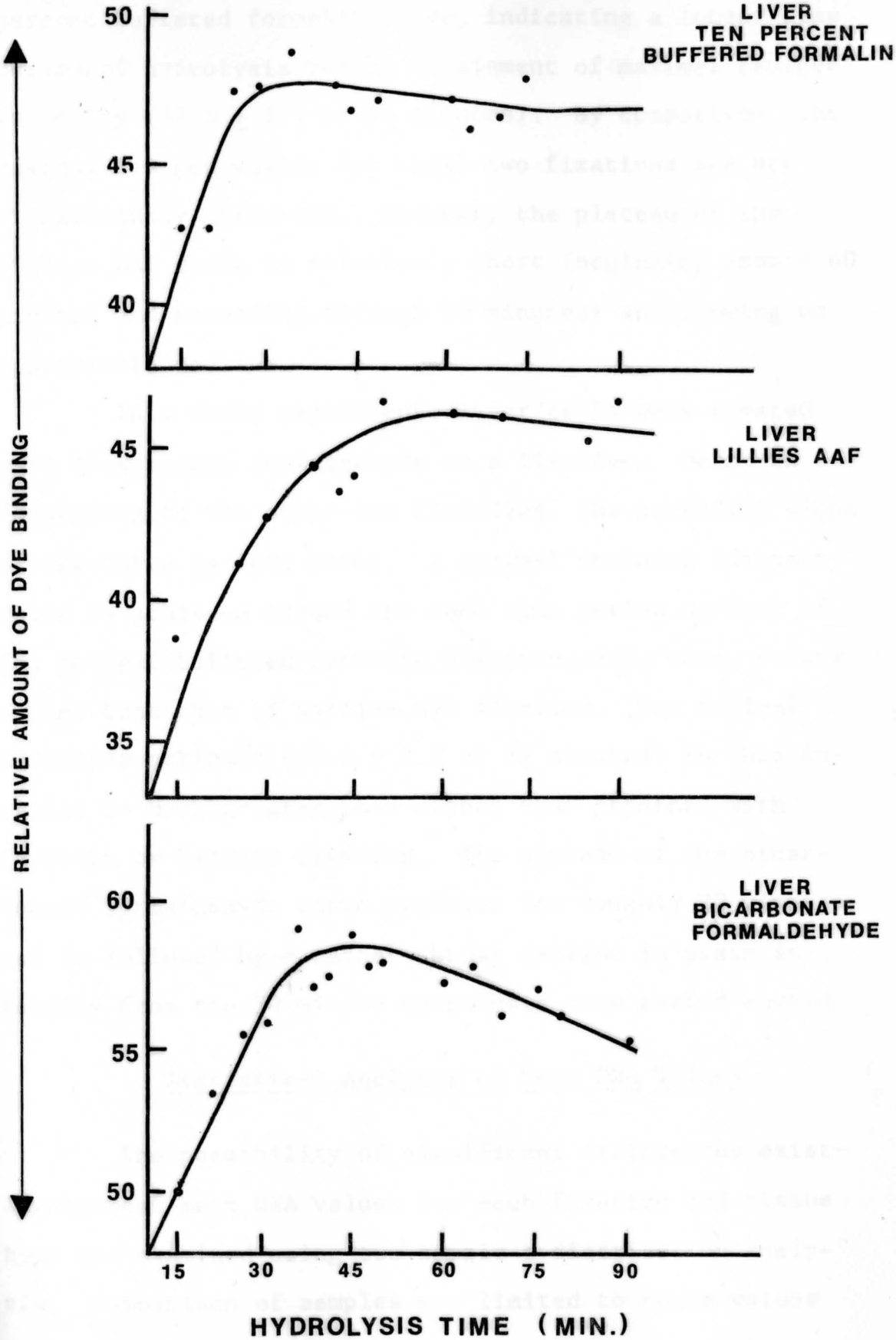
Analysis of F-DNA in Liver Nuclei

The resultant hydrolysis curves of liver tissue fixed in ten percent buffered formalin, Lillies alcohol-acetic acid-formalin, and bicarbonate formaldehyde are presented in Figure 3.

Treatment of liver cells with ten percent buffered formalin produced a moderately steep ascending slope of the hydrolysis curve when compared to those of the other two fixatives. Maximal stain intensity (47.6 $\pm$ 1.5 at 30 minutes) is followed by a slight decrease in the slope of the line, leveling at 75 minutes and continuing through as such to the 90 minute hydrolysis period.

Liver cells fixed in Lillies AAF exhibit an ascending slope which is less steep than that of the ten




LIVER
TEN PERCENT
BUFFERED FORMALIN
50
45
40
LIVER
LILLIES AAF
45
40
35
LIVER
BICARBONATE
FORMALDEHYDE
60
55
50
15
30
45
60
75
90
HYDROLYSIS TIME (MIN.)
RELATIVE AMOUNT OF DYE BINDING

percent buffered formalin curve, indicating a longer time period of hydrolysis before attainment of maximal Feulgen intensity (47.3 $\pm$ 1.1 at 50 minutes). By comparison, the maximal Feulgen values for these two fixatives are not significantly different. However, the plateau of the Lillies AAF curve is relatively short (beginning around 60 minutes and extending through 90 minutes) and showing no appreciable decline.

In a third experiment, liver cells were treated with bicarbonate formaldehyde as a fixative. Here, in comparison to the other two fixatives, the ascending slope of the curve is less steep. A maximal staining intensity value is achieved around the same time period as that of ten percent buffered formalin fixation, and, thus, occurs sooner that that of Lillies AAF fixation. The maximal intensity achieved (58.8 $\pm$ 2.2 at 35 minutes) in this instance is much greater than either that attained with formalin or Lillies fixation. The plateau of the bicarbonate formaldehyde curve persists for roughly 20 minutes and is followed by a rather abrupt decline in stain intensity from the 50 minute hydrolysis time period onward.

Statistical Analysis of Mean DNA Values

The possibility of significant differences existing between mean DNA values for each fixative and tissue type was examined using two sample t-distribution analysis. Comparison of samples was limited to those values

representing the initial hydrolysis time period, the period of maximal staining intensity, and, finally, that of the initial decline (plateau) of staining intensity. In each case, analysis of the initial periods and that of the maximal periods indicated significant differences between these values, making the choice of a hydrolysis time period within these limits to represent maximal stain binding unfeasible. Comparison of the maximal periods to that of plateau revealed no significant differences occurring between these values, indicating that maximum intensity could be attained and maintained throughout that particular time continuum of hydrolysis. These statistical findings are presented for both liver nuclei and myxamoebal nuclei in Tables III and IV. These significant differences are also readily shown in the frequency distribution histograms of Figures 4-9.

Myxamoebal Nuclei

Statistical analysis of the mean DNA values of myxamoebae fixed in ten percent buffered formalin (Table III) for the hydrolysis time periods of 15 and 35 minutes shows a significant difference in values, whereas, comparison of the mean DNA values for the 35 and 65 minute periods indicate no significant differences at a confidence level of 0.05. The difference which does occur is readily noticeable when comparing histograms of these time periods (Figure 4) in which the relative amounts of DNA

TABLE III

TWO SAMPLE t-DISTRIBUTION COMPARISON OF MEAN DNA VALUES FOR AMOEBAE TISSUE FIXED WITH THREE DIFFERENT FIXATIVES

Fixative	Hydrolysis Periods Compared (Min.)	$(X_1 - X_2)^2$	est $\sigma_{X1} - \sigma_{X2}$	t
Ten Percent Buffered Formalin	15 35	32.92 10.47	0.2689	5.95
	35 65	10.47 32.87	0.2687	0.37
Lillies AAF	15 35	26.12 8.90	0.2415	3.72
	35 90	8.90 5.27	0.1536	0.65
Bicarbonate Formaldehyde	15 38	6.66 2.97	0.1266	6.31
	38 60	2.97 7.04	0.1291	0.77

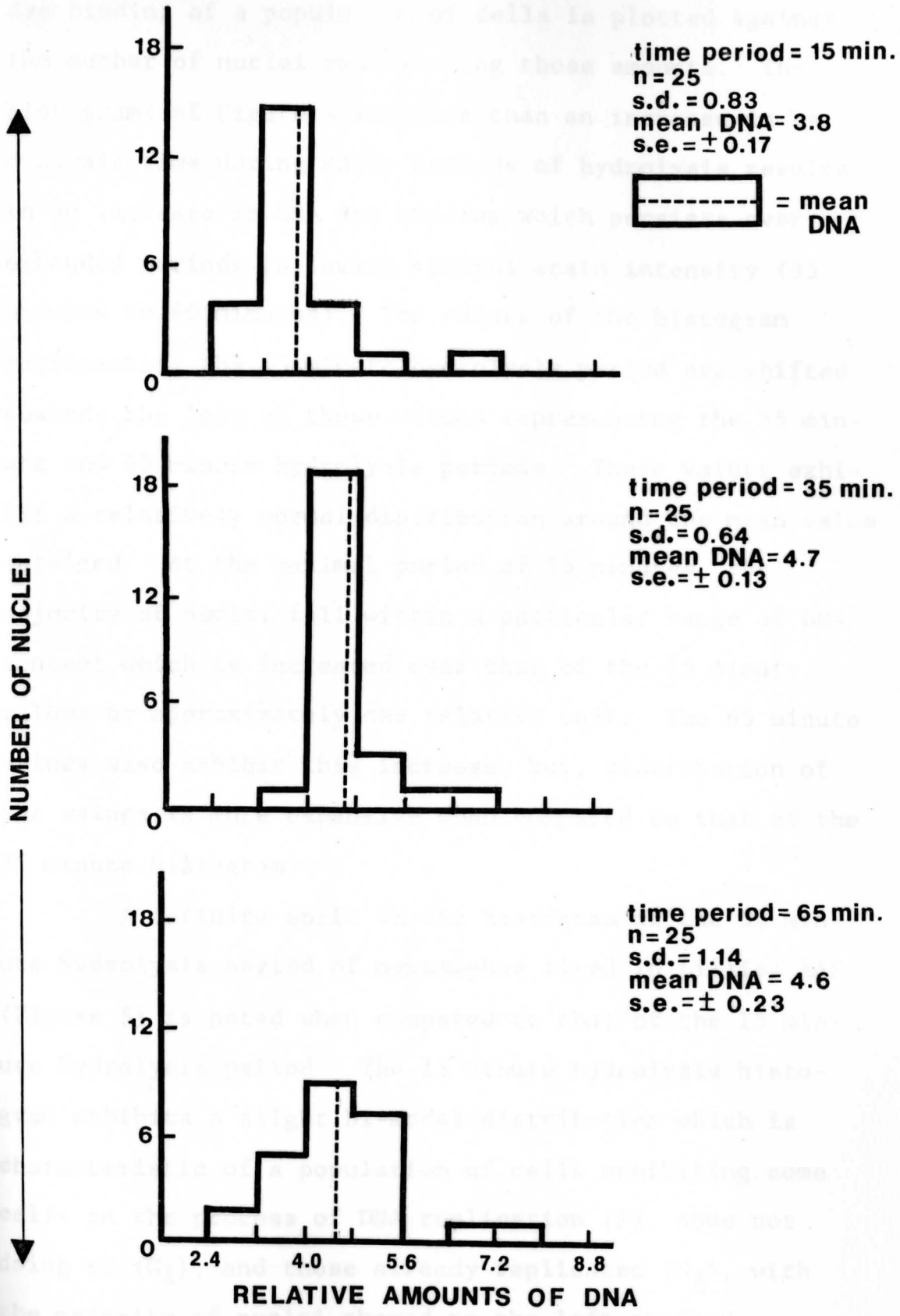
df = 48
α = 0.05

TABLE IV

TWO SAMPLE t-DISTRIBUTION COMPARISON OF MEAN DNA VALUES
FOR LIVER TISSUE FIXED WITH THREE DIFFERENT FIXATIVES

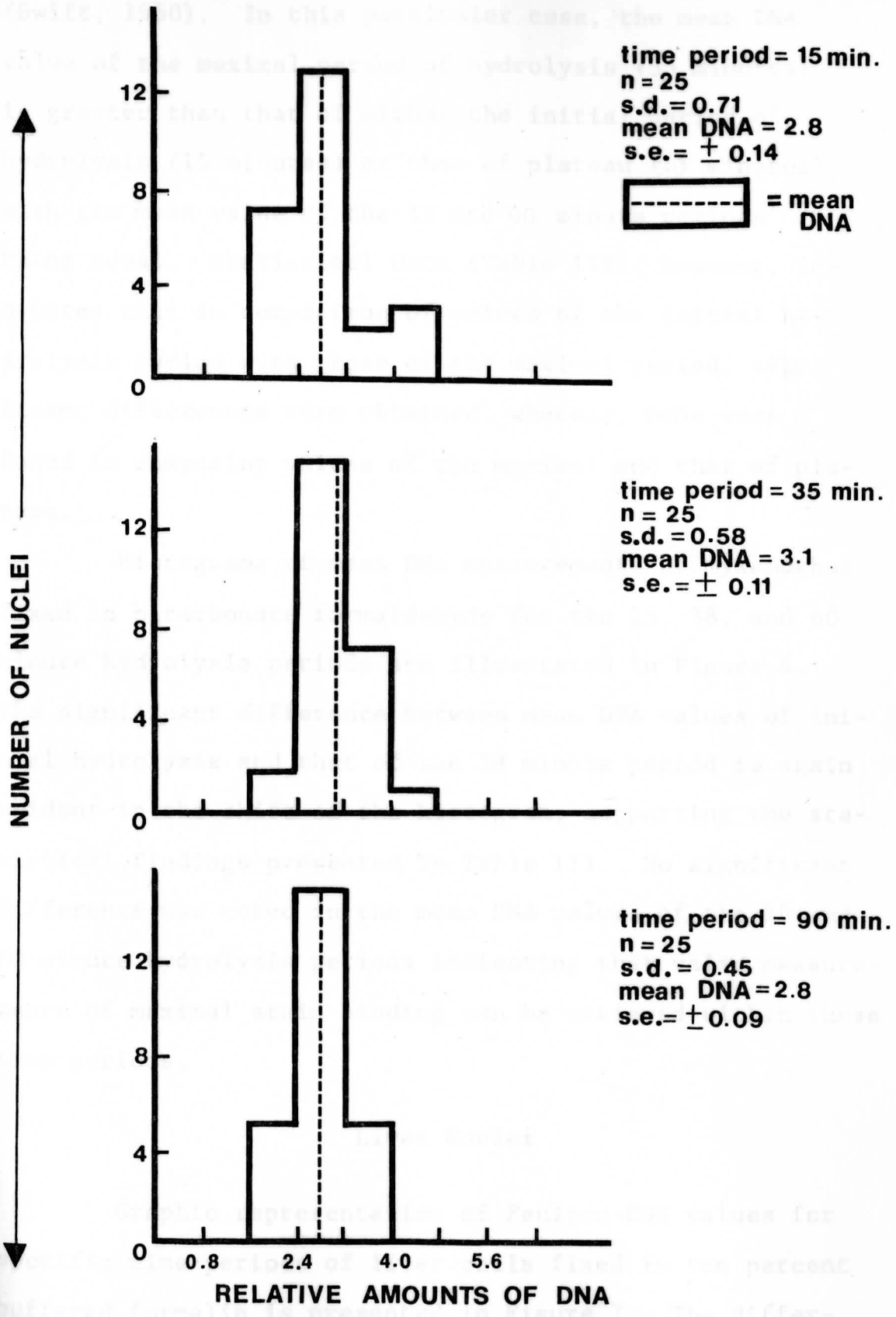
Fixative	Hydrolysis Periods Compared (Min.)	$(X_1 - X_2)^2$	est $\sigma_{X_1} - \sigma_{X_2}$	t
Ten Percent Buffered Formalin	15 30	106.62 59.74	0.5265	11.77
	30 60	59.74 80.97	0.4842	0.41
Lillies AAF	15 50	223.30 70.39	0.6996	13.29
	50 90	70.39 105.56	0.5414	0.18
Bicarbonate Formaldehyde	15 35	184.46 157.14	0.7545	9.93
	35 65	157.14 206.14	0.7781	0.38

df = 48
α = 0.05


time period = 15 min.
n = 25
s.d. = 0.83
mean DNA = 3.8
s.e. = ± 0.17
= mean DNA
time period = 35 min.
n = 25
s.d. = 0.64
mean DNA = 4.7
s.e. = ± 0.13
time period = 65 min.
n = 25
s.d. = 1.14
mean DNA = 4.6
s.e. = ± 0.23
NUMBER OF NUCLEI
18
12
6
0
2.4
4.0
5.6
7.2
8.8
RELATIVE AMOUNTS OF DNA

dye binding of a population of cells is plotted against the number of nuclei representing those amounts. The histograms of Figure 4 indicate than an increase in hydrolysis time during early periods of hydrolysis results in an increase in DNA dye binding which persists over extended periods following maximal stain intensity (35 minutes to 60 minutes). The values of the histogram representing the 15 minute hydrolysis period are shifted towards the left of those values representing the 35 minute and 65 minute hydrolysis periods. These values exhibit a relatively normal distribution around the mean value obtained. At the maximal period of 35 minutes, the majority of nuclei fall within a particular range of DNA content which is increased over that of the 15 minute values by approximately one relative unit. The 65 minute values also exhibit this increase, but, distribution of the values is more expansive when compared to that of the 35 minute histogram.

A definite shift in the histogram of the 35 minute hydrolysis period of myxamoebae fixed in Lillies AAF (Figure 5) is noted when compared to that of the 15 minute hydrolysis period. The 15 minute hydrolysis histogram exhibits a slight bi-modal distribution which is characteristic of a population of cells exhibiting some cells in the process of DNA replication (S), some not doing so (G_1), and those already replicated (G_2), with the majority of nuclei skewed to the left or in G_1

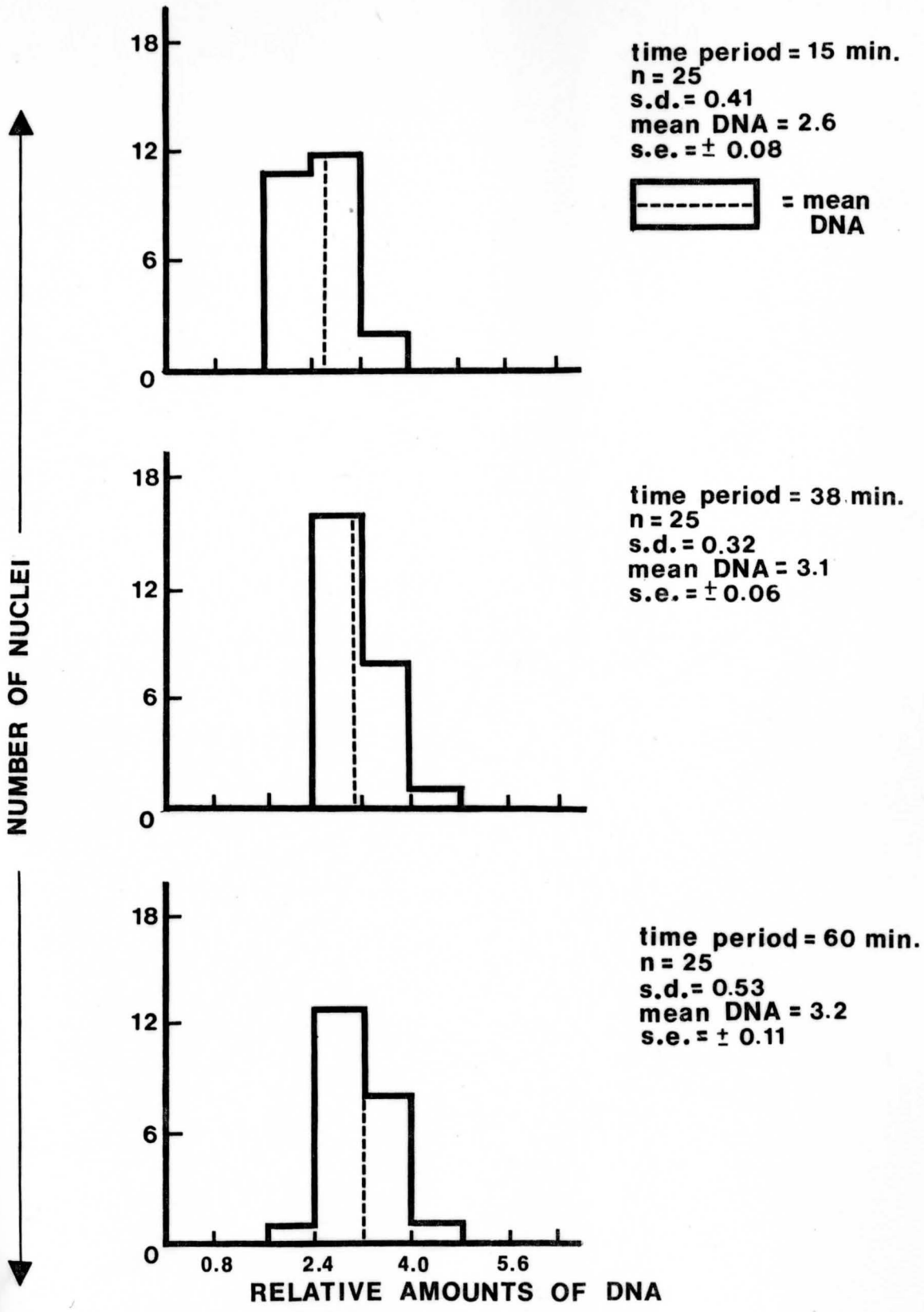

time period = 15 min.
n = 25
s.d. = 0.71
mean DNA = 2.8
s.e. = ± 0.14
= mean DNA
time period = 35 min.
n = 25
s.d. = 0.58
mean DNA = 3.1
s.e. = ± 0.11
time period = 90 min.
n = 25
s.d. = 0.45
mean DNA = 2.8
s.e. = ± 0.09
NUMBER OF NUCLEI
0
4
8
12
0.8
2.4
4.0
5.6
RELATIVE AMOUNTS OF DNA

(Swift, 1950). In this particular case, the mean DNA value of the maximal period of hydrolysis (35 minutes) is greater than that of either the initial period of hydrolysis (15 minutes) or that of plateau (90 minutes), with the mean value of the 15 and 90 minute periods being equal. Statistical data (Table III), however, indicates that in comparison of values of the initial hydrolysis period with those of the maximal period, significant differences were obtained, whereas, none were found in comparing values of the maximal and that of plateau.

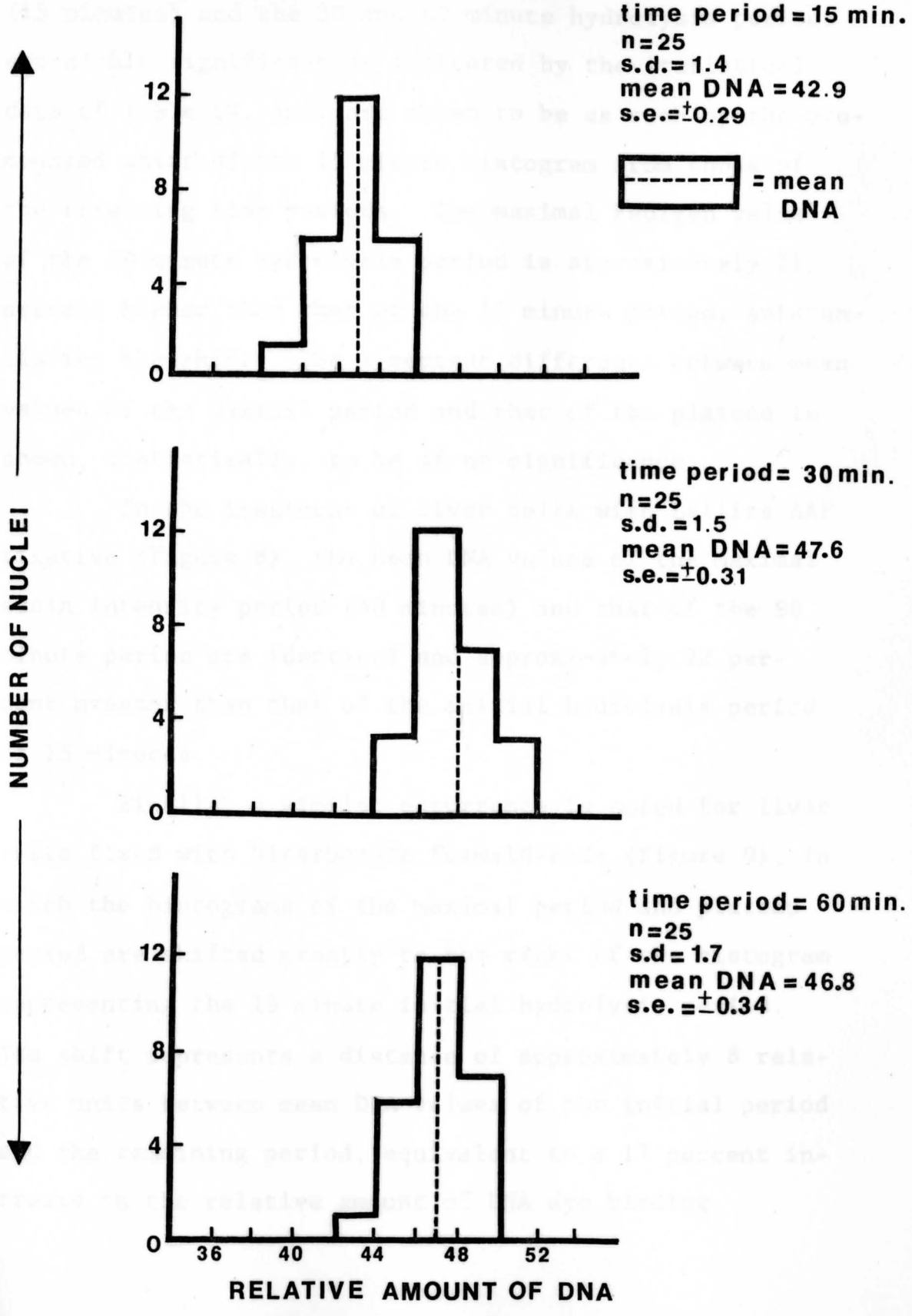
Histograms of mean DNA measurements of myxamoebae fixed in bicarbonate formaldehyde for the 15, 38, and 60 minute hydrolysis periods are illustrated in Figure 6. The significant difference between mean DNA values of initial hydrolysis and that of the 38 minute period is again evident in the shift of the histogram, supporting the statistical findings presented in Table III. No significant difference was noted in the mean DNA values of the 38 and 60 minute hydrolysis periods indicating that valid measurements of maximal stain binding can be achieved within these time periods.

Liver Nuclei

Graphic representation of Feulgen-DNA values for specific time periods of liver cells fixed in ten percent buffered formalin is presented in Figure 7. The differ-


18
12
6
0
time period = 15 min.
n = 25
s.d. = 0.41
mean DNA = 2.6
s.e. = ± 0.08
= mean DNA
18
12
6
0
time period = 38 min.
n = 25
s.d. = 0.32
mean DNA = 3.1
s.e. = ± 0.06
18
12
6
0
time period = 60 min.
n = 25
s.d. = 0.53
mean DNA = 3.2
s.e. = ± 0.11
NUMBER OF NUCLEI
0.8
2.4
4.0
5.6
RELATIVE AMOUNTS OF DNA

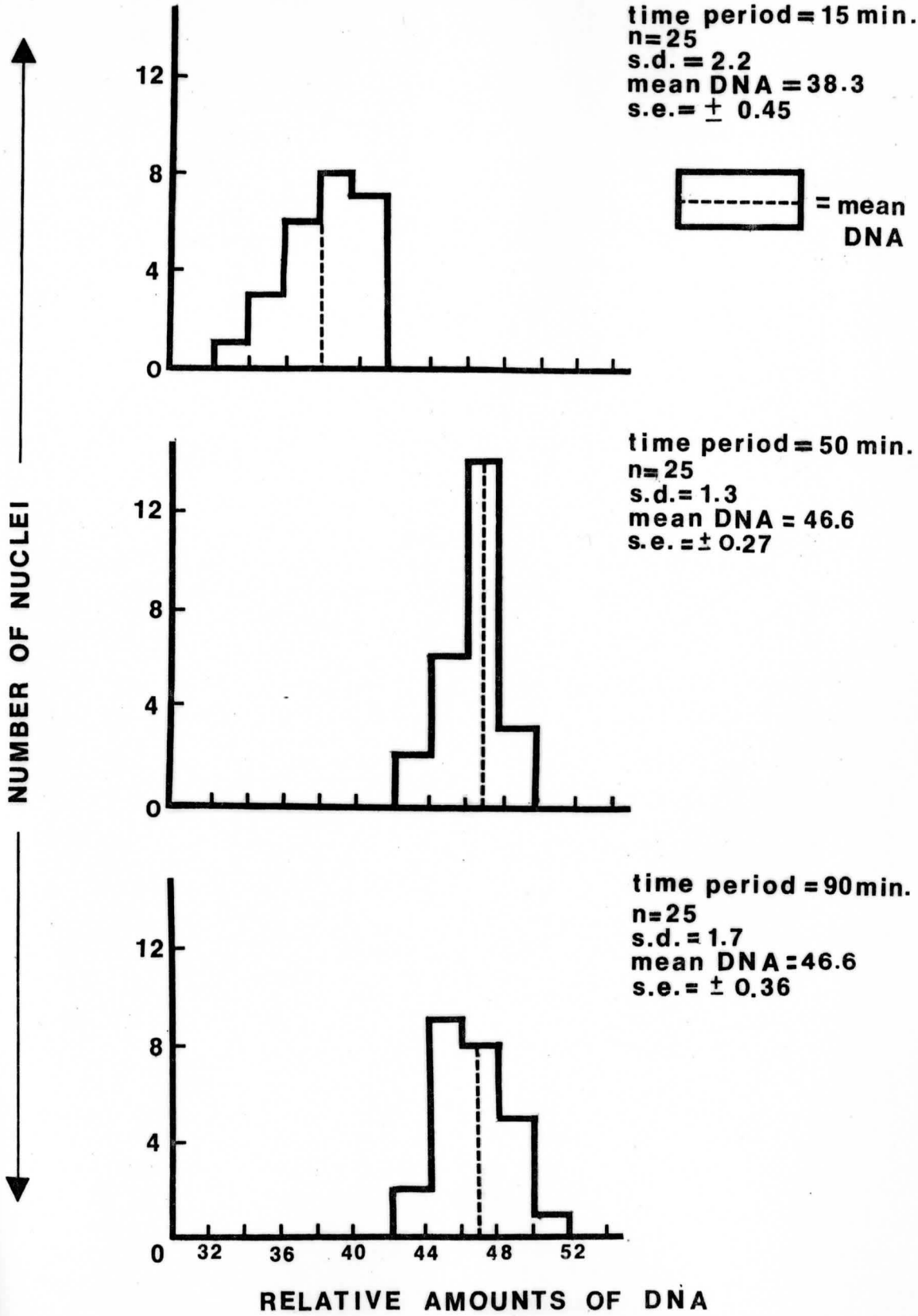
RELATIVE AMOUNT OF DNA


time period = 15 min.
n = 25
s.d. = 1.4
mean DNA = 42.9
s.e. = ±0.29
= mean DNA
time period = 30 min.
n = 25
s.d. = 1.5
mean DNA = 47.6
s.e. = ±0.31
time period = 60 min.
n = 25
s.d = 1.7
mean DNA = 46.8
s.e. = ±0.34
NUMBER OF NUCLEI
0
4
8
12
36
40
44
48
52
RELATIVE AMOUNT OF DNA

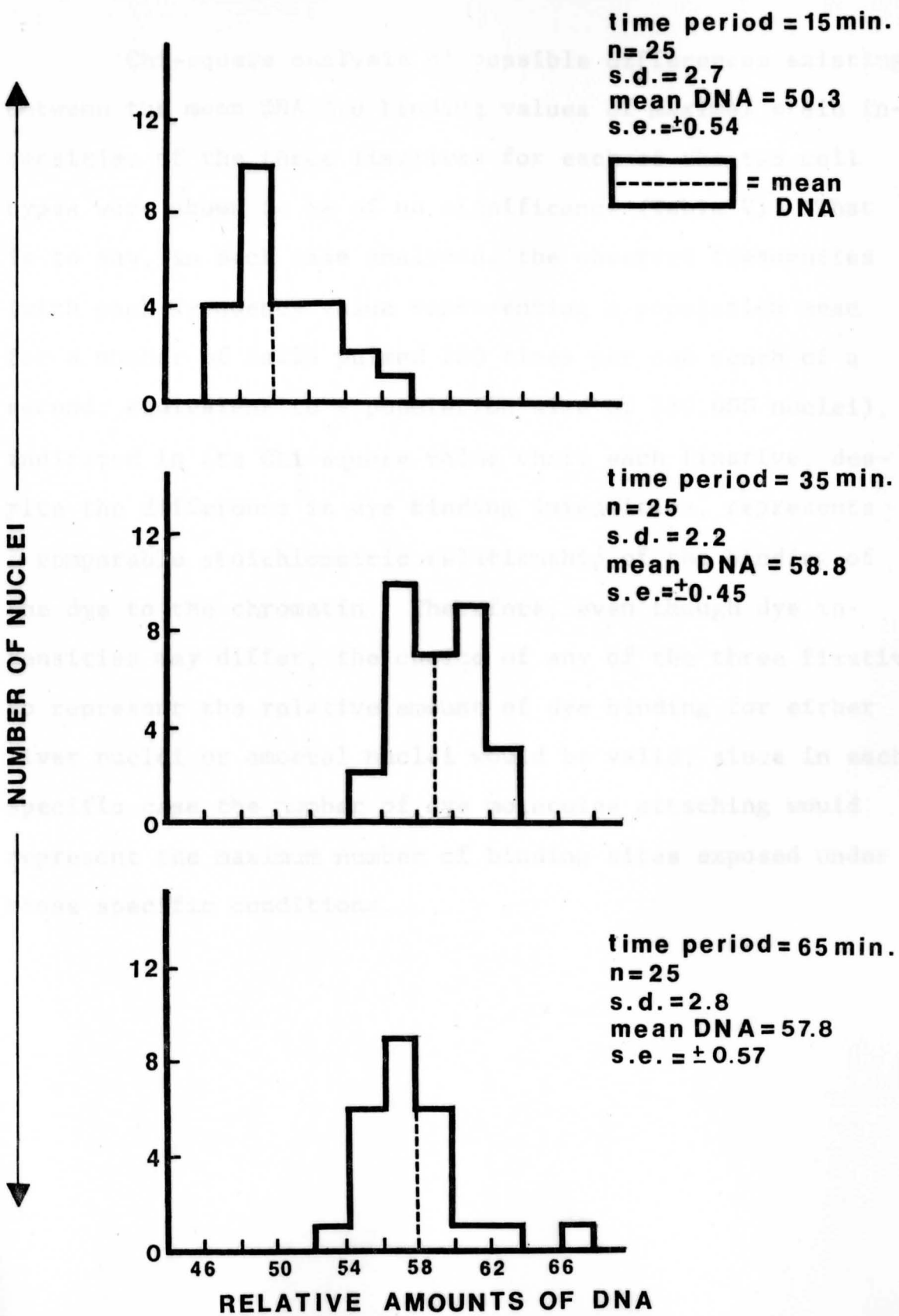
ences occurring between the initial hydrolysis period (15 minutes) and the 30 and 60 minute hydrolysis periods are highly significant as indicated by the statistical data of Table IV, and, are shown to be as such by the pronounced shift of the 15 minute histogram from those of the remaining time periods. The maximal Feulgen value of the 30 minute hydrolysis period is approximately 11 percent higher than that of the 15 minute period, substantiating the shift. The 2 percent difference between mean values of the maximal period and that of the plateau is shown, statistically, to be of no significance.

In the treatment of liver cells with Lillies AAF fixative (Figure 8), the mean DNA values of the maximal stain intensity period (50 minutes) and that of the 90 minute period are identical and approximately 22 percent greater than that of the initial hydrolysis period of 15 minutes.

Finally, a similar occurrence is noted for liver cells fixed with bicarbonate formaldehyde (Figure 9), in which the histograms of the maximal period and plateau period are shifted greatly to the right of the histogram representing the 15 minute initial hydrolysis period. The shift represents a distance of approximately 8 relative units between mean DNA values of the initial period and the remaining period, equivalent to a 17 percent increase in the relative amount of DNA dye binding.


time period = 15 min.
n=25
s.d. = 2.2
mean DNA = 38.3
s.e. = ± 0.45
= mean DNA
time period = 50 min.
n=25
s.d. = 1.3
mean DNA = 46.6
s.e. = ± 0.27
time period = 90 min.
n=25
s.d. = 1.7
mean DNA = 46.6
s.e. = ± 0.36
NUMBER OF NUCLEI
0
4
8
12
32
36
40
44
48
52
RELATIVE AMOUNTS OF DNA

time period = 15 min.
n = 25
s.d. = 4.2
mean DNA = 60.3
s.e. = 0.84
mean DNA
time period = 35 min.
n = 25
s.d. = 3.2
mean DNA = 52.8
s.e. = 0.64
time period = 65 min.
n = 25
s.d. = 2.5
mean DNA = 52.8
s.e. = 0.57
RELATIVE AMOUNTS OF DNA


time period = 15 min.
n = 25
s.d. = 2.7
mean DNA = 50.3
s.e. = ±0.54
= mean DNA
time period = 35 min.
n = 25
s.d. = 2.2
mean DNA = 58.8
s.e. = ±0.45
time period = 65 min.
n = 25
s.d. = 2.8
mean DNA = 57.8
s.e. = ± 0.57
NUMBER OF NUCLEI
0
4
8
12
46
50
54
58
62
66
RELATIVE AMOUNTS OF DNA

Chi-square Analysis of Mean DNA Values

Chi-square analysis of possible differences existing between the mean DNA dye binding values of maximal stain intensities of the three fixatives for each of the two cell types were shown to be of no significance (Table V). That is to say, in each case analyzed, the observed frequencies (with each frequency value representing a population mean for a number of cells pulsed 280 times per one tenth of a second, equivalent to a population size of 350,000 nuclei), indicated in its Chi-square value that, each fixative, despite the difference in dye binding intensities, represents a comparable stoichiometric relationship of the binding of the dye to the chromatin. Therefore, even though dye intensities may differ, the choice of any of the three fixatives to represent the relative amount of dye binding for either liver nuclei or amoebal nuclei would be valid, since in each specific case the number of dye molecules attaching would represent the maximum number of binding sites exposed under those specific conditions.

TABLE V

CHI-SQUARE ANALYSIS OF LIVER AND MYXAMOEBAL MEAN DNA VALUES AT MAXIMAL STAIN INTENSITIES FOR THREE FIXATIVES

Myxamoebal Tissue

X	Observed Frequency	Expected Frequency	Deviation	Deviation^2/Expected
1	4.70	3.63	1.06	0.30924
2	3.10	3.63	-0.53	0.07331
3	3.10	3.63	-0.53	0.07331
Totals	10.90	10.89	0	0.46386

Liver Tissue

1	47.6	51.0	-3.40	0.22666
2	46.6	51.0	-4.40	0.37960
3	58.8	51.0	7.80	1.19294
Totals	153.0	153.0	0	1.79920

1 = Ten Percent Buffered Formalin
2 = Lillies AAF
3 = Bicarbonate Formaldehyde

df = 2

α = 0.05

DISCUSSION

The results of this investigation substantiate the observations of previous investigators which indicate that the intensity of the Feulgen nucleal reaction varies with: (1) the duration of hydrolysis, increasing to a maximum and decreasing as hydrolysis proceeds (DiStefano, 1948); (2) that the time at which optimum staining intensity is reached is a function of the fixative and tissue employed (Bauer, 1932; Hillary, 1939); (3) and that the height of the maximum absorption peak varies with each different fixative (Sibatani and Fukuda, 1953). The aforementioned observations are readily illustrated in the hydrolysis curves for both Feulgen stained liver and myxamoebal nuclei using three different fixatives in this study.

The results of liver tissue fixation suggest that the best maximal staining intensity is obtained with bicarbonate formaldehyde as a fixative. In the case of myxamoebal nuclei, ten percent buffered formalin produced the best results in the attainment of maximal values when compared to the two remaining fixatives. The maximal stain intensity attained reflects the ability of DNA to withstand the Feulgen hydrolysis procedures, e.g., chromatin stability. Chromatin stability, in turn, is dependent upon the type of fixative em-

ployed. It was the original intent of this study to determine how a new fixative, bicarbonate formaldehyde, would compare to two established fixatives frequently used in quantitative Feulgen cytophotometry studies, ten percent buffered formalin and ethanol-acetic acid-formalin, in stabilizing nuclear chromatin, and, to determine whether a given fixative can generally be used with any tissue type or whether a specific fixative must be used to produce maximal stain intensity for distinctly different cell types.

For the myxamoebal nuclei, the slopes of the ascending curves and the times at which the maximal Feulgen values were reached did not differ significantly among experiments. The longest plateau was found in fixation with both Lillies alcohol-acetic acid-formalin and bicarbonate formaldehyde. However, as was stated previously, the highest maximal Feulgen intensity value was obtained in fixation with ten percent buffered formalin. In comparison, the details of the hydrolysis curves for liver nuclei differ with respect to the time at which maximal intensity was attained and the degree of persistence of plateau. Maximal Feulgen intensity was attained fastest and persisted longest in fixation with ten percent buffered formalin, yet, the highest maximal Feulgen values attained occurred in bicarbonate formaldehyde fixation.

A reasonable explanation for the persistence of the plateau, and of the stain intensity for liver nuclei fixed in ten percent buffered formalin, and for the attainment of high maximal Feulgen values in formalin-fixed myxamoebal nuclei is

perhaps the same. Formalin, as a fixative, has been found to penetrate tissue rapidly and to have a profound stabilizing effect on DNA (Ericsson and Beberfeld, 1967; Andersson and Kjellstrand, 1974).

In considering the Feulgen reaction procedure, mild hydrolysis of DNA with hydrochloric acid is essential to stain binding. Initially in hydrolysis, the glycosidic link between the sugars and purines is broken resulting in purine liberation from the DNA molecule (depurinization), exposure of the aldehyde dye binding sites, and, the transformation of DNA into apurinic acid. Prolonged hydrolysis results in the destruction of the dye binding sites and/or the degradation or depolymerization of apurinic acid into fragments which can leave the cell by diffusion (removal of stainable groups), and leads to a reduction in Feulgen reaction intensity (Jordanov, 1963; Dutt, 1971). Hillary (1939) and Ross and Ely (1949) proposed that the release of purine bases from DNA is accompanied by a solubilization of a part of the apurinic acid. Cytophotometric studies (DiStefano, 1948; Ris and Mirsky, 1949) also indicate that when solubility of the apurinic acid begins, the transition of the remaining DNA molecules to apurinic acid terminates (termination of the formation of aldehyde dye binding sites). Such occurrences indicate that a part of the Feulgen positive material can be lost during initial hydrolysis procedures or fail to be evidenced at all during staining. The degree of the elimination of stainable groups and the dissolution of the DNA molecule is, therefore, considerably dependent

upon chromatin stability toward mineral acid hydrolysis in addition to other factors such as length of a single DNA molecule and the state of the diffusion barrier in preventing the extraction of such high molecular weight constituents (Andersson and Kjellstrand, 1971). The state of the nuclear DNA-protein complex (DPN) determines chromatin stability. The less soluble the complex, the more stable the chromatin. Formalin fixation produces such a low solubility DNA-protein complex (highly polymerized DNA molecule) via salt links between nuclear proteins and by binding directly to the amino groups of nuclear DNA bases. Through fixation with formalin, the resultant polymerized DNA molecule is rendered more highly resistant to deploymerization, retains a much larger proportion of aldehyde, and, thus, allows for enhanced staining intensity (Dutt, 1971). Sibatani and Fukuda (1953) showed that DNA was more readily liberated from non-fixed nucleohistone than from formalin-fixed nucleohistone, in that, formalin fixation rendered the nucleohistone less soluble in acid media and allowed DNA to resist dissolution more strongly. The ability of formalin to denature DNA molecules and to maintain such denatured states is also evidenced in the inability of DNase and mineral acids to extract formalin-treated DNA (Swift, 1966; Greenwood and Berlyn, 1968).

The ability of formalin to adequately stabilize DNA in order to attain and maintain maximal stain intensity over extended hydrolysis time periods in the case of liver nuclei is evident in the extended plateau period of the hydrolysis

curve. The slight decrease in the slope of the descending curve is statistically insignificant with respect to differences in stain intensity values attained at the hydrolysis periods within this range when contrasted to maximal stain intensity as this study has demonstrated. Therefore, hydrolysis of tissue at any of these time periods would reflect the maximal values attainable under the specified fixation and reaction conditions. Yet, in contrast, the hydrolysis curve representing myxamoebal nuclei fixed in formalin shows a significant decrease in the descending slope of the curve following maximal stain intensity; this data, as interpreted, reflects a tissue specific difference. Even though formalin fixation is recommended by most authors in quantitative studies involving the Feulgen reaction procedure as providing the most desirable results, it seems that the type of tissue being fixed is also an important consideration. It is important to add that overfixation with this fixative can cause the loss of detectable amounts of proteins and nucleic acids as was reported by Sibatani and Fukuda (1953), Baker and McCrae (1966), and Chambers (1968), and as was supported in this study. A survey of the literature indicates that a variety of formalin fixation periods for various tissue, including those of minimum times of 2 to 6 hours (Abramczuk, 1971) and maximum periods of 18 to 24 hours (Deitch, 1966), have been employed to obtain desirable results. This study has conclusively shown that a 12 hour period (or longer) of formalin fixation results in the overfixation of the myxamoebal tissue,

rendering the nuclear material more susceptible to hydrolytic dissolution, and, hence, decreased staining intensity. The data presented demonstrates that shorter fixation periods provide the best results with respect to maximal Feulgen intensities attained for myxamoebal nuclei within its 15 minute maximal stain intensity plateau ranging between 35 and 50 minutes of hydrolysis. Utilization of hydrolysis periods restricted to this range is recommended to ensure attainment of maximal stain intensity. Hydrolysis periods outside this range, prior to 35 minutes and succeeding 50 minutes, represent depurinization (not all stainable groups unmasked) and DNA depolymerization (loss of stainable groups), respectively, and are not recommended as valid periods to represent maximal stain intensity.

The results of fixation of cells in Lillies alcohol-acetic acid-formalin suggest that the choice of Lillies AAF as a fixative for liver nuclei is a poor one when the resultant hydrolysis curves of each fixative are compared. It can be seen that longer periods of hydrolysis are required to attain maximal stain intensity. This results in the prolonging of the reaction procedure to a period which dangerously approaches the period at which destruction of the material to be stained occurs. In addition, prolonged hydrolysis periods, in order to obtain maximal Feulgen values with this fixative, results in stain intensity well below the maximal values attained rather quickly with the remaining fixatives. Since the curve does not reflect a distinct demarkation of maximum

intensity, it is more difficult to determine the time period at which this event does occur.

On the other hand, the hydrolysis curve for myxamoebal nuclei fixed in Lillies AAF indicates that the fixative may conceivably offer an alternative to formalin fixation, in that, the maximum stain intensity is readily defined, achieved early, and persists as such for extended periods of time. Still, as in the case of liver nuclei fixed in Lillies AAF, the maximal stain intensity value attained falls short of those values attained by the remaining fixatives. A drawback to the use of this fixative is that it is rather harsh on tissue preparations. Cytoplasmic and nuclear shrinkage was evident in both cell types fixed in Lillies AAF, which agrees with the findings of Greenwood and Berlyn (1968) in their study involving the effect of formal-acetic acid-ethanol as a fixative on Feulgen intensity. The decrease in the maximal Feulgen intensities attained for both myxamoebal and liver nuclei fixed in Lillies AAF when compared to the remaining fixatives may be due possibly to two factors. The first suggests that fixation with Lillies AAF does not produce as profound a stabilizing effect on the DNA molecule as formalin fixation does e.g., that the DNA may occur in somewhat of a different condition (more susceptible to the deleterious effects of hydrolysis) when fixed in AAF. For example, the treatment of Carnoy's (absolute ethanol:glacial acetic acid) fixed tissue and tissue fixed with similar fixatives (including AAF) with DNase removed all or most of the

Feulgen positive material present, whereas, formalin-fixed material was affected to a very limited degree (Swift, 1950). Deitch (1967) reported that the extent of loss of DNA was variable in using methanol-acetic acid-formalin as a fixative for several cell types undergoing Feulgen reaction procedures and minimal after fixation with formalin. A second factor which may account for the decreased maximal values observed is that nuclear shrinkage results in a higher degree of nuclear material condensation; fewer aldehyde residues are exposed for dye-binding than would be if the chromatin existed in a less condensed state (Andersson and Kjellstrand, 1974).

A rapid rate of tissue penetration and homogeneous denaturation of soluble proteins characterize the fixation effects of a relatively new fixative, bicarbonate formaldehyde. The especially high fixation rate is presumably due to the presence of bicarbonate ions which penetrate cells readily (Cantarow and Shepartz, 1967) and act as a buffer towards the hydrogen ions liberated when formaldehyde interacts with the free amino groups of proteins. Such buffering action allows for the formation of an irreversible formaldehyde/protein interaction, and, thus, continually decreases the number of formaldehyde molecules present in the tissue as the molecules bind to the protein. The decrease maintains a difference between the folmaldehyde concentration of the tissue and of the fixative solution, preventing deceleration of the diffusion rate (Artvinli, 1975). In view of the fact that formalin fixation has been shown to produce such profound effects

on stabilizing nuclear DNA, and, that the presence of bicarbonate ions tends to facilitate and increase the amount of fixed chromatin, an enhanced maximal staining intensity would be expected to occur when using bicarbonate formaldehyde as a fixative. Analysis of the Feulgen hydrolysis curve for liver tissue fixed in this material tends to support this contention. However, the curve also reflects a significant and abrupt decline in maximal staining intensity following the twenty minute hydrolysis plateau period. Such an abrupt decline is not evidenced in the descending slope of the hydrolysis curve for myxamoebal nuclei, nor, in comparison with the other fixatives, does fixation with bicarbonate formaldehyde, in all cases, produce the best maximal stain intensity as is demonstrated in its use with liver tissue. One would not expect similar Feulgen intensities to occur between two distinct cell types when using the same fixative as already noted mainly because of the differences in the type and amount of DNA and the manner in which the particular nucleic acid interacts with the fixative in producing its unique stearically altered molecular configuration for dye-binding. These differences are evidenced in comparison of the shapes of the hydrolysis curves of both liver and myxamoebal nuclei for each particular fixative. In turn, similarities in maximal stain intensity may or may not occur in using different fixatives. In the case of both the liver and myxamoebal nuclei, the latter was found to be true in comparing the maximal stain intensities achieved for each

fixative. It was statistically shown, however, that these differences do not affect the stoichiometric measurements of dye binding, and, hence, the material to be measured. That is to say, each fixative, for the same type of tissue, will produce a characteristic molecular configuration of DNA determining its own stoichiometric relationship between the number of available binding sites and the total amount of dye binding provided the proper hydrolysis time is observed. Therefore, even though stain intensities may differ, the choice of any of these fixatives in order to accurately represent the maximal stain intensity possible for a specific tissue type is valid under the specified reaction conditions, and, hence, is purely subjective on the part of the investigator depending upon what each procedure has to offer in view of the tissue type used and the requirements set for a particular research problem. For example, bicarbonate formaldehyde fixation for liver tissue and ten percent buffered formalin fixation for amoebal nuclei may appeal to some investigators because of their ability to provide intense staining, making microscopic detection of nuclei relatively easy in comparison with lightly stained nuclei. Time factors, such as length of fixation period and length of hydrolysis time before maximal stain intensity is achieved and/or length of its persistance (plateau), may also influence the choice.

In recent years, much time and study has been devoted to quantitative Feulgen analyses of nuclear DNA changes in the hope of elucidating the mechanisms by which cellular

growth and differentiation occur. The model systems utilized have expanded over the years to include inumerable cellular systems possessing unique characteristics reflected through the singular way in which each system is affected by Feulgen reaction procedures in providing comparable values of the relative DNA content of cell nuclei. The data and findings presented in this study represent the effects of three specific fixatives on the Feulgen staining intensity of two distinct cell types and implies that the occurrence of similar results for any other tissue type using these fixatives would occur. Despite the differences in maximal staining intensities obtained between fixatives, any fixation procedure utilized in this study can accurately represent the relative amount of nuclear DNA present in cell nuclei depending upon the effects of that specific reaction procedure on the nucleic acid. For example, if intense staining is desired, the results indicate that the choice of ten percent buffered formalin for amoebal nuclei and of bicarbonate formaldehyde for liver nuclei as fixatives would be appropriate.

Thus, in view of the fact that the same fixative may have different effects on the staining intensity of the Feulgen nucleal reaction for distinct cell types, and, that, diverse cellular systems are now being employed in such quantitative studies, careful consideration should be given toward the type of fixative used and the reaction parameters employed in providing the best means of obtaining the desired results

for a particular cell type.

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