AN ABSTRACT OF THE THESIS OF

FRANK JOSEPH LONGO for the M.S. in Zoology
(Name) (Degree) (Major)

Date thesis is presented August 20, 1964

Title CYTOPHOTOMETRIC DETERMINATIONS OF NUCLEAR DNA CONTENT IN TISSUES OF THE WAX MOTH LARVA, GALLERIA MELLONELLA LINNAEUS.

Abstract approved _____
(Major professor)

The present study was undertaken to determine, by measurement of nuclear DNA, the degrees and frequencies of ploidy in the last larval instar of the moth Galleria mellonella Linnaeus. While such data have been amassed for a wide variety of vertebrate tissues, few studies have been concerned with invertebrates and especially insects where somatic polyploidy is developed to an unusually high degree.

Relative amounts of DNA were determined cytophotometrically on individual Feulgen-stained nuclei of hypodermis, ventriculus, fat cells, blood cells, anterior silk gland, and proventriculus. Graphs of the determined DNA values indicated the presence of nuclear classes falling into polyploid ratios.

The majority of blood cell and fat cell nuclei had class I values (= diploid), while class II and III (= tetraploid and octoploid)

DNA values represented about 17 to 20 percent of the total nuclear population. The majority of DNA values of the ventriculus nuclei fell into the second class while 6.8 percent were considered as class I. The hypodermal nuclei were mostly of class I, while class II values represented 6.6 percent of the total number of determinations.

Nuclei of the proventriculus and the anterior silk gland showed a broad spectrum of DNA values ranging from classes IV to VIII including many intermediate values.

The method of measurement was analyzed for sources of error. Various interpretations were offered for the variations in DNA values including the role of endomitosis, definitive polyploidy, aneuploidy, differential gene replication, etc. In the case of the anterior silk gland and the proventriculus a possible relationship between DNA amounts and cell activity is indicated.

CYTOPHOTOMETRIC DETERMINATIONS OF NUCLEAR DNA CONTENT IN TISSUES OF THE WAX MOTH LARVA, GALLERIA MELLONELLA LINNAEUS

by

FRANK JOSEPH LONGO

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1965

APPROVED:

Redacted for Privacy

Professor and Chairman of Department of Zoology

In Charge of Major

Redacted for Privacy

Dean of Graduate School

Date thesis is presented August 20, 1964

Typed by Nancy Kerley

ACKNOWLEDGMENTS

Heartfelt thanks are expressed to Dr. Ernst J. Dornfeld for recommending this study, for his critical advice and encouragement, and for his help in the preparation of this thesis.

Sincere gratitude is acknowledged to Dr. A. Owczarzak for his technical instruction and assistance and his critical guidance.

Appreciation is also expressed to Dr. G. C. Wittig for providing the specimens; to John Belton for his direction in the preparation of the many photomicrographs necessary to this study; and to my wife, Kitty, for her assistance in the numerous calculations and her constant encouragement.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
MATERIALS AND METHODS	4
Fixation and Staining	4
Photometric Apparatus	5
Method of Measurement	6
RESULTS	9
Hypodermal Nuclei	9
Ventriculus Nuclei	10
Fat Cell Nuclei	10
Blood Cell Nuclei	11
Proventriculus Nuclei	12
Anterior Silk Gland Nuclei	12
DISCUSSION AND CONCLUSIONS	14
Specificity of the Feulgen Stain	14
Sources of Error in Cytospectrophotometry	15
Analysis of Results	17
SUMMARY	22
BIBLIOGRAPHY	25
APPENDIX	30
Derivation of the Photometric Formula	30
Plates	32

CYTOPHOTOMETRIC DETERMINATIONS OF NUCLEAR DNA CONTENT IN TISSUES OF THE WAX MOTH LARVA, GALLERIA MELLONELLA LINNAEUS

INTRODUCTION

It is a well established fact that desoxyribose nucleic acid (DNA) constitutes a major component of the hereditary nuclear material and can be taken as an index of the chromatin content within a cell. Quantitative determinations of DNA are therefore useful in establishing phases of chromosome replication and degrees of ploidy or polyteny within cell nuclei. For such studies Lessler (1953) considers the Feulgen nucleal reaction to be the most reliable. This reaction was first described by Feulgen and Rossenback in 1924 and since that time it has been the most widely used quantitative cytochemical test for DNA.

Early quantitative studies on DNA were based on biochemical analysis of mass nuclear suspensions. The method was first used by Bovin, Vendrely, and Vendrely (1948) on mammalian tissues and subsequently employed by Mirsky and Ris (1949) on investigations of fish, amphibian, reptile, and bird tissues. Results from such work indicated that the nuclear DNA content is specific for any one species and that it may be viewed as a possible genetic component. However, the nuclear DNA content determined with similar methods by Mirsky and Ris (1949) for different beef tissues demonstrated results

inconsistent with this theory. It was suggested that since the nuclear suspension method yields determinations representing average values for a great number of nuclei no insight could be gained as to the DNA composition of specific cells and therefore variations between individual nuclei were lost in the mass examinations.

Photometric measurements of individual nuclei present a more complete picture of the variations in the DNA content between nuclei of the same and different tissue types. The first cytospectrophotometric method, introduced by Caspersson (1936), demonstrated that the purines and pyrimidines of nucleic acids have maximum absorbencies when examined with ultraviolet light at 260 mμ. The method of measuring the absorption of visible light by Feulgen stained nuclei was later developed by Pollister and Ris (1947) and by Pollister and Moses (1949). Although the method is simple in theory, in practice it is limited by heterogeneous chromatin distribution and by variation in nuclear size and shape. In 1952 Patau (1952) and Ornstein (1952) independently devised a method to circumvent the above difficulties. Later, in 1958 and 1961, Mendelsohn (1958b; 1961) modified the newer method by reducing the number of operational steps and providing tables of standard calculations. Essentially the method devised by Patau and Ornstein employs two wavelengths of light and is not dependent on chromatin distribution and nuclear geometry.

Through the use of the photometric method considerable

attention has been given to the DNA variation in the mitotic and meiotic cycles, the degree and frequency of ploidy, and DNA variations accompanying physiological changes. Photometric measurements of ploidy in interphasic nuclei are particularly valuable, since chromosome counts cannot be carried out and estimates based on nuclear size are unreliable. Increase in nuclear volume does not necessarily indicate an increase in chromosome number or in DNA content. Polyploid classes and their frequencies have been determined for a host of animal tissues, most of which have been vertebrate types. With regard to studies on invertebrate tissues rather little has been done. In the latter group may be mentioned the work of Merriam and Ris (1954) on honey-bee tissues; Leuchtenberger and Schrader (1952) on the salivary glands of Helix; Lison and Pasteels (1951) on blastomeres of the sea urchin; Swift and Kleinfeld (1953) on grasshopper spermatogenesis, oögenesis, and cleavage; Kurnick and Herskowitz (1952) on salivary gland nuclei of Drosophila; and Stich and Naylor (1958) on polytene chromosomes of chironomids.

The present study was undertaken to extend this rather limited body of information by producing data on the nuclear distribution of DNA in various larval tissues of the wax moth, Galleria mellonella Linnaeus.

MATERIALS AND METHODS

Fixation and Staining

Last instar larvae of Galleria mellonella were obtained from the Forest Science Laboratory of Oregon State University. They average about 3.0 cm in length and 0.5 cm in diameter, are relatively unornamented, gray in color, and with few bristles. In a preliminary histological examination a nuclear size inventory was made on the larval tissues. The material for investigation was prepared in the following manner: The larvae were injected with Carnoy's fluid (three parts of absolute ethanol to one part glacial acetic acid). The head and part of the posterior end were removed and the animals were bisected into anterior and posterior halves. The bisected portions were placed into vials containing Carnoy's fluid and fixed under vacuum for five hours. The fluid was changed three times to compensate for the change in concentration of the fixative while under a decreasing partial pressure. Following fixation the larvae were dehydrated and embedded in paraffin in the usual histological manner. Cross sections were made of the bisected halves according to the results of the nuclear size inventory, specifically, at thicknesses equal to one and one half times the size of the nuclear type under study. Sections were cut at 25 μ for anterior silk gland and

proventriculus; 10 μ for hypodermis, blood cells, and ventriculus; and 12 μ for fat cells. Staining with the Feulgen reagent followed a modified method described by Stowell (1945). Tissue sections were hydrolyzed in 1N HCl at 60° C for ten minutes followed by staining in sulphurous fuchsin reagent for one hour. Unhydrolyzed sections were also stained for controls. Any one slide contained sections of only one thickness. Hypodermal cells were found in all sections and were used as a standard of comparison. All the slides were stained with the same stock Feulgen reagent kept colorless by storage in a tightly closed container at 5° C.

Photometric Apparatus

The photometric apparatus (Plate VII) consisted essentially of a microscope and a Farrand photomultiplier unit. The light source was a 115-volt tungsten coiled filament mounted in a Spencer illuminator connected through a Raytheon voltage stabilizer to a 110-volt line source. One coil of the lamp was used as a point source of light. It was focused on the entrance slit of a Bausch and Lomb grating monochromator set at 0.7 mm. The magnified image of the microscope field was projected through the bellows of a Leitz Aristophot camera and viewed with a telescope carried on a modified plate carrier similar to the photohead assembly designed by Birge (1959). The nuclear sizes and field areas could be measured in

microns with an ocular micrometer in the telescope. By sliding the telescope out of position the image was directed to an RCA 1P21 phototube. An iris diaphragm placed directly under the phototube limited the projection on the photosensitive area to the desired field area.

All absorption measurements were taken at 490 mμ and 514 mμ isolated from the light source by the Bausch and Lomb monochromator. Bausch and Lomb 4 mm and 28 mm achromatic objectives, N. A. 0. 65 and 0. 08, respectively, were used together with 8X, 10X, and 15X Leitz periplan oculars depending on the magnification required. The objectives were mounted on a centerable nose piece. A Leitz aplanic, achromatic centerable condenser, N. A. 1. 40, completed the optical set-up. Photocurrents were read on an RCA ultra-sensitive DC microammeter of 0 to 1000 μamp range and 50 megohms maximum input resistance.

Method of Measurement

The following data were recorded (see sample data sheet, Plate VIII):

1) Code number of specific nuclei (#)

2) Area of the microscope field in microns (B) containing the whole nucleus and a border of cytoplasm

3) Microammeter deflection when light of 490 mμ passed through the nucleus (I_{11})

4) Microammeter deflection when light of 490 mμ passed through the cytoplasm near the nucleus (I_{10})

5) Transmission (T_1) of light (490 mμ), given by I_{11}/I_{10}

6) Reciprocal of T_1 (L_1), given by $1-T_1$

7) Microammeter deflection when light of 514 mμ passed through the nucleus (I_{22})

8) Microammeter deflection when light of 514 mμ passed through the cytoplasm near the nucleus (I_{20})

9) Transmission (T_2) of light (514 mμ), given by I_{22}/I_{20}

10) Reciprocal of T_2 (L_2), given by $1-T_2$

11) Factor (Q) expressing the product of L_2/L_1

12) Correction factor (C) which is a function of Q. This factor is given in chart form by Patau (1952) and can be computed from the following equation:

$$C = \frac{1}{2-Q} \ln \frac{1}{Q-1}$$

13) Relative amount of the absorbing substance (γ), or DNA in the whole field area (B). This is calculated from the formula used by Patau (1952), viz.,

$$\gamma = KBL_1C$$

where γ is the amount of DNA in arbitrary units, K is a constant

equal to $\frac{1}{k_1}$ ln 10 (where k_1 is the extinction coefficient) and may be neglected when relative amounts of γ are desired, B is the area of the microscope field containing the nucleus, L_1 is the reciprocal of $1-T_1$, and C is the correction factor given by Patau. Derivation of this formula is given in the appendix.

14) Factor (M) representing the average relative amount of DNA for a series of two readings.

Care was taken to measure only entire nuclei as determined by critically focusing beyond their upper and lower surfaces. Nuclei which were completely out of focus when other objects remained sharply in focus were assumed to be whole. Errors involved in the measurements of incomplete nuclei were thus avoided.

Magnification at the level of the phototube was adjusted in such a way that the diameter of the measured nucleus would not exceed the range of two mm to ten mm. Two millimeters was the lower limit of uniform sensitivity of the phototube while ten mm was the upper limit. Field areas were measured to the nearest tenths of all magnifications. Microammeter deflection was estimated to the nearest thousandth.

RESULTS

Five last instar larvae were used for the present study. For each larva about 15 nuclei of each cell type were measured for DNA content. Analysis of the DNA content per cell type was made on an individual larva as well as a collective basis. Examination of the graphs on Plates X, XII, XIV, XVI, XVIII, and XX demonstrates that no real significant differences exist among the five larvae. Therefore the results will be discussed collectively by the tissue type.

Microscopic appearances of nuclei of the ventriculus, proventriculus, blood cells, fat cells, hypodermis, and anterior region of the silk gland are shown in Plates II through VI.

Hypodermal Nuclei

Fifteen nuclei of the hypodermis were measured in each of the five larvae. Distributions of the relative amounts of DNA are graphically recorded in Plates IX and X. The majority of DNA values, ranging from 1.0 to 3.0, form a rather sharply delimited and fairly symmetrical unimodal curve. A few DNA values, 6.6 percent of the total number of determinations, range from 3.5 to 4.0. DNA values of hypodermal nuclei were thus grouped into two classes. The peak value for class I nuclei (diploid) is 2.0 while the mean and standard deviation are 1.94 and 0.14, respectively. Class II nuclei (tetraploid),

which include DNA values double the class I peak, show a mean and standard deviation of 3.8 and 0.26.

Ventriculus Nuclei

The number of ventriculus nuclei examined was 73. The results are graphed on Plates XI and XII. The majority of DNA values, ranging from 3.0 to 6.5, form a fairly symmetrical unimodal curve with a peak value of 4.0. These DNA values fall into class II and have a mean and standard deviation of 4.25 and 0.89, respectively. A few DNA values, 6.8 percent of the total number of determinations, ranging from 1.5 to 2.5, fall into class I; mean and standard deviation are 2.0 and 0.36.

Fat Cell Nuclei

The distribution of DNA values for 76 fat cell nuclei are shown on Plates XIII and XIV. The results obtained are essentially similar to those found in the hypodermal nuclei. Within the range of 0.50 to 3.0 the DNA values form a high unimodal curve with a peak value of 1.5 (class I nuclei). Some DNA values, 12 percent of the total, are found in the range of 3.5 to 5.5 (class II nuclei), while a few DNA values, 5.2 percent of the total number of determinations, are found in the range of 6.0 to 7.5 (class III, or octoploid nuclei). The mean and standard deviation of the class I values are 1.47 and 0.60,

respectively. The slightly lower mean values of the class I nuclei can be accounted for by the degeneration of the fat cells as the larvae approach metamorphosis. Mention of this phenomenon will be made in the discussion. For class II nuclei, the mean and standard deviation are 4.61 and 0.80, respectively; for class III they are 7.0 and 0.55.

Blood Cell Nuclei

Distribution of DNA values for 47 blood cell nuclei are given on Plates XV and XVI. Due to the difficulty in finding these cells in tissue sections 75 measurements (15 measurements per larva) could not be made. Random measurements were made of various blood cell nuclei without distinction of possible types, which proved insurmountably difficult. DNA values of class I nuclei form a bimodal curve ranging from 1.0 to 3.0 with peaks at 1.5 and 2.5. The mean and standard deviation are 1.82 and 0.67, respectively, for the bimodal range. Lack of a symmetrical unimodal curve may be due to an insufficient number of readings and/or the inability of classifying the various blood cell types. Fourteen percent of the total number of determinations range from 3.5 to 6.0 and are considered to be class II nuclei. Mean and standard deviation of the class II nuclei are 4.21 and 0.88, respectively. Class III nuclei, making up 6.4 percent of the total determinations, range from 6.5 to 8.5. Mean and

standard deviation for the class III nuclei are 8.0 and 0.86.

Proventriculus Nuclei

Due to the large size and overlapping disposition of the proventricular nuclei difficulty was encountered in attempting to measure large numbers of single, entire nuclei. Distribution of the 52 DNA values (graphs on Plates XVII and XVIII) form a rather wide spectrum from 23 to 143 with no evidence of symmetrical unimodal curves. The bulk of the measurements (62.5 percent) tend to fall into the range of 44 to 82. Placement of the DNA values into nuclear classes is at best difficult. A possible grouping would include in class IV the values 12 to 24, in class V 32 to 49, class VI 50 to 95, and class VII 98 to 143. Such a breakdown would yield successive doubling of mean values (IV = 23.0; V = 44.0; VI = 64.3; and VII = 111.6). Further consideration of this broad range of DNA values is deferred to the discussion.

Anterior Silk Gland Nuclei

The number of anterior silk gland nuclei measured was 54 (graphs on Plates XIX and XX). As in the proventriculus, the silk gland nuclei reach gigantic size and tend to overlap in sections; hence the standard quota of measurable nuclei could not be reached. Distribution of the DNA values form a broad spectrum, from 13 to

201.5, reminiscent of the values found in the proventriculus. The bulk of the measurements (59.2 percent) fall in the range of 29 to 76. There exists no clear evidence of unimodal curves or sequential doubling of DNA values. Again placement of the DNA values into nuclear classes is at best difficult. Possible groupings would include in class IV 13 to 28, class V 29 to 49, class VI 50 to 95, class VII 97 to 160, and class VIII 169 to 201. Such a breakdown would roughly yield successive doubling of mean values (IV = 13.0; V = 37.6; VI = 70.0; VII = 113.0; and VIII = 201.5). Further consideration of this situation will be deferred to the discussion.

DISCUSSION AND CONCLUSIONS

Specificity of the Feulgen Stain

The exact mechanism of the Feulgen nucleal reaction for DNA has not been fully established. However, it is generally agreed that a positive Feulgen reaction after acid hydrolysis under controlled conditions coupled with a negative reaction without hydrolysis is a specific test for the presence of DNA-protein.

Ris and Mirsky (1949) have demonstrated the quantitative nature of the Feulgen reaction by comparing results obtained on cytological preparations with DNA values determined biochemically. Work by Tamm, Hodes, and Chargaff (1952), Overend (1950), and Overend and Stacey (1949) give evidence that controlled mild acid hydrolysis removes purines from DNA without significant loss of polymerization of the remaining molecule, thus providing specific loci of attachment for leucofuchsin. Swift (1950) and Hoover and Thomas (1951) have demonstrated that the color intensity of Feulgen preparations varies directly with the thickness of the absorbing layer, obeying the requirements of the Beer-Lambert law. The constancy in DNA values of Feulgen-stained nuclei, as well as the 1:2:4 ratios reported in numerous studies of polyploid series offers good indication of the quantitative nature of this staining reaction. Further

studies on the Feulgen reaction are reviewed by Lessler (1953) and Novikoff (1955) who have concluded the reaction to be highly reliable.

Conditions such as temperature of the hydrolytic solution, hydrolysis time, pH, and duration of staining in the Feulgen reagent must be controlled if reproducible results are to be obtained. In the course of this study standardized conditions were maintained.

Sources of Error in Cytospectrophotometry

The various sources of error found in the two-wavelength method have been discussed and estimated by Patau (1952), Ornstein (1952), Pollister and Ornstein (1955), Mendelsohn (1958a; 1958b; 1961), and Garcia (1962a; 1962b).

Essentially, there exist in photometry two major sources of optical error which may be considered as the most important. The first is the "distributional error" which is caused by non-uniformity of the dye distribution in the object and the second is the "stray light error" which is due to extraneous light waves entering the photo-receiver.

In principle the "distributional error" can be eliminated by using a wavelength of light at which the stained object is so highly transparent that all dye "particles" are exposed to the same flux of photons and there is no effective shadowing. Thus the amount of light absorbed out of a given light flux will be proportional to the number

of dye "particles" present. In effect the two-wavelength method utilizes such a principle, correcting for the "distributional error." Patau (1952) has demonstrated that this error (δ) is equal to $\frac{\gamma-\gamma'}{\gamma'}$, where γ is an approximation of the true amount of γ' dye. With increasing transparency (brought about by changes of the wavelengths) δ will converge towards zero. Computation of this formula also demonstrates that the field area (B) has no influence on γ. Analytical treatment of the "distributional error" (δ) appears to be a hopeless undertaking in view of the infinite number of possible dye distributions. However, through the use of nuclear models that depict selected and varied chromatin distributions it can be demonstrated that the two-wavelength method may have a "distributional error" of less than two percent.

Stray light or the scattering of light into the measured beam by the surrounding tissues [referred to as the Schwarzschild-Villiger effect and discussed by Naora (1951; 1952; 1955)] was reduced by closing down the vertical slits of the monochromator to 0.7 mm so that the illuminated field was a few diameters larger than the nucleus to be measured. By employing wavelengths somewhat off the Feulgen absorption peak errors arising from high extinction values were further minimized.

Other errors that may occur in photometry may take the form of non-specific light loss, measurement of incomplete nuclei,

and non-linearity of phototube response. Non-specific light loss may occur due to the scattering of light out of the measured beam by colloidal particles and by large bodies of different refractive indices from the surrounding medium. The degree of this light loss may be determined by measuring undyed tissue otherwise identical to the test object. Swift (1950) has reported that such measurements may yield negative absorption. In this case correction for such non-specific light was found unnecessary.

Measurement of incomplete nuclei was avoided by the method previously explained. It is unlikely that any incomplete nuclei were measured.

Linearity of phototube response was checked before experimental measurements were made. As discussed earlier, linearity was obtained at focal plane (level of the phototube) diameters of two to ten millimeters. Magnifications were adjusted for measurements within these limits.

Analysis of Results

In the data presented, the measurements form curves essentially similar to those usually obtained in Feulgen photometric studies of nuclei. Nuclear DNA classes indicated by the curves suggest a successive doubling of chromatin material. Class II nuclei may be considered as interphasic doubling of DNA prior to chromatid

separation were it not for the fact that mitotic figures do not occur in the examined tissue sections. Therefore it is likely that these nuclei are definitively tetraploid or in an equivalent polytene condition.

Variation within classes may be accounted for by the overall error involved in the cytophotometric method. The exact amount of this error can not be assessed with certainty. Patau (1952) calculates that under the proper conditions this error may be kept below three percent of the true dye content. Some of the variations may be accounted for by incomplete synthesis of DNA during endomitosis or be attributed to aneuploidy. Without actual chromosome counts aneuploidy can not be established with confidence, but it has been chromosomally demonstrated in vertebrate tissues by Hsu and Pomerat (1953), Walker and Boothroyd (1954), and others.

Low polyploid frequencies are found in the blood cells, hypodermis, ventriculus, and fat cells in contrast to the anterior silk gland and proventriculus where these frequencies are high. The low frequencies, however, may be largely spurious and due to mitotic cycling.

The development of fat cells and their histolysis during insect growth and metamorphosis has been discussed by Nakahara (1917), Bishop (1922; 1923), and Perez (1910); a general discussion is given by Wigglesworth (1953). As an insect approaches metamorphosis fat

cell nuclei become intensely active. The nuclear surface may be increased by amitosis (Nakahara, 1917) or by the elongation or branching of the nucleus (Bishop, 1922; 1923). These phenomena may be accompanied by the breakdown of the nuclear membrane and the discharge of basophilic Feulgen-negative granules from the nucleus (Wigglesworth, 1953). Examination of Plate IV will show the fat cell nucleus undergoing several of these changes prior to pupation. Lower DNA values tend to indicate the destruction or removal of chromatin material as cited by Bishop (1922; 1923) and Paillot and Noel (1937). However, to establish this change of nuclear content unequivocally a photometric study would be necessary which would follow the course of development and histolysis in the fat cells.

The proventriculus and anterior silk gland nuclei present rather interesting cases. In view of the great number of nuclei with apparent intermediate DNA values, it may be that DNA replication or chromatin duplication is not wholly euploid. Similar cases have been observed by Truong and Dornfeld (1955) and Leuchtenberger and Schrader (1952). The possibility also exists that the excessive number of intermediate DNA values reflect a high rate of endomitotic activity thus resulting in a picture similar to that obtained when examining tissues with high mitotic rates.

Work by Breuer and Pavan (1955), Beermann and Bahr (1954), and Mechelke (1952) has demonstrated that at certain stages of

dipteran larval development specific bands of polytene chromosomes show enlargements called chromosomal puffs or Balbiani rings. Investigations with the use of tritiated thymidine by Ficq and Pavan (1957) have shown that there is a build-up of DNA followed by the synthesis of RNA at these chromosomal sites. The possibility of the same or a similar phenomenon being responsible for the number of intermediate DNA values in the larvae of Galleria mellonella can not be ignored. It is conceivable that during development some genes or groups of genes have enhanced physiological activities and are engaged in excessive replication. Thus different DNA values may be obtained from cells of the same tissue type depending on variables in the times and degrees of their activity.

Nuclear and cytoplasmic changes accompanying the various phases of secretory activity in the silk glands of the larvae of Galleria mellonella have been described by Wu (1930). It was suggested, although without certitude, that the anterior portion has little or no role in secretory activity. Intense activity, however, was demonstrated for the middle and posterior portions of the gland. The high DNA values found in this study to occur in the anterior portion of the silk gland nevertheless suggest some kind of synthetic activity. The highly branched and attenuated nuclei of the middle and posterior regions were, unfortunately, not amenable to DNA measurement, but it is quite obvious that they represent degrees of ploidy far in excess

of those in the anterior region (cf. Plate VI). It may be pertinent to comment that similar nuclei from salivary glands of the heteropteran water-strider, Gerris lateralis, were found, by x-chromosome count, to be around 2048-ploid (Geitler, 1938).

SUMMARY

1) Relative DNA amounts were measured photometrically on individual Feulgen-stained nuclei of hypodermis, ventriculus, fat cells, blood cells, anterior silk gland, and proventriculus of the last instar larvae of Galleria mellonella Linnaeus. Graphs of the determined DNA values indicated the presence of nuclear classes falling into, and in some cases between, polyploid ratios.

2) Seventy-five hypodermis nuclei were measured in five larvae. The majority of DNA values fell into the first (or diploid) class while a few values, 6.6 percent of the total number of determinations, constituted class II (tetraploid).

3) The number of ventriculus nuclei examined was 73. The majority of the DNA values fell into class II while a few (6.8 percent) were considered as class I nuclei.

4) The number of DNA values determined for the fat cell nuclei was 76. The bulk of the DNA values fell into class I. The mean value of the class I nuclei here was slightly lower than that experienced in the ventriculus and hypodermis (1.47 as compared to 2.0 and 1.94, respectively). This lower value was attributed to the presence of histolysis prior to metamorphosis. Class II and class III values were present also, making up 12 percent and 5.2 percent, respectively.

5) Only 47 blood cell nuclei were measured, due to the difficulty in finding these cells in tissue sections. Random measurements were made without distinction of possible cell types. The majority of the DNA values fell into the first class while 14 percent and 6. 4 percent of the total determinations were considered to be classes II and III, respectively.

6) Due to their large size and over-lapping disposition only 52 proventriculus nuclei could be measured. A broad spectrum of DNA values was found ranging from 23 to 143, with a great number of intermediate values and no evidence of unimodal curves. This spectrum encompassed classes IV through VII. Various explanations were proposed to account for this phenomenon.

7) The number of anterior silk gland nuclei measured was 54. Again a broad spectrum of DNA values was found ranging from 13 to 201. 5, with a great number of intermediate values and no evidence of unimodal curves. The highly branched, attenuated, and overlapping nuclei of the middle region of the silk gland were unmeasurable, but their DNA values very likely extend to class XI (2048-ploid).

8) The sources of error in the measurements were analyzed. Various interpretations were given for variations in DNA values which included endomitosis, definitive polyploidy, aneuploidy, differential gene replication, etc. In the case of the silk gland and the

proventriculus a strong relationship between DNA content and cell activity appears to exist. The nature of this relationship needs separate study.

BIBLIOGRAPHY

Beermann, W. and G. F. Bahr. The submicroscopic structure of the Balbiani ring. Experimental Cell Research 6:195-201. 1954.

Birge, W. J. A photometer head assembly for use in microphotometry. Journal of Histochemistry and Cytochemistry 7:395-397. 1959.

Bishop, G. H. Cell metabolism in the insect fat-body. I. Cytological changes accompanying growth and histolysis of the fat-body in Apis mellifica. Journal of Morphology 36:567-601. 1922.

Bishop, G. H. Cell metabolism in the insect fat-body. II. A functional interpretation of the changes in structure in the fat-body cells of the honey bee. Journal of Morphology 37:533-553. 1923.

Boivin, A., R. Vendrely and C. Vendrely. L'acide désoxyribonucléique du noyau cellulaire, dépositaire des caractères héréditaires; arguments d'ordre analytique. Comptes Rendus des Séances de l'Académie des Sciences (Paris) 226:1061-1063. 1948.

Breuer, M. E. and C. Pavan. Behavior of polytene chromosomes of Rhynchosciara angelae at different stages of larval development. Chromosome 7:371-386. 1955.

Caspersson, T. Über den chemischen Aufbau des Zellkerns. Skandinavisches Archiv für Physiologie 73(suppl. 8):1-15. 1936.

Ficq, A. and C. Pavan. Autoradiography of polytene chromosomes of Rhynchosciara angelae at different stages of larval development. Nature 180:983-984. 1957.

Garcia, A. M. Studies on DNA in leucocytes and related cells of mammals. I. On microspectrophotometric errors and statistical models. Histochemie 3:170-177. 1962a.

Garcia, A. M. Studies on DNA in leucocytes and related cells of mammals. II. On the Feulgen reaction and two wavelength microspectrophotometry. Histochemie 3:178-194. 1962b.

Geitler, L. Über den Bau des Ruhekerns mit besonderer Berücksichtigung der Heteropteren und Dipteren. Biologisches Zentralblatt 58:152-178. 1938.

Hoover, C. R. and L. E. Thomas. Microspectrophotometric studies of the Feulgen and arginine histochemical reactions. Journal of the National Cancer Institute 12:219-223. 1951.

Hsu, T. C. and C. M. Pomerat. Mammalian chromosomes in vitro. III. On somatic aneuploidy. Journal of Morphology 93:42-54. 1951.

Kurnick, N. B. and I. H. Herskowitz. The estimation of polyteny in Drosophila salivary gland nuclei based on determination of DNA content. Journal of Cellular and Comparative Physiology 39:281-300. 1952.

Lessler, M. A. The nature and specificity of the Feulgen reaction. International Review of Cytology 2:231-247. 1953.

Leuchtenberger, C. and F. Schrader. Variation in the amounts of DNA in cells of the same tissue and its correlation with secretory function. Proceedings of the National Academy of Science 38:99-105. 1952.

Lison, L. and J. Pasteels. Études histophotometrique sur la teneur en acide desoxyribonucléique des noyaux au cours du development embryonnaire chez l'oursin Paracentrotus lividus. Archives de Biologie 62:1-64. 1951.

Mechelke, F. Die Entstehung der polyploiden Zellkerne des Antherentapetums bei Antirrhinum majus L. Chromosoma 5: 246-295. 1952.

Mendelsohn, M. L. The two-wavelength method of microspectrophotometry. I. A microspectrophotometer and tests on model systems. Journal of Biophysical and Biochemical Cytology 4: 407-414. 1958a.

Mendelsohn, M. L. The two-wavelength method of microspectrophotometry. II. A set of tables to facilitate the calculations. Journal of Biophysical and Biochemical Cytology 4:415-424. 1958b.

Mendelsohn, M. L. The two-wavelength method of microspectrophotometry. III. An extension based on photographic color transparencies. Journal of Biophysical and Biochemical Cytology 4:425-451. 1958c.

Mendelsohn, M. L. The two-wavelength method of microspectrophotometry. IV. A new solution. Journal of Biophysical and Biochemical Cytology 11:509-513. 1961.

Merriam, R. W. and H. Ris. Size and DNA content of nuclei in various tissues of male, female, and worker honey bees. Chromosoma 6:522-538. 1954.

Mirsky, A. E. and H. Ris. Variable and constant components of chromosomes. Nature 163:666-667. 1949.

Nakshara, W. Studies of amitosis: Its physiological relations in the adipose cells of insects, and its probable significance. Journal of Morphology 30:483-525. 1917.

Naora, H. Microspectrophotometry and cytochemical analysis of nucleic acids. Science 114:279. 1951.

Naora, H. Schwarzschild-Villiger effect in microspectrophotometry. Science 115:248-249. 1952.

Naora, H. Microspectrophotometry of cell nucleus stained by Feulgen reaction. I. Microspectrophotometric apparatus without Schwarzschild-Villiger effect. Experimental Cell Research 8:259-278. 1955.

Novikoff, A. B. Histochemical and cytochemical staining methods. In: Analytical Cytology, ed. R. C. Mellors. McGraw-Hill Book Co., Inc., 1955. p. 1-63.

Ornstein, L. The distributional error in microspectrophotometry of cells. Laboratory Investigation 1:250-262. 1952.

Overend, W. G. Desoxy-sugars. Part XIII. Some observations of the Feulgen nucleal reaction. Journal of the Chemical Society, 1950, p. 2769-2774.

Overend, W. G. and M. Stacey. Mechanism of the Feulgen nucleal reaction. Nature 163:538-540. 1949.

Paillot, A. and R. Noel. Nouvelles recherches sur l'histophysiologie du corps adipeux et des tissus ectodermiques du Bomby du mûrier perdant la mue. Competes Rendus des Séances de l' Académie des Sciences (Paris) 205:1095-1096. 1937.

Patau, K. Absorption microphotometry of irregular-shaped objects. Chromosoma 5:341-362. 1952.

Pérez, C. Recherches histologiques sur le métamorphose des Muscides: Calliphora erythrocephala Mg. Archives de Zoologie Expérimentale et Générale 44:1-274. 1910.

Pollister, A. W. and M. J. Moses. A simplified apparatus for photometric analysis and photomicrography. Journal of General Physiology 32:567-577. 1949.

Pollister, A. W. and L. Ornstein. Cytophotometric analysis in the visible spectrum. In: Analytical Cytology, ed. R. C. Mellors. McGraw-Hill Book Co., Inc., 1955, p. 1-71.

Pollister, A. W. and H. Ris. Nucleoprotein determination in cytological preparations. Cold Spring Harbor Symposia on Quantitative Biology 12:147-157. 1947.

Ris, H. and A. E. Mirsky. Quantitative cytochemical determinations of desoxyribonucleic acid with the Feulgen nucleal reaction. Journal of General Physiology 33:125-146. 1949.

Stich, H. F. and J. M. Naylor. Variation of desoxyribonucleic acid content of specific chromosome regions. Experimental Cell Research 14:442-445. 1958.

Stowell, R. E. Feulgen reaction for thymonucleic acid. Stain Technology 20:45-58. 1945.

Swift, H. H. The desoxyribose nucleic acid content of animal nuclei. Physiological Zoology 23:169-198. 1950.

Swift, H. H. and R. Kleinfeld. DNA in grasshopper spermatogenesis, oogenesis, and cleavage. Physiological Zoology 26:301-311. 1953.

Tamm, C., M. E. Hodes, and E. Chargaff. The formation of apurinic acid from the desoxyribose nucleic acid of calf thymus. Journal of Biological Chemistry 195:49-63. 1952.

Truong, S. and E. J. Dornfeld. Desoxyribose nucleic acid content in the nuclei of salamander somatic tissues. Biological Bulletin 108:242-251. 1955.

Walker, B. E. and E. R. Boothroyd. Chromosome numbers in somatic tissues of mouse and man. Genetics 39:210-219. 1954.

Wigglesworth, V. B. the principles of insect physiology. 5th ed. London, Methuen and Co., 1953. p. 289-308.

Wu, Chao-Fa. Cytological studies on the spinning glands of the larvae of Galleria mellonella. Journal of Morphology 49:509-534. 1930.

APPENDIX

DERIVATION OF THE PHOTOMETRIC FORMULA $\gamma = KBL_1C$*

λ_1 and λ_2 have been selected so that the test object yields extinctions $E(\lambda_2) = 2E(\lambda_1)$. If these measurements are absolutely accurate, if there is not stray light, and if the test object is a uniformly stained layer, then $k_2 = 2k_1$ (where k is the extinction coefficient). Let, instead, the true ratio of extinction coefficients be $k_1:k_2 = 1:2(1+\Delta)$. The small unknown error (Δ) is composed of three parts: $\Delta = \Delta_1 + \Delta_2 + \Delta_3$. Δ_1 is the sampling error of the galvanometer readings and can be made very small by taking a sufficient number of repeated readings. Δ_2 is approximately the difference of the relative stray light errors committed in measurement of $E(\lambda_2)$ and $E(\lambda_1)$. These are the stray light errors of the conventional method. Δ_3 is caused by a non-uniform dye distribution within the test object. Distributional errors are caused by the existence of different light paths along which photons have different probabilities of being absorbed.

It is assumed that an object to be determined is uniformly absorbing and that the illuminating beam consists of parallel light with normal incidence then the following may be demonstrated. Let τ_v be the unknown transmission of the object at $\lambda_v (v = 1, 2)$. Let

---

*This derivation is condensed from Patau (1952).

$F \leq B$ be the unknown total area of the object. The flux through F in the absence of the object is $F/B\ I_{v0}$; that with the object is $I_{v1} - \frac{B-F}{B} I_{v0}$.

Hence:

$$\eta_v = 1 - \frac{B}{F} L_v \tag{1}$$

The object has, at λ_v, the extinction $E_v = -\log \eta_v$. Because of $k_2 = 2(1 + \Delta)k_1$ it is $E_2 = 2(1 + \Delta)E_1$ and $\eta_2 = \eta_1^{2(1+\Delta)}$.

This in conjunction with equation (1) leads to

$$Q = \frac{L_2}{L_1} = 1 + \eta_1 \frac{1 - \eta_1^{1+2\Delta}}{1-\eta_1} .$$

Introducing

$$q(\Delta) = \frac{1 - \eta_1}{1 - \eta_1^{1+2\Delta}} \tag{2}$$

there is obtained $\eta_1 = (Q - 1)_q (\Delta)$. Hence:

$$E_1 = \log \frac{1}{Q-1} - \log q (\Delta) \tag{3}$$

Equation (1) yields $F = BL_1 \frac{1}{1-\eta_1}$ and

$$F = BL_1 \frac{1}{1-(Q-1)_q (\Delta)} \tag{4}$$

Because of $\gamma = \frac{1}{k_1} FE_1$, Equations (3) and (4) combine to

$$\gamma = KBL_1 \frac{1}{1-(Q-1)_q (\Delta)} \left[\ln \frac{1}{Q-1} - \ln q (\Delta)\right] \tag{5}$$

For $\Delta = 0$, that is $q (\Delta) = 1$, equation (5) becomes

$$\gamma = KBL_1 C$$

PLATE I

Dorsal aspect of dissected last instar larva of Galleria mellonella L. (x7). Fat bodies have been removed for greater morphological detail.

ABBREVIATIONS

ASG . . . . . Anterior region of the silk gland

C . . . . . Crop

Co. . . . . Colon

I . . . . . Ileum

M . . . . . Malphigian tubule

MSG . . . . . Mid-region of the silk gland

PSG . . . . . Posterior region of the silk gland

Pv. . . . . Proventriculus

R . . . . . Rectum

Sal G . . . . . Salivary gland

T . . . . . Tracheae

V . . . . . Ventriculus

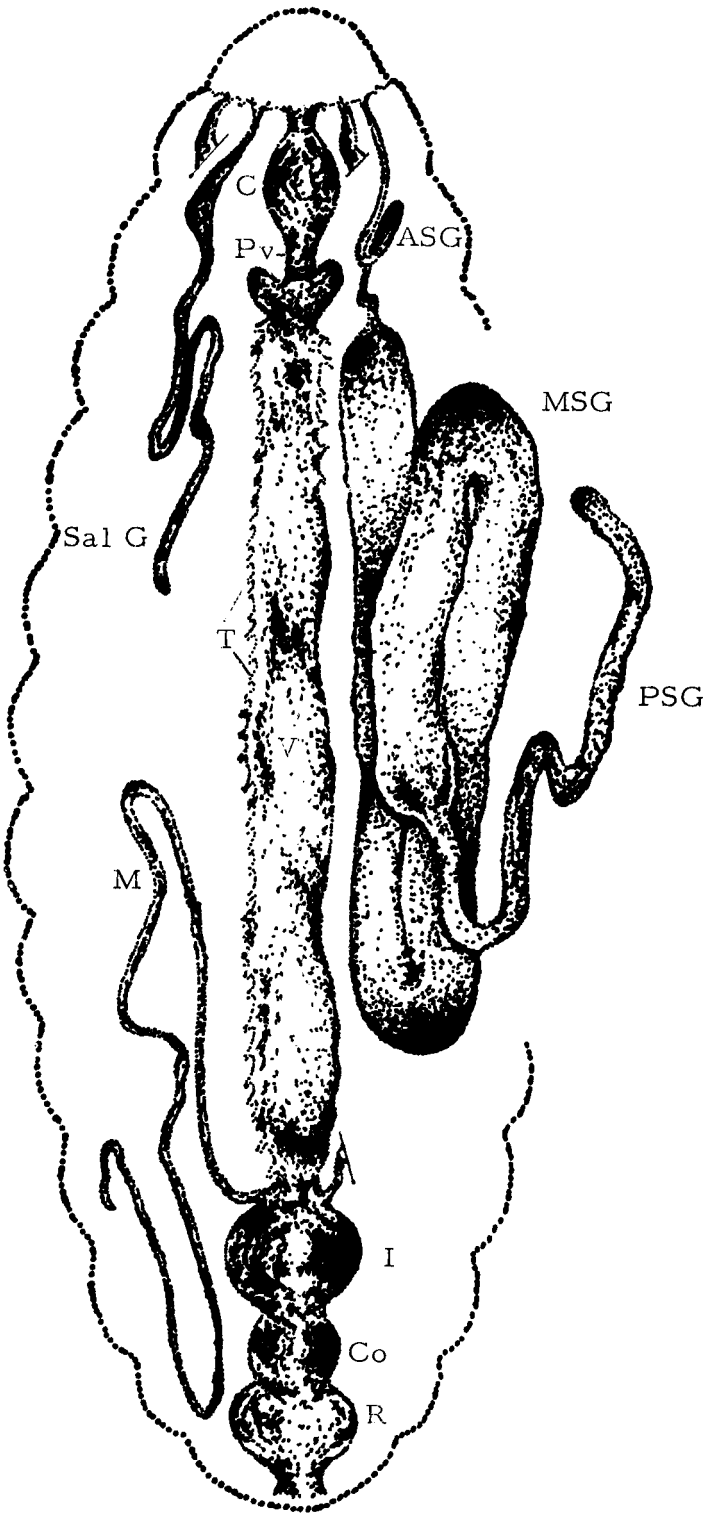

C
Pv
ASG
MSG
Sal G
T
PSG
V
M
A
I
Co
R

PLATE II

Cross section of last instar larva of Galleria mellonella L. through the preventriculus. (x30)

ABBREVIATIONS

ASG . . . . . Anterior region of the silk gland

C . . . . . Cuticle

FB . . . . . Fat body

H . . . . . Hypodermis

MB . . . . . Muscle band

Pv . . . . . Proventriculus

Sal G . . . . . Salivary gland

T . . . . . Trachea

NC . . . . . Nerve cord

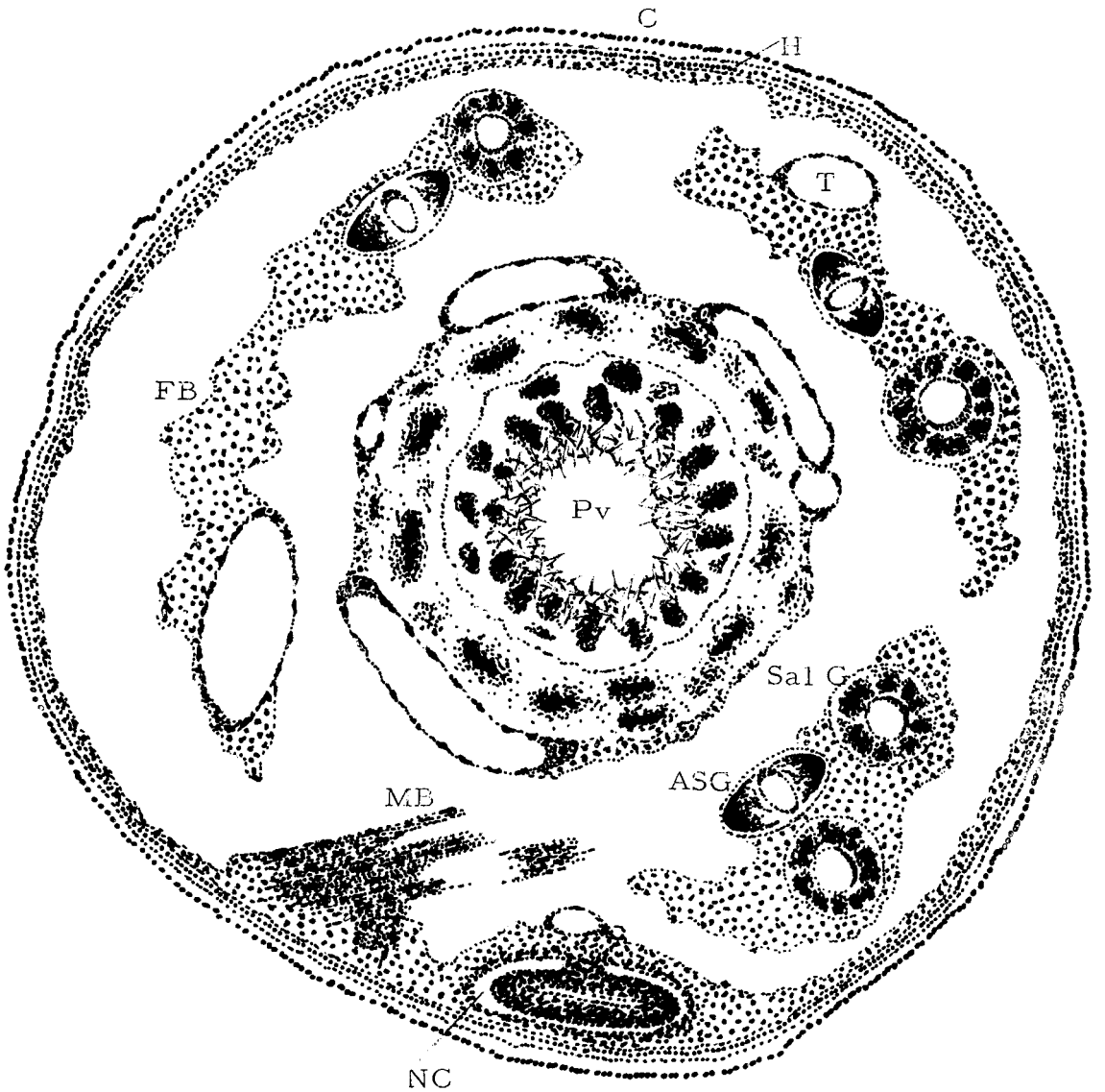

C
H
T
FB
Pv
Sal G
ASG
MB
NC

PLATE III

Cross section of last instar larva of Galleria mellonella L. through the ventriculus. (x30)

ABBREVIATIONS

C . . . . . Cuticle

FB . . . . . Fat body

H . . . . . Hypodermis

MB . . . . . Muscle band

MSG . . . . . Mid-region of the silk gland

T . . . . . Trachea

V . . . . . Ventriculus

NC . . . . . Nerve cord

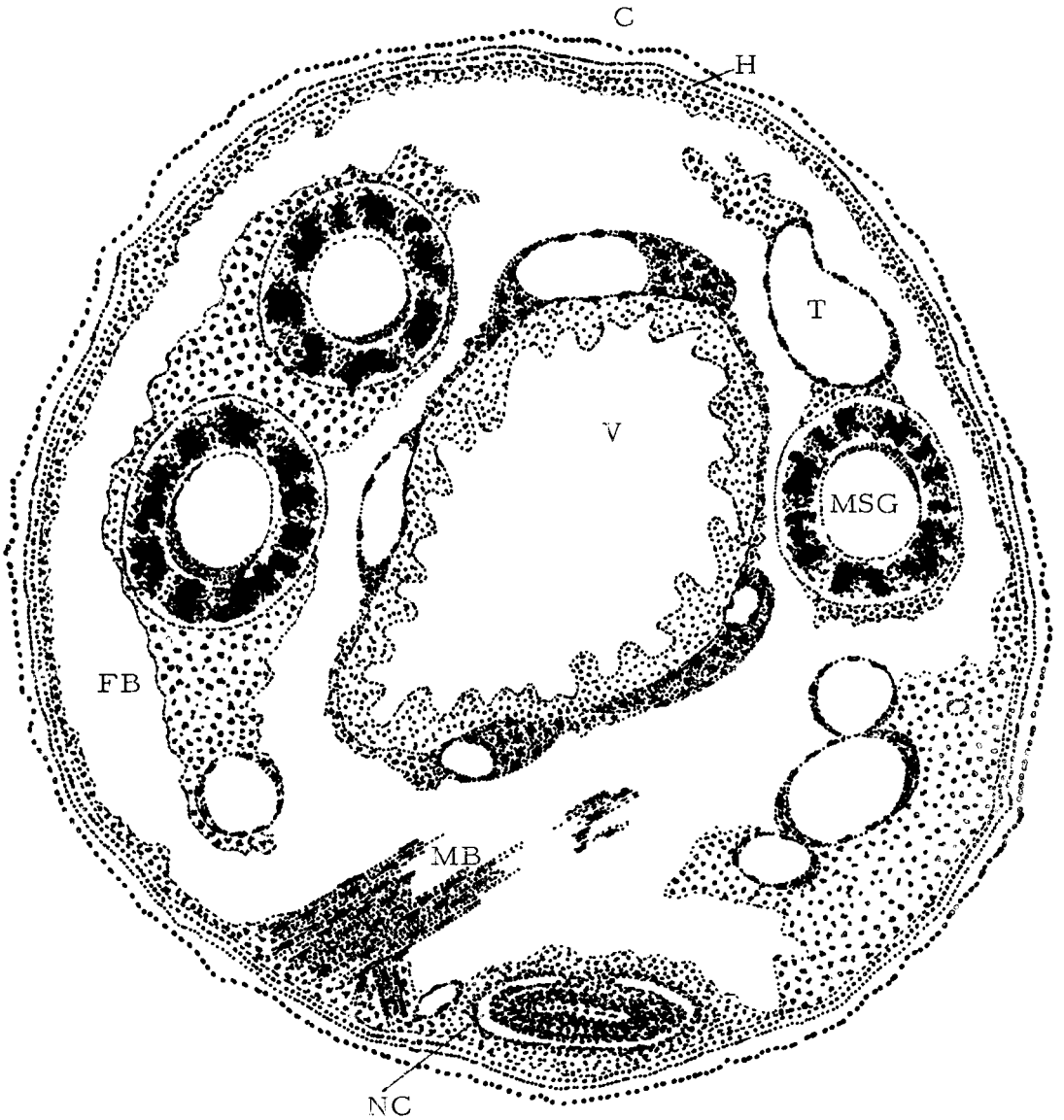

C
H
T
V
MSG
FB
MB
NC

PLATE IV

Figure 1. Blood smear stained with Wright's stain. Magnification 1000x.

Figure 2. Cuticle (C) and hypodermis nuclei (H). Feulgen stain. Section thickness 10 μ. Magnification 1000x.

Figure 3. Fat cell nuclei stained with Feulgen reagent. Section thickness 12 μ. Magnification 1000x.

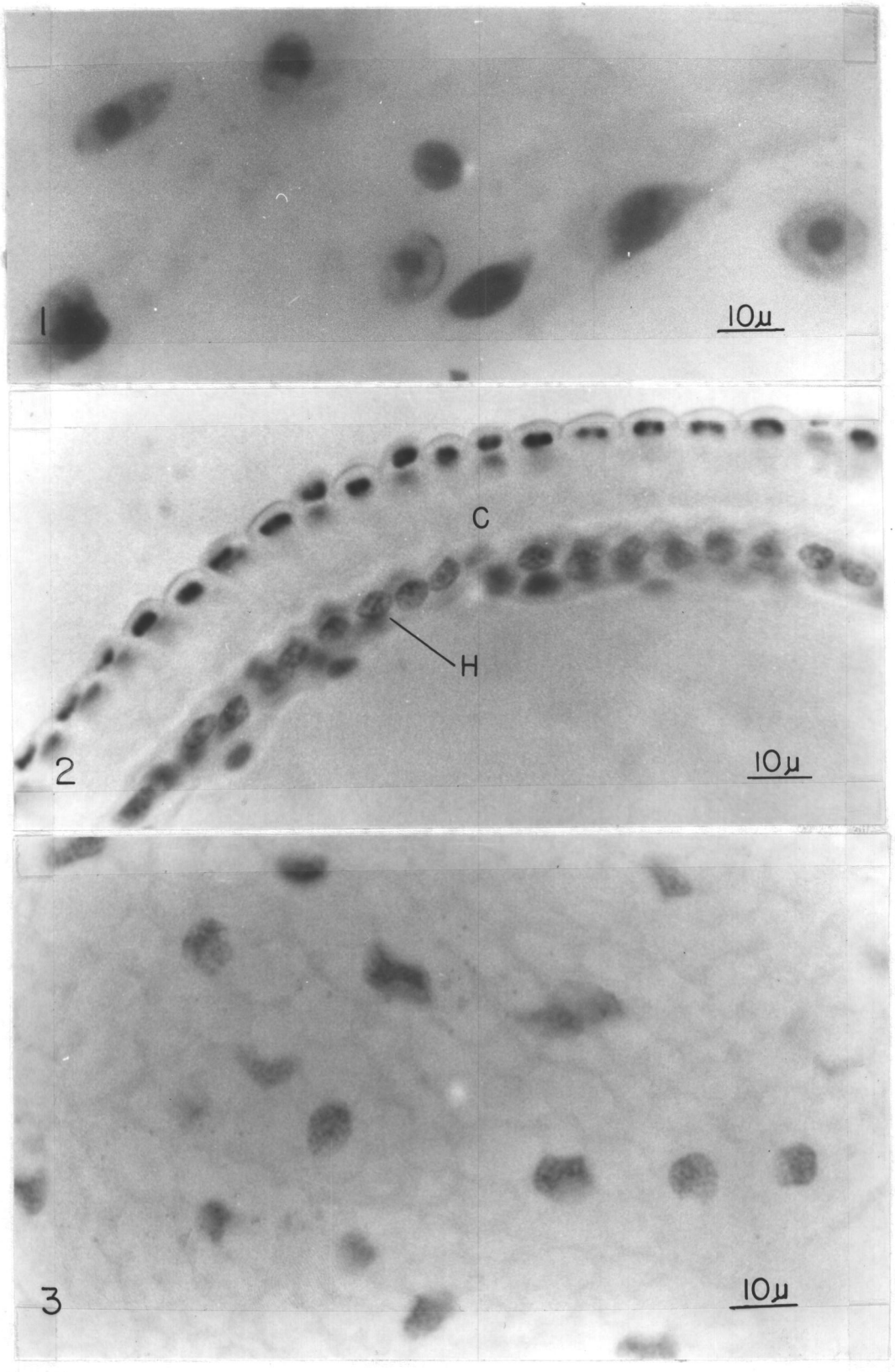
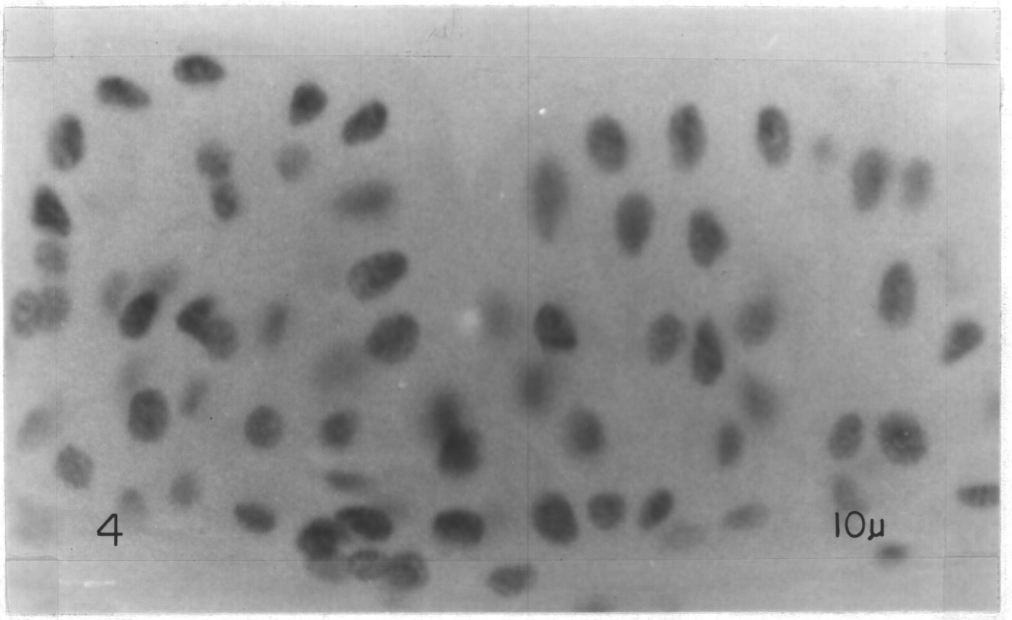

1
10μ
C
H
2
10μ
3
10μ

PLATE V

Figure 4. Ventriculus nuclei stained with Feulgen reagent. Section thickness 10 μ. Magnification 1000x.

Figure 5. Nuclei of the proventriculus stained with Feulgen reagent. Section thickness 25 μ. Magnification 200x.


4
10μ

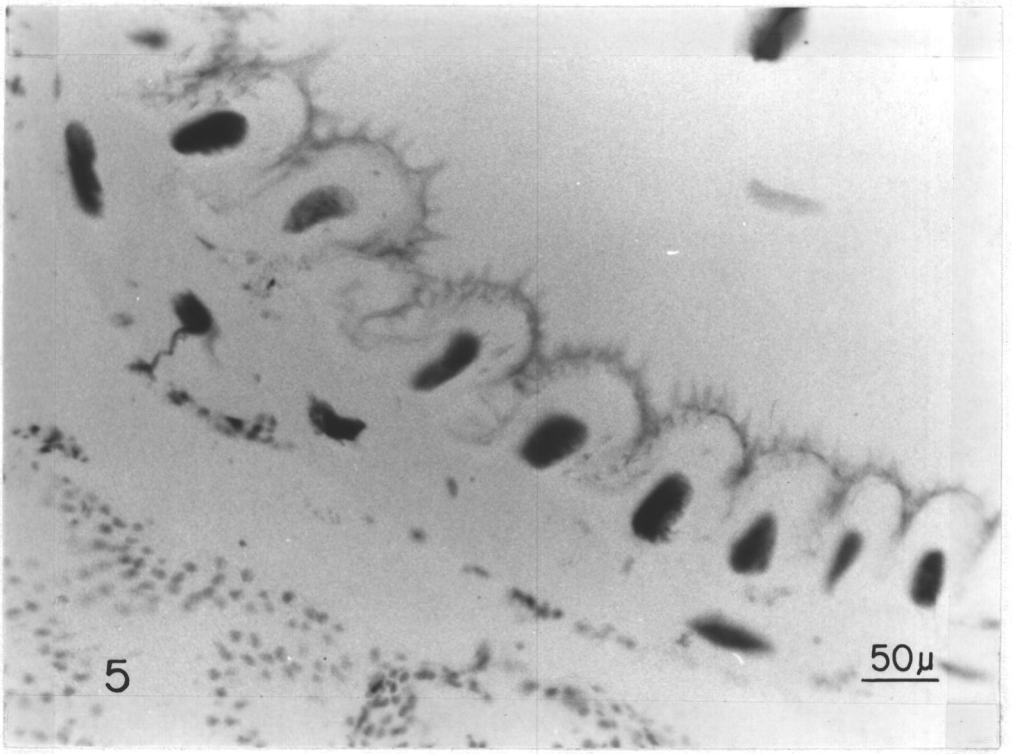
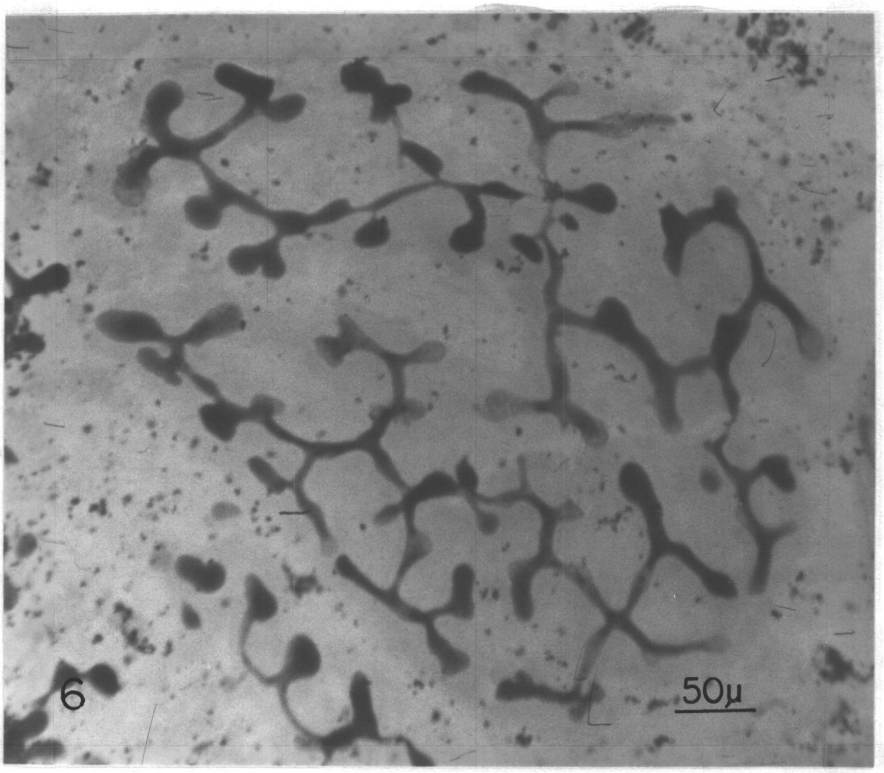

5
50μ

PLATE VI

Figure 6. Squash preparation of mid-region of the silk gland nuclei stained with aceto-orcein. Magnification 200x.

Figure 7. Nuclei of the anterior region of the silk gland (left) and salivary gland (right) stained with Feulgen reagent. Section thickness 25 μ. Magnification 1000x.


6
50μ

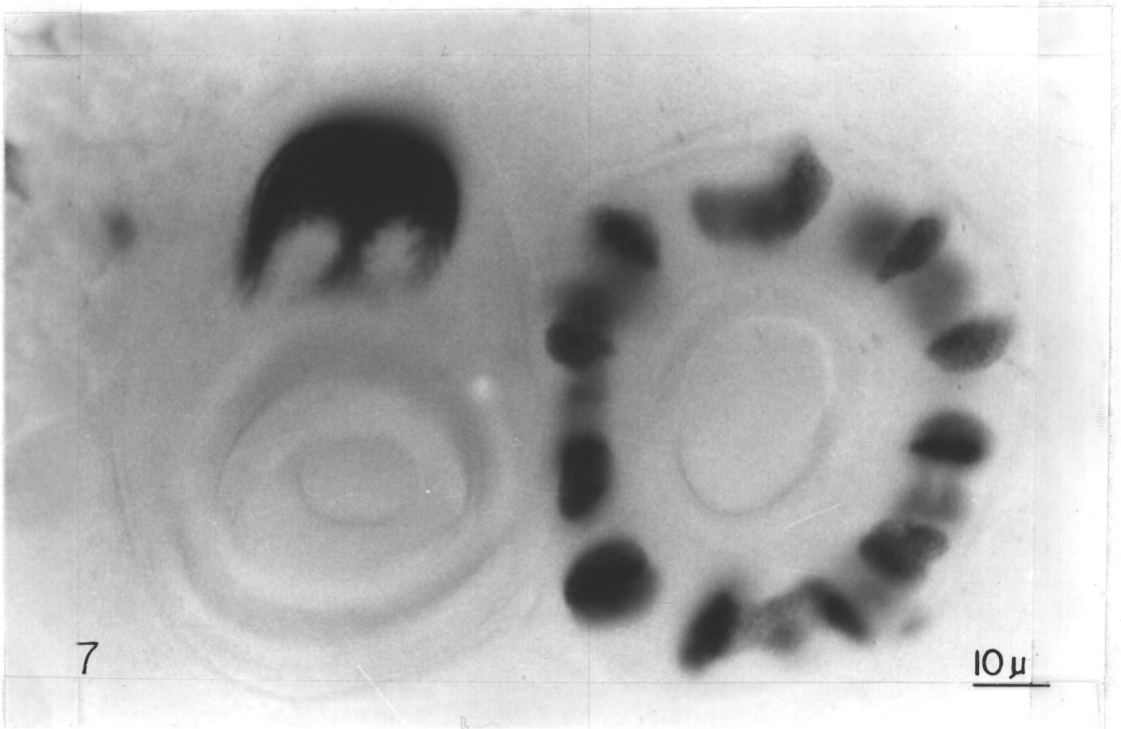

7
10μ

PLATE VII

Cytospectrophotometric apparatus used in this study. Description is given in the text.

ABBREVIATIONS

L . . . . . Light source (enclosed in black box)

M . . . . . Monochromator

P . . . . . Phototube

PS . . . . Power supply

R . . . . . Microammeter

T . . . . . Telescope

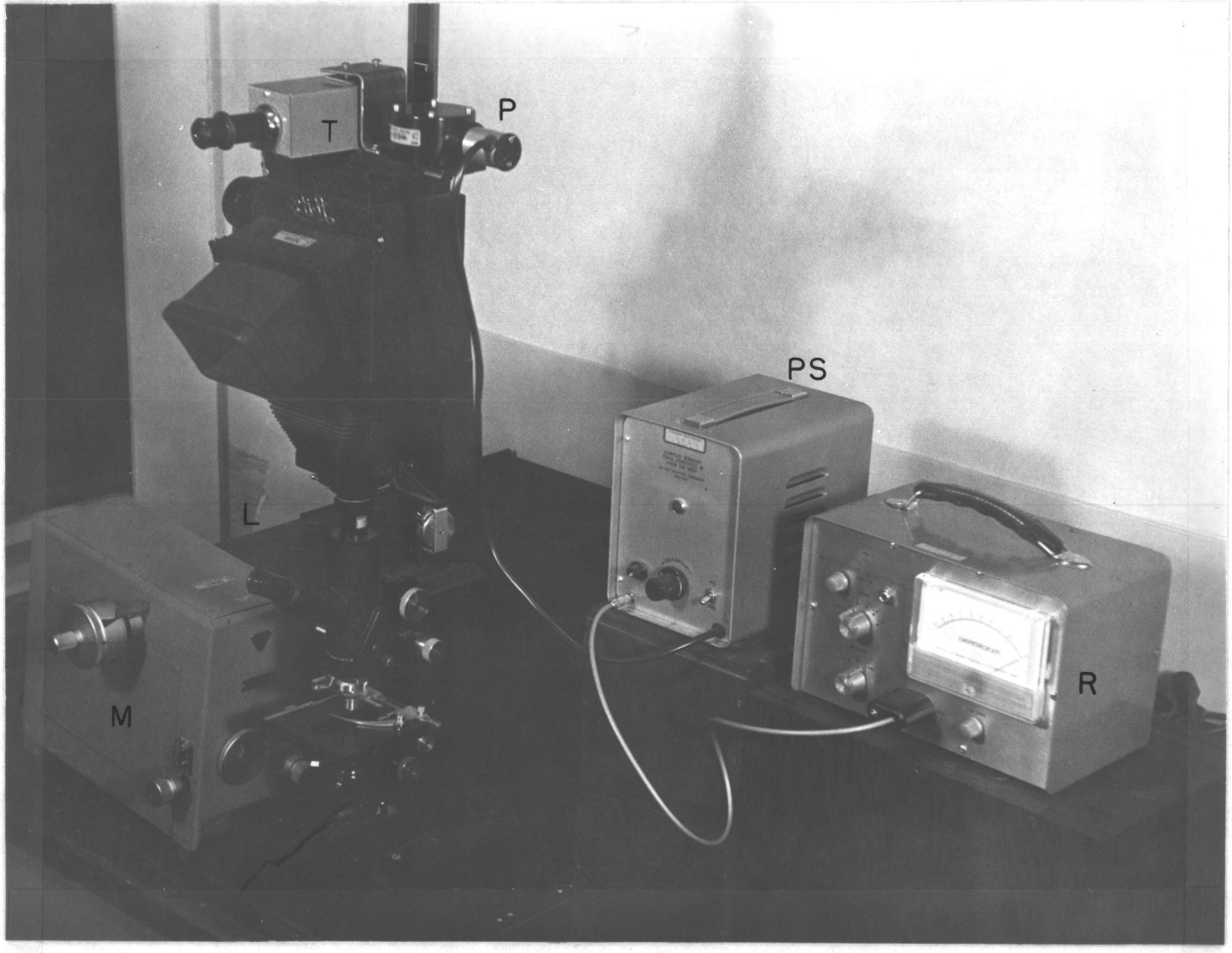
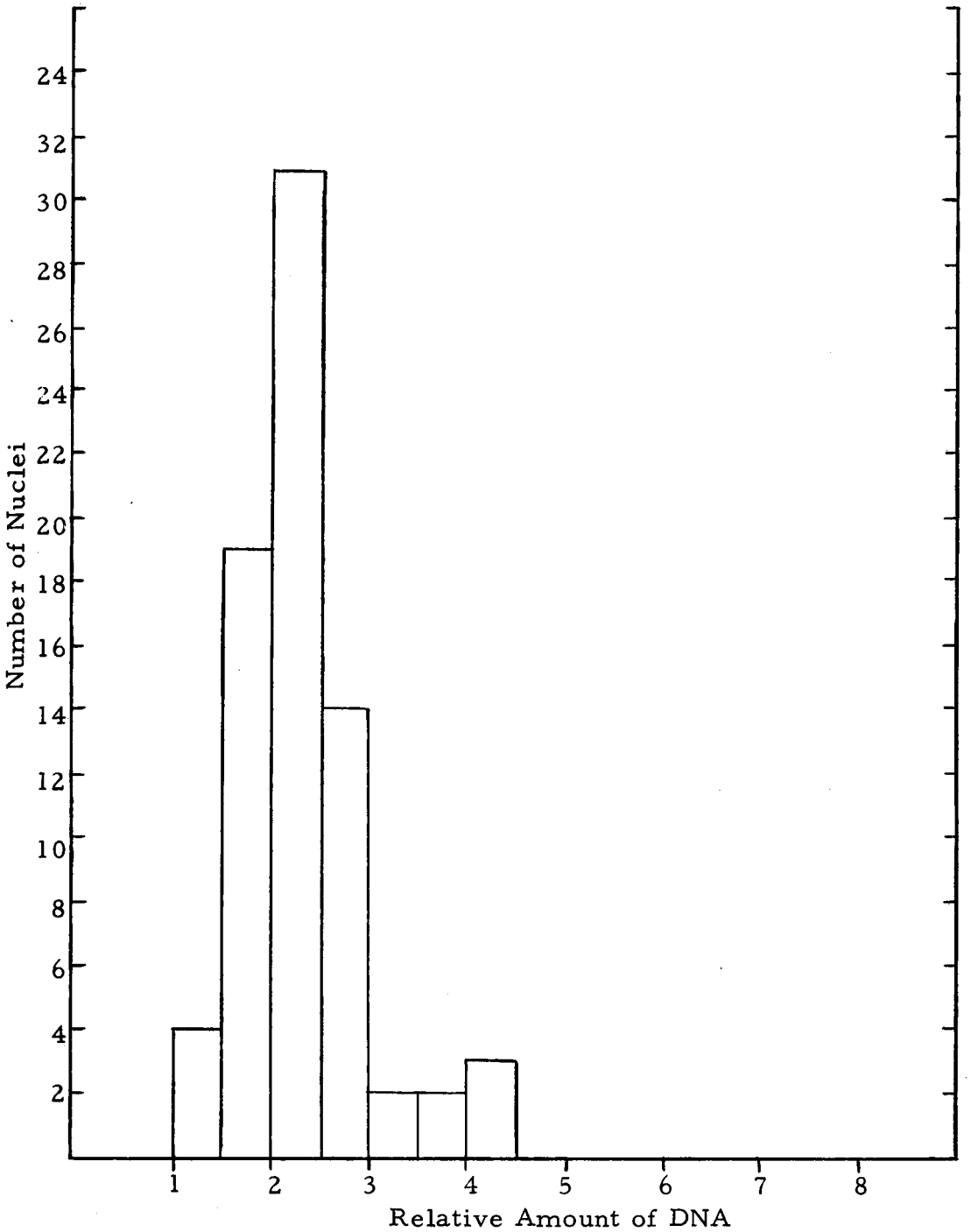

T
P
PS
L
M
R

PLATE VIII

6/14/64		λ = 490 mμ				λ = 514 mμ						
Fat Cell				$T_1 = I_{11}/I_{10}$	$L_1 = 1-T_1$			$T_2 = T_{22}/T_{20}$	$L_2 = 1-T_2$	$Q = L_2/L_1$	C	$\gamma = KBL_1C$
#	B	I_{11}	I_{10}			I_{22}	I_{20}					
B9 - 3A - 2	3.8	.505	.563	.8970	.1030	.473	.570	.8269	.1731	1.681	1.205	4.363
		.505	.563			.468	.568					
		1.010	1.126			.941	1.138					
	35.16	.503	.560	.8955	.1045	.465	.568	.8213	.1787	1.710	1.181	4.339
		.500	.560			.465	.568					
	5.93	1.003	1.120			.933	1.136					
											M:	4.351
B9 - 3A - 15	3.8	.530	.565	.9424	.0576	.510	.578	.8849	.1151	1.998	1.005	1.977
		.533	.565			.513	.578					
		1.063	1.128			1.023	1.156					
	35.16	.530	.565	.9397	.0602	.508	.575	.8835	.1165	1.935	1.031	2.182
		.530	.563			.508	.575					
	5.93	1.060	1.128			1.016	1.150					
											M:	2.079
B9 - 3A - 14	3.8	.523	.565	.9257	.0743	.500	.578	.8676	.1324	1.782	1.129	3.263
		.523	.565			.503	.578					
		1.046	1.130			1.003	1.156					
	35.16	.520	.563	.9246	.0754	.503	.578	.8699	.1301	1.725	1.166	3.091
		.523	.565			.500	.575					
	5.93	1.043	1.128			1.003	1.153					
											M:	3.177

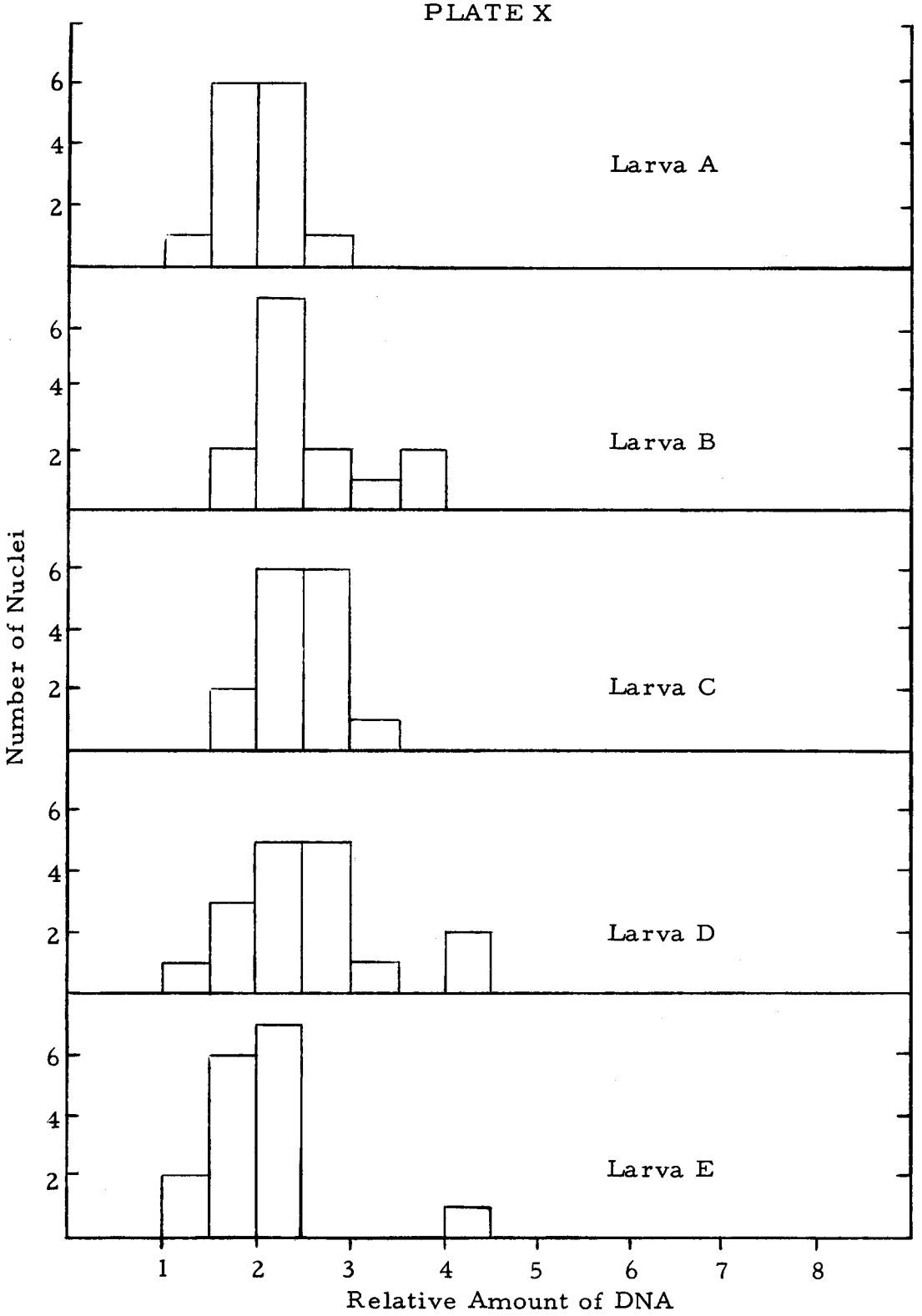
SAMPLE DATA SHEET

PLATE IX



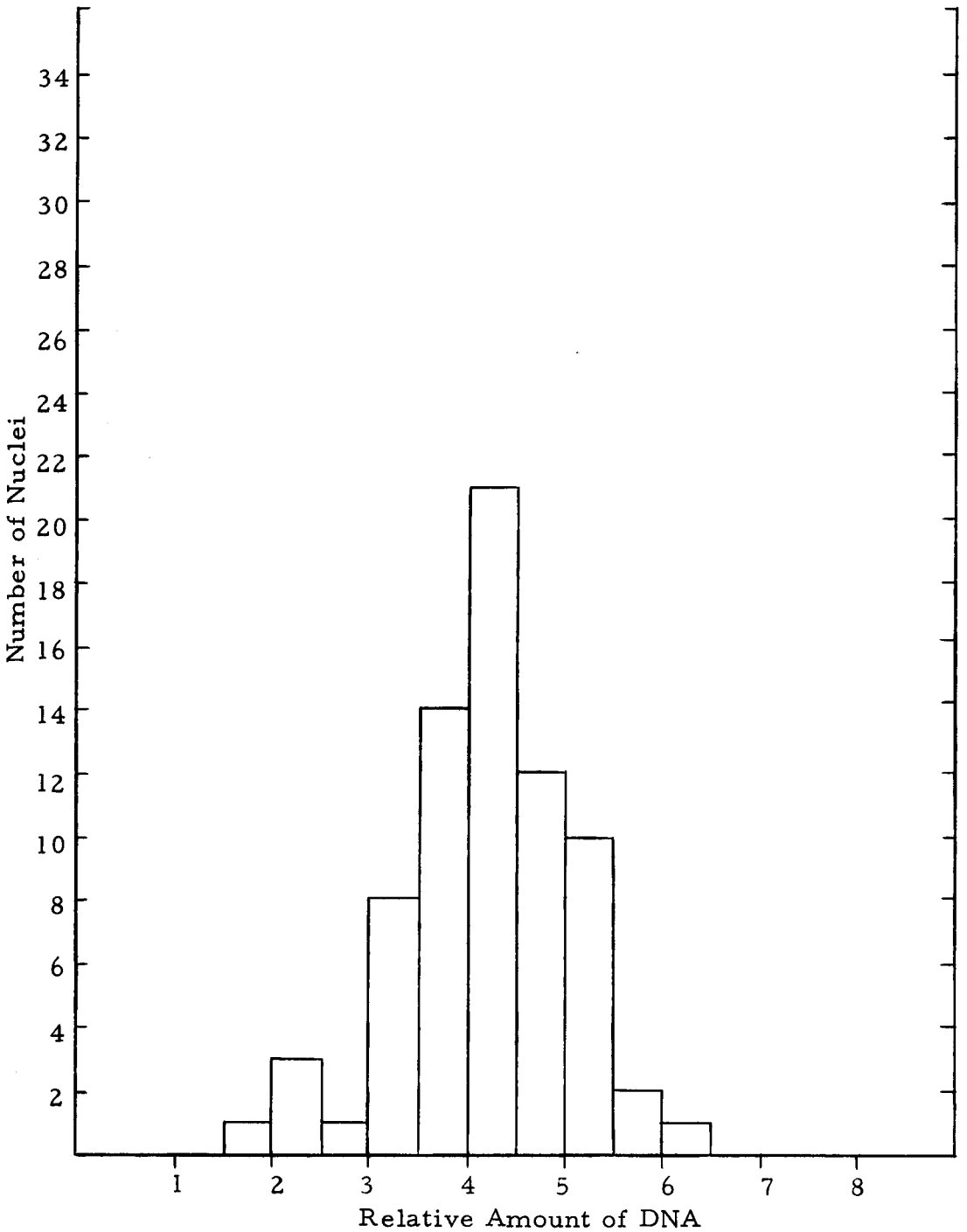
Distribution of DNA in the hypodermis nuclei of five larvae.

PLATE X



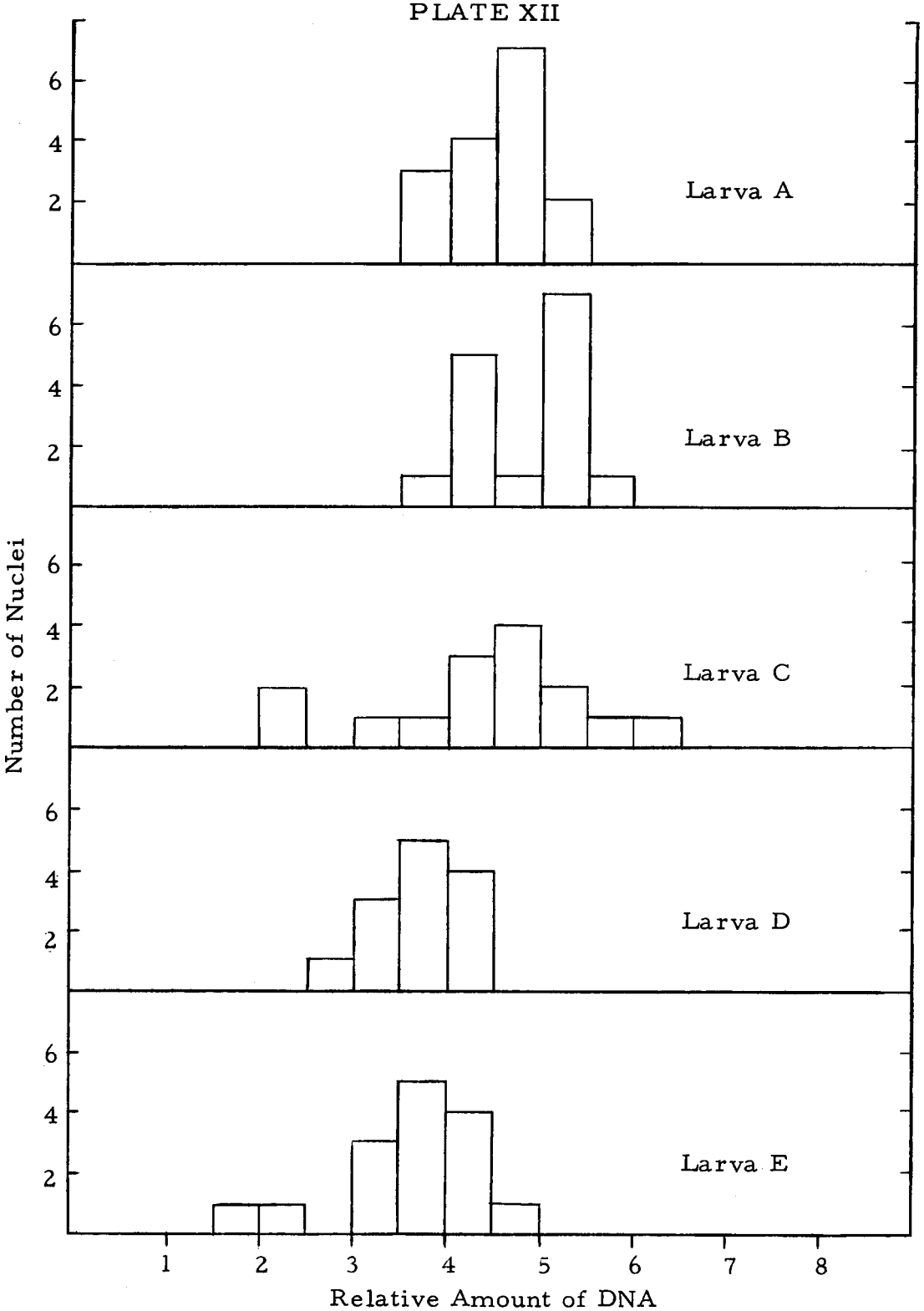
Distribution of DNA in the hypodermis nuclei of each larva.

PLATE XI



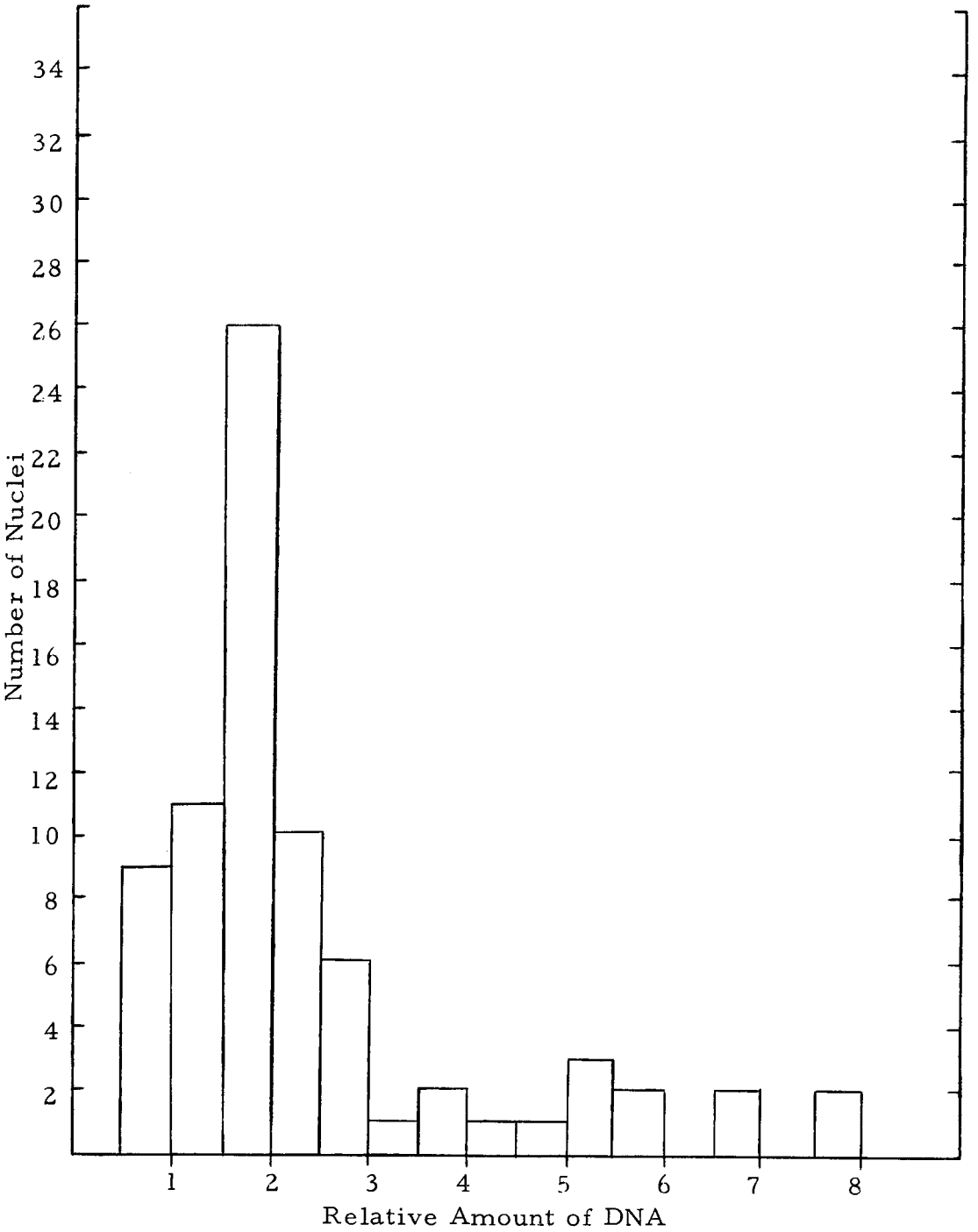
Distribution of DNA in the ventriculus nuclei of five larvae.

PLATE XII



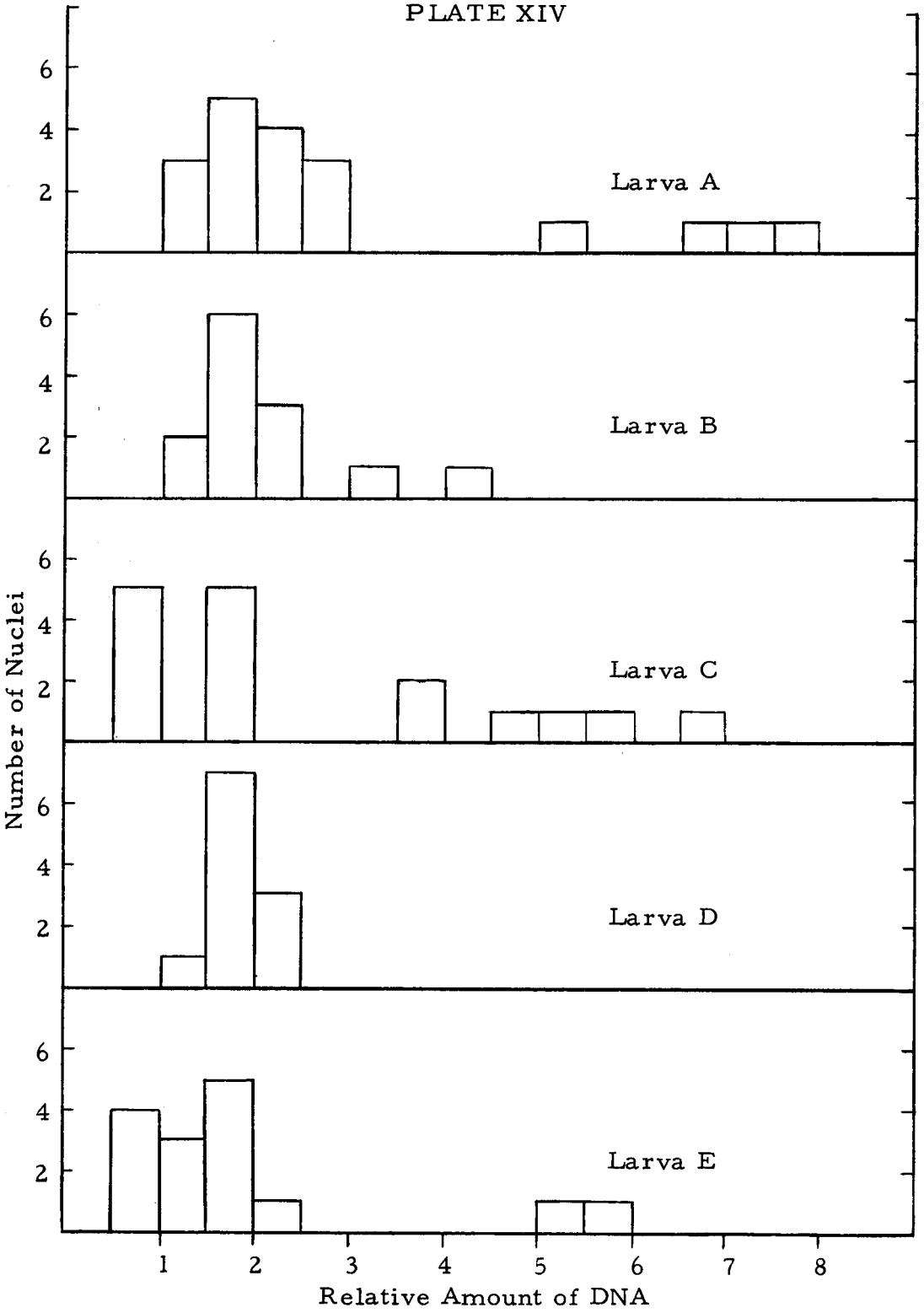
Distribution of DNA in the ventriculus nuclei of each larva.

PLATE XIII



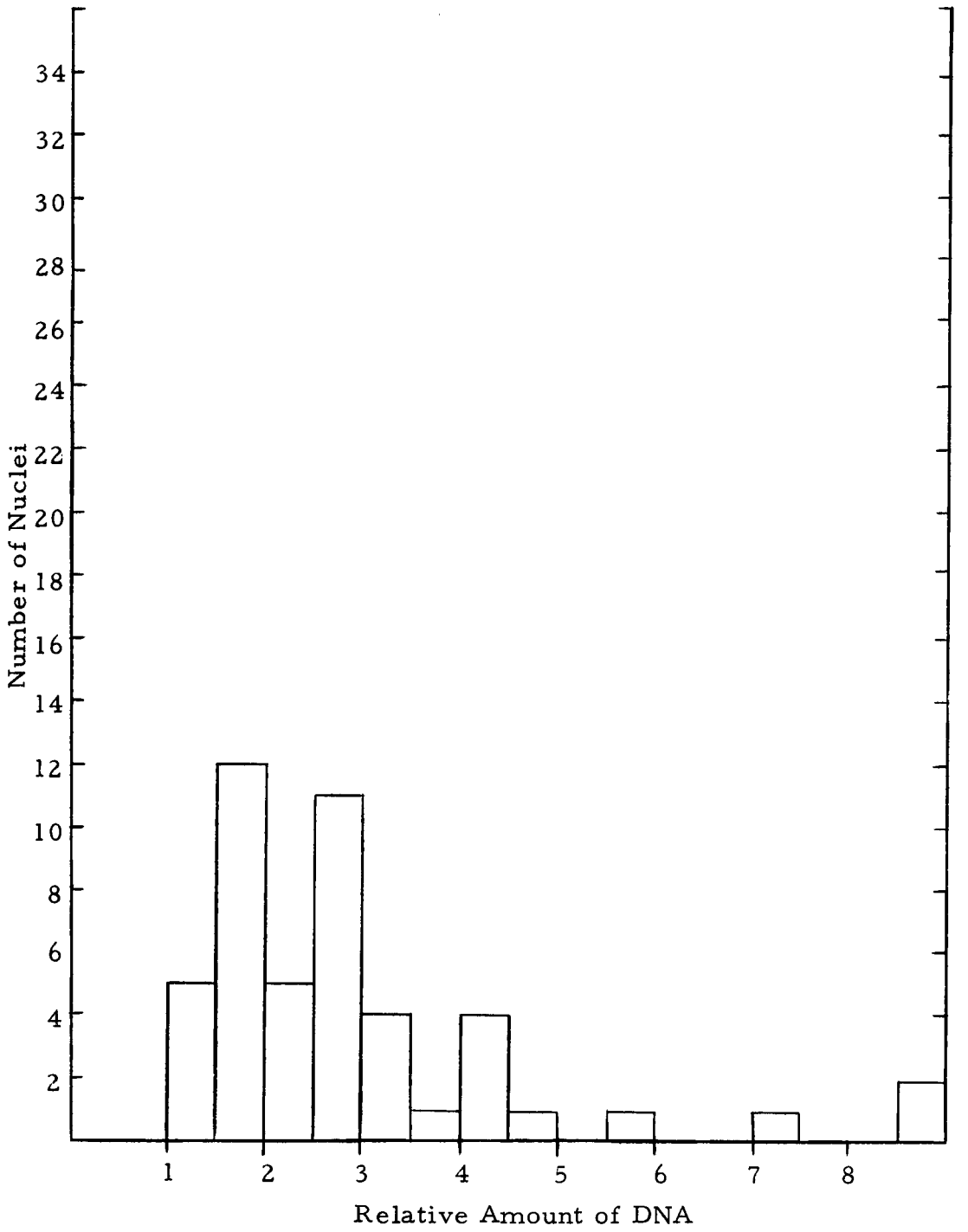
Distribution of DNA in the fat cell nuclei of five larvae.

PLATE XIV

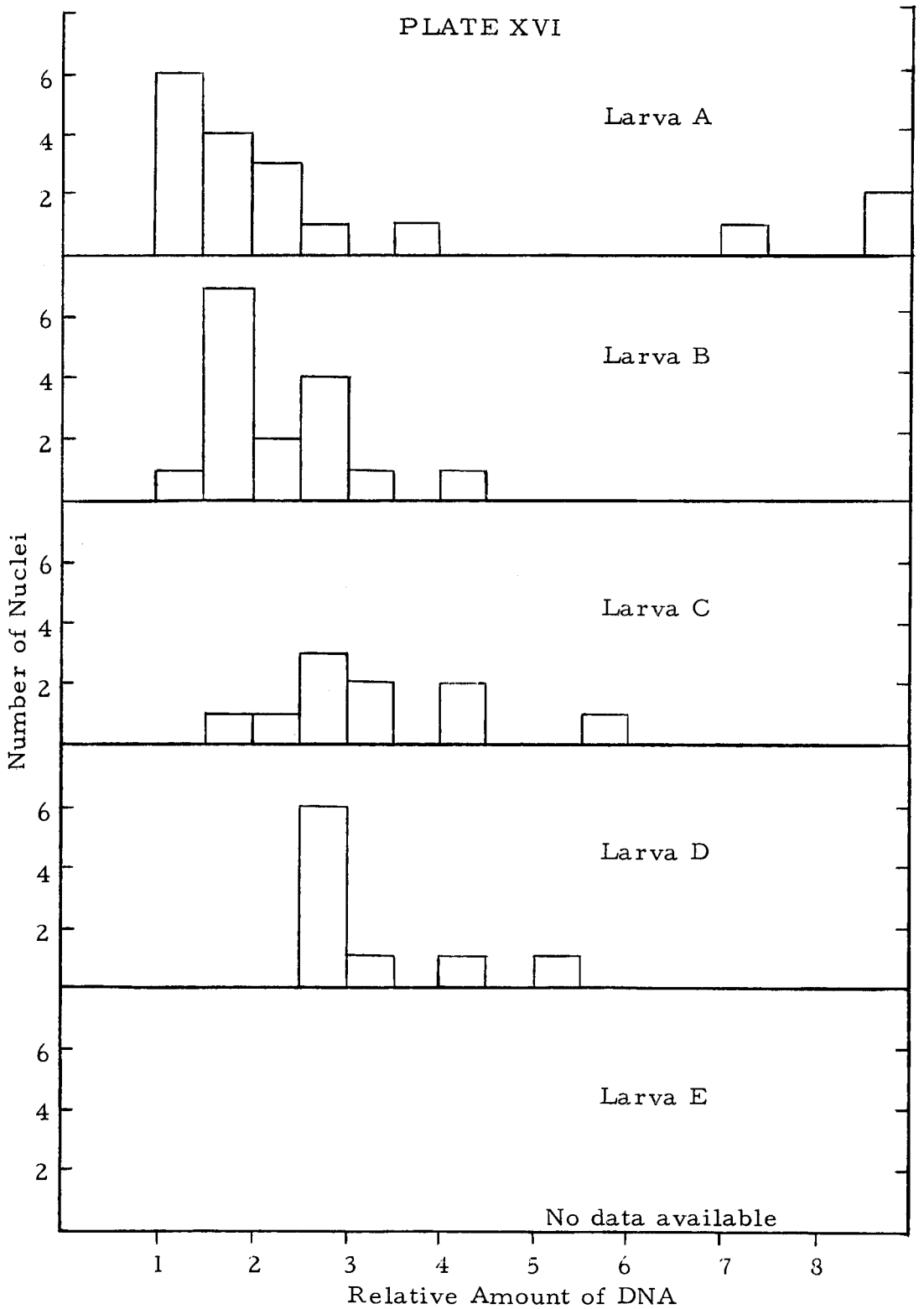


Distribution of DNA in the fat cell nuclei of each larva.

PLATE XV

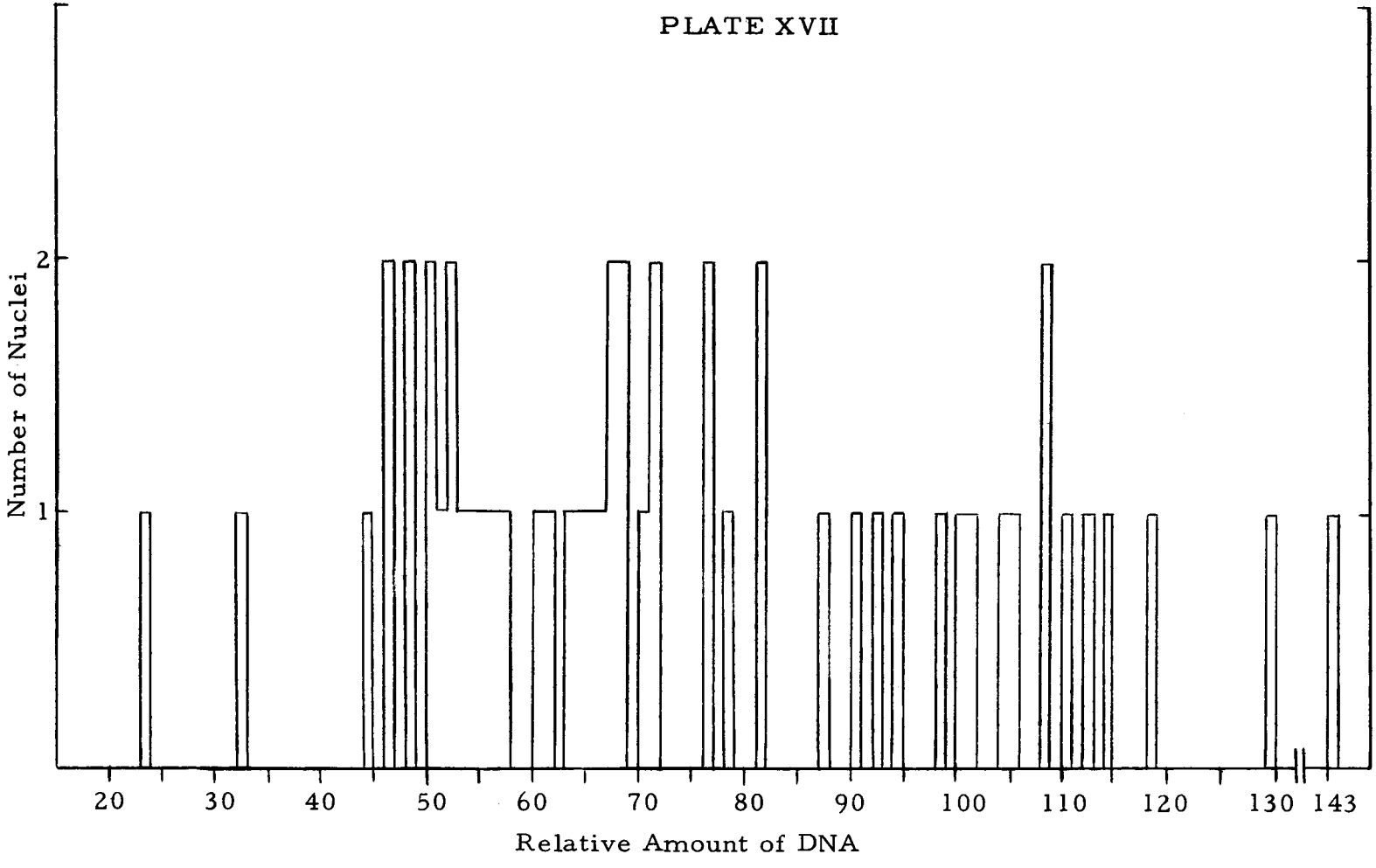


Distribution of DNA in the blood cell nuclei of four larvae.



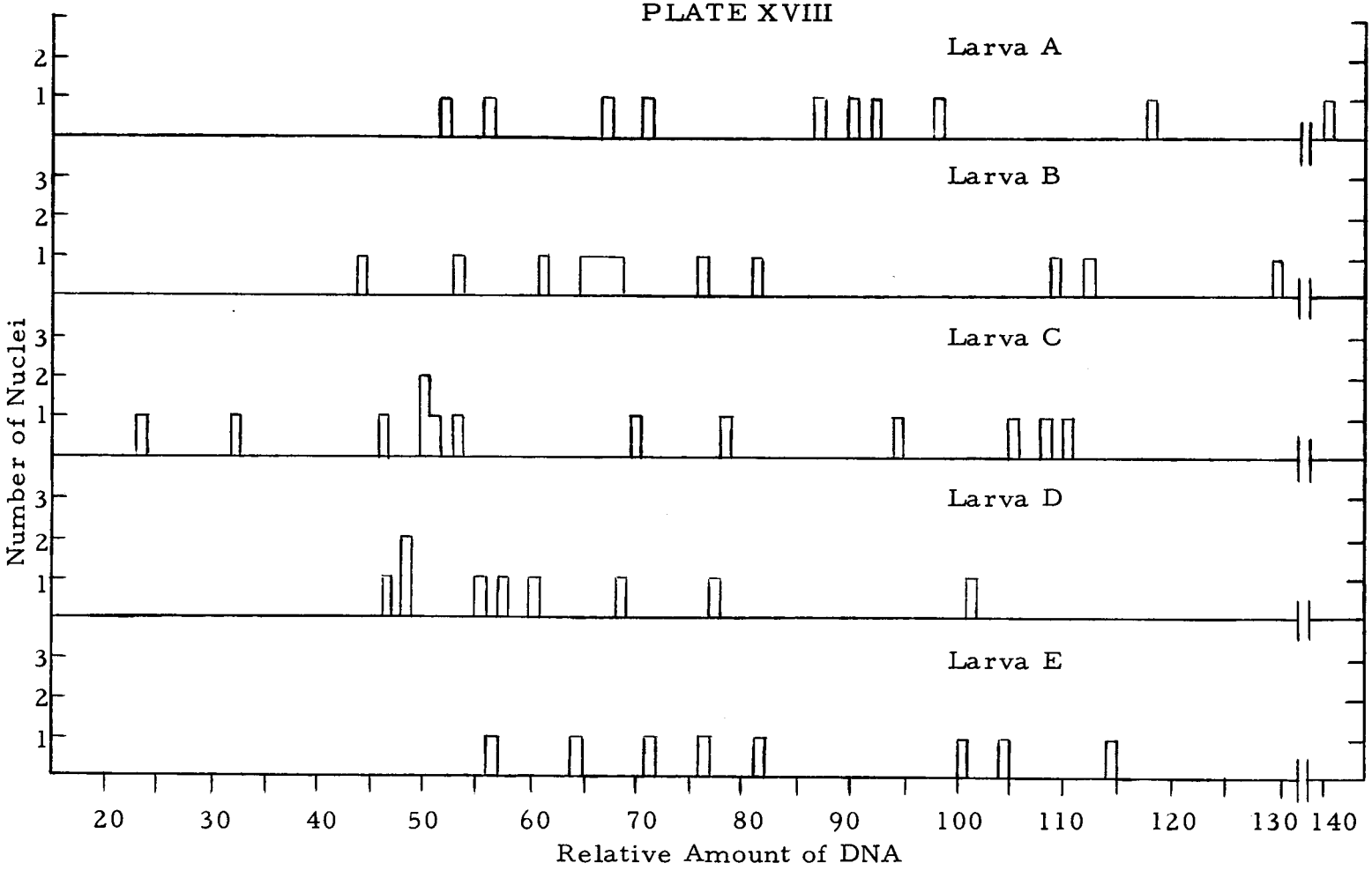
Distribution of DNA in the blood cell nuclei of each larva.

PLATE XVII



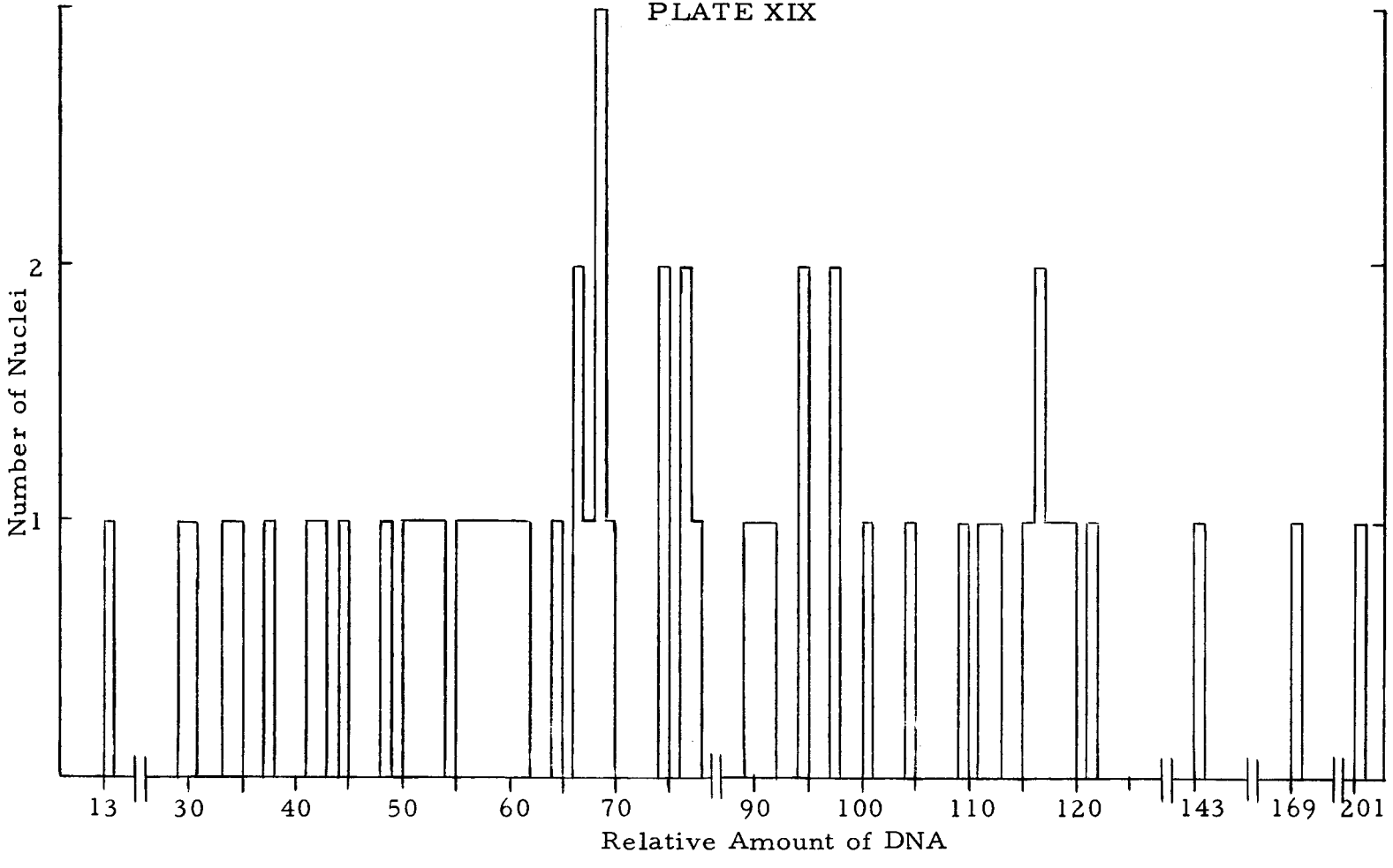
Distribution of DNA in the proventriculus nuclei of five larvae.

PLATE XVIII



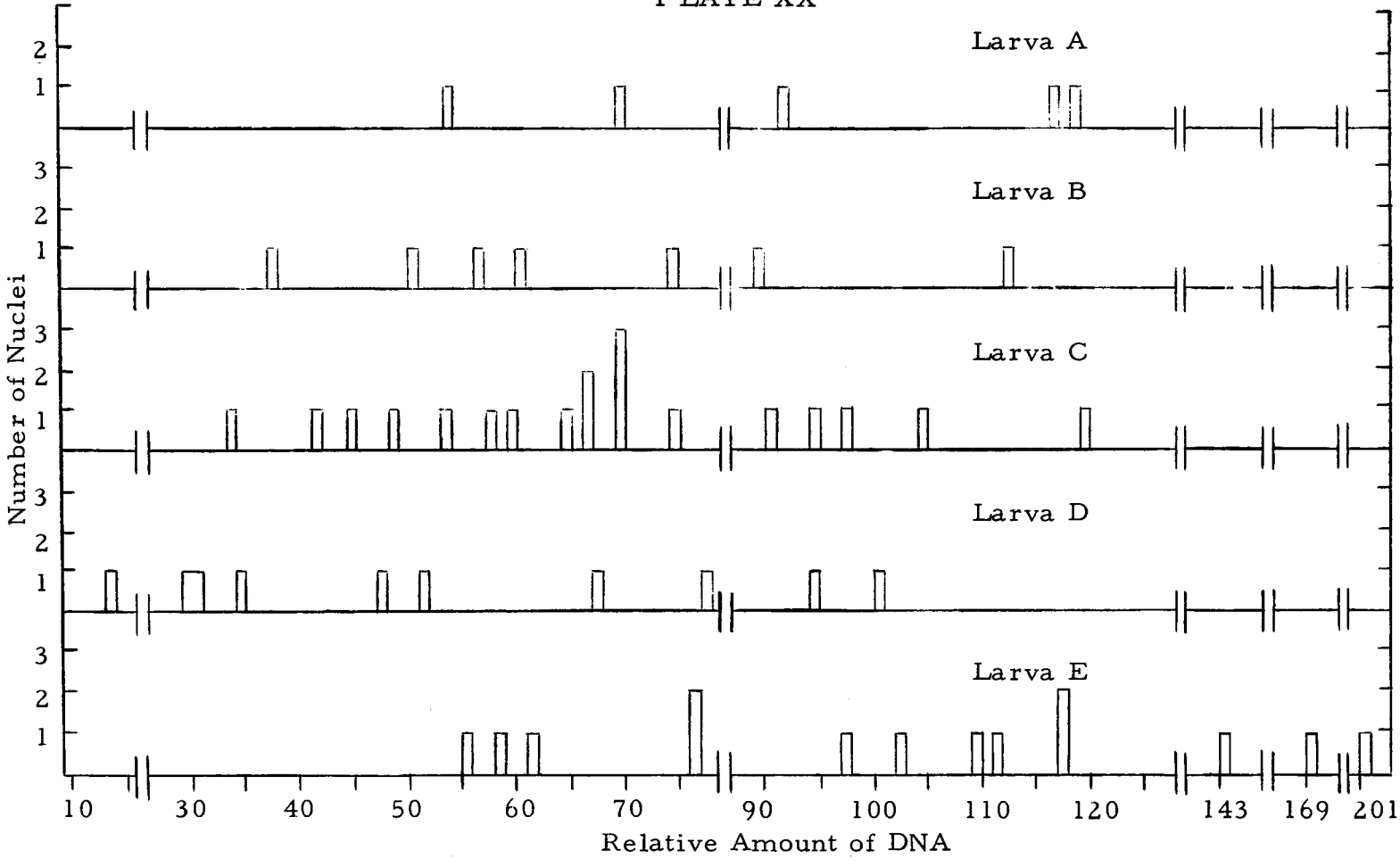
Distribution of DNA in the proventriculus nuclei of each larva.

PLATE XIX



Distribution of DNA in the anterior silk gland nuclei of five larvae.

PLATE XX



Distribution of DNA in the anterior silk gland nuclei of each larva.