CELL CYCLES IN CERCARIAE OF TRICHOBILHARZIA OCELLATA

CELL CYCLE ANALYSIS IN DEVELOPING CERCARIAE OF *TRICHOBILHARZIA OCELLATA* (TREMATODA: SCHISTOSOMATIDAE)

by

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SCOPE AND CONTENTS:

Experiments described in this study were carried out in order to (a) determine the parameters of the cell cycle and (b) examine the mode of reproduction during cercarial development of Trichobilharzia ocellata.

The average duration of t_C was estimated to be 15.2 hours in which t_M lasts 1.6 hours; t_{G_1}, 5.6 hours; t_S, 4.8 hours; and t_{G_2}, 3.2 hours. Beginning at the tail-bud stage, an increasing proportion of the cells cease to proliferate and become non-dividing cells; at this stage the first appearance of differentiating body structures was observed.

SCOPE AND CONTENTS (continued):

Nuclei were found to vary in morphology during interphase; these variations and the examinations of cells in the division phase of the cell cycle have been used to support the suggestion that all cells reproduce asexually throughout all stages of cercarial development.

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1. INTRODUCTION

Trichobilharzia ocellata (La Valette, 1855) Brumpt, 1931, a causative agent of schistosome dermatitis (swimmer's itch) is a digenetic trematode. This parasite follows a definite life cycle pattern involving an alternation between two hosts: the adult stage is found in the duck, Anas s.p., the definitive host; and the immature or juvenile stages are found in the snail, Lymnaea stagnalis, the intermediate host (Talbot, 1936) (Fig. 1). The adults live in the portal system of wild ducks and lay spindle-shaped eggs. These eggs then pass through the gut wall of the host and are freed with the faeces. Upon contact with water a miracidium hatches. This free-swimming form is attracted to and subsequently penetrates either the foot or the mantle of the snail. Near the point of penetration, the miracidium metamorphoses into the primary germinal sac, viz. the mother sporocyst. Secondary germinal sacs, daughter sporocysts, emerge from the mother sporocyst and migrate to the snail's digestive gland. Each daughter sporocyst in turn gives rise to numerous fork-tailed cercariae. The cercariae escape as free-swimming forms from the snail about six weeks after the initial infection and upon penetrating the definitive host

develop into adults, a process requiring two to three weeks (Brackett, 1940; McMullen and Beaver, 1945).

Studies on the cellular events during the life cycle of the digenetic trematodes have dealt predominantly with the development of the egg, the miracidium, and the primary and secondary germinal sacs. In comparison cercarial development has been investigated only to a limited extent. In general, the morphogenesis of the cercariae has been considered to parallel the pattern of development of the free-living miracidium (Rees, 1940; see reviews by Hyman, 1951; Dawes, 1956). Specific studies of cercarial morphogenesis have concentrated on the events that occur in either the first phase, that is from one to about the 30 cell stage (Chen, 1937; Pieper, 1953; Guilford, 1958; James and Bowers, 1967b) or the terminal phases of cercarial growth when organ formation occurs (Rees, 1940; Maldonado and Matienzo, 1947; Cheng, 1960; Cheng and James, 1960; Pan, 1965). Recently Cheng and Bier (1972) examined the complete phasing of cercarial development in Schistosoma mansoni. They divided cercarial development into seven successive stages based on number of cells and on the overall shape of the cellular masses: stage I, single cell; stage II, a naked cell aggregate which is composed of two or more cells; stage III, spherical germ ball; stage IV, elongating germ ball; stage V, tail-bud; stage VI, developing cercarial structure and organs; stage VII, mature cercaria (Fig. 1).

For many species of digenetic trematodes, the cercaria is initiated from a single progenitor cell (for reviews see Cort, 1944; Cort et al., 1954). This single cell which has also been called a germinal cell (Cort, 1944; Cort et al., 1948) divides: one daughter initiates the somatic cell line and the other daughter cell is the progenitor of the germ cell line. The two major processes of cercarial development are the formation of the cercaria and the establishment of the genital primordium. The cells of the genital primordium are the presumptive meiocytes that will give rise to the gametes in the sexually mature forms within the definitive host. At present there is no information about the pattern of somatic and germinal cellular proliferation with respect to estimates of cell cycle parameters, organization of specific growth centers or proportions of proliferating and non-proliferating cells during the growth and development of the cercariae.

The hazards to the trematodes of transfer from host to host are considerable; as a result few individuals survive to maturity. Probably a compensation for the high mortality rate in the digenetic trematodes is an extraordinary reproductive ability; adults produce large numbers of eggs and each miracidium produces large numbers of cercariae. It is generally accepted that spermatogenesis, oögenesis and zygote formation are similar in the adults of a number of species

(Hyman, 1951; Dawes, 1956). Controversy still centers, however, around the form of reproduction in successive larval stages; some workers suggest that it is an asexual process, others suggest that it involves meiosis.

Morphological investigations (Brooks, 1930; Cable, 1934; Chen, 1937; Rees, 1940; Pieper, 1953; Cirordia, 1956; Guilford, 1958; and the numerous works of Cort and his co-workers, for reviews see Cort et al., 1948, 1954) have lent support to a theory, first presented by Leuckart (1879), of germinal lineage whereby the germinal cells persist through the successive stages of the life cycle. That is, only the germinal cells give rise to the next stage of the life cycle, the somatic cells disappear without descendants (Fig. 2). These workers have suggested that the first cleavage of the fertilized egg in the adult, or the first division of the germinal cell in the primary or secondary germinal sacs produces one germinal cell and one somatic cell. These cells proliferate, the somatic cell forming the soma of the next generation and the germinal cell forming the progenitive components. This form of asexual multiplication has been called polyembryony (Marchal, 1904; Döllfus, 1919; Brooks, 1930; and reviewed by Cort et al., 1954). According to this view, since the progenitive components of the primary and secondary germinal sacs are direct descendants of the germinal cell arising at the first cleavage of the

fertilized egg, the multiplication of these components is really a polyembryony of the fertilized egg extending throughout the development of the germinal sacs within the intermediate host.

Some objections can be raised in applying the term polyembryony to explain reproduction within the intermediate stages of the trematode life cycle. First, in the classical examples displaying polyembryony, e.g. some of the parasitic Hymenoptera and armadillos, two or more individuals arise very early in the development of the single zygote (Patterson, 1927). In contrast, in the Digenea, from each zygote only one miracidium is formed; production of the hundreds of daughter sporocysts and cercariae does not normally occur until after the miracidium has penetrated into the intermediate host. Secondly, in all organisms in which polyembryony was originally described all embryos arising from the fertilized ovum not only undergo similar developmental processes, but they also form individuals which tend to be morphologically identical. Although the early processes leading to the formation of daughter sporocysts and cercariae are thought to parallel miracidial development, it is obvious that the structures formed bear little or no resemblance to the original miracidium. Thus the mechanisms regulating the terminal phases of development, such as body organization and organ formation, must be different for each stage of life

cycle. In sum, the question that arises is whether polyembryony as defined by Patterson, accurately describes reproduction in the digenean germinal sacs, or whether the available evidence would lend support to an alternative explanation.

An additional modification concerning the method of reproduction within the intermediate stages has recently been provided. A number of investigators have interpreted the results of their cytological investigations as evidence that meiotic events are involved in the formation of the progenitive components (Fig. 2). Bednarz (1962) described germinal cells in the process of extruding polar bodies; Khalil and Cable (1968) have reported germinal cells with nuclei in the zygotene or the diakinesis stages of meiosis. In both reports meiosis was considered to be incomplete and as a result cells retained a diploid chromosome complement. According to Bednarz, the bivalent chromosomes divide longitudinally at diakinesis, one sister chromatid of each bivalent is then extruded in the form of a polar body; the remaining chromatids re-duplicate and the cell subsequently undergoes a mitotic division. Khalil and Cable suggest that only the diakinesis stage of meiosis is reached, the nucleus then returns to interphase prior to dividing mitotically. These authors were of the opinion that reproduction, in the intramolluscan stages, was by diploid

parthenogenesis. Szidat (1962), James (1964), Cable (1965), James and Cable (1965), and James and Bowers (1967b) also concluded that reproduction in the species studied by them was by diploid parthenogenesis, although they gave no supporting cytological evidence.

The above mentioned authors have used the definition of Suomalainen (1950) that diploid parthenogenesis is "the development of the egg-cell into a new individual without fertilization." Accordingly, the progenitive components located within the germinal sacs have been referred to as diploid parthenogenetic ova (James and Bowers, 1967b) or as egg cells (Bednarz, 1962), or as oögonia prior to and as oöcytes after displaying meiotic phenomena (Khalil and Cable, 1968). Acceptance of this theory implies that the germinal cells located within the germinal sacs are equivalent to the unfertilized egg cells located within the ovaries of the adult. The following consideration suggests that diploid parthenogenesis does not occur in some species of digenetic trematodes.

For T. ocellata and other species of Schistosomatidae the adults are dioecious, that is the male and female gametes are produced in separate individuals, also the females are morphologically distinct from the males (Fig. 1). Sex determination occurs at fertilization since all schistosome cercariae, arising from a single miracidium, upon infecting

the vertebrate host develop into adults which are of only one sex (Stirewalt, 1951; Short, 1952a,b). Thus for those miracidia destined to produce male progeny it seems unlikely that within the primary and secondary germinal sacs, the progenetive components would display meiotic phenomena characteristic of a developing egg cell; while in the adult the descendants of these components would undergo normal gonadogenesis. It may be supposed that some investigators were mistaken in their interpretation of the cytological evidence presented, in which case a re-interpretation of the data is necessary.

The present study is concerned with an analysis of cellular proliferation during cercarial development. For this purpose the schistosome, Trichobilharzia ocellata, was chosen because it is easily maintained within the laboratory and because each infected snail host readily yields numerous cercariae at all developmental stages. The cercarial stage was chosen since it is this phase of the trematode life cycle about which the least is known. Furthermore, a study of cellular proliferation and differentiation during cercarial development is necessary before any attempts can be made at determining the factors which control these processes. An understanding of the basic developmental processes may

also aid in establishing an effective biological control method for the cercarial stage of the life cycle.

The present study reveals that prior to the tail-bud stage, cells contained within the developing cercarial larvae were involved in cellular proliferation. Nuclei undergo extensive changes in morphology while in interphase. The variations in nuclear morphology have been classified and correlated with the cell's position in interphase. The duration of the cell cycle was estimated to be 15.2 hours, in which mitosis lasts 1.6 hours and DNA synthesis 4.8 hours. Beginning with the tail-bud stage an increasing proportion of the cells were no longer found to be cycling: at this stage, the first appearance of the differentiating body structures was observed.

The study reported here also examines the mode of reproduction during cercarial development. The cytological evidence obtained from cells in division together with the observed morphological changes in the interphase nuclei support the suggestion that all proliferating cells divide by mitotic divisions.

2. MATERIALS AND METHODS

2.1. MATERIALS

The schistosome, T. ocellata, was maintained within the laboratory in its intermediate host, the snail, L. stagnalis. The snails were kept in large canisters containing tap water previously conditioned by vigorous aeration for one to two days. Water temperature was maintained at 23°C. Snails were reared on fresh green lettuce leaves and chalk.

Miracidia were obtained from the faeces of Pekin ducks 15 to 20 days after exposure to T. ocellata cercariae. The faecal material was mixed with distilled water and placed in a three litre side-arm flask. About 30 minutes after the addition of the distilled water miracidia collected in the side arm (McMullen and Beaver, 1945). Individual snails were exposed to six miracidia for four to five hours and then returned to the tanks. About 85% of the exposed snails released cercariae six weeks later.

The shells of infected snails were broken open with small dissecting scissors and the digestive glands were removed, cut into small pieces and fixed in pre-cooled acetic

acid:absolute alcohol (1:3 v/v) containing a few drops of formalin. The material was left in this fixative at 4°C for 24 hours, then transferred to 70% alcohol and stored at room temperature.

2.2 MICROSPECTROPHOTOMETRY

Microspectrophotometric measurements of Feulgen-DNA content per nucleus were performed on samples of cells from germinal balls of increasing size. Pieces of digestive gland were washed for 20 minutes in two changes of distilled water and then hydrolyzed in 5 N HCl at 23°C (DeCosse and Aiello, 1966; Deitch et al., 1968; Murgatroyd, 1968). An optimum hydrolysis time of 22 minutes had previously been determined by plotting absorption at 550-560 nm against time. Subsequently the material was stained in freshly prepared Schiff's reagent for 60 minutes, then washed in three 15 minute sulfite rinses (Drury and Wallington, 1967).

Individual cercarial germinal balls were dissected out of the daughter sporocysts and placed in a drop of 45% acetic acid on a pre-cleaned glass slide. Individual cells were separated by rolling the germinal ball along the slide with a 27 gauge 1" hypodermic needle. The slides were air dried, dehydrated in an alcohol series, cleared in

xylene, and mounted in oil. The refractive index, n_D, of the oil (n_D = 1.568, Cargille Laboratories, Inc., Cedar Grove, New Jersey) closely matched that of the unstained cytoplasm. The effects of non-specific light loss, due to scatter from unstained material in the cytoplasm, are minimized when the refractive indices of the oil and cytoplasm are matched (Swift and Rasch, 1956; Pollister and Ornstein, 1959).

All photometric measurements were made with a Leitz MPV microscope photometer. Monochromatic light was provided by a Leitz in-line monochromator illuminated by a quartz-iodine lamp stabilized by a Leitz transformer. Total Feulgen dye content per nucleus was measured using the two-wavelength method of Ornstein (1952) and Patau (1952) and reviewed by Pollister et al. (1969). The size of the cytophotometric field for each reading was determined photometrically using the method described by Garcia (1962) and Garcia and Iorio (1966). The relative DNA content of the nuclei was computed from two-wavelength tables (Pollsiter et al., 1969).

2.3 AUTORADIOGRAPHY

For studies of DNA synthesis, infected snails of

approximately equal weight, 2.5 gms, were injected with 10 µCi of ^{3}H-TdR (Amersham/Searle Corp., Specific Activity 5 Ci/mM). The relative purity of the supply of ^{3}H-TdR was determined by ascending paper chromatography. Three separate solvent systems were used:

(a) n-butanol:glacial acetic acid:water (50:25:25).

(b) n-butanol:formic acid:water (70:10:13).

(c) water adjusted to pH 10 with NH_4OH.

In each case the chromatogram contained one spot of UV absorbing material. The position of the spot matched the R_f value for the location of thymidine in control chromatograms and when counted in a Beckman scintillation counter (Model LS-150) contained over 95% of the radioactivity.

The technique for injection of the labelled thymidine into the snails was a modified version of the procedure described by Friedel (1961). Snails were washed in distilled water and placed on toweling to absorb excess moisture. The shell was then wiped with 70% alcohol. A perforation through the shell was made, using a sterilized dissecting needle, adjacent to the yellowish albumin gland. The ^{3}H-TdR was introduced into the digestive gland by injection with a sterilized microlitre syringe through the perforation. Immediately after injection, the shell opening was covered with a thin layer of gum arabic to prevent loss of body fluids and bacterial contamination. Snails were sacrificed

hourly, from one hour to 24 hours, after introduction of ^{3}H-TdR. Slides of cercarial germinal balls were prepared as previously described and dipped in NTB-2 liquid emulsion (Kodak, Rochester, New York). Following 10-14 days exposure, at approximately 4°C, the autoradiographs were developed for 4 minutes in Kodak D-19 (17 gm/200 ml distilled water) at 20°C. They were dipped in distilled water and fixed for 5 minutes in Kodak Rapid Fixer. The slides were washed for 30 minutes in running tap water, dehydrated in a graded series of ethanol, cleared in xylene and mounted with a coverslip in Permount.

2.4 HISTOLOGY

Pieces of a fixed digestive gland from an infected snail were dehydrated in an alcohol series, cleared with xylene and embedded in Tissuemat (M.P. 52°C). Sections were cut at 8-10 μ thickness and stained with either Harris' haematoxylin and eosin or Feulgen's reagent and counterstained with fast green.

2.5 STATISTICAL ANALYSES

The frequencies of mitotic cells, nuclear types and ^{3}H-TdR labelled nuclei in individual germinal balls were scored. The germinal balls ranged in size from 20 to 1900 cells; germinal balls of similar size were grouped together. Scores obtained for balls of similar size were averaged and, unless otherwise indicated, the Chi Square test was used to provide estimates of the range in scores observed with each average value. The Chi Square test was considered to be an appropriate parametric test to apply to the grouped data. The procedure used is presented in Appendix I.

To determine the relationship between each of the above mentioned parameters and size of the germinal ball, individual scores were plotted against the respective germ ball size. A best fit line through the data was obtained by the method of least squares. The Chi Square test was used to define the limits about the best fit line. These limits define an interval such that 95% of repeated samplings would give measurements within that range.

3. RESULTS

3.1 NUCLEAR MORPHOLOGY

Preparations of germinal balls were examined and a variation in nuclear morphology was observed. On the basis of two distinguishing characteristics, size and stain intensity, seven kinds of interphase nuclei were found. They were classified as follows:

	Nuclear Size	Stain Intensity	Class Designation
1	Large	Light	L.L.
2	Large	Medium	L.M.
3	Medium	Light	M.L.
4	Medium	Medium	M.M.
5	Medium	Dark	M.D.
6	Small	Dark	S.D.
7	Very Small	Intensely stained	Bleb

Quantitative data on the DNA content and area for each morphological class will be presented later.

The organization of chromatin varied considerably among the different classes of nuclei (Pl. I, Fig. 1A-F). In the large and medium sized nuclei three distinct patterns of chromatin condensation were recognized: Type I: the Feulgen

stained material appeared fairly homogeneous. The chromatin was made up of fine threads, more or less equally distributed throughout the nucleus, though with occasional small clumps of Feulgen-positive material. Type II: the chromatin was condensed into granules of varying sizes and these were randomly dispersed throughout the nucleus. In some cells the granules were connected by threads. Type III: the chromatin was condensed into well-defined threads. The length and thickness of these threads varied considerably in the different cells. In the large medium and medium dark classes of nuclei, the thickness of the threads suggested that the cell was probably about to enter prophase.

The Bleb class of nuclei were distinctive and appeared as small, compact, intensely-staining bodies. Most of these bodies were regular in outline; but in some there were varying numbers of protrusions extending from one nuclear surface. This latter type of configuration resembled the morphology of cells in late anaphase or early telophase. In the small dark class of nuclei small, densely staining regions alternated with lightly staining regions. In many of these nuclei the denser regions had a banded appearance.

Prophases, metaphases and some anaphases and telophases were also observed. An attempt was made to obtain a chromosome count for this species of trematode. Because of the minute size of the metaphase chromosomes a precise count

was difficult to make. The chromosome number was estimated to be between 12 and 14.

Nuclei from seven germinal balls with from 49 to 1092 cells were measured microspectrophotometrically. Each nucleus, for which the DNA content was determined, was also classified in terms of its nuclear size and relative staining intensity. The data indicated that, regardless of the size of the germinal ball, the relationships between and among the DNA amounts and nuclear areas for each morphological class were identical. The results for the 513, 890, and 1092-celled germinal balls are presented in Tables I-III and Figures 3-8. The data obtained for the 1092-celled germinal ball are described in detail.

The median 2C DNA content for this sample, based on photometric data, was 27.8 ± 1.1 (±S.D.) arbitrary Feulgen-DNA units; the 4C median DNA content was 53.7 ± 1.1 units. The average DNA amount for the Bleb, S.D., M.L. and L.L. nuclear classes was approximately 2C, while the amount of DNA for the L.M. and M.D. nuclear classes was equivalent to 4C (Table III). The mean value for the M.M. class was intermediate between 2C and 4C.

The DNA values for each class have been converted into a frequency distribution of the relative DNA contents (Fig. 7). Each class of nucleus is somewhat restricted in its DNA content, e.g. none of the classes have DNA contents

ranging from 2C to 4C. However, comparisons between nuclear classes reveals that they differ in their range of values and the extent of overlap. The DNA values for the L.L. class range from a 2C to about a 3C DNA content, while the values for the L.M. class range from about a 3C to a 4C value. Overlap between these two classes of nuclei occurred in those cells whose DNA values were intermediate between 2C and 4C. DNA values for the M.M. nuclear class are all in the range of about 2.5 to 3.5C; their lowest DNA values overlap with the highest values obtained for the M.L. class, while their highest values overlap with the lowest values recorded for the M.D. class. The Bleb and S.D. nuclear classes have DNA contents which overlap each other considerably; their values also overlap with the lower values observed for the M.L. and L.L. classes, but not with the values for either the M.M., M.D. or L.M. classes.

With respect to nuclear area, the smallest nuclei were the Blebs (Table III). The S.D. class was the next largest and occupied twice the area of the Blebs. All medium-sized nuclei, regardless of their stain intensity, have an average area which is approximately four times that of the Blebs. The two large classes of nuclei, L.L. and L.M., have ten times the nuclear area of the Blebs.

A comparison of nuclear areas reveals some degree of overlap between the different nuclear morphology classes.

(Fig. 8). The area values for the S.D. nuclear class overlap with the higher values of the Blebs and with the lower values of the three classes of medium-sized nuclei. The values for the large-sized nuclei overlap with those of the medium-sized nuclei but not with the values of either the Bleb or S.D. classes. The range in area values increases as nuclear size increases. For example, the area values ranged from 2.6 to 5.6 μ^2 for the Blebs and from about 30 to 80 μ^2 for the two large-sized classes. Four classes of nuclei in which many of the cells contained 2C DNA contents were, in order of increasing area: Bleb, S.D., M.L. and L.L. classes. Nuclear classes of similar area but in which the DNA contents ranged from 2C to 4C were, in order to increasing staining reaction: L.L., L.M., and the M.L., M.M., M.D. classes.

It appears that the morphology of a nucleus changes, in part, with changes in DNA content. To examine these changes further the stage of each nuclear class within the cell cycle was examined qualitatively. Cells undergoing DNA synthesis were marked with ^{3}H-TdR and their progress through the cell cycle was then determined by an examination of germinal balls 1, 3, 5 and 7 hours after the introduction of the label.

One hour after the injection of the radioactive thymidine the morphological classes of labelled nuclei included some of the M.L., L.L., L.M. and many of the M.M.

classes. By three hours, labelled nuclei had not yet reached mitosis, however, a large proportion of the M.D. and L.M. nuclear classes were observed to be labelled. At five hours, labelled prophases, metaphases, anaphases, telophases and Blebs were observed. By seven hours, S.D. nuclei became labelled.

Taking into consideration the relationships between nuclear class, size, DNA content, and pattern of ^{3}H-TdR labelling it is suggested that: (a) the Blebs represent structures of a late mitotic cell; the six remaining nuclear classes are in interphase, (b) as the cells marked with ^{3}H-thymidine passed from S through G_2, mitosis and G_1, their nuclear morphology changed, (c) the morphological classes of nuclei which became labelled at 1, 3, 5 and 7 hours represent a population of proliferating cells, (d) at the 7 hour fixation the majority of the M.L. and L.L. nuclear classes were still unlabelled. While it might be suggested that these two classes represent a population of non-dividing cells, it seems likely that with subsequent fixations some of these nuclei would have become labelled, (e) the frequency of each morphological class can be used to determine the proportion of cells located within each phase of the cell cycle.

The relative frequencies of the six different morphological classes of nuclei and of cells in mitosis were

determined in 85 germinal balls consisting of 18 to 1829 cells. In all cases, except the M.L. class, the relative frequencies expressed as a percentage of the total number of cells decreased as total cell number increased (Figs. 9-15). In contrast, the M.L. class increased. In the smallest germinal balls scored the frequency of mitotic cells was ≈ 20%. The frequency of the non-mitotic cells was ≈ 80%, of these the six interphase classes were: S.D., 6%; M.L., 30%; M.M., 12%; M.D., 8%; L.L., 14%; and L.M., 10%. At the 1800 cell stage approximately 97% of the nuclei appeared as the M.L. class and the other classes contributed with about each frequency to the remaining 3%.

The evidence derived from the observed changes in the relative frequency of each nuclear class during cercarial development indicates that: (a) the proportion of cells in the S, G_2 and mitotic phases of the cycle decreased, (b) the proportion of cells appearing in the G_1 phase increased, (c) cells accumulating in the G_1 phase appeared as the M.L. nuclear type. Additional support for these suggestions was also gained from examining the changes that occurred in the absolute number of cells appearing in all nuclear classes.

From the 25 to about the 750 cell stage the absolute number of mitotic and non-mitotic classes increased (Figs. 16-22). The number of mitotic cells increased from

3 to 40 and the increases for each interphase nuclear class were: S.D. from 3 to 25; M.L. from 7 to 495; M.M. from 3 to 70; M.D. from 3 to 40; L.L. from 3 to 45; and L.M. from 3 to 35. All classes except for the M.L. nuclear class reached their maximum frequency at about the 750 cell stage. Between the 750 and 850 cell stages the numbers of cells with M.M., M.D., L.L., and L.M. nuclei decreased. A decrease in the number of mitotic and S.D. cells was not detected until the 1000 cell stage. Subsequently all classes except for the M.L. class continued to fall until in the largest cercarial larvae scored, the frequency for each class was: mitotic, 5; S.D., 10; M.M., 20; M.D., 15; L.L., 20; and L.M., 5. The remaining 1750 cells all appeared as the M.L. type. Thus, the above evidence indicates that: (a) up to the 750 cell stage the number of cells appearing in all phases of the cell cycle increased, (b) the proliferative condition found in the small germinal balls is reduced starting at about the 750 cell stage, (c) the drop in the number of proliferating cells begins in the G_1 phase and the majority of cells still in S and G_2 proceed into mitosis, and (d) cells which were no longer cycling must have appeared with a M.L. nuclear morphology since only the frequency of this nuclear class continued to increase beyond the 1000 cell stage.

3.2 SYNCHRONY

If cercarial development is initiated from a single cell, the question arises as to the extent of synchrony during development. When the mitotic indices (number of mitotic cells/total number of cells X 100) of 165 germinal balls were compared evidence of partial synchrony was obtained (Fig. 23). Extreme variability in the MI was observed in the germinal balls consisting of 100 or fewer cells; MI ranged from 3 to 40. With an increase in total cell number variability was reduced, e.g. within the four largest larvae examined, in which the total cell number was $\approx$ 1800, the MI ranged from 0.3 to 0.9.

In part this variability could result from errors in scoring, due to overlapping of nuclei or from breakage of some nuclei during slide preparation. In small germinal balls errors of this nature could introduce large fluctuations in MI. In germinal balls with increased cell number these errors would have a reduced effect. To determine the influence of sampling error on MI a best fit line was constructed by the method of least squares. This line represents the theoretical MI expected for a germinal ball of any size (Fig. 23). A range of values due to sampling errors would lie about this line. Estimates of these ranges were calculated by the Chi Square technique.

In the small germinal balls a significant portion of the MI values fall outside these boundaries, indicating that the observed variability is probably due to a biological cause. If complete synchrony had occurred in the proliferating cells, MI would have ranged from 0 to 100.0; the observed range was 3 to 40 (Fig. 23). The cells show only partial synchrony. As the cercariae grow the range in MI values falls and by about the 250 cell stage the range in values can be accounted for by sampling errors. Thus during cercarial development the degree of synchrony decreases and by the 200 to 300 cell stage, which represents about eight consecutive divisions of one progenitor cell, the partial synchrony is lost. The degree of synchrony generally falls because of variations appearing within the individual cell cycle times. It seems therefore that individual cell cycle times vary shortly after the cercariae begin to develop since the partial synchrony is not maintained for very long.

3.3 LABELLING INDEX

To determine the proportion of cells in the S phase at different stages of cercarial development, labelling indices (number of labelled interphase nuclei/total number of interphase cells X 100) were obtained from germinal balls

at ½, 1, 3, 5 and 7 hours after injection of ^{3}H-TdR. When snails were sacrificed ½ hour after exposure to the radioactive precursor no labelled nuclei were observed, but at 1 hour labelled nuclei were present. In the germinal balls composed of 25 to 250 cells the LI ranged from 4.2 to 75.0 (Table IV, Fig. 24). Sampling errors did not account for the range in LI values (Fig. 25). The variation in labelling indices are in agreement with the MI data, and they also indicate partial synchrony, in this case of cells in the S phase. In the previous section, it was shown that with an increase in the size of the germinal balls the extent of synchrony is reduced. The degree of synchrony of cells in S at any time shows a similar reduction with an increasing number of cells per germinal ball; also the range in LI values becomes smaller as the cercariae increase in cell number. For example, the difference between the maximum and minimum values decreased from an initial value of 70.8 in the 25 to 250 cell group to 45.5 in the 250 to 500 cell group to 22.2 in the 500 to 1100 cell group and to 9.6 in the 1100 to 1600 cell group. A similar change in LI was observed in germinal balls of different cell numbers scored at 3, 5 and 7 hours (Table IV, Fig. 24).

If partial synchrony had been the only factor which accounted for the variation in labelling patterns, then one would expect that in the 25 to 250 group, for example, the

range in LI values would be the same at 1, 3, 5 or 7 hours. The range in LI values, however, decreased at 3 to 7 hours. This decrease resulted primarily from a rise in the lowest LI value observed. For example, at one hour the lowest LI value was 4.2, at 3 hours it was 23.7 and at 5 and 7 hours the lowest values were 29.3 and 52.0. A similar trend was found in the 250 to 500, 500 to 1100 and 1100 to 1600 cell groups (Table IV, Fig. 24). These results indicate that all the ^{3}H-TdR supplied to the infected snails had neither been fully incorporated nor totally destroyed after injection; the labelled precursor remained within the snail and continued to become incorporated into nuclei undergoing DNA synthesis.

Moreover, in the four groups of germinal balls, the highest LI occurred at 7 hours after exposure to the ^{3}H-TdR (Table IV, Fig. 24). Theoretically, if the radioactive thymidine had been available for incorporation into S phase nuclei and quickly dissipated, then the absolute number of labelled cells would have remained constant at each successive sampling period. During the sampling intervals cells which had been in G_2 or mitosis at the time the label was present would have completed mitosis and thereby increased the absolute number of cells per germinal ball. If this were the case, then in comparison to the LI at the first hour the LI at subsequent sampling

times would be lower. The fact that the highest LI values at 1, 3 and 5 hours remained constant supports the suggestion that the ^{3}H-TdR treatment was not a pulse.

The continued movement of cells into S from the G_1 phase together with their incorporation of the labelled precursor has been described, up until this point, as the only mechanism responsible for an increase in the number of labelled cells. Division of the labelled cells would also lead to an increase. The absence of labelled cells at 1 and 3 hours and the appearance of only labelled prophases and metaphases at 5 hours (Section 3.1) indicates that the division of labelled cells had no influence on the LI values observed at these fixations. Between 5 and 7 hours some of the labelled cells observed at 5 hours would have divided. The dramatic increase in the highest LI recorded between the 5 and 7 hour fixations reflects an increase in the number of labelled cells which resulted from not only the movement and incorporation of label into cells entering the S phase, but also from the divisions of labelled cells. Thus, the highest LI values scored at 1, 3, 5 and, in particular, 7 hours indicate that the proliferating cells continued to pass through the cell cycle.

LI data can normally be used to provide an estimate of the fraction of total cycle time (t_c) spent in S (t_s/t_c). From the results presented above, it can be concluded that

several factors influence the LI observed for each germinal ball scored. In the germinal balls scored at 3, 5 or 7 hours the absolute number of labelled cells had increased relative to the frequency of labelled cells obtained at 1 hour. Hence the corresponding LI values represent an over-estimate of the true frequency of cells in S. The values obtained in 1 hour were used. To take into account the synchrony described for the proliferating cells within the small germinal balls, the LI values were obtained from the best fit line (Fig. 25). LI decreased with an increase in total cell number, e.g. in the germinal balls consisting of 100 or fewer cells the LI was 45.5; in the 1200-cell germinal balls the LI was only 10. The results seem to indicate that, with an increase in total cell number, nuclei spend a decreasing proportion of the total cycle undergoing DNA synthesis.

3.4 PERCENTAGE LABELLED MITOSES (PLM)

The technique of the percentage labelled mitoses curve, devised by Quastler and Sherman (1959), is used in the analysis of cell cycle kinetics. However, to obtain meaningful results, two important assumptions must be examined: (a) Treatments with ^{3}H-TdR should not distort the duration of any phases of the cell cycle or total cycle time. For

many animal cells TdR concentrations between 10^{-5} and 10^{-4} M are inhibitory (Cleaver, 1967). Growth is apparently unaffected when the concentration is below 10^{-5} M. The concentration of thymidine used in this study was 10^{-7} M, well below the inhibitory level. Moreover, additional support that the addition of 10^{-7} M ^{3}H-TdR appeared to have no effect on the proliferating cells came from the LI patterns (Sections 3.1 and 3.3). In all germinal balls examined at successive intervals after exposure to the radioactive precursor, the proliferating cells had continued to proceed through interphase and into mitosis. The data fails to reveal, however, if the proliferating cells continued to cycle at rates that were unchanged by treating with the labelled thymidine. (b) The PLM technique depends upon pulse labelling cells in S immediately after injecting the labelled thymidine. If the precursor is not instantaneously incorporated into the DNA of these cells or destroyed then (i) a lag occurs prior to the first cells becoming labelled, (ii) the frequency of labelled nuclei will continue to increase in the period after injection because the precursor is available continuously. Such is the case in the cells of the germinal balls. Notwithstanding this limitation, the progression of the first labelled cells from the S phase until the first peak of labelled mitoses is reached is independent of continuous labelling of cells as they enter the S phase. From this

portion of the curve two important estimates can be obtained: (i) the value obtained for the maximum percentage of labelled mitoses gives a partial indication of the extent of heterogeneity in cell cycle times and variability in the duration of the G_2 phase, (ii) the time at which the percentage of labelled mitoses reaches 50% of the maximum value is equal to the mean duration of G_2 + M/2. This estimate must, however, be corrected to compensate for the one-half hour lag which occurs between the time of injection and the first appearance of labelled cells.

3.4.1 Heterogeneity

If a population of proliferating cells is homogeneous for the duration of G_2 the maximum PLM value observed will be 100. In the present system the average PLM peak value for 65 germinal balls was 78.9 ± 7.7 (±S.D.) at 7 hours. PLM values less than 100 indicate variability in the duration of G_2.

As noted in Section 3.3, there are several factors that can influence the LI of each germinal ball. These factors also affect the PLM values. To investigate the nature of these factors, it was necessary to examine the individual PLM scores (Fig. 26). (a) Scoring Errors: It could be argued that 7 hours was not a sufficient duration in which to reach the highest PLM possible. Cells fixed

at 8 and 9 hours after exposure to labelled thymidine were also examined. No increase was observed in the average value of the PLM curve, that is, the peak reached at 7 hours was approximately constant for at least two additional hours.

Some difficulty was encountered in scoring the slides prepared at 7 hours. The heavy density of grains made it extremely difficult, in some cases, to discern whether or not a labelled cell was in mitosis. Unlabelled cells, especially mitotic cells, were easily scored. Hence the individual PLM values probably represent underestimates of the true values. Further analysis with the highest PLM values obtained would tend to minimize the effects of scoring errors.

(b) Partial Synchrony: The presence of partial synchrony in the small germinal balls and the loss of this synchrony in the larger balls accounts, in part, for: (i) the considerable range in PLM values recorded in the group of germinal balls consisting of 25 to 250 cells, and (ii) the decrease in the range of PLM values in balls of increasing size scored at 5 or 7 hours (Table V). Selection of only the highest PLM values observed at 5 or 7 hours identifies the germinal balls which had a high frequency of cells in S during the ^{3}H-TdR exposure, and in which many of the labelled cells had progressed into mitosis.

In germinal balls of 25 to 250, 250 to 500, 500 to 1100 and 1100 to 1600 cells, scored at 7 hours, some had a

PLM value of 100 (Fig. 26). of the 65 germinal balls scored at 7 hours about 25% had values $\geq$ 95. The high PLM values recorded in each of the four groups differing in total cell number indicates that (i) the proliferating cells were homogeneous for the duration of G_2 and mitosis, and (ii) there is little variability in the duration of G_2 or mitosis at different stages during cercarial development.

3.4.2 Estimates of G_2 + M/2

Two sets of data were used to provide estimates of G_2 + M/2: the highest and the average PLM value obtained for germinal balls of similar size (Figs. 27A and B). The first ascending curves derived from the highest PLM values were based on one germinal ball per point. For these individual PLM values (a) the effects of synchrony would be maximal, that is, many proliferating cells were in the S phase at the time of exposure to the ^{3}H-TdR and many of the cells had progressed into mitosis, (b) the effects of scoring errors would be minimal, that is, labelled mitotic cells would readily be distinguished from labelled interphase cells. The second ascending curve was based on the average PLM values and would include all PLM values affected by these errors.

The estimates of the duration of G_2 + M/2 using the highest PLM values were similar to the estimates using the

average PLM values (Table VI); for example, in the 25 to 250 cell group the estimates from the highest and average PLM values were 3.9 to 4.1 hours. Similarly, for the 250 to 500 cell group the estimates were 4.1 and 4.2 hours. These results indicate that the effect of the errors on the average PLM values must have remained constant between 5 and 7 hours. Since the estimates of G_2 + M/2 (Table VI) were approximately constant for all sizes of germinal balls examined, it is suggested that the duration of G_2 + M/2 remained constant in the germinal balls ranging in size from 25 to 1540 total cells.

3.5 MICROSPECTROPHOTOMETRY

To determine the relative durations of G_1, S and G_2 in developing germinal balls, the DNA contents in samples of interphase nuclei from individual germinal balls were measured microspectrophotometrically. Seven germinal balls ranging in size from 42 to 1092 cells were analyzed. The data obtained for the 513 celled germinal ball was described in detail.

The frequency distribution for the relative Feulgen-DNA contents of 191 nuclei shows that the values cover a wide range; the peak in the highest relative Feulgen-DNA contents is about double the peak in the lowest values (Fig. 28).

The lowest values probably represent nuclei which have not undergone DNA synthesis and are thus in the G_1 phase of the cycle, while the higher values probably represent nuclei which have completed DNA synthesis and are thus in the G_2 phase. Intermediate values also indicate that the population probably includes cells in the S phase. At present, the following assumption is made regarding the photometric data: the lower relative Feulgen-DNA amounts represent nuclei containing a 2C DNA content whereas the higher values represent nuclei containing a 4C DNA content.

Coherent populations of 2C, intermediate, 4C nuclei and the limits for these classes were determined by graphical methods (Appendix II). The data were first plotted as a linear distribution of absorption values against percentage cumulative frequency. A better fit to the straight lines, describing the 2C and 4C subpopulations, was obtained when logarithms of Feulgen-DNA contents were plotted.

Since the 2C subpopulation constituted 39.5% of the nuclei measured, cells spend 39.5% of interphase in G_1 (Fig. 29). The log estimates for the mean and standard deviation of this subpopulation were 1.422 and 0.036. The 4C subpopulation represented 36.5% of the nuclei scored. Thus cells spend 36.5% of interphase in G_2. Log estimates of its mean and standard deviation were 1.685 and 0.048. In the remaining 24.0% of the nuclei their DNA contents were intermediate

between 2C and 4C; thus cells spend 24.0% of interphase in S.

Sample estimates of G_1, S and G_2 can be used to provide estimates describing the entire population. It is possible to evaluate whether or not the sample estimates accurately describe the entire population. The method involves a comparison between the sample and population distributions for each of the six different nuclear morphology classes. The results indicate that the microspectrophotometric sample was not representative of the entire population (Table VII). Discrepancies between the sample and population distributions for each nuclear morphology class are present in all germinal balls examined. For example, the 513 celled germinal ball contained 44.2% of the M.L. nuclear type while the sample contained only 35.6%.

The discrepancies may have resulted from a non-random selection of nuclei to be measured microspectrophotometrically. Nuclei were selected on the basis of appearance. If a nucleus had been damaged during slide preparation or if one nucleus overlapped another they were not measured. In the germinal balls consisting of 41 to 227 cells, the sample size (Table VII) represents the maximum number of interphase nuclei suitable for measurement. Furthermore, with a large population of cells, 200 nuclei are normally considered to be a good sample size to measure photometrically. In the three largest examples described in this report a sample size of

approximately 200 nuclei was measured and constituted 39.0% of the total number of cells for 513 celled germinal ball, 22.9% for the 874 celled germinal ball, and only 18.3% for the 1092-celled germinal ball. With larger populations therefore a smaller proportion of the nuclei were sampled thereby increasing the chance of obtaining a non-random sample.

Correction of the data for graphical analysis refines the estimates for the cell cycle outlined above. The data was corrected by weighting the individual photometric values for each morphological type of interphase nucleus to represent its distribution within the population. For example, in the 513 celled germinal ball, the sample contained 35.6% and the population contained 44.2% medium light type of nuclei. The individual photometric values for the medium light nuclei were weighted so these values constituted 44.2% of the total population. From the weighted sample data a new cumulative frequency plot was constructed (Fig. 30). From this plot, estimates of the three parts of interphase were determined for the entire population: G_1 constitutes 51.9% of total interphase; S, 20.5%; and G_2, 27.6%. The mitotic index for this germinal ball was 9.4; thus, the value for mitosis and for the interphase classes expressed as percentages of total cycle time were:

	% Total Cycle
Mitosis	9.4
G_1	47.0
S	18.6
G_2	25.0

The estimate of G_2 + M/2 derived from the PLM data has been used to convert these percentages into durations (Appendix III): t_C was estimated to last 13.9 hours in which t_{G_1} was 6.5 hours, t_S was 2.6 hours, t_{G_2} was 3.5 hours, and t_M was 1.3 hours.

For each of the 7 germinal balls analyzed, the sample and population estimates of interphase composition were not identical (Table VIII), e.g. the percentage difference between the sample and population estimates for the G_1 phase were:

Sample	1	2	3	4	5	6	7
% Difference	9.9	7.0	17.8	17.2	24.3	31.3	54.9

This variation resulted from the sampling errors introduced when the nuclei were originally selected for measurement. The degree of variation would depend upon the extent to which each morphological class was weighted and on the DNA contents for each morphological class of interphase nuclei. In the smaller germinal balls (Samples 1-4) the majority of the M.L. nuclei were originally measured photometrically, whereas in the larger germinal balls (samples 5-7) a large discrepancy

existed between the sample and population frequencies. For the larger masses the individual DNA readings obtained for the M.L. nuclei would be weighted to a greater extent than for any of the five remaining interphase classes, e.g. M.M. nuclei which showed almost identical frequencies for the sample and the population estimates (Table VII). Weighting the individual sample DNA values for the M.L. class would tend to increase the percentage of cells located in the G_1 phase, since almost all of the M.L. readings represented a 2C DNA content (Section 3.1). In contrast weighting the values for M.M. class would tend to influence the fraction of cells appearing in S since the M.M. DNA values ranged from 2.5C to 3.5C (Section 3.1).

In the five samples consisting of 513 or fewer cells the estimates for the relative duration of each phase were approximately constant (Table IX). The average value for t_C was $\tilde{\sim}$ 15.2 hours and the durations of the individual phases were 1.6 hours for t_M, 5.6 hours for t_{G_1}, 4.8 hours for t_S, and 3.2 hours for t_{G_2}. On the other hand, in the two largest germinal balls analyzed photometrically, that is the 874 and the 1092 cell germinal balls, the durations of t_C and t_{G_1} were about two to three times the durations observed for the germinal balls composed of 513 of fewer cells. The durations of mitosis, S and G_2 were, however, similar to those of the smaller masses. The increase in

the duration of t_{G_1} reflects the increase in the relative proportion of the G_1 nuclei (Table VIII); and in particular from an increase in the absolute number of the M.L. type of nuclei (Table VII).

Within any germinal ball the fraction of the total population of nuclei containing polyploid DNA contents can be estimated by the graphical method. Points of inflection beyond the 4C population indicate nuclei containing polyploid DNA contents. No evidence for polyploidy was found in the five germinal balls consisting of 41 to 513 cells. The two largest germinal balls contained varying proportions of nuclei with DNA contents greater than 4C. In sample no. 6 with 974 cells, 0.85% of the nuclei contained DNA contents greater than 4C of which 0.32% fell between 4C and 8C and 0.52% were 8C (Fig. 31). In sample no. 7 with 1092 cells, 0.58% of the nuclei had values between 4C and 8C (Fig. 32). The morphological class of nucleus containing polyploid DNA contents was always the large medium type.

The incidence of polyploidy was low and variable; nevertheless, the presence of polyploid nuclei could be used to describe the developmental processes occurring within this system. Since polyploid nuclei are detected by photometry only when the nuclei are at least at the 5C DNA level, they must have become committed at least 1½ cycles prior to the time of fixation, i.e. at a time when the larvae contained

fewer cells than recorded at the time of fixation. "Committed" implies that these cells are different from other proliferative cells. The difference is that the polyploid type of nuclei have gone through a replicative cycle which was not followed by a division cycle. This some cells are being committed at a stage of larval development which is similar to the stage at which previous results have shown that many other cells are also leaving the proliferating pool to become non-dividing cells.

3.6 HISTOLOGY OF DEVELOPING CERCARIAL LARVAE

A histological study was carried out in order to determine the position of dividing cells within the developing cercariae. Such an analysis is not possible in squash preparations. The distributions of the morphologically different types of interphase nuclei were also examined.

Prior to the 1100 cell stage, mitotic figures were scattered apparently at random throughout each germinal ball (Fig. 33; Pl. II, Figs. 2,3,7). When the germinal balls consisted of ≈ 1100 cells, two foci of proliferative activity were found; mitotic figures were restricted to the anterior and posterior regions of the body (Pl. II, Fig. 6). In the larger germinal balls, the posterior region of proliferative

activity is lost, while the anterior one is retained.

The large and medium-sized classes of non-mitotic nuclei were also scattered throughout the germinal balls composed of 1100 or fewer cells (Pl. II, Figs. 2,3,7). At the tail bud stage (Stage V; Cheng and Bier, 1972) the tail region was composed of nuclei which were all of the medium light type (Pl. II, Figs. 4,5). The body of the developing cercaria consisted predominantly of the medium light type whereas the large-sized class were localized in the mid-body region. Gland cells eventually form in this region. These gland cells contain nuclei which although large are morphologically different from the large interphase nuclei of young germinal balls. The former type contained densely staining chromatin bodies and a large nucleolus (Pl. II, Figs. 4,5); the latter type has been described previously (Section 3.1). It may be supposed that some of the cells with large interphase nuclei, located in the mid-region of the developing cercariae, are destined to form gland cells.

In the mature cercariae the average number of nuclei contained within the tail was 280 of which 40 nuclei were located in each fork of the tail. It was impossible, from examining the sectioned material, to obtain an accurate estimate for the total number of cells eventually contained within the body. Even the technique used to separate the individual cells failed to separate all the cells of a mature

cercaria. The largest masses accurately scored by this latter procedure consisted of ≈ 1800 cells. Since their MI was less than 1.0 (Section 3.4), a reasonable estimate for the total number of cells contained within a mature cercaria is about 2000.

Nuclei appeared to be in close proximity with adjacent nuclei in the Feulgen and Fast Green stained sectioned material of germinal balls consisting of 1100 or fewer cells (Pl. II, Figs. 2,6). The absence of a positive cytological staining reaction was also observed when sections of similar sized germinal balls were stained with haematoxylin and eosin (Pl. II, Figs. 3,5,7). The histological evidence indicates that since the cells have almost no cytoplasm there can be little or no synthesis of cytoplasmic components. When the cercarial larvae consisted of about 1100 or more cells a positive eosin reaction could be detected in the cells of the elongating tail and in many cells of the body (Pl. II, Figs. 4,5). This observed positive eosin staining reaction indicates that some of the cells were engaged in the synthesis of cytoplasmic components; these cells were apparently differentiating into specialized cells. Some cells apparently remain undifferentiated; in the mature cercariae the genital primordium appeared as an undifferentiated mass of small cells (Pl. II, Fig. 8).

The above results support the suggestion made previously, that within the small germinal balls the majority of cells were actively engaged in cellular proliferation. However, when the germinal balls contained approximately 1100 cells, one-half the number found in a mature cercaria, many of the cells were no longer proliferating. The cytological evidence indicates that many of these non-cycling cells were differentiating.

TABLES

TABLE I

Relative Feulgen-DNA contents and areas for the morphologically different classes of nuclei from a 513 cell germinal ball. The 2C and 4C median DNA contents were 26.4 ± 1.1 (±S.D.) and 48.1 ± 1.1 arbitrary units.

TABLE I

Nuclear Class	DNA Content		Area		Sample Size
	Mean	Standard Deviation	Mean	Standard Deviation	
Large Light	35.4	9.0	49.1	14.6	14
Large Medium	50.5	6.4	38.8	10.2	27
Medium Light	28.2	5.3	18.5	6.2	68
Medium Medium	44.1	6.8	19.7	4.9	34
Medium Dark	52.4	2.7	19.2	6.5	13
Small Dark	28.0	3.0	9.5	3.0	37
Bleb	32.0	1.9	4.5	1.1	25

TABLE II

Relative Feulgen-DNA contents and areas for the morphologically different classes of nuclei from a 905 cell germinal ball. The 2C and 4C median DNA contents were 29.5 ± 1.1 (±S.D.) and 60.0 ± 1.1 arbitrary units.

TABLE II

Nuclear Class	DNA Content		Area		Sample Size
	Mean	Standard Deviation	Mean	Standard Deviation	
Large Light	38.3	7.3	47.6	13.8	21
Large Medium	61.2	4.2	44.8	9.5	31
Medium Light	30.3	4.8	17.3	4.5	55
Medium Medium	50.9	4.3	22.1	4.6	33
Medium Dark	63.2	3.9	22.6	7.1	16
Small Dark	30.2	3.2	10.9	2.0	39
Bleb	32.0	3.3	3.7	1.0	23

TABLE III

Relative Feulgen-DNA contents and areas for the morphologically different classes of nuclei from a 1092 cell germinal ball. The 2C and 4C median DNA contents were 27.8 ± 1.1 (±S.D.) and 53.7 ± 1.1 arbitrary units.

TABLE III

Nuclear Class	DNA Content		Area		Sample Size
	Mean	Standard Deviation	Mean	Standard Deviation	
Large Light	33.8	5.0	41.8	8.5	22
Large Medium	53.7	6.5	41.8	13.6	33
Medium Light	30.1	2.8	16.8	5.4	24
Medium Medium	43.7	3.4	19.8	6.9	30
Medium Dark	55.1	3.6	18.7	4.9	23
Small Dark	29.0	2.5	8.9	1.4	30
Bleb	27.6	2.2	4.0	1.4	30

TABLE IV

Labelling indices of germinal balls at various times after exposure to ^{3}H-TdR.

TABLE IV

Hours after exposure to ^{3}H-TdR	Germinal ball cell group	Smallest-Largest germinal ball (cells)	Number of germinal balls scored	Labelling Index				
				Mean	Std. Dev.	Lowest	Highest	Range
1	25-250	28-246	33	45.7	16.5	4.2	75.0	70.8
	250-500	270-491	8	35.6	11.0	12.3	57.8	45.5
	500-1100	545-1043	19	21.5	7.7	12.0	34.2	22.2
	1100-1600	1154-1396	5	8.4	3.9	3.7	13.3	9.6
3	25-250	71-246	15	48.1	13.3	23.7	79.5	55.8
	250-500	254-488	14	42.9	12.3	28.0	61.5	33.5
	500-1100	505-930	12	35.7	8.6	19.5	39.5	20.0
	1100-1600	1137-1395	9	19.2	5.9	10.1	27.5	17.4
5	25-250	30-319	22	49.4	12.7	29.3	70.0	40.7
	250-500	251-497	12	49.7	12.6	32.3	79.0	46.7
	500-1100	565-988	12	29.9	5.8	21.8	37.0	15.2
	1100-1600	1178-1536	4	20.4	6.2	12.2	27.4	15.2
7	25-250	44-211	20	72.9	14.2	52.0	92.5	40.5
	250-500	255-481	20	58.2	14.3	37.6	73.3	35.7
	500-1100	521-1061	15	38.9	12.9	23.0	47.8	24.8
	1100-1600	1108-1586	10	24.6	6.3	17.1	38.4	21.3

TABLE V

Percentage labelled mitoses of germinal balls at various times after exposure to ^{3}H-TdR.

TABLE V

Hours after exposure to ^{3}H-TdR	Germinal ball cell group	Smallest-Largest germinal ball (cells)	Number of germinal balls scored	Percentage Labelled Mitoses				
				Mean	Std. Dev.	Lowest	Highest	Range
5	25-250	30-219	22	45.3	12.5	21.0	75.0	54.0
	250-500	251-497	12	46.9	11.0	30.5	64.0	33.5
	500-1100	565-988	12	46.1	13.2	34.0	63.0	29.0
	1100-1600	1178-1536	4	38.7	9.7	25.0	47.5	22.5
7	25-250	44-211	20	71.8	24.4	23.0	100.0	77.0
	250-500	255-481	20	78.3	15.9	45.5	100.0	54.5
	500-1100	521-1061	15	78.9	15.6	48.5	100.0	51.5
	1100-1600	1108-1586	10	82.9	9.5	71.5	100.0	28.5

TABLE VI

Estimates of G_2 + M/2 derived from PLM curves of germinal balls differing in cell number.

TABLE VI

Germinal Ball Cell Group	Estimate of G_2 + M/2 (Hours) from highest PLM at 5 and 7 hours	from average PLM at 5 and 7 hours
25-250	4.4 (3.9)[a]	4.6 (4.1)
250-500	4.6 (4.1)	4.7 (4.2)
500-1100	4.6 (4.1)	4.7 (4.2)
1100-1600	5.1 (4.6)	5.2 (4.7)

[a] Number in parenthesis denotes a corrected value which is derived by subtracting ½ hour from the observed value.

TABLE VII

Relationship between the number of nuclei measured photometrically and the absolute total number of nuclei within each nuclear morphology class for seven germinal balls.

a. Total number of cells in the germinal ball.

b. Number of nuclei for each morphological class scored photometrically.

c. Actual number for each morphological class present in the germinal ball.

d. [Number of nuclei scored for each morphological class]/[Combined total number of nuclei scored for all morphological classes] X 100.

e. [Actual number for each morphological class]/[Combined total number of nuclei for all morphological classes] X 100.

TABLE VII

Nuclear Morphology Class	Sample No. 1 (41 cells)[a] Number of Nuclei in Each Morphological Class		Sample No. 1 (41 cells)[a] Percentage of Each Morphological Class		Sample No. 2 (61 cells) Number of Nuclei in Each Morphological Class		Sample No. 2 (61 cells) Percentage of Each Morphological Class	
	Sam.[b]	Pop.[c]	Sam.[d]	Pop.[e]	Sam.	Pop.	Sam.	Pop.
Large Light	2	4	10.0	10.5	2	6	5.9	11.8
Large Medium	3	6	15.0	15.8	6	7	17.6	13.7
Medium Light	6	12	30.0	31.6	14	23	41.2	45.1
Medium Medium	5	9	25.0	23.7	7	8	20.6	15.7
Medium Dark	2	3	10.0	7.9	3	4	8.8	7.8
Small Dark	2	4	10.0	10.5	2	3	5.9	5.9
Total Interphase Nuclei	20	38	100.0	100.0	34	51	100.0	100.0

TABLE VII (Continued)

Nuclear Morphology Class	Sample No. 3 (129 cells)				Sample No. 4 (227 cells)			
	Number of Nuclei in Each Morphological Class		Percentage of Each Morphological Class		Number of Nuclei in Each Morphological Class		Percentage of Each Morphological Class	
	Sam.	Pop.	Sam.	Pop.	Sam.	Pop.	Sam.	Pop.
Large Light	2	8	2.3	7.0	5	20	4.2	9.6
Large Medium	13	14	15.1	12.3	10	20	8.4	9.6
Medium Light	30	38	34.9	33.3	47	87	39.5	41.8
Medium Medium	17	24	19.8	21.1	26	36	21.8	17.3
Medium Dark	12	17	14.0	14.9	13	20	10.9	9.6
Small Dark	12	13	14.0	11.4	18	25	15.1	12.0
Total Interphase Nuclei	86	114	100.0	100.0	119	208	100.0	100.0

TABLE VII (Continued)

Nuclear Morphology Class	Sample No. 5 (513 cells)				Sample No. 6 (874 cells)			
	Number of Nuclei in Each Morphological Class		Percentage of Each Morphological Class		Number of Nuclei in Each Morphological Class		Percentage of Each Morphological Class	
	Sam.	Pop.	Sam.	Pop.	Sam.	Pop.	Sam.	Pop.
Large Light	12	34	6.3	7.7	21	39	10.8	5.0
Large Medium	27	37	14.1	8.4	31	36	15.9	4.6
Medium Light	68	195	35.6	44.2	55	539	28.2	68.9
Medium Medium	34	71	17.8	16.1	33	81	16.9	10.4
Medium Dark	13	34	6.8	7.7	16	23	8.2	2.9
Small Dark	37	70	19.4	15.9	39	64	20.0	8.2
Total Interphase Nuclei	191	441	100.0	100.0	195	782	100.0	100.0

TABLE VII (Continued)

Nuclear Morphology Class	Sample No. 7 (1092 cells) Number of Nuclei in Each Morphological Class		Percentage of Each Morphological Class	
	Sam.	Pop.	Sam.	Pop.
Large Light	22	49	13.6	4.9
Large Medium	33	51	20.4	5.1
Medium Light	24	530	14.8	53.4
Medium Medium	30	142	18.5	14.3
Medium Dark	23	60	14.2	6.1
Small Dark	30	160	18.5	16.1
Total Interphase Nuclei	162	992	100.0	100.0

TABLE VIII

Estimates of the relative durations of the phases of the cell cycle expressed as percentages of the total cycle in germinal balls differing in cell numbers.

[a]Determined from the graphical analysis of the nuclei measured microspectrophotometrically.

[b]Determined from the graphical analysis of the individual photometric values weighted to represent their distribution within the population:

TABLE VIII

Sample No.		1	2	3	4	5	6	7
Number of Cells/ Germinal Ball		41	61	129	227	513	874	1092
No. of Nuclei Measured		20	34	86	119	191	195	162
Sample Estimates of Interphase Composition[a]	G_1	37.5	39.7	47.1	31.5	39.3	49.5	28.4
	S	35.0	38.2	27.9	53.8	24.0	17.9	31.5
	G_2	27.5	22.1	25.0	14.7	36.7	32.6	40.1
Population Estimates of Interphase Composition[b]	G_1	41.2	37.1	40.0	36.9	51.9	72.1	63.0
	S	33.3	40.8	35.5	44.6	20.5	17.9	22.4
	G_2	25.5	22.1	24.5	18.5	27.6	10.0	14.6
M.I.		7.3	16.4	11.6	8.4	9.4	5.2	4.3
Population Cell Cycle Parameters (Expressed as a percentage of total cycle)	M	7.3	16.4	11.6	8.4	9.4	5.2	4.3
	G_1	38.2	31.0	35.4	33.8	47.0	68.4	60.3
	S	30.9	34.1	31.4	40.8	18.6	17.0	21.3
	G_2	23.6	18.5	21.6	17.0	25.0	9.5	14.0

TABLE IX

Estimates of the durations of the phases of the cell cycle in germinal balls differing in cell number.

TABLE IX

Sample No.	Number of Cells/ Germinal Ball	t_C	t_M	t_{G_1} (hours)	t_S	t_{G_2}
1[a]	41	14.3	1.0	5.5	4.4	3.4
2	61	14.6	2.4	4.5	5.0	2.7
3	129	14.2	1.6	5.0	4.5	3.1
4	227	18.4	1.6	6.2	7.5	3.1
5[b]	513	13.9	1.3	6.5	2.6	3.5
6	874	33.8	1.8	23.1	5.7	3.2
7	1092	25.4	1.1	15.3	5.4	3.6

[a]Samples 1 to 4, $G_2 + M/2 = 3.9$ hours.

[b]Samples 5 and 6, $G_2 + M/2 = 4.1$ hours.

FIGURES

FIGURE 1

Diagrammatic outline of the life cycle of the schistosome *Trichobilharzia ocellata*.

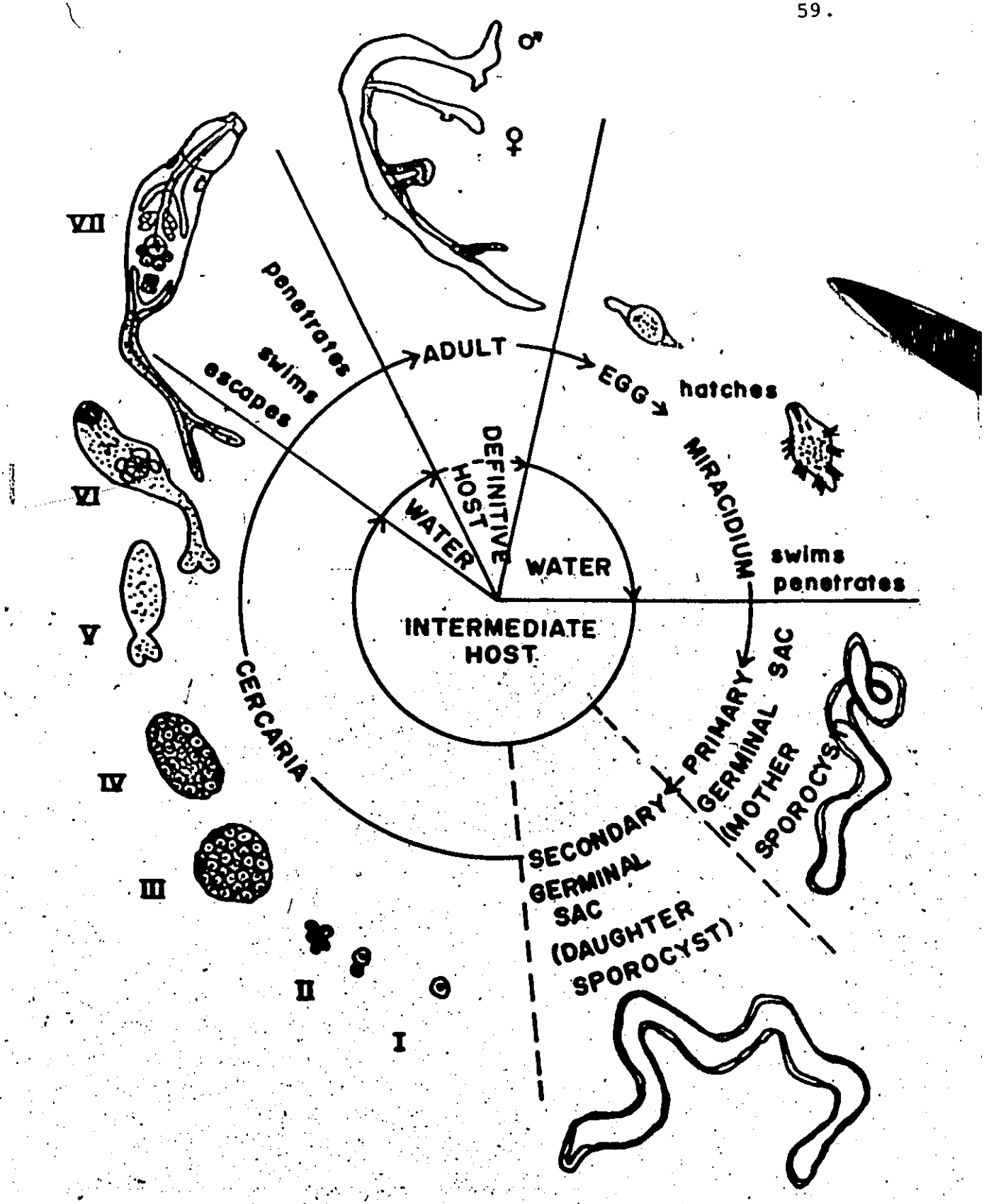

♂
♀
VII
VI
V
IV
III
II
I
penetrates
swims
escapes
ADULT
EGG
hatches
DEFINITIVE HOST
WATER
WATER
MIRACIDIUM
swims
penetrates
INTERMEDIATE HOST
CERCARIA
PRIMARY GERMINAL SAC (MOTHER SPOROCYST)
SECONDARY GERMINAL SAC (DAUGHTER SPOROCYST)

FIGURE 2

Diagram summarizing development during the life cycle of *Trichobilharzia ocellata*.

○ Cells of the germinal line.

● Somatic cells which form the tissues and disappear without descendants.

• Indicates the stage of germinal development at which incomplete meiotic divisions would occur if the germinal cells reproduced parthenogenetically.

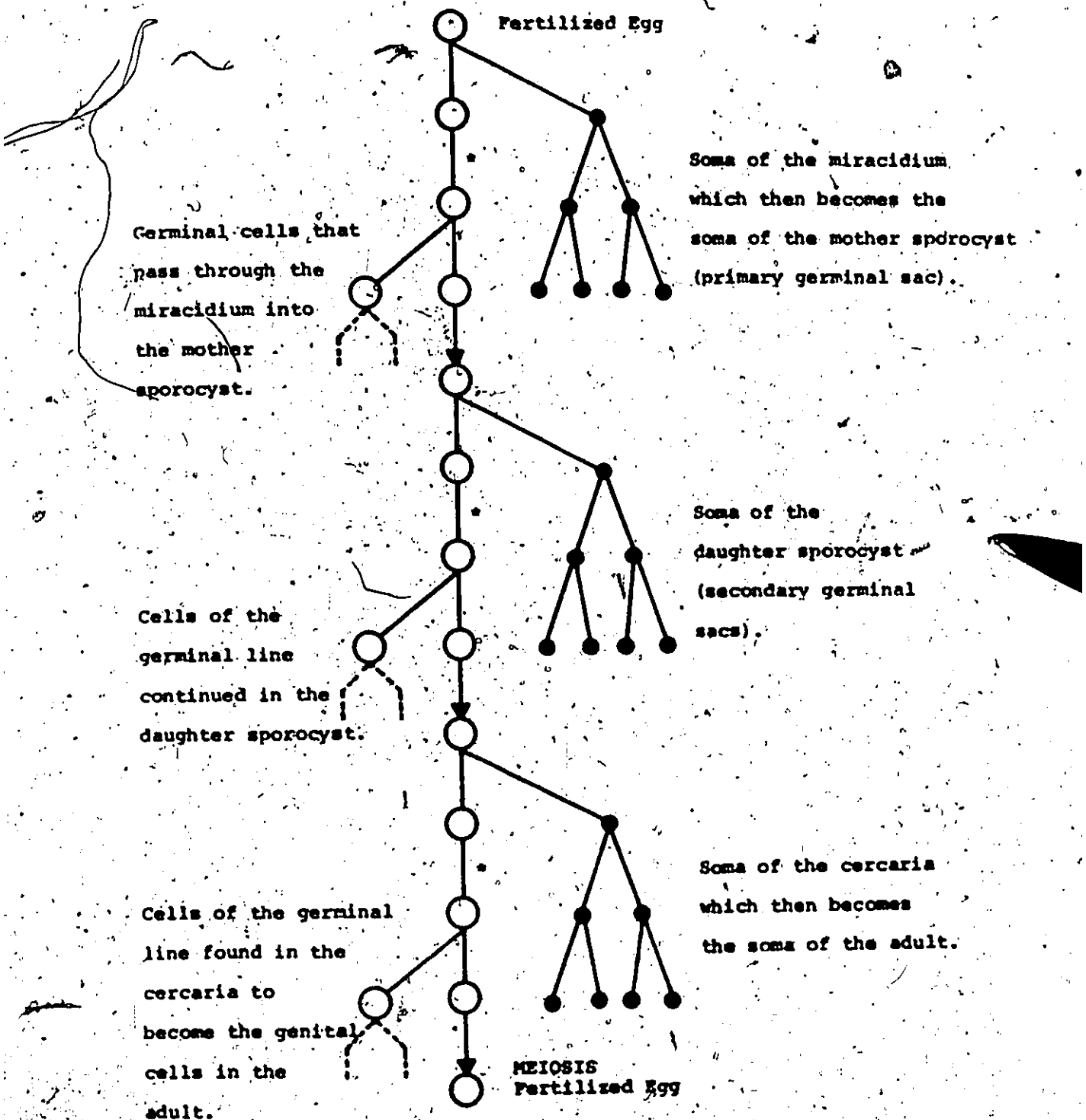

Fertilized Egg
Soma of the miracidium which then becomes the soma of the mother sporocyst (primary germinal sac).
Germinal cells that pass through the miracidium into the mother sporocyst.
Soma of the daughter sporocyst (secondary germinal sacs).
Cells of the germinal line continued in the daughter sporocyst.
Soma of the cercaria which then becomes the soma of the adult.
Cells of the germinal line found in the cercaria to become the genital cells in the adult.
MEIOSIS
Fertilized Egg

FIGURE 3

Frequency distribution of relative Feulgen-DNA contents of each nuclear morphology class from a 513 cell germinal ball. Arrows indicate the 2C and 4C median DNA contents.

Panel	Nuclear Morphology Class
A	Bleb
B	Small Dark
C	Medium Light
D	Medium Medium
E	Medium Dark
F	Large Light
G	Large Medium

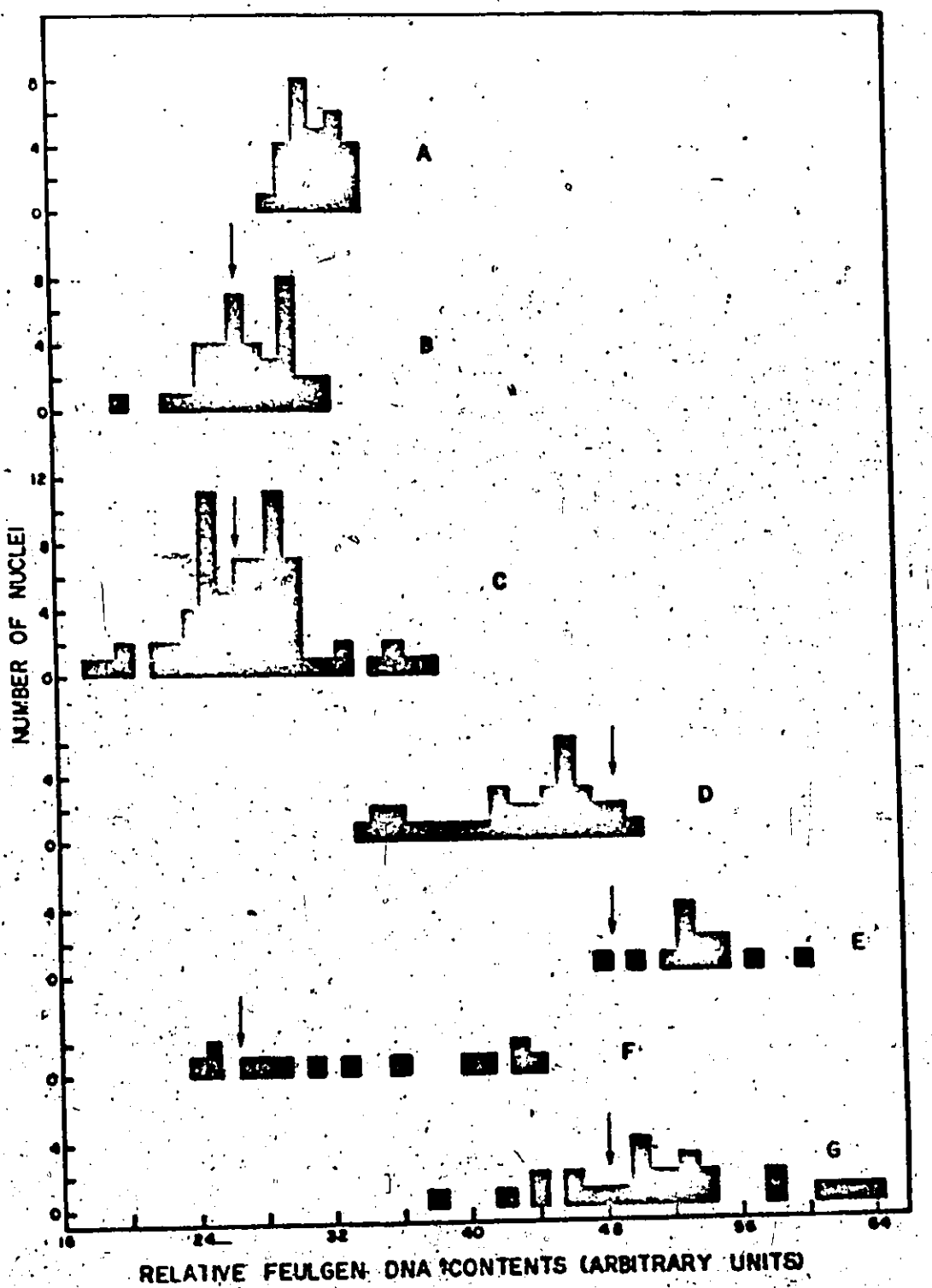
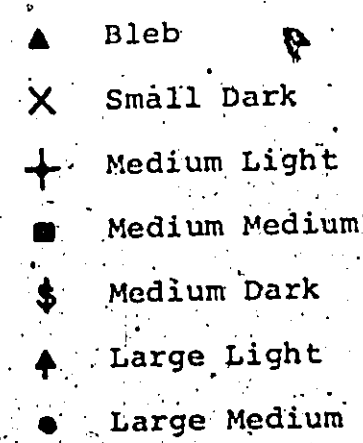

A
B
C
D
E
F
G
NUMBER OF NUCLEI
RELATIVE FEULGEN DNA CONTENTS (ARBITRARY UNITS)

FIGURE 4

Scatter plot of nuclear area and relative Feulgen-DNA content for each nuclear morphology class from a 513 cell germinal ball.



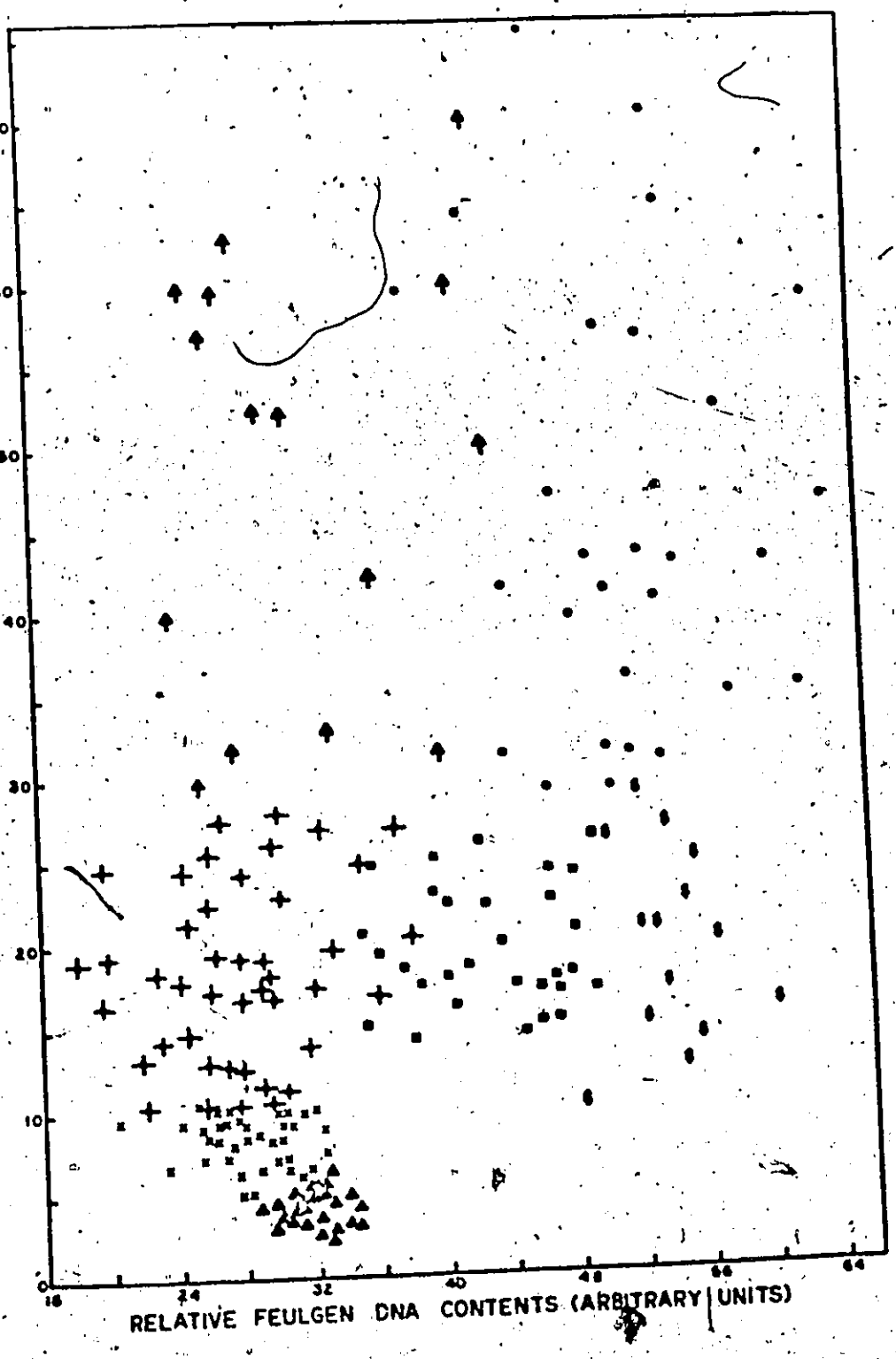

AREA (μ²)
70
60
50
40
30
20
10
0
16
24
32
40
48
56
64
RELATIVE FEULGEN DNA CONTENTS (ARBITRARY UNITS)

FIGURE 5

Frequency distribution of relative Feulgen-DNA contents of each nuclear morphology class from a 905 cell germinal ball. Arrows indicate the 2C and 4C median DNA contents.

Panel	Nuclear Morphology Class
A	Bleb
B	Small Dark
C	Medium Light
D	Medium Medium
E	Medium Dark
F	Large Light
G	Large Medium

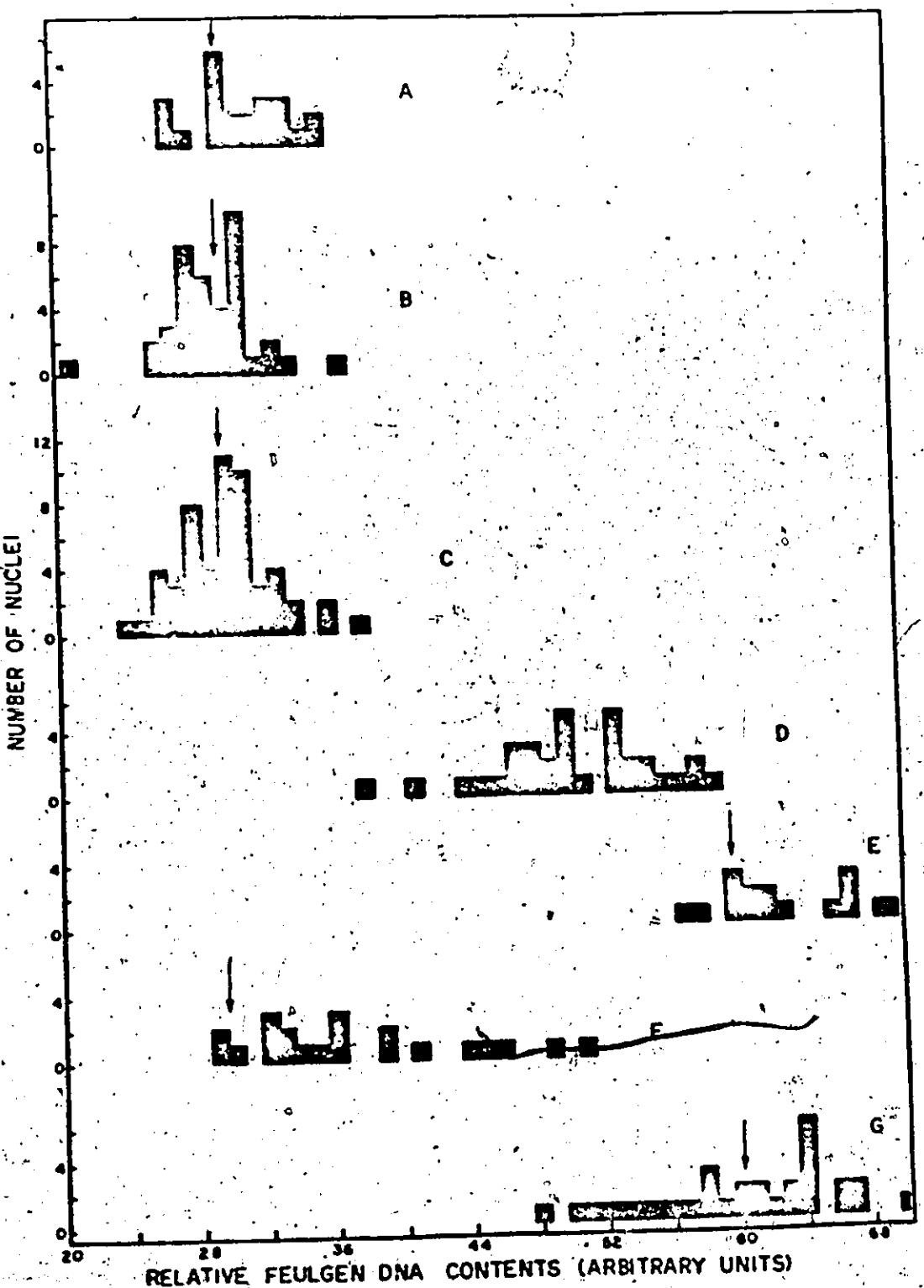

A
B
C
D
E
F
G
NUMBER OF NUCLEI
20
28
36
44
52
60
68
RELATIVE FEULGEN DNA CONTENTS (ARBITRARY UNITS)

FIGURE 6

Scatter plot of nuclear area and relative Feulgen-DNA content for each nuclear morphology class from a 905 cell germinal ball

▲ Bleb

× Small Dark

+ Medium Light

■ Medium Medium

$ Medium Dark

▲ Large Light

● Large Medium

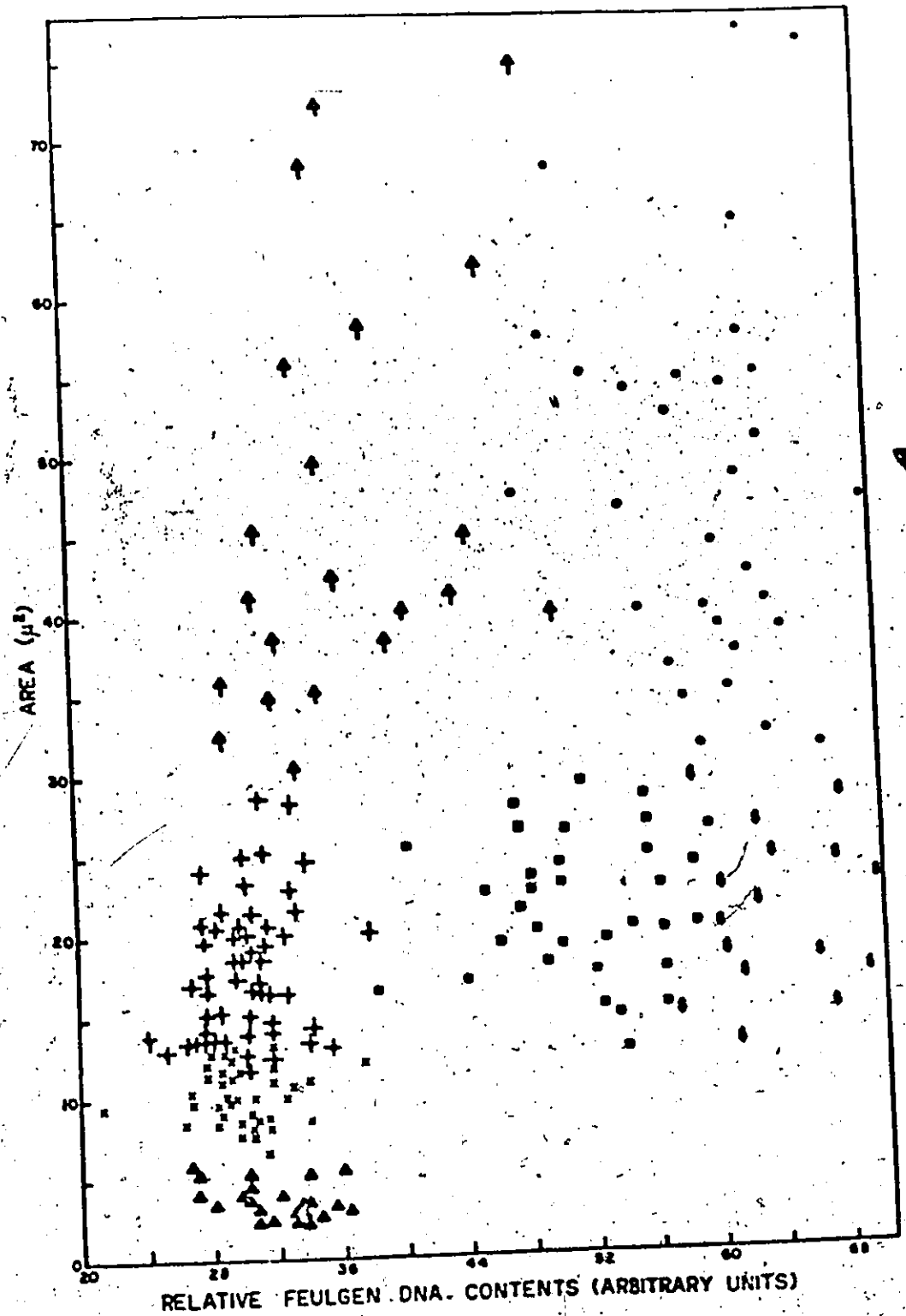

AREA (μ²)
70
60
50
40
30
20
10
0
20
28
36
44
52
60
68
RELATIVE FEULGEN DNA CONTENTS (ARBITRARY UNITS)

FIGURE 7

Frequency distribution of relative Feulgen-DNA contents of each nuclear morphology class from a 1092 cell germinal ball. Arrows indicate the 2C and 4C median DNA contents.

Panel	Nuclear Morphology Class
A	Bleb
B	Small Dark
C	Medium Light
D	Medium Medium
E	Medium Dark
F	Large Light
G	Large Medium

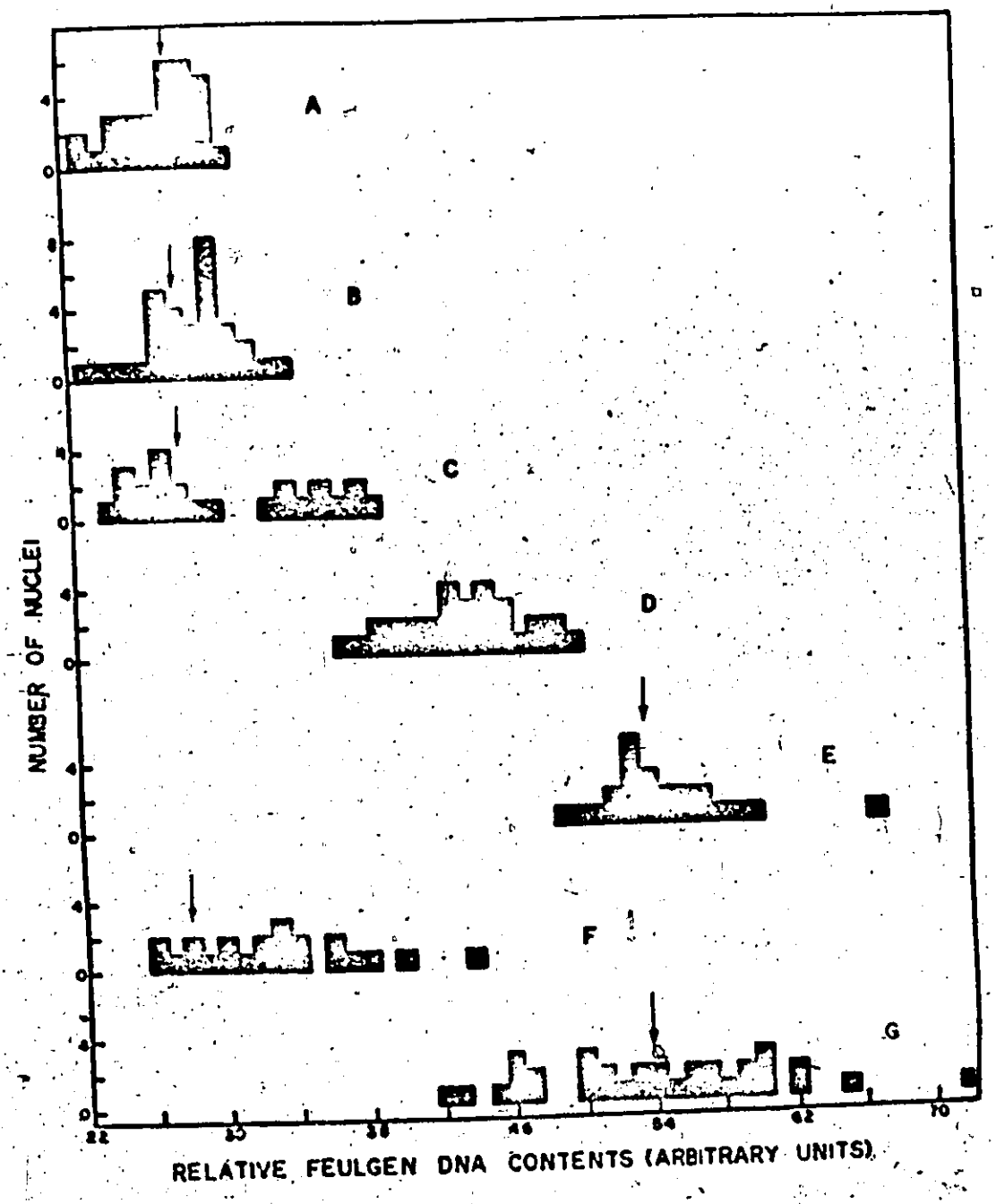
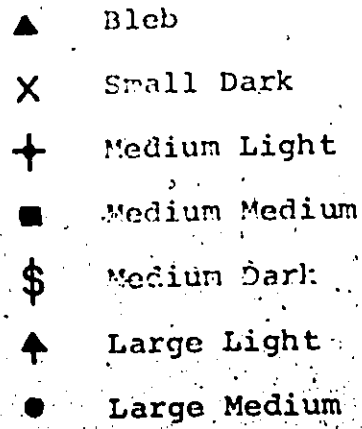

A
B
C
D
E
F
G
NUMBER OF NUCLEI
0
22
33
38
46
54
62
70
RELATIVE FEULGEN DNA CONTENTS (ARBITRARY UNITS)

FIGURE 8

Scatter plot of nuclear area and relative Feulgen-DNA content for each nuclear morphology class from a 1092 cell germinal ball.



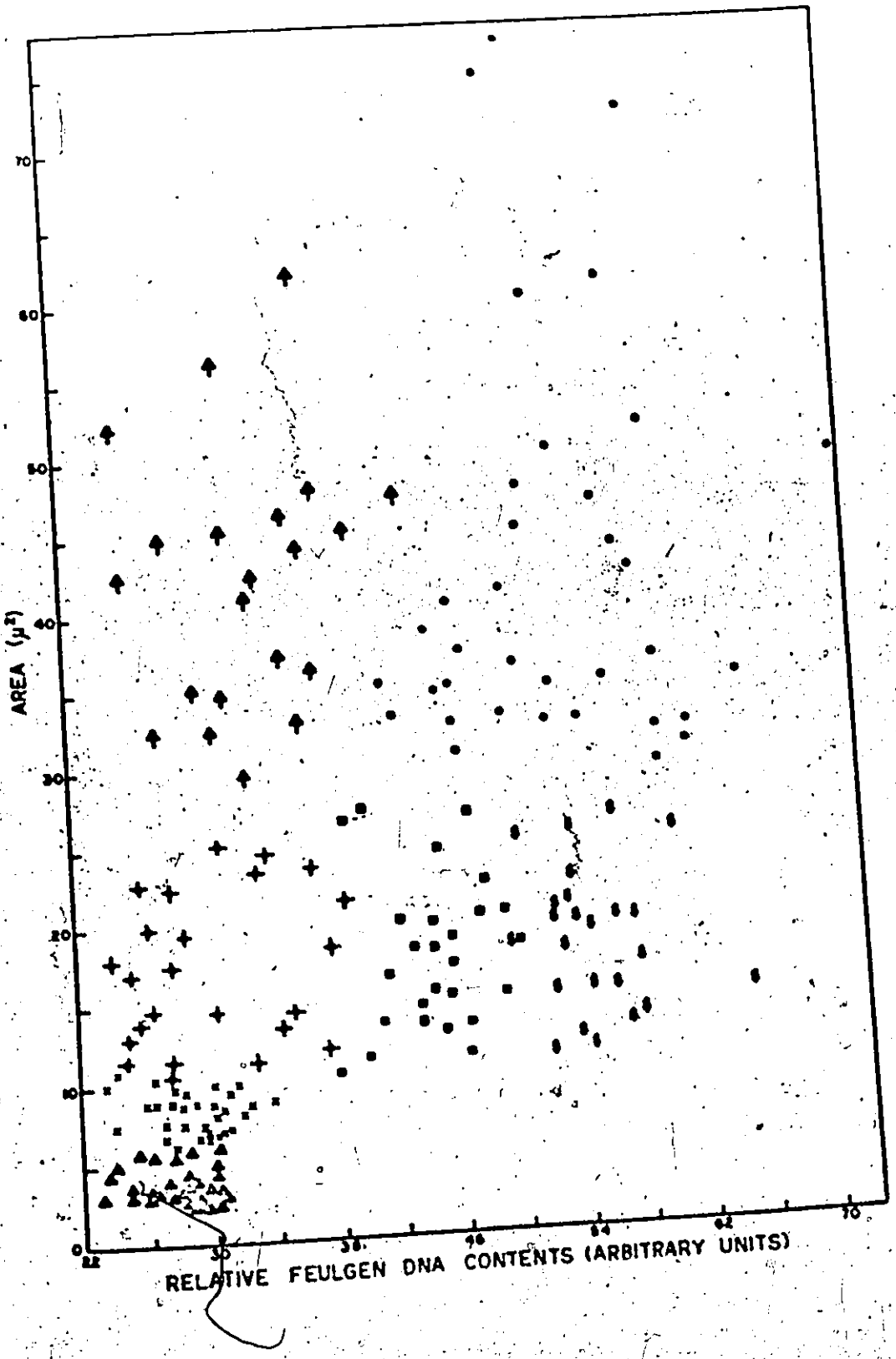

AREA (μ²)
70
60
50
40
30
20
10
0
RELATIVE FEULGEN DNA CONTENTS (ARBITRARY UNITS)

FIGURE 9

Percent distribution of mitotic cells in germinal balls of increasing cell number.

x designates the average value for the grouped data.

+ designates the upper limit for the grouped data.

- designates the lower limit for the grouped data.

Number above each average value (x) denotes the group number.

Group No.	Smallest-Largest Germinal scored (cells)	No. of Balls Scored
1	12-25	5
2	28-50	14
3	54-72	7
4	76-97	7
5	101-193	12
6	230-279	3
7	319-392	4
8	408-487	3
9	503-521	3
10	654-693	4
11	717-768	6
12	810-840	4
13	942-972	3
14	1044-1064	4
15	1315-1376	2
16	1718-1780	3
17	1829	1

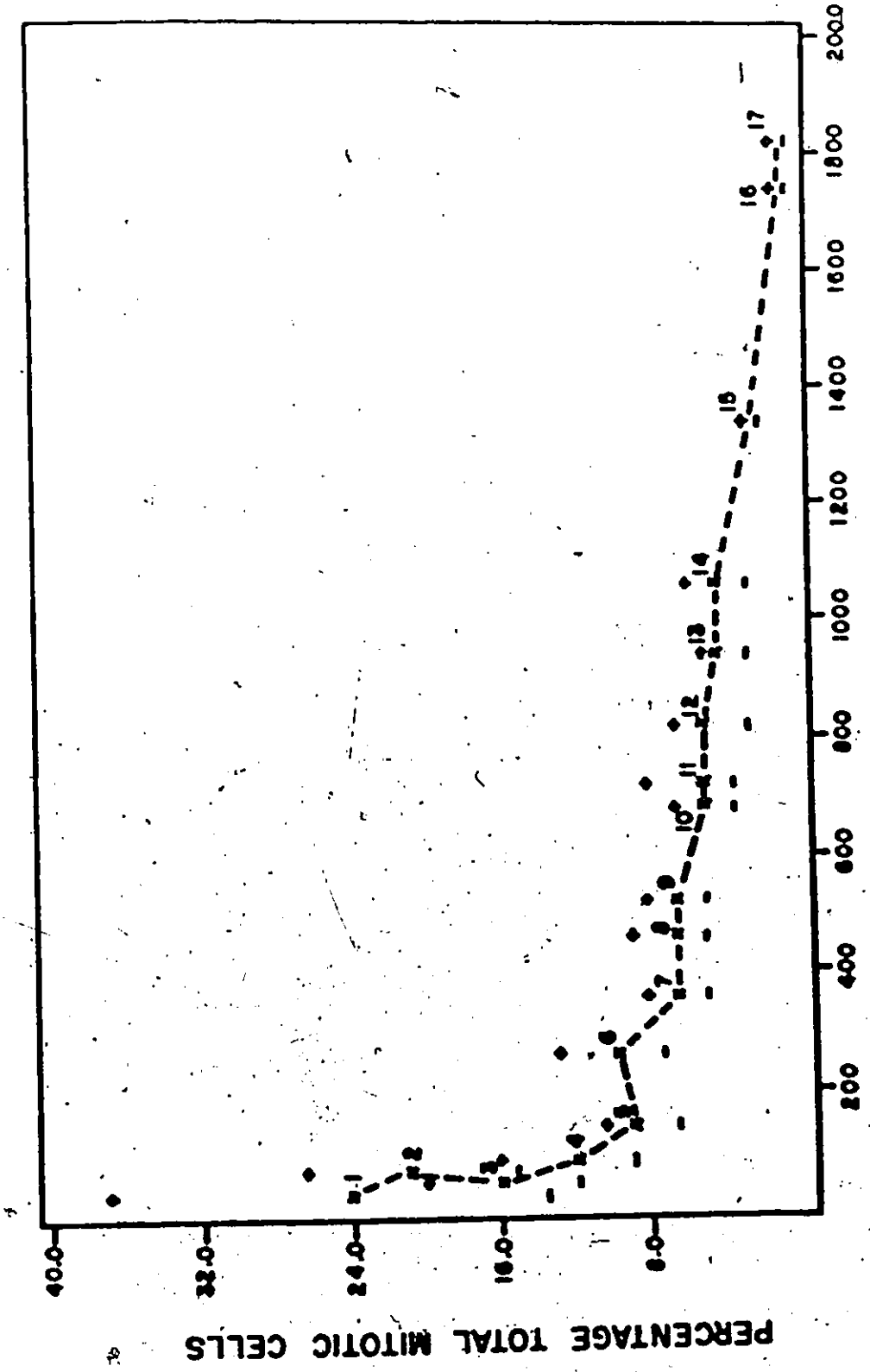

PERCENTAGE TOTAL MITOTIC CELLS
40.0
32.0
24.0
16.0
8.0
TOTAL CELL NUMBER
200
400
600
800
1000
1200
1400
1600
1800
2000

FIGURE 10

Percent distribution of the Small Dark nuclear morphology class in germinal balls of increasing cell number. See Figure 9 for details.

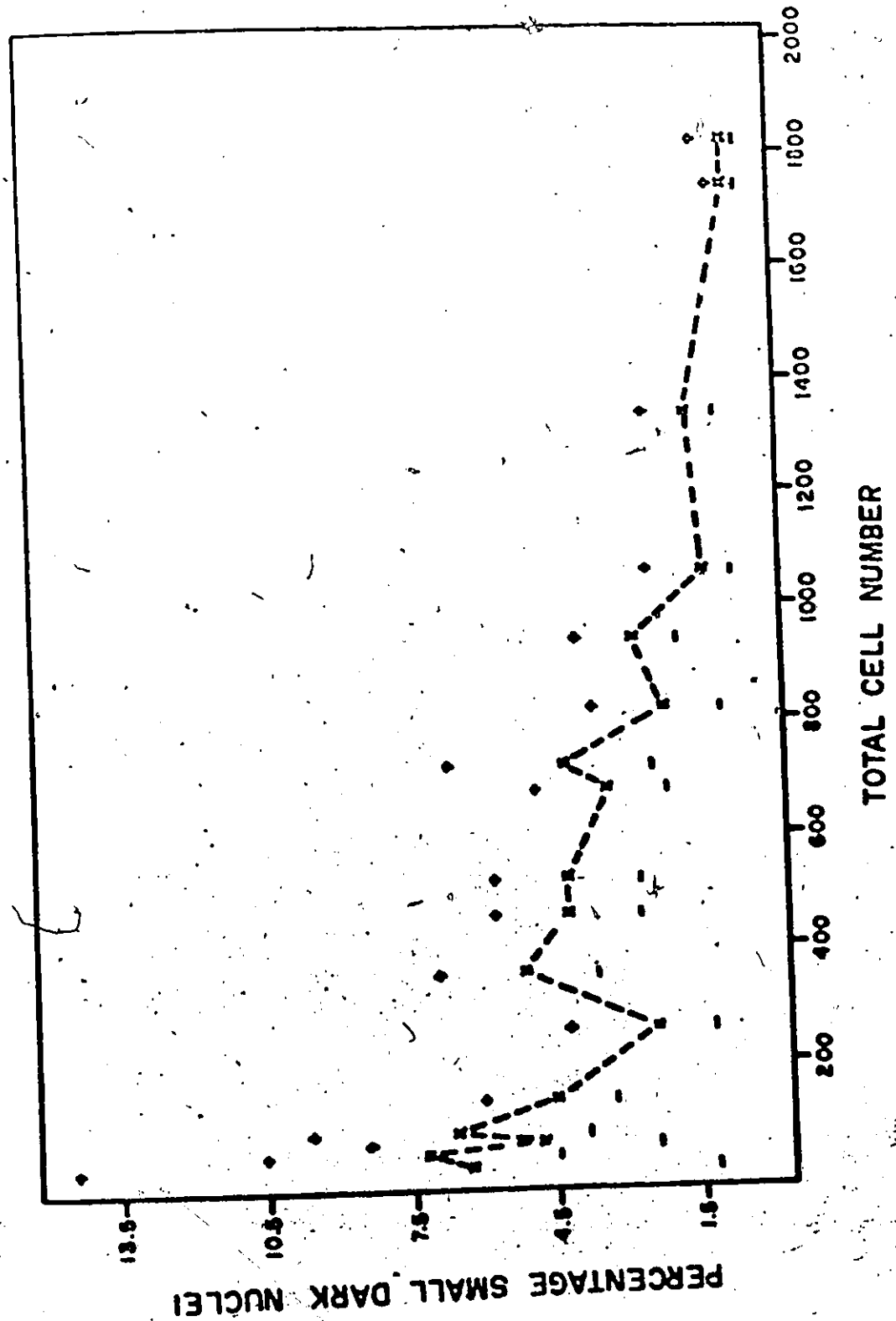

PERCENTAGE SMALL DARK NUCLEI
13.5
10.5
7.5
4.5
1.5
TOTAL CELL NUMBER
200
400
600
800
1000
1200
1400
1600
1800
2000

FIGURE 11

Percent distribution of the Medium Light nuclear morphology class in germinal balls of increasing cell number. See Figure 9 for details.

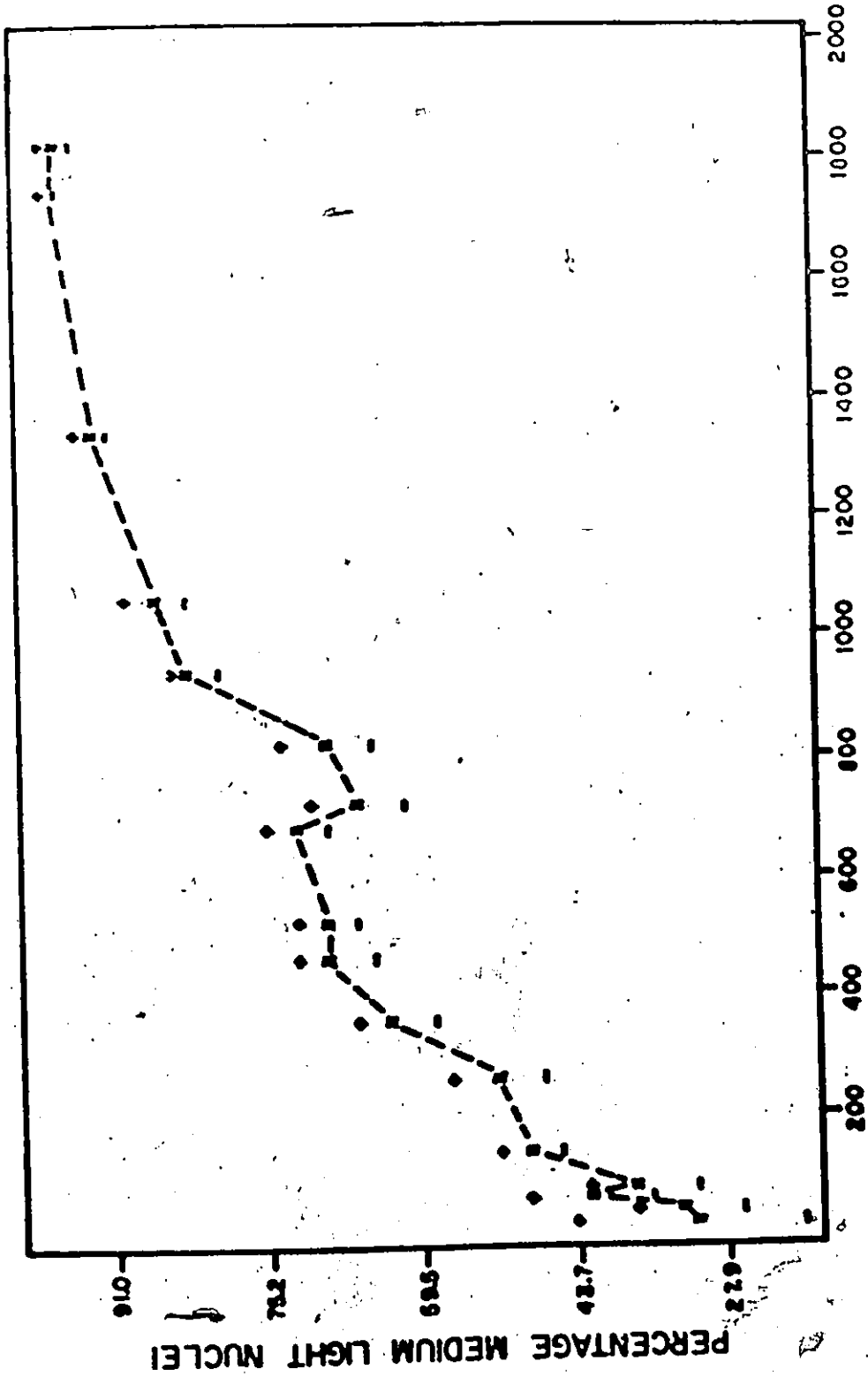

PERCENTAGE MEDIUM LIGHT NUCLEI
TOTAL CELL NUMBER
200
400
600
800
1000
1200
1400
1600
1800
2000

FIGURE 12

Percent distribution of the Medium Medium nuclear morphology class in germinal balls of increasing cell number. See Figure 9 for details.

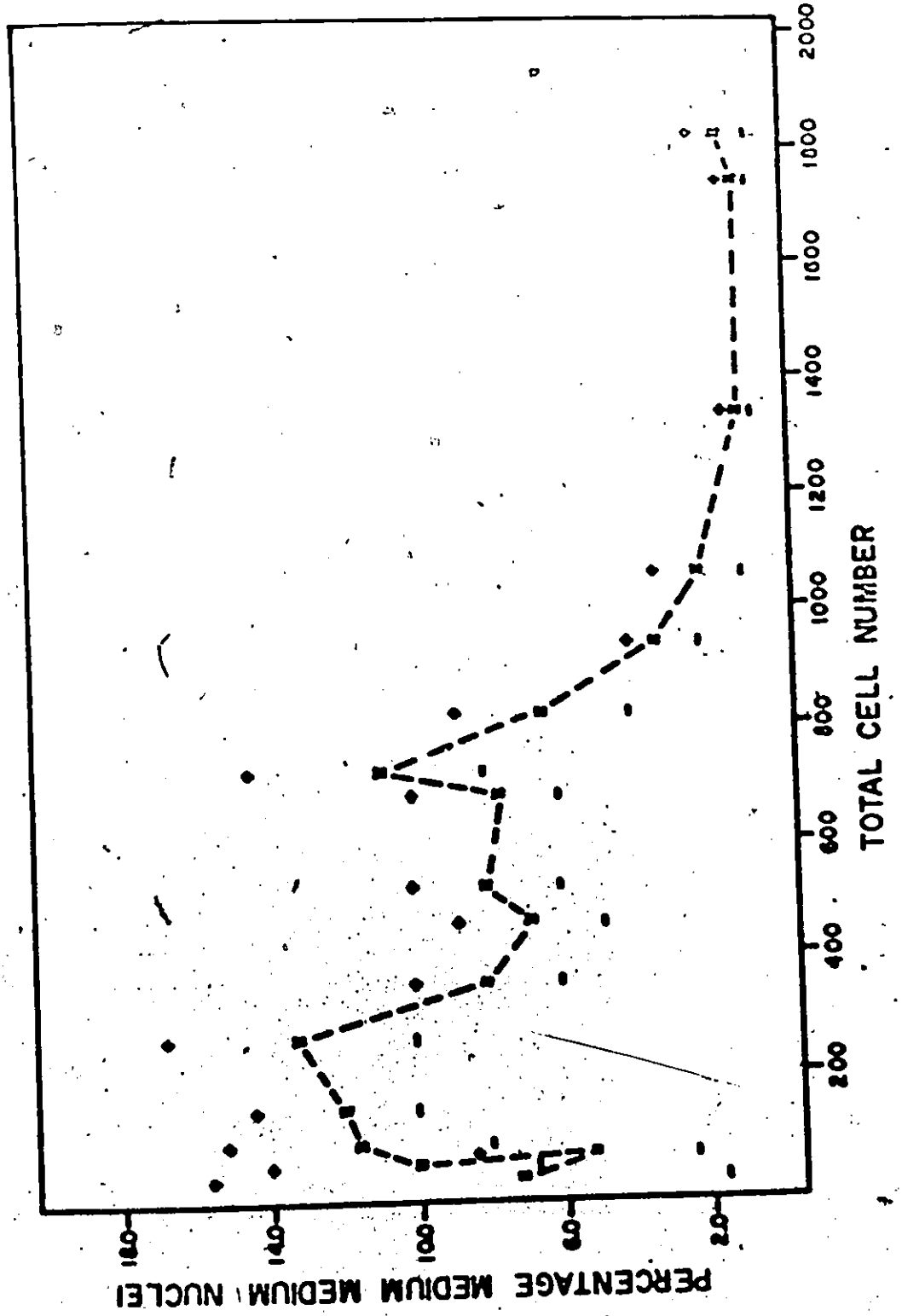

PERCENTAGE MEDIUM MEDIUM NUCLEI
0
5
10
15
20
TOTAL CELL NUMBER
200
400
600
800
1000
1200
1400
1600
1800
2000

FIGURE 13

Percent distribution of the Medium Dark nuclear morphology class in germinal balls of increasing cell number. See Figure 9 for details.

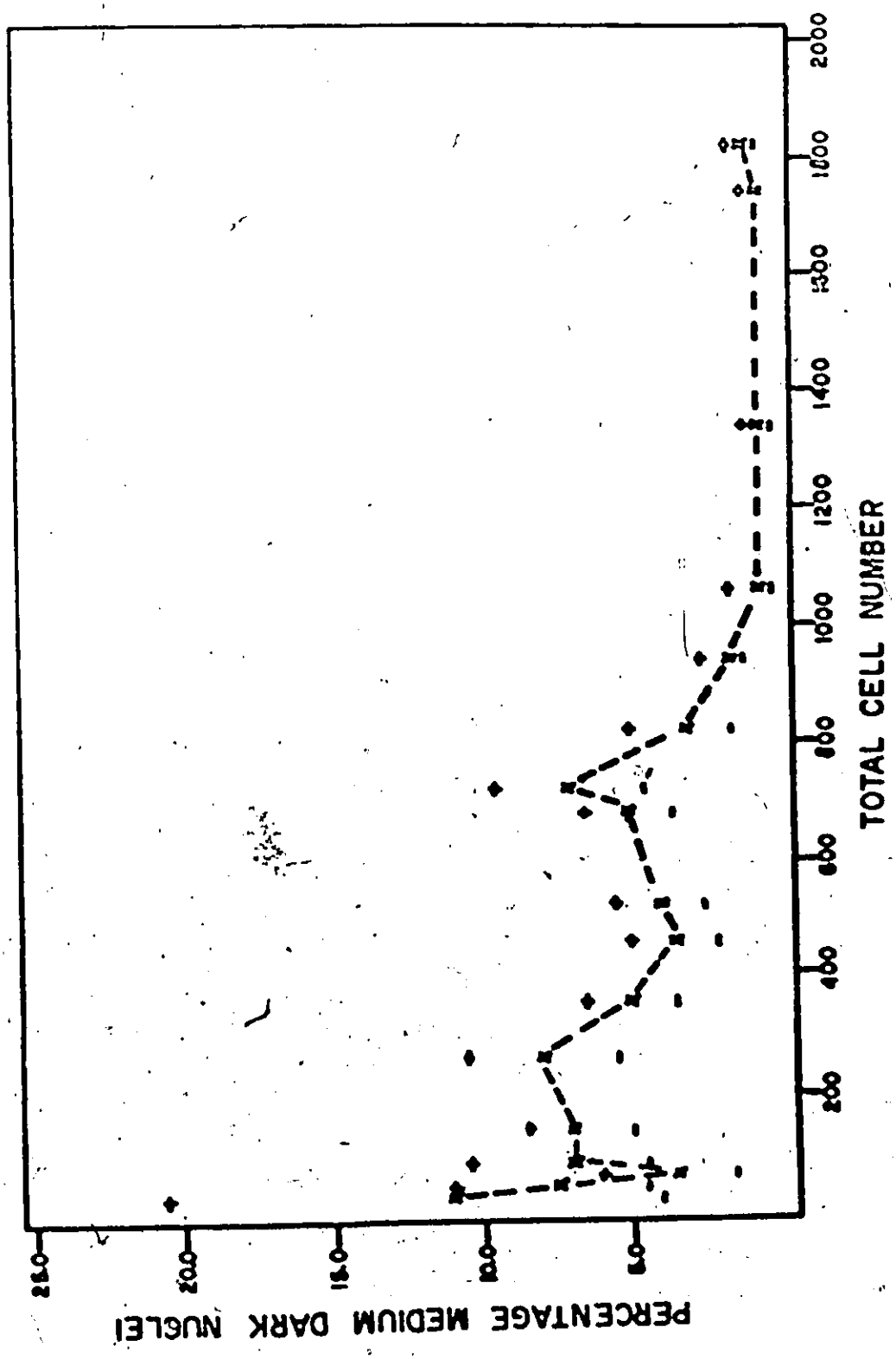

PERCENTAGE MEDIUM DARK NUCLEI
25.0
20.0
15.0
10.0
5.0
TOTAL CELL NUMBER
200
400
600
800
1000
1200
1400
1600
1800
2000

FIGURE 14

Percent distribution of the Large Light nuclear morphology class in germinal balls of increasing cell number. See Figure 9 for details.

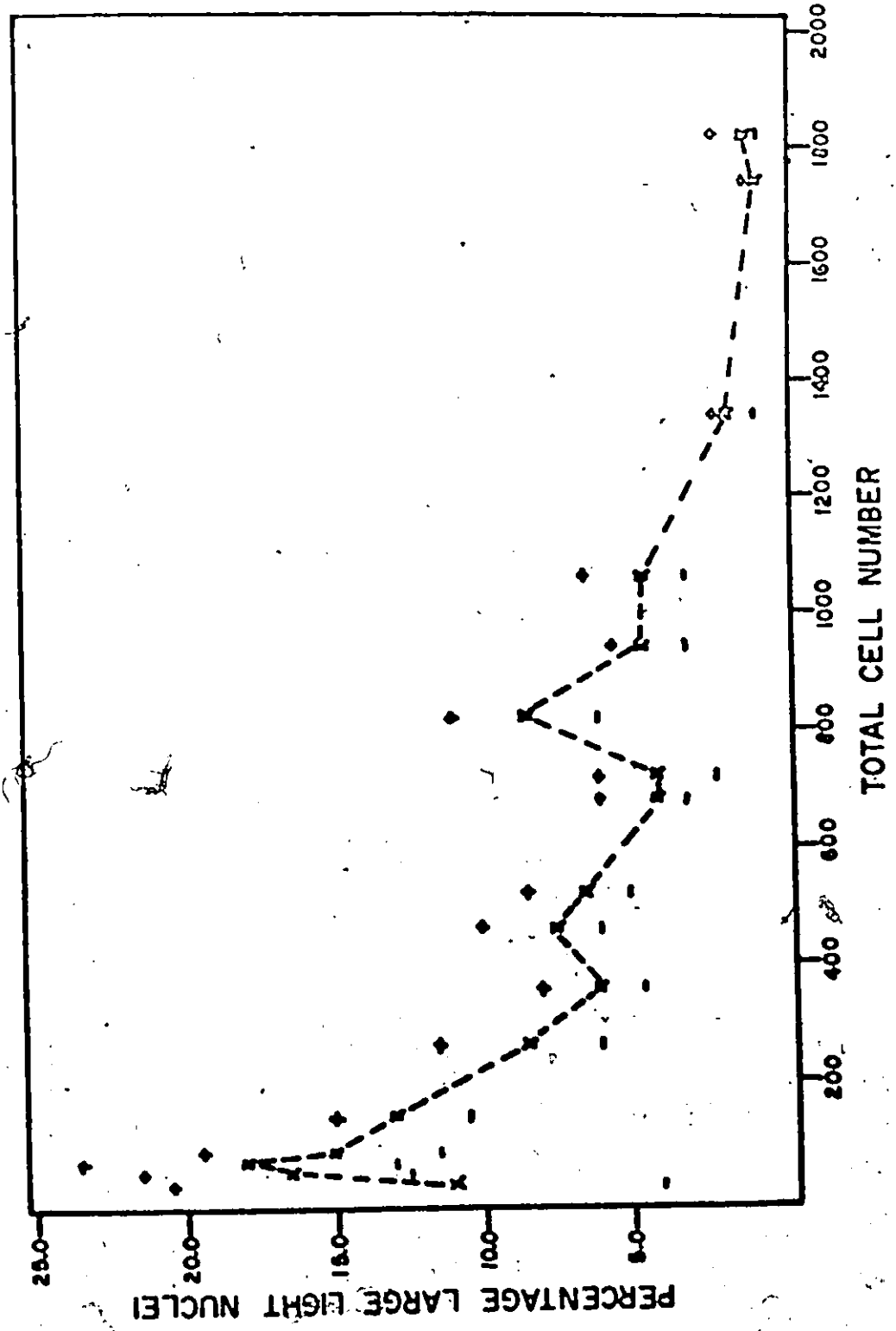

PERCENTAGE LARGE LIGHT NUCLEI
25.0
20.0
15.0
10.0
5.0
TOTAL CELL NUMBER
200
400
600
800
1000
1200
1400
1600
1800
2000

FIGURE 15

Percent distribution of the Large Medium nuclear morphology class in germinal balls of increasing cell number. See Figure 9 for details.

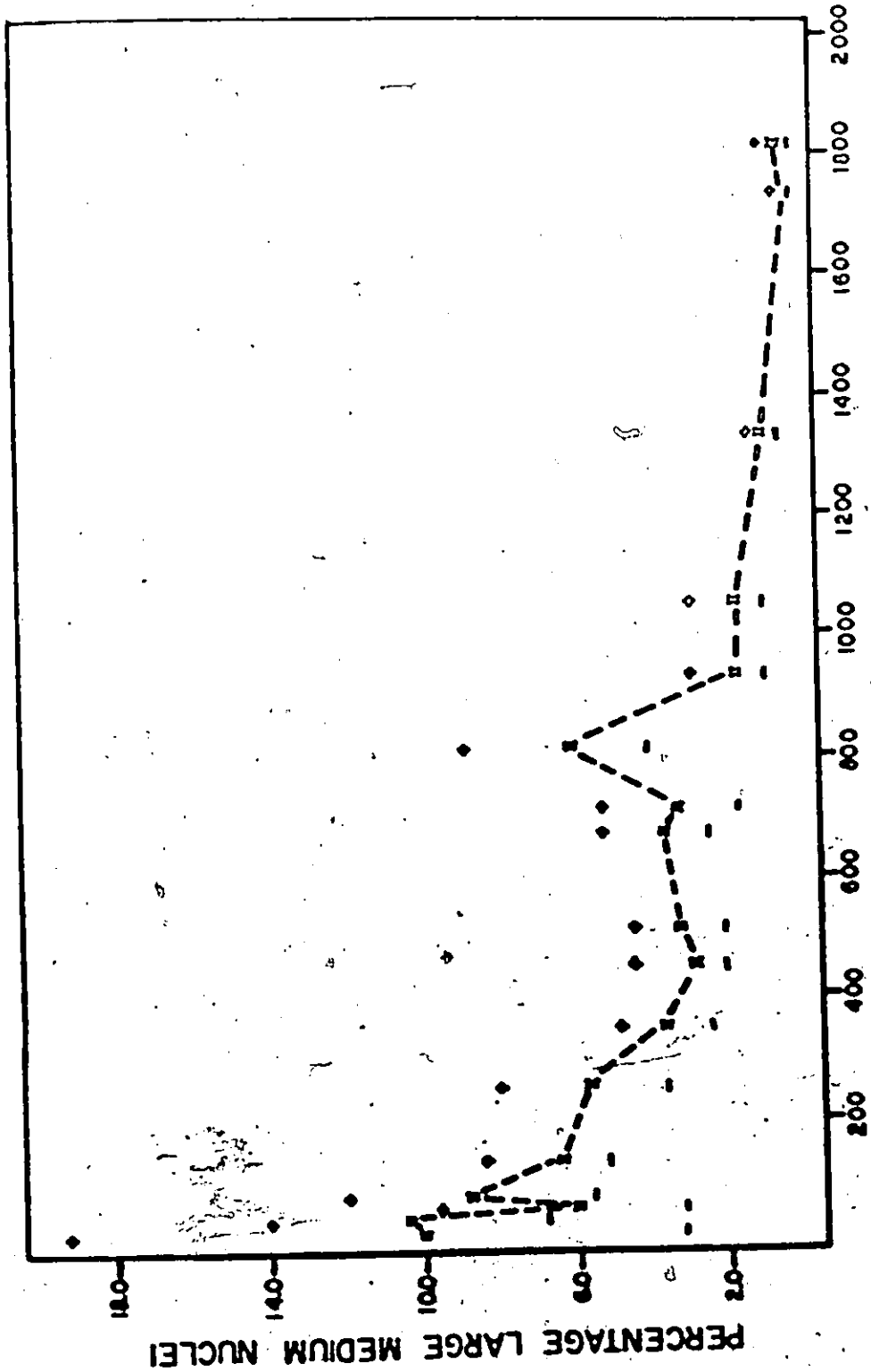

PERCENTAGE LARGE MEDIUM NUCLEI
140
120
100
80
60
TOTAL CELL NUMBER
200
400
600
800
1000
1200
1400
1600
1800
2000

FIGURE 16

Absolute number of mitotic cells in germinal balls of increasing cell number. See Figure 9 for details.

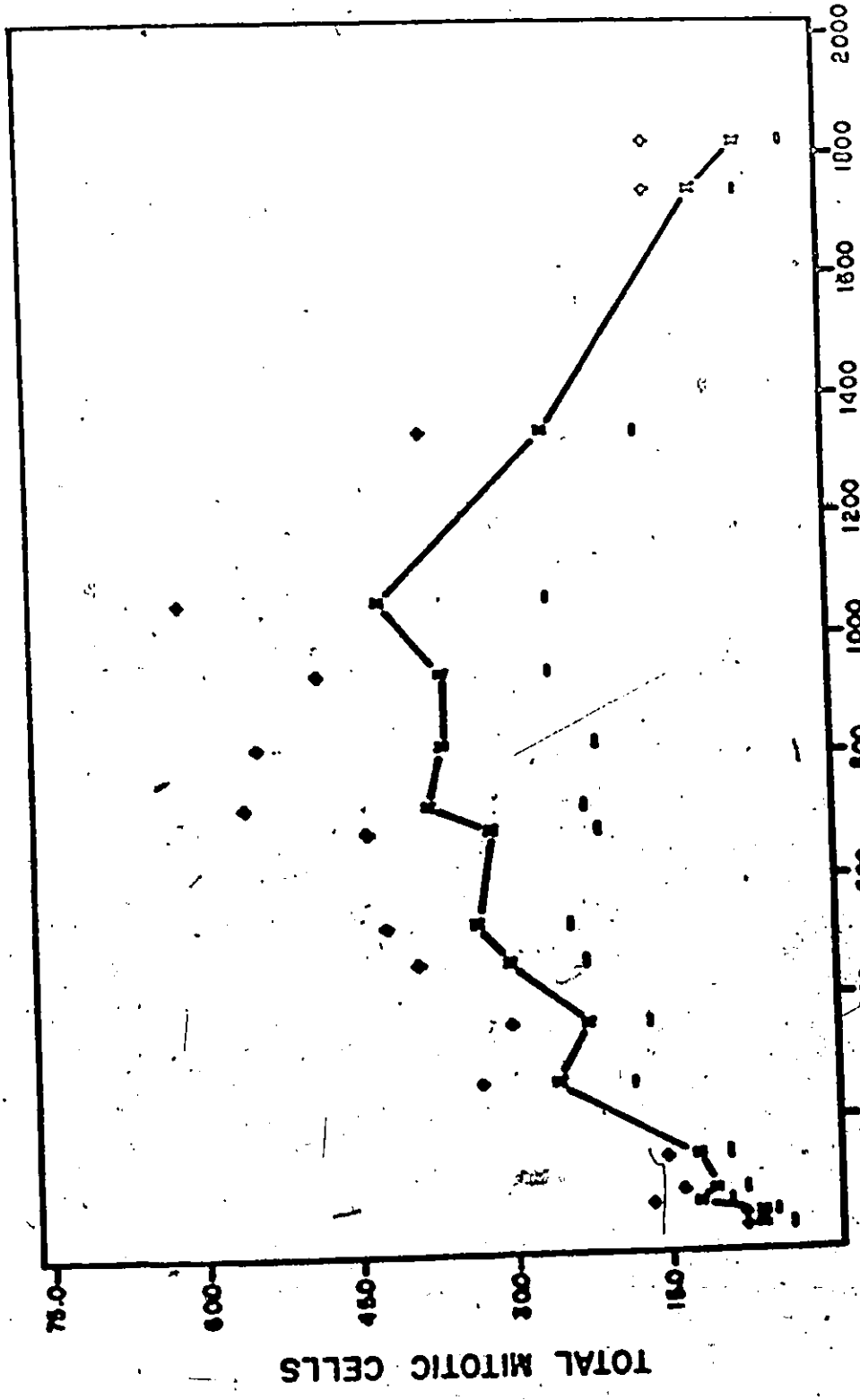

TOTAL MITOTIC CELLS
TOTAL CELL NUMBER
200
400
600
800
1000
1200
1400
1600
1800
2000

FIGURE 17

Absolute number of Small Dark nuclei in germinal balls of increasing cell number. See Figure 9 for details.

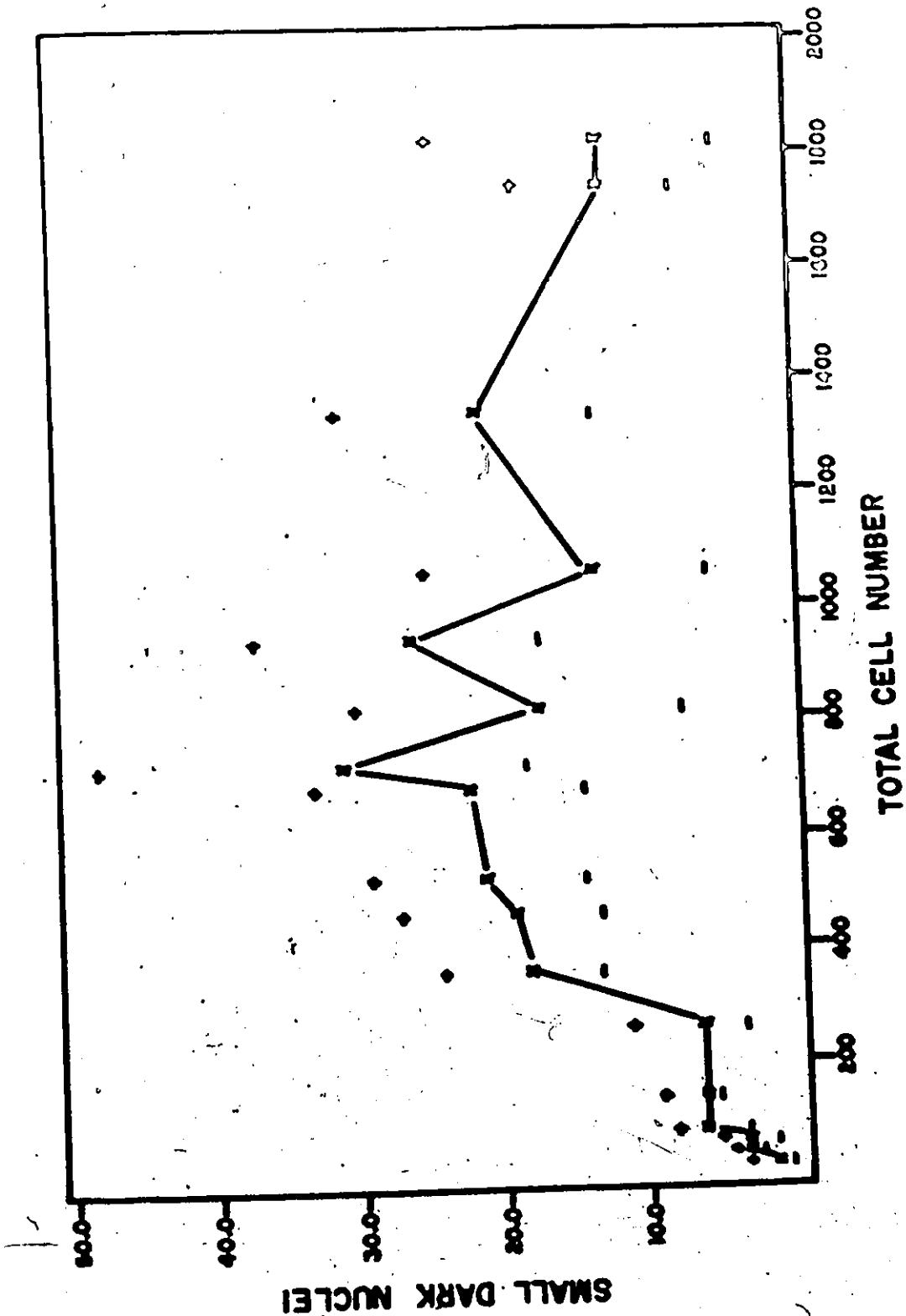

SMALL DARK NUCLEI
50.0
40.0
30.0
20.0
10.0
TOTAL CELL NUMBER
200
400
600
800
1000
1200
1400
1600
1800
2000

FIGURE 18

Absolute number of Medium Light nuclei in germinal balls of increasing cell number. See Figure 9 for details.

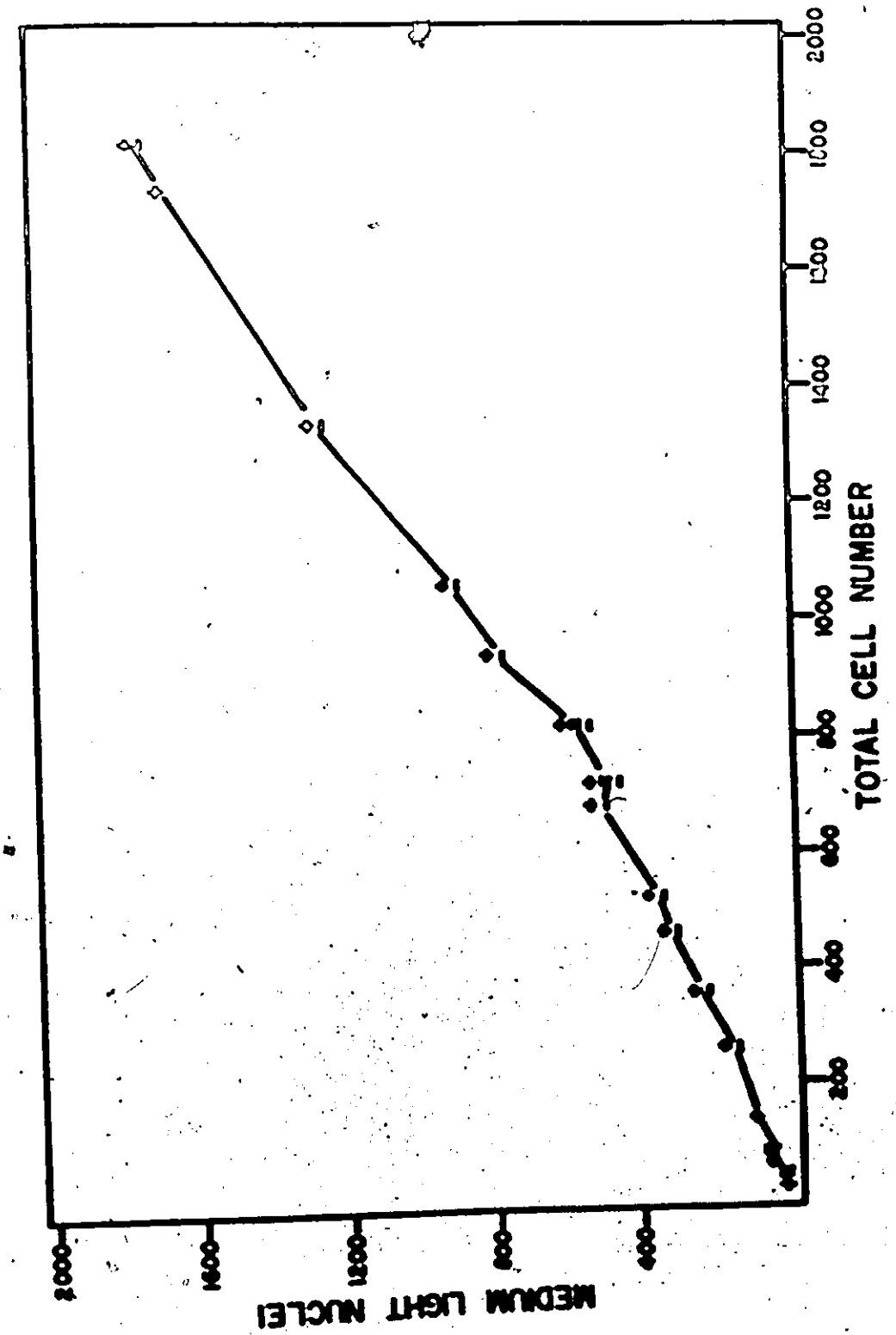

MEDIUM LIGHT NUCLEI
TOTAL CELL NUMBER
2000
1600
1200
800
400
200
400
600
800
1000
1200
1400
1600
1800
2000

FIGURE 19

Absolute number of Medium Medium nuclei in germinal balls of increasing cell number. See Figure 9 for details.

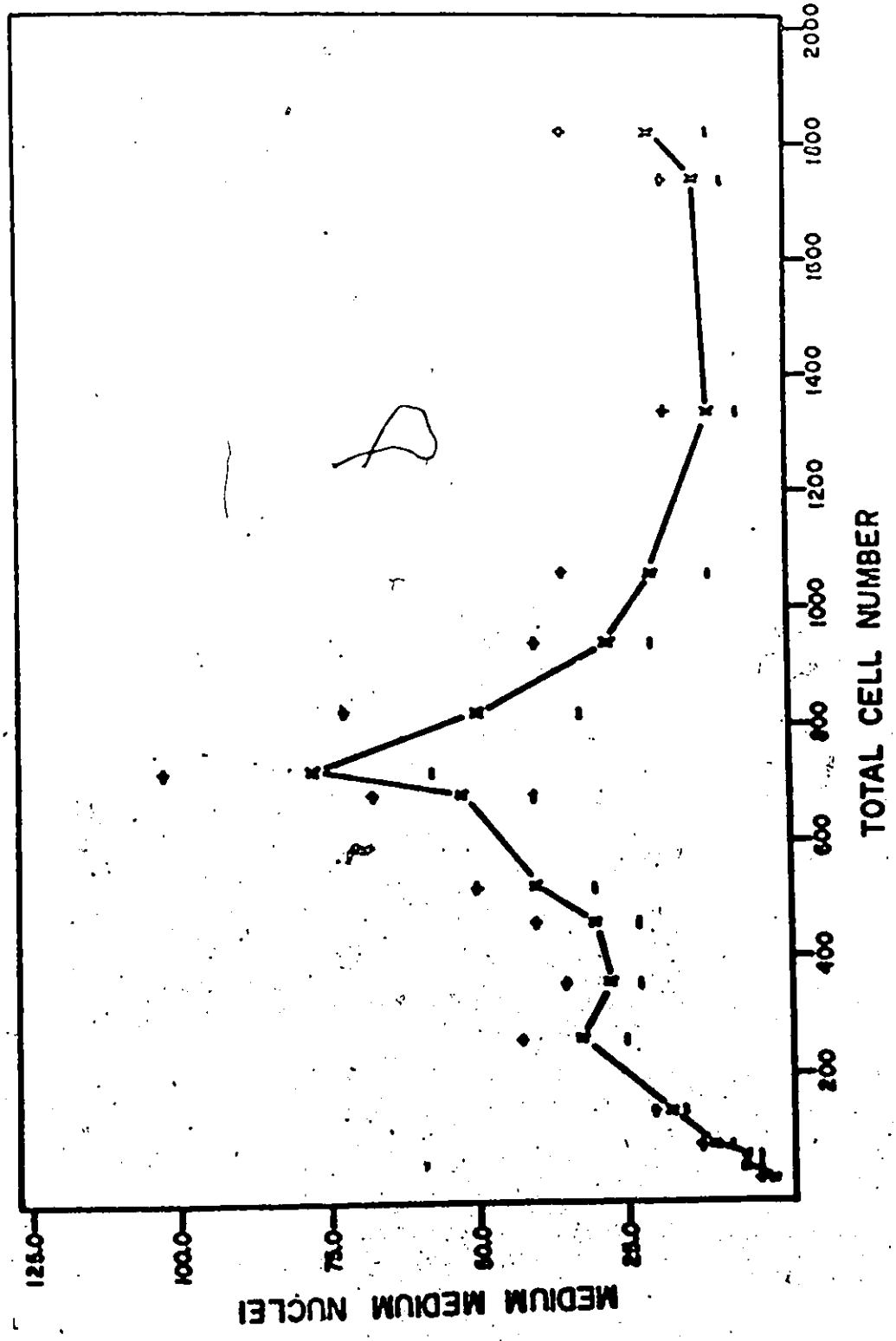

MEDIUM MEDIUM NUCLEI
125.0
100.0
75.0
50.0
25.0
TOTAL CELL NUMBER
200
400
600
800
1000
1200
1400
1600
1800
2000

FIGURE 20

Absolute number of Medium Dark nuclei in germinal balls of increasing cell number. See Figure 9 for details.

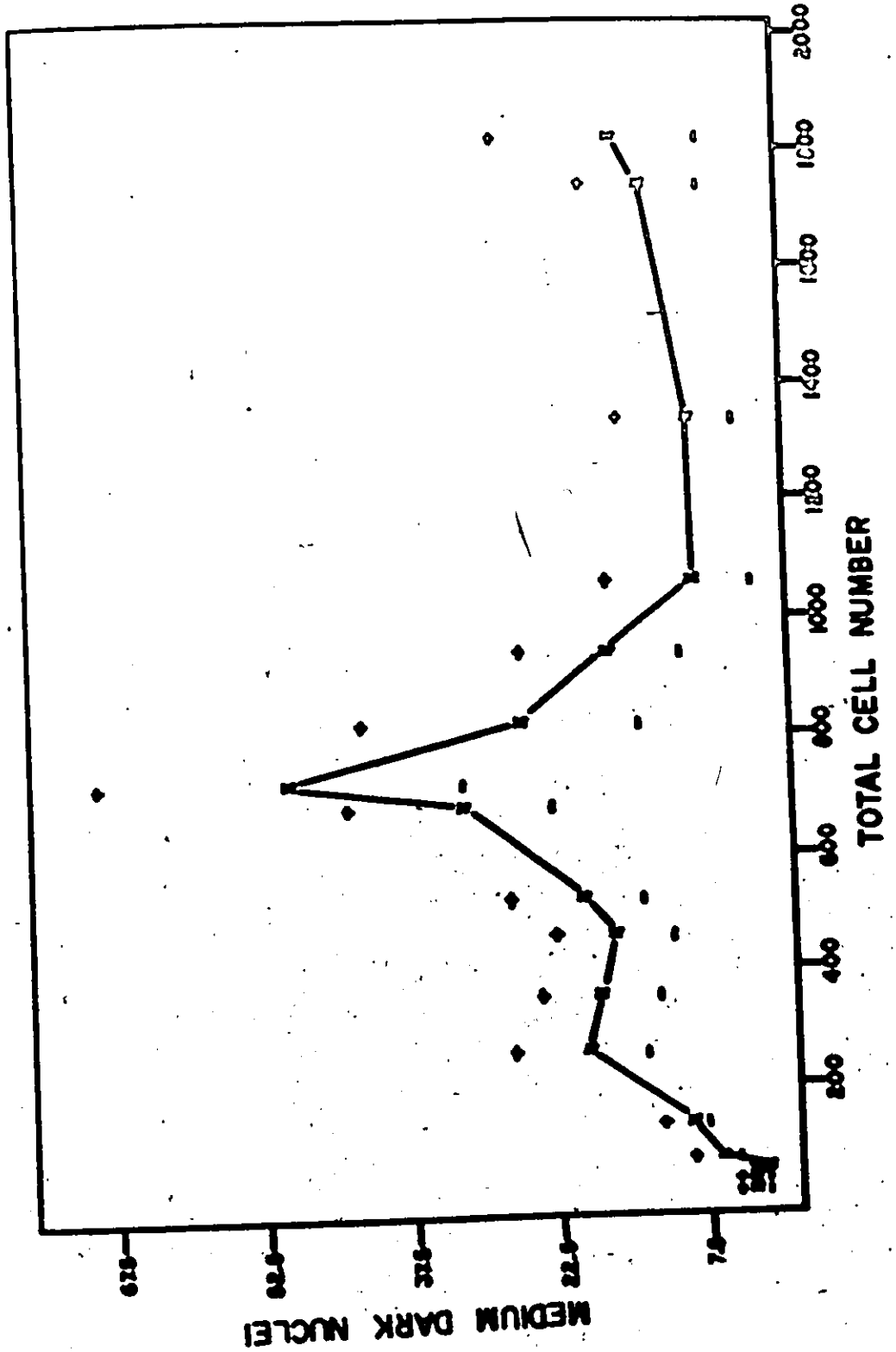

MEDIUM DARK NUCLEI
TOTAL CELL NUMBER
200
400
800
1000
1400
1800
2000

FIGURE 21

Absolute number of Large Light nuclei in germinal balls of increasing cell number. See Figure 9 for details.

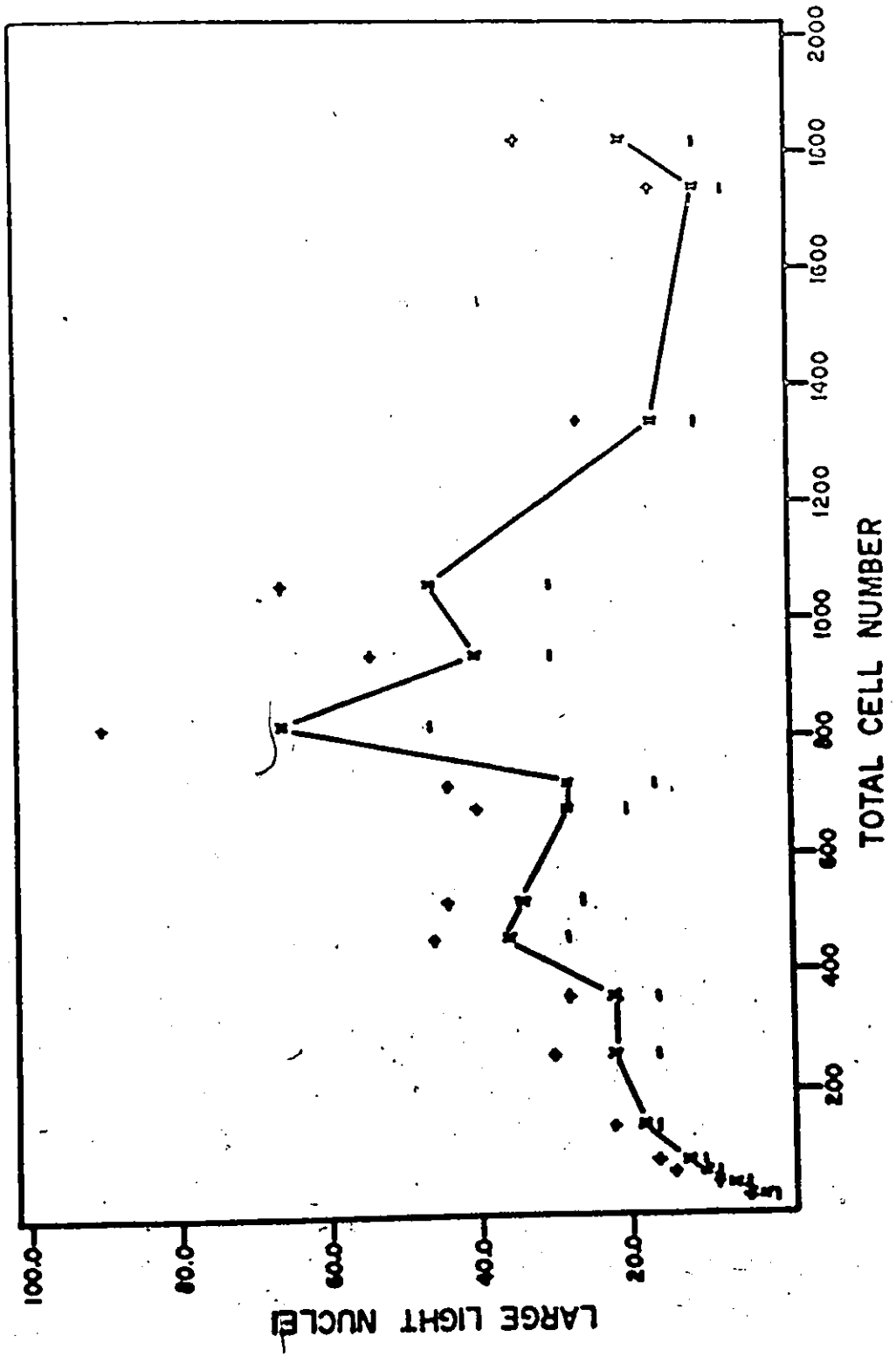

LARGE LIGHT NUCLEI
100.0
80.0
60.0
40.0
20.0
TOTAL CELL NUMBER
200
400
600
800
1000
1200
1400
1600
1800
2000

FIGURE 22

Absolute number of Large Medium nuclei in germinal balls of increasing cell number. See Figure 9 for details.

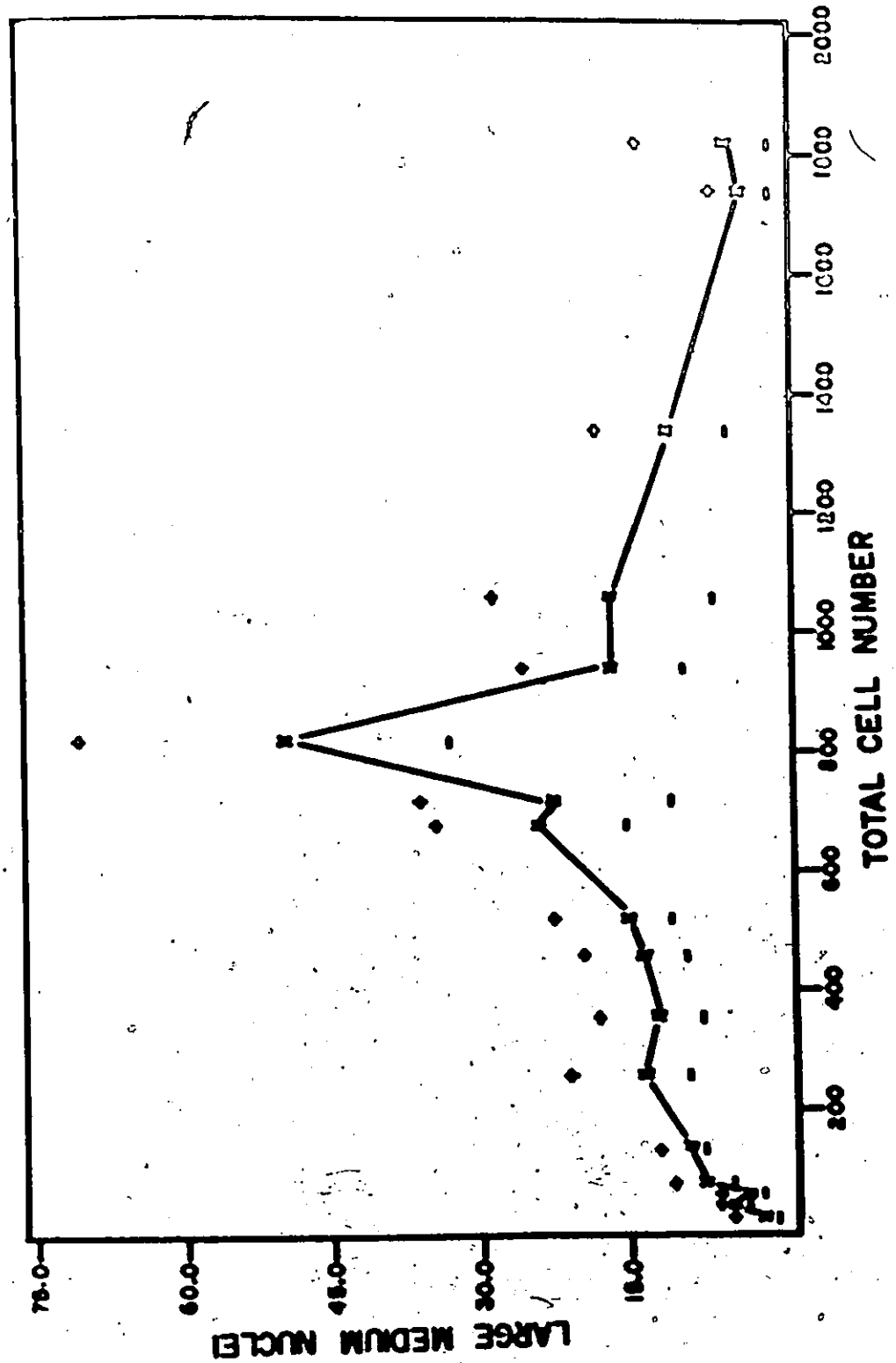

LARGE MEDIUM NUCLEI
75.0
60.0
45.0
30.0
15.0
TOTAL CELL NUMBER
200
400
600
800
1000
1200
1400
1600
1800
2000

FIGURE 23

Loss of partial synchrony in germinal balls of increasing cell number. The Best Fit Line was obtained by the method of least squares (coefficient of correlation r = 0.75). Upper and lower limits for sampling errors were calculated by the Chi Square technique presented in MATERIALS AND METHODS.

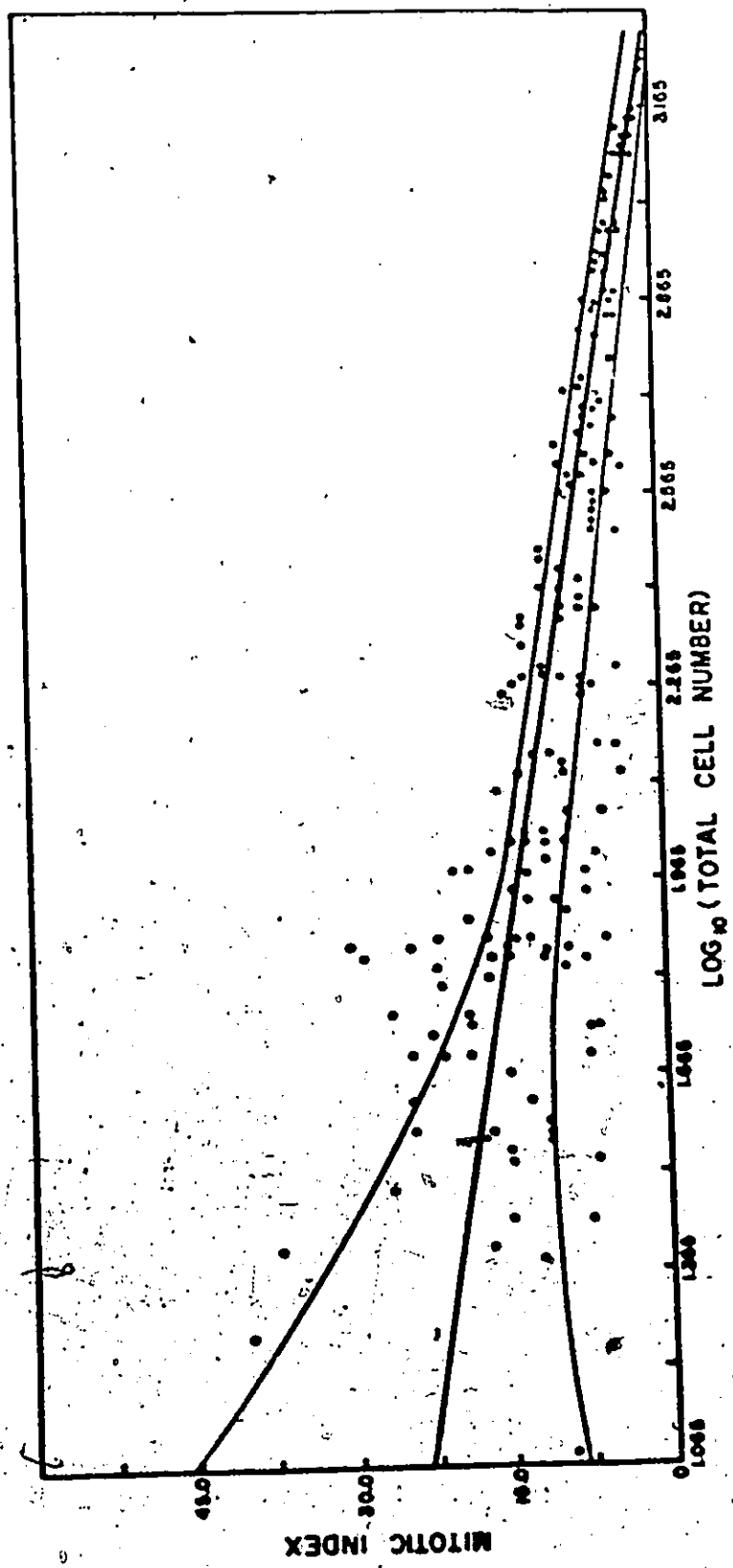

3.165
2.865
2.565
2.265
1.965
1.665
1.365
1.065
LOG10 (TOTAL CELL NUMBER)
40.0
30.0
20.0
0
MITOTIC INDEX

FIGURE 24

Labelling index for germinal balls of different cell number at various times after administration of ^{3}H-thymidine.

Hours After Exposure to ^{3}H-TdR

o 1

o 3

□ 5

△ 7

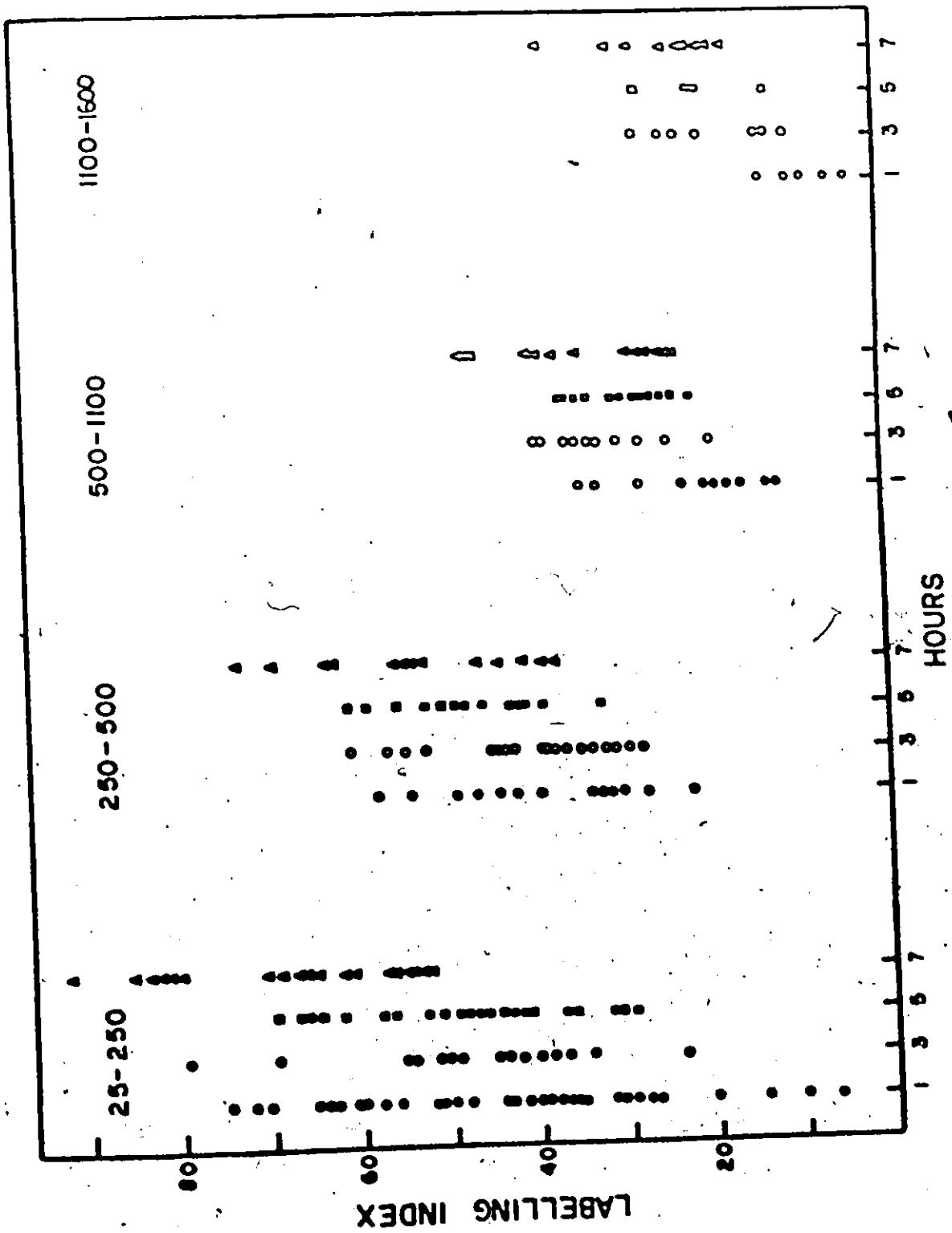

1100-1600
500-1100
250-500
25-250
HOURS
1 3 5 7
LABELLING INDEX
0
0
40
20

FIGURE 25

Percentage labelled cells in germinal balls of increasing cell number 1 hour after exposure to ^{3}H-TdR. The best fit line was obtained by the method of least squares (coefficient of correlation $r = 0.69$). Upper and lower limits for sampling errors were calculated by the Chi Square technique described in MATERIALS AND METHODS.

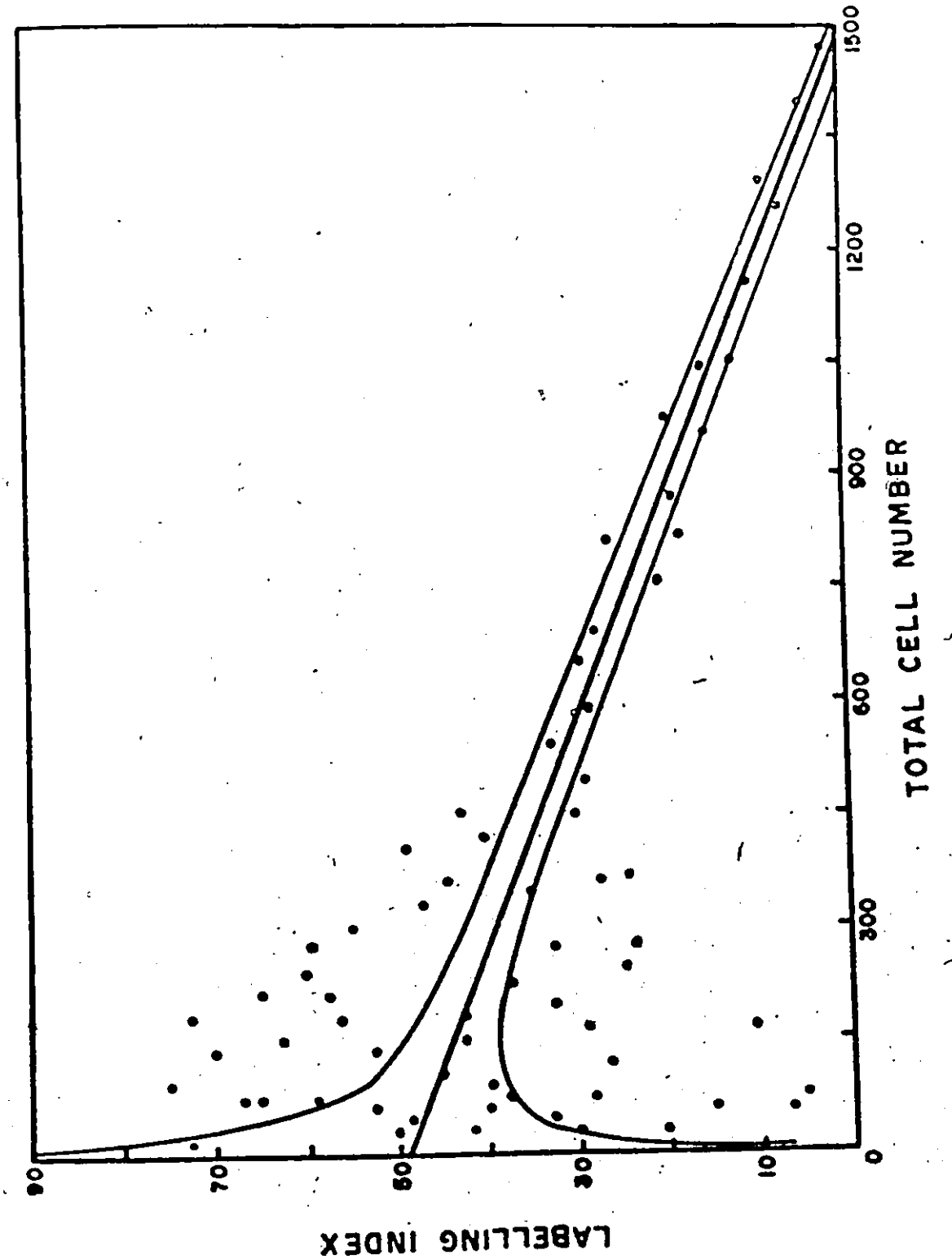

LABELLING INDEX
90
70
50
30
10
0
TOTAL CELL NUMBER
1500
1200
900
600
300

FIGURE 26

Percentage labelled mitoses for germinal balls of different cell number at various times after administration of ^{3}H-TdR.

Hours After Exposure to ^{3}H-TdR

o 5

o 7

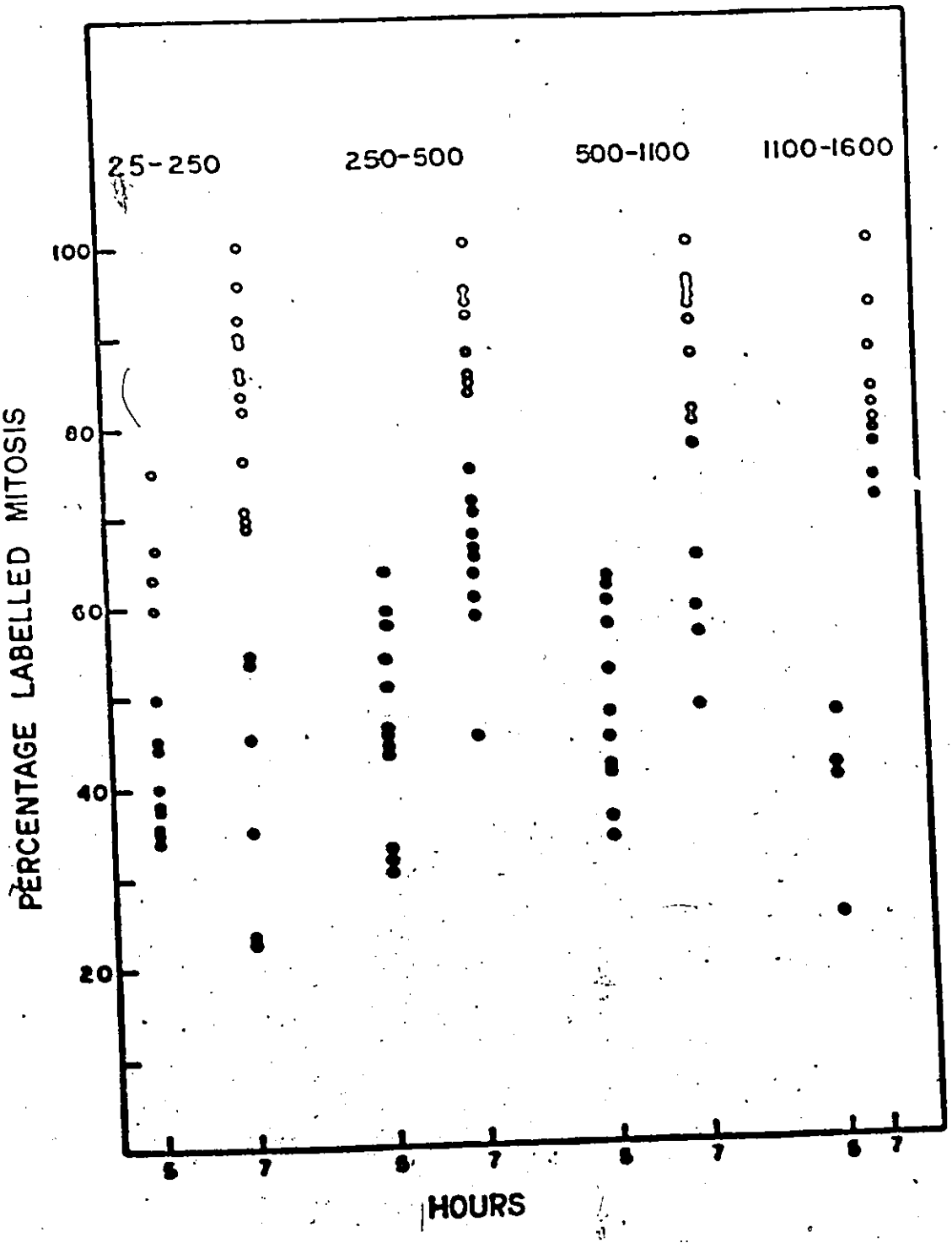

25-250
250-500
500-1100
1100-1600
100
80
60
40
20
PERCENTAGE LABELLED MITOSIS
5
7
5
7
5
7
5
7
HOURS

FIGURE 27

Percentage labelled mitoses curves for germinal balls of similar cell number.

Panel A. Points plotted represent the highest PLM values from each germinal ball group differing in cell number.

25-250 — — — — —

250-500 —·—·—·—·—

500-1100 ----------

1100-1600 ————————

Panel B. Points plotted represent the average PLM values from each germinal ball group differing in cell number.

25-250 — — — — — —

250-500 —·—·—·—·—

500-1100 ----------

1100-1600 ————————

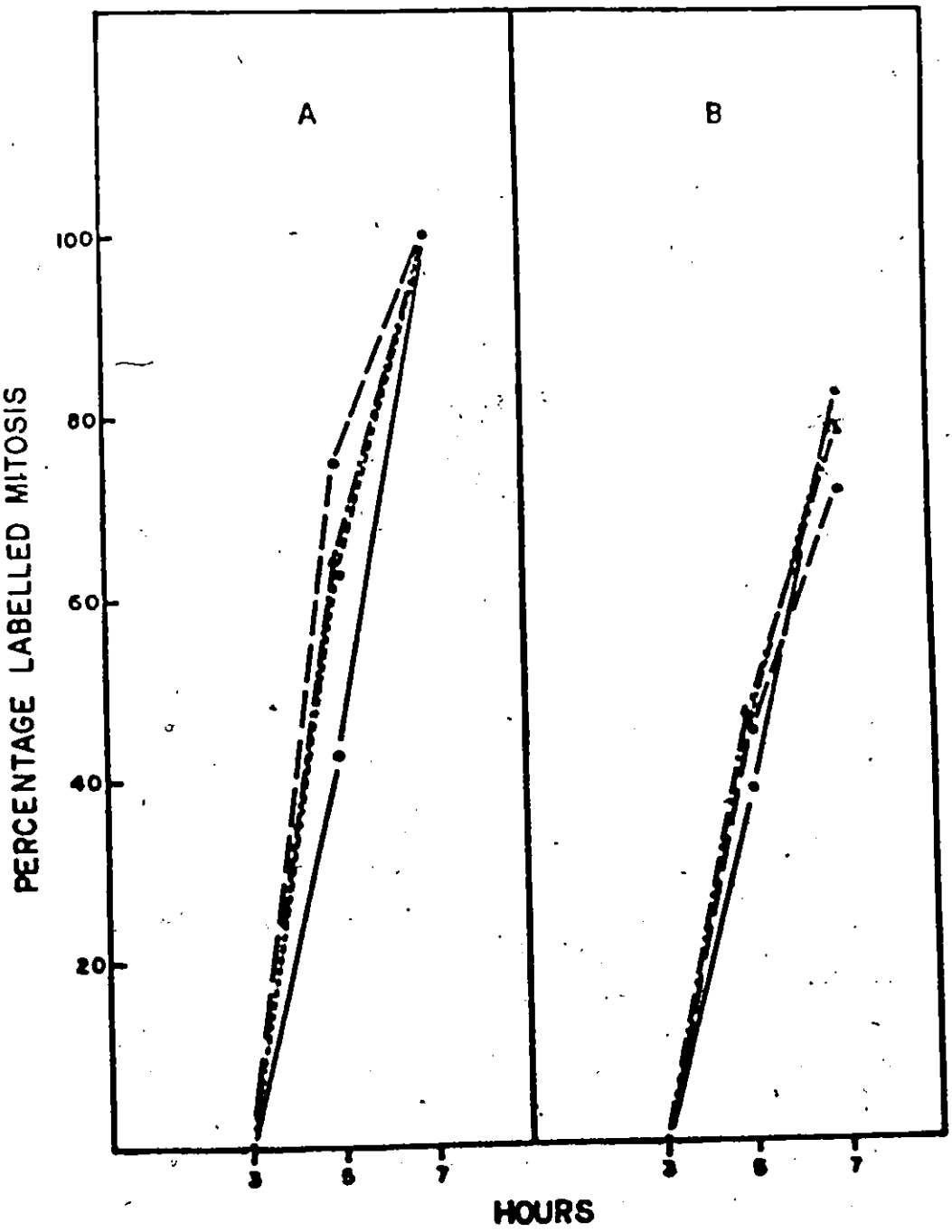

A
B
100
80
60
40
20
PERCENTAGE LABELLED MITOSIS
3
5
7
3
5
7
HOURS

FIGURE 28

Frequency distribution of relative Feulgen-DNA values from 191 nuclei of a 513 cell germinal ball.

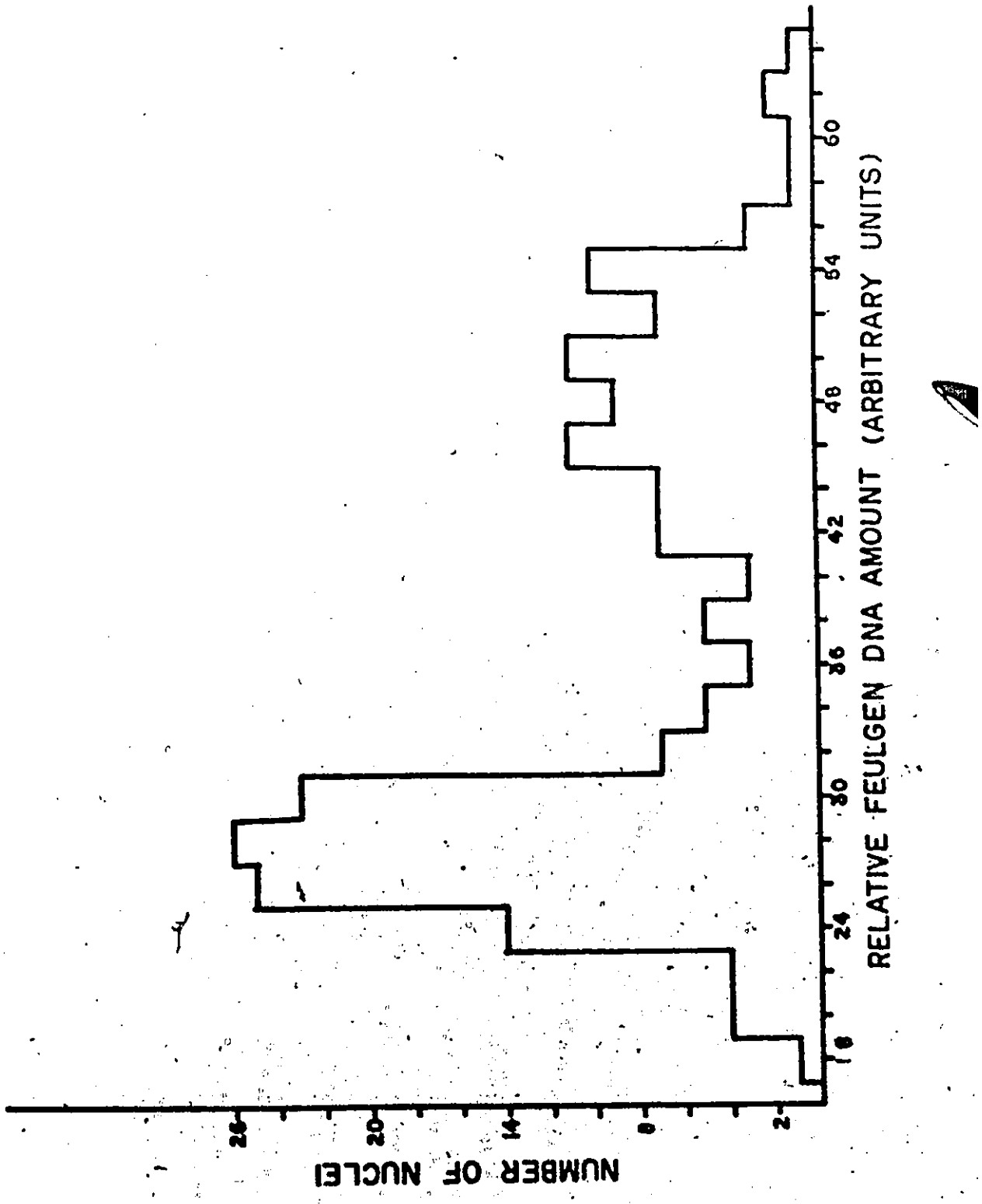

NUMBER OF NUCLEI
20
20
14
8
2
RELATIVE FEULGEN DNA AMOUNT (ARBITRARY UNITS)
18
24
30
36
42
48
54
60

FIGURE 29

Frequency distributions of logarithm relative Feulgen-DNA contents from 191 nuclei of a 513 cell germinal ball.

Upper Figure. $\log_{10}$ relative-Feulgen-DNA contents expressed as percent cumulative frequency. The straight lines represent the 2C subpopulation (left) and the 4C subpopulation (right).

Lower Figure. Histogram of $\log_{10}$ relative Feulgen-DNA contents. The area under the two Gaussian distributions represents distribution of nuclei with 2C (left) and 4C (right) DNA contents.

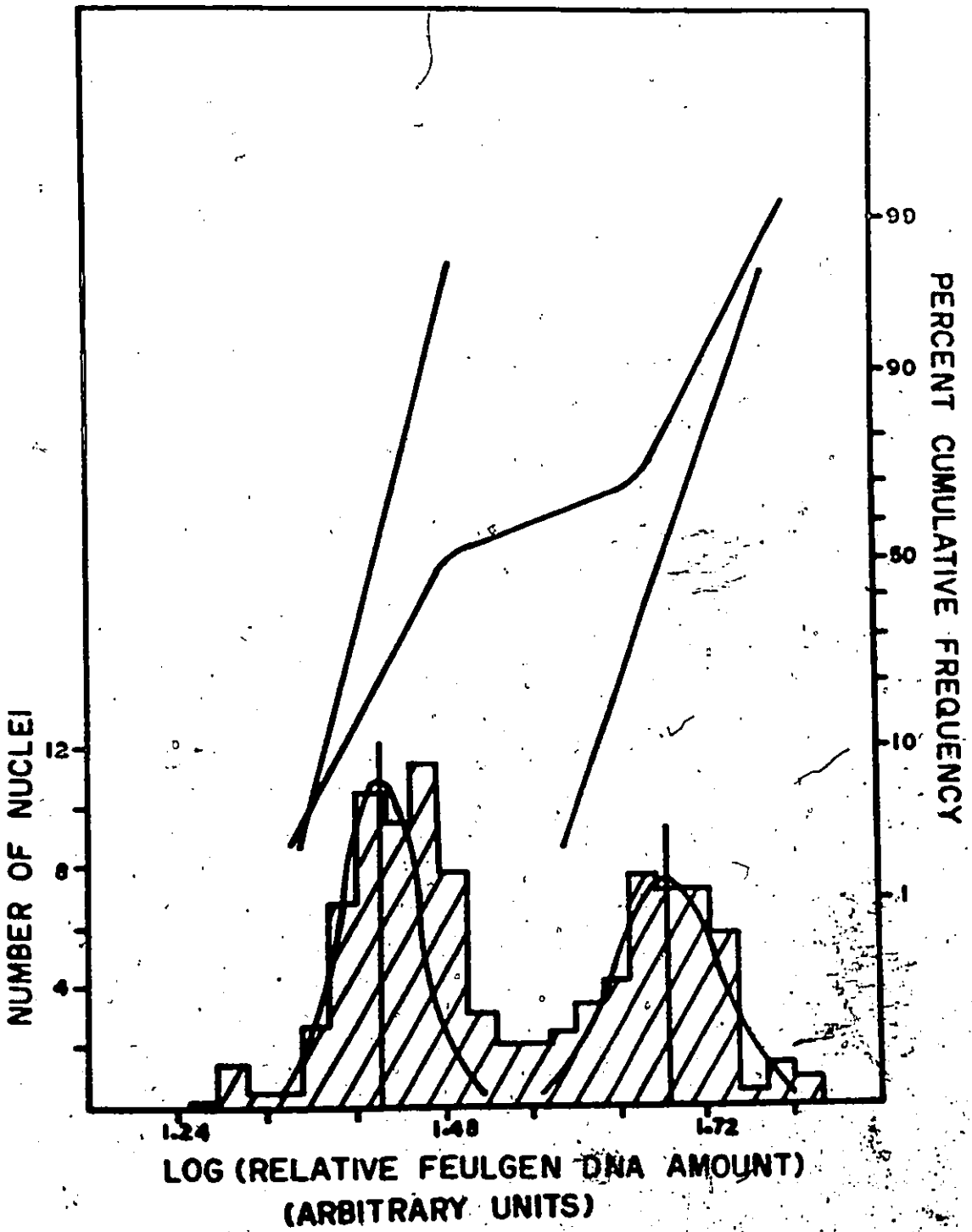

NUMBER OF NUCLEI
12
8
4
90
90
50
10
1
PERCENT CUMULATIVE FREQUENCY
1.24
1.48
1.72
LOG (RELATIVE FEULGEN DNA AMOUNT)
(ARBITRARY UNITS)

FIGURE 30

Frequency distributions of logarithm relative Feulgen-DNA contents from 191 nuclei of a 513 cell germinal ball. Each individual photometric value was weighted to represent its distribution within the population. See Figure 29 for details.

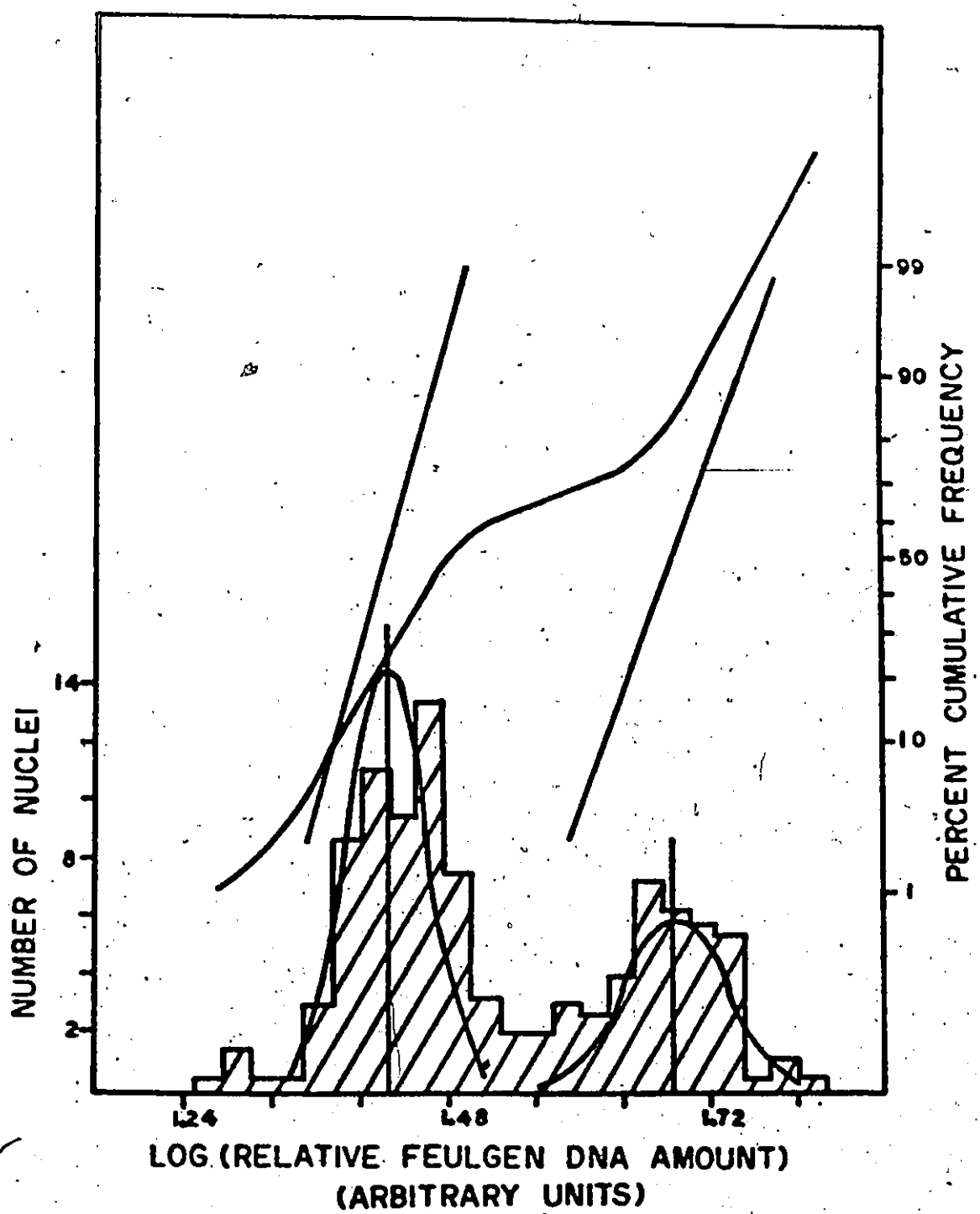

NUMBER OF NUCLEI
14
8
2
99
90
50
10
1
PERCENT CUMULATIVE FREQUENCY
L24
L48
L72
LOG (RELATIVE FEULGEN DNA AMOUNT)
(ARBITRARY UNITS)

FIGURE 31

Frequency distributions of logarithm relative Feulgen-DNA contents from 195 nuclei of a 915 cell germinal ball. Each individual photometric value was weighted to represent its distribution within the population. See Figure 29 for details.

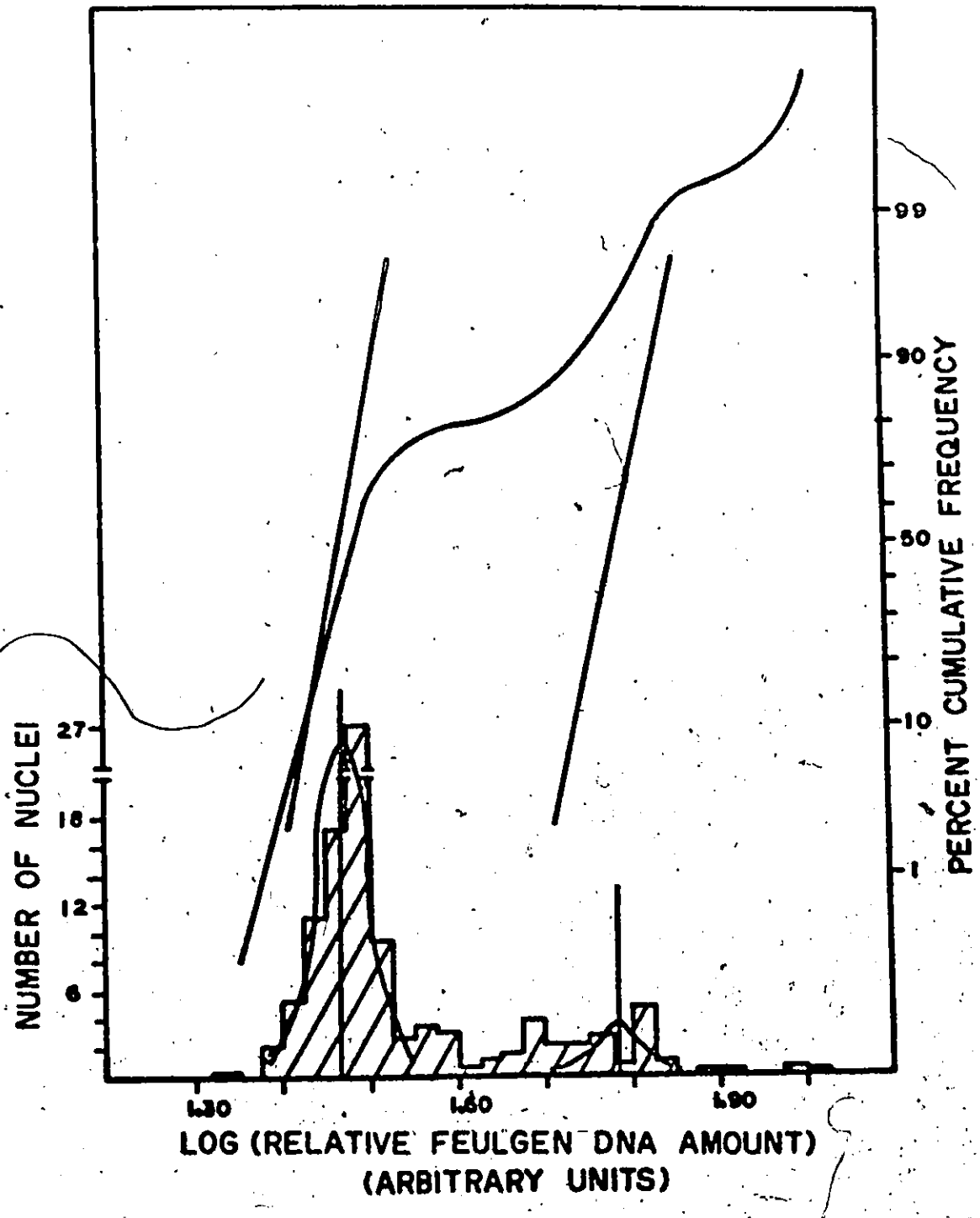

NUMBER OF NUCLEI
27
18
12
6
99
90
50
10
1
PERCENT CUMULATIVE FREQUENCY
1.30
1.60
1.90
LOG (RELATIVE FEULGEN DNA AMOUNT)
(ARBITRARY UNITS)

FIGURE 32

Frequency distributions of logarithm relative Feulgen-DNA contents from 162 nuclei of a 1092 cell germinal ball. Each individual photometric value was weighted to represent its distribution within the population. See Figure 29 for details.

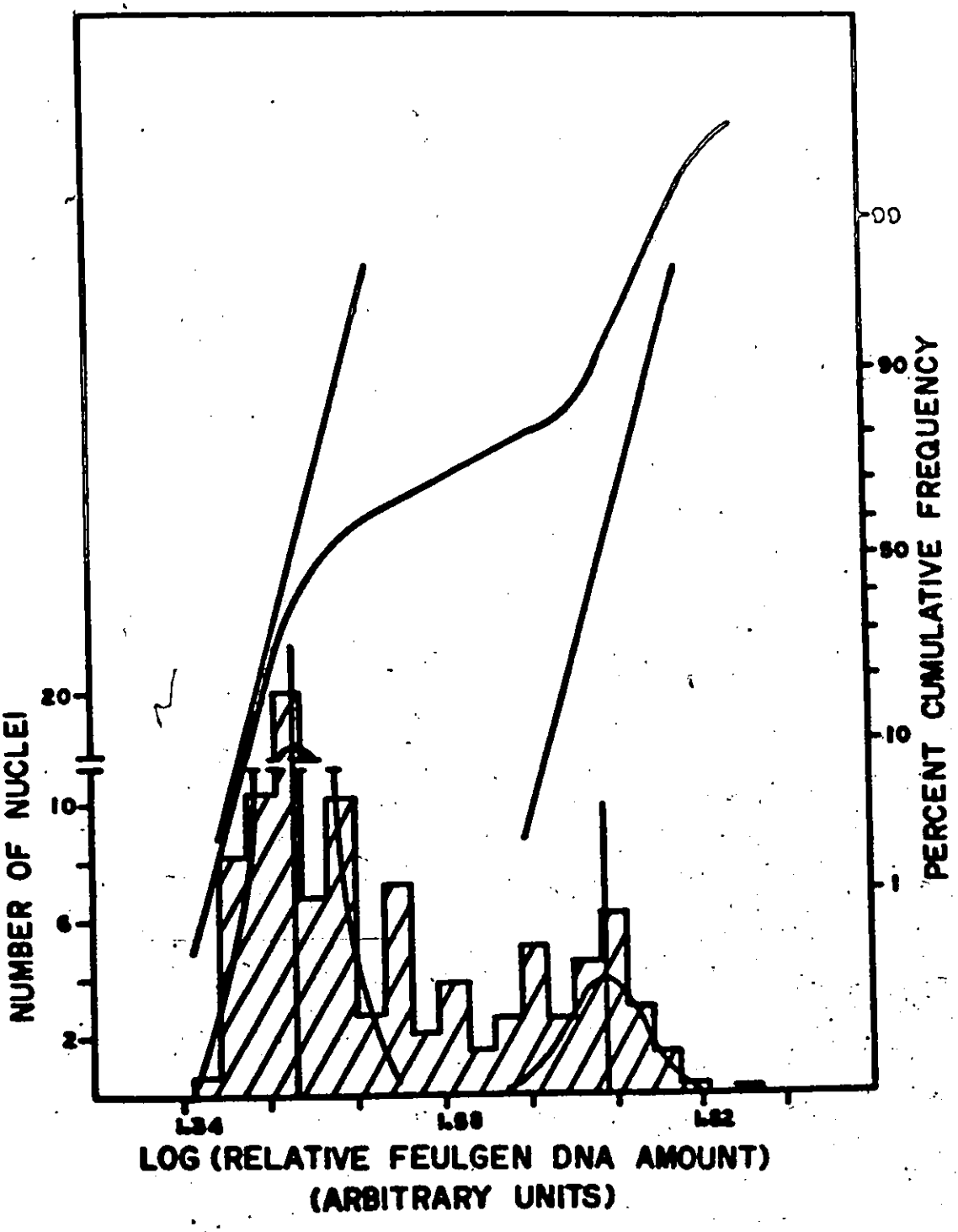

NUMBER OF NUCLEI
20
10
6
2
100
90
50
10
1
PERCENT CUMULATIVE FREQUENCY
1.84
1.88
1.92
LOG (RELATIVE FEULGEN DNA AMOUNT)
(ARBITRARY UNITS)

FIGURE 33

Distributions of mitotic figures in germinal balls differing in cell number. For each germinal ball the sequence was drawn from serial sections. Scores indicate the approximate total number of cells in each germinal ball.

M = metaphase.

T = telophase.

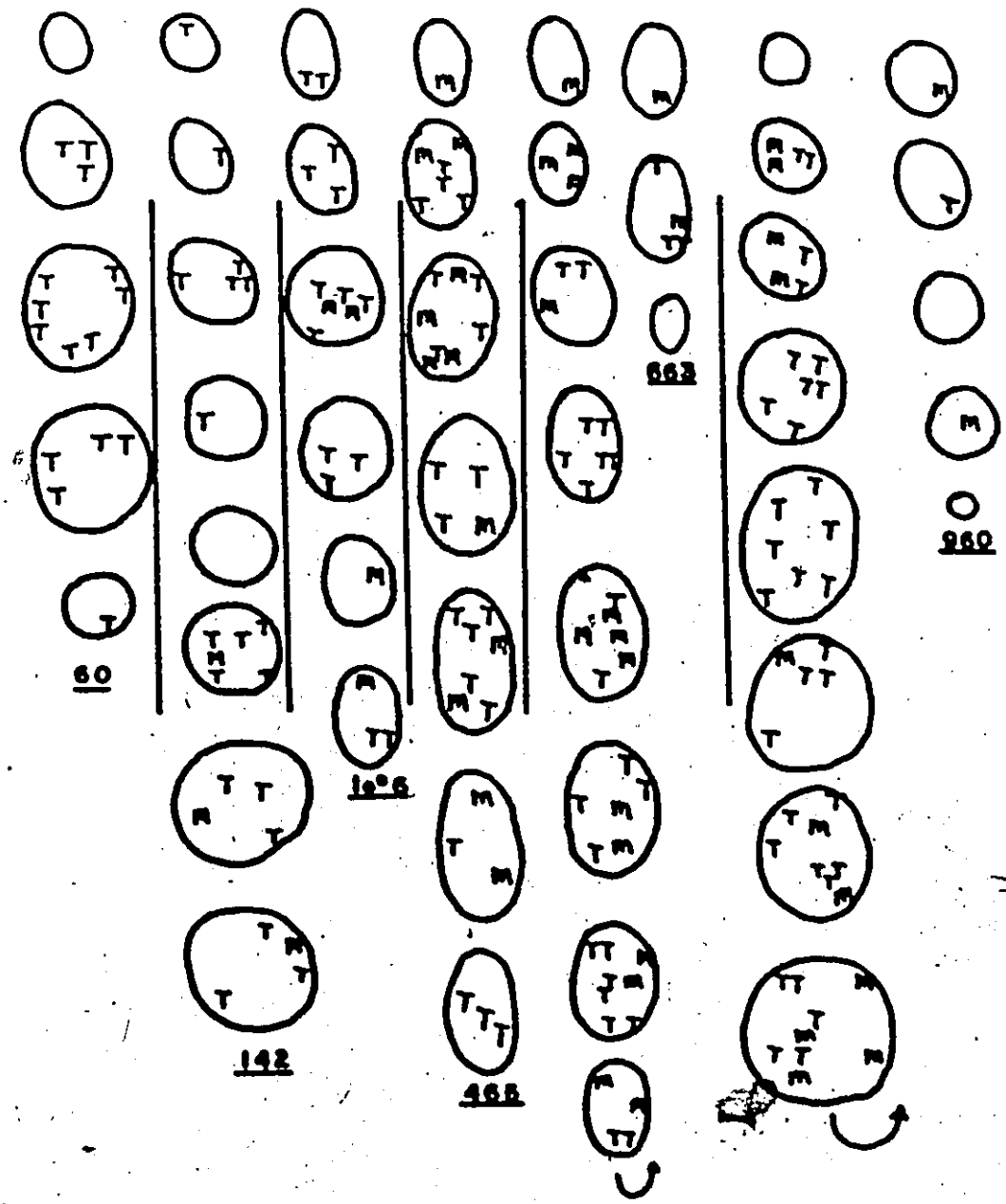
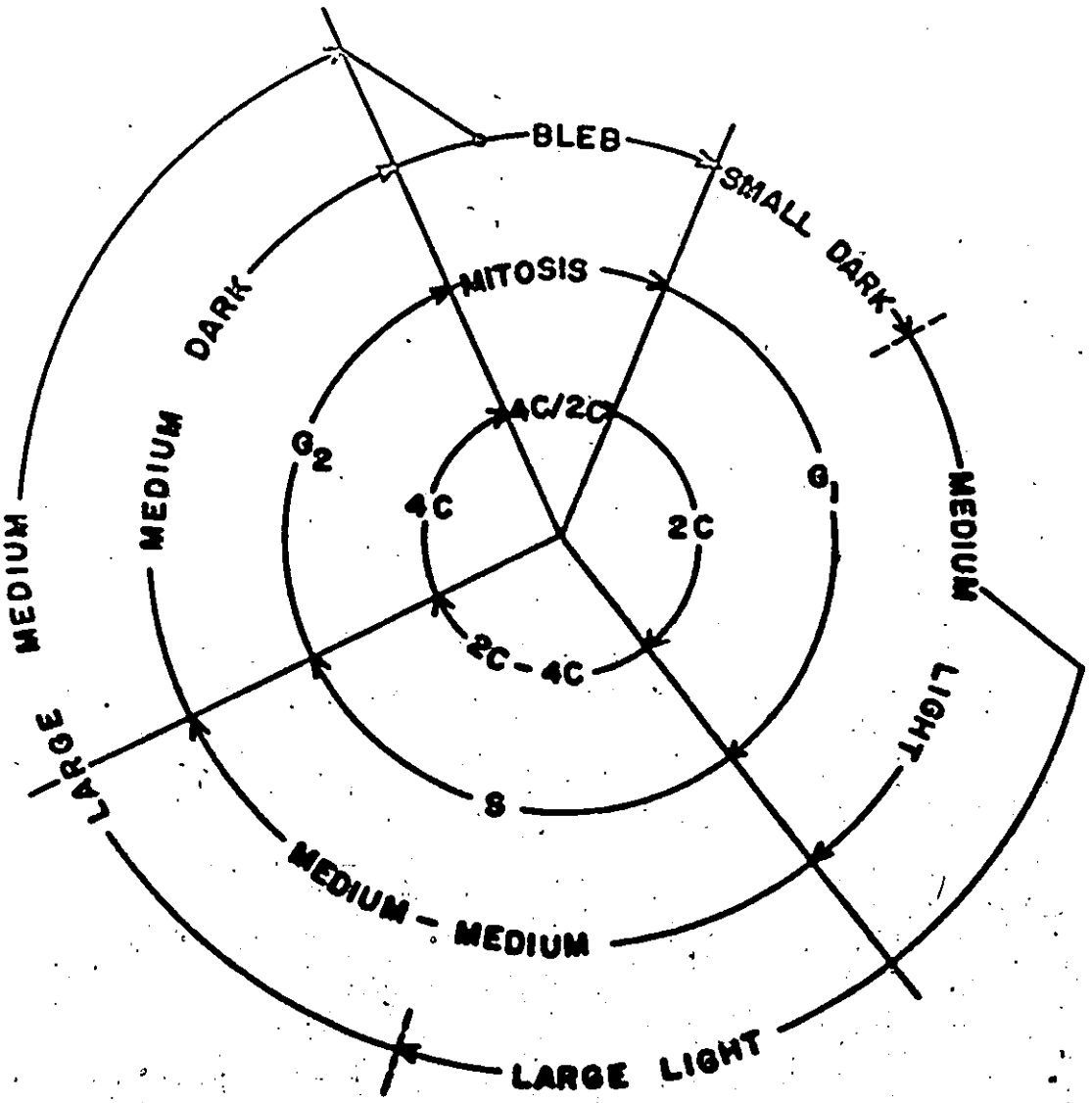

60
142
1006
466
663
960

FIGURE 34

Diagrammatic outline of the distributions of the nuclear morphology classes throughout the cell cycle.


BLEB
SMALL DARK
MEDIUM
LIGHT
LARGE LIGHT
MEDIUM - MEDIUM
LARGE
MEDIUM
MEDIUM DARK
MITOSIS
4C/2C
G2
G1
4C
2C
2C - 4C
S

PLATES

PLATE 1

Figure 1. Variation in the nuclear morphologies in developing T. ocellata cercariae.

Abbreviations

1	Large-Light
2	Large-Medium
3	Medium-Light
4	Medium-Medium
5	Medium-Dark
6	Small-Dark
7	Bleb
P	Prophase
M	Metaphase
A	Anaphase
T	Telophase

Feulgen-stained.

Panels A-B. Magnification X640.

Panels C-F. Magnification X800.

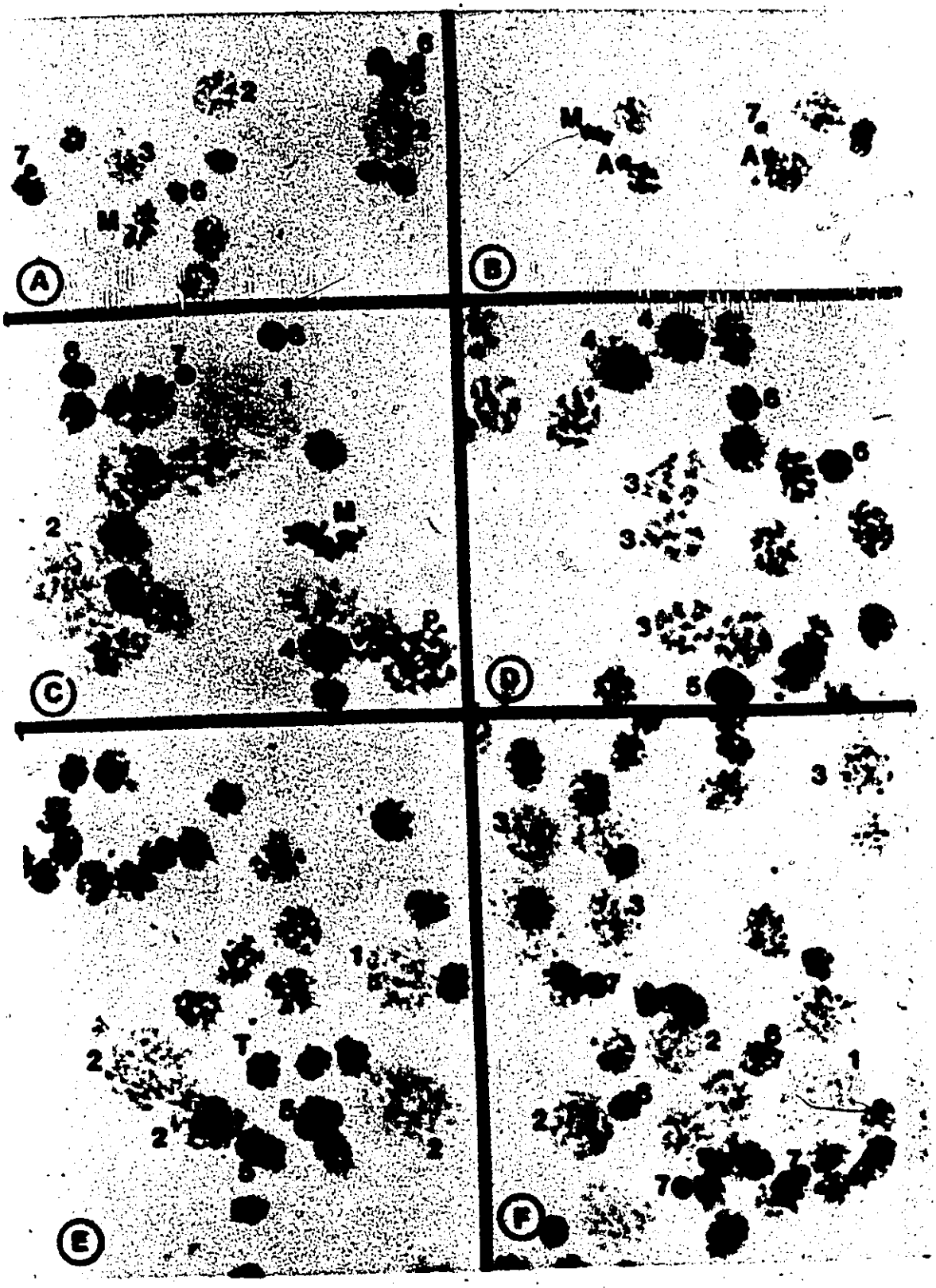

A
B
C
D
E
F

PLATE 2

Figure 2. Distribution of mitotic figures in a 300-celled germinal ball. M = Metaphase; B = Bleb. Feulgen and Fast Green stained. Magnification X580.

Figure 3. Distribution of mitotic figures and large-size nuclei in a 600-cell germinal ball. B = Bleb; L = Large-size nucleus. Haematoxylin and eosin stained. Magnification X580.

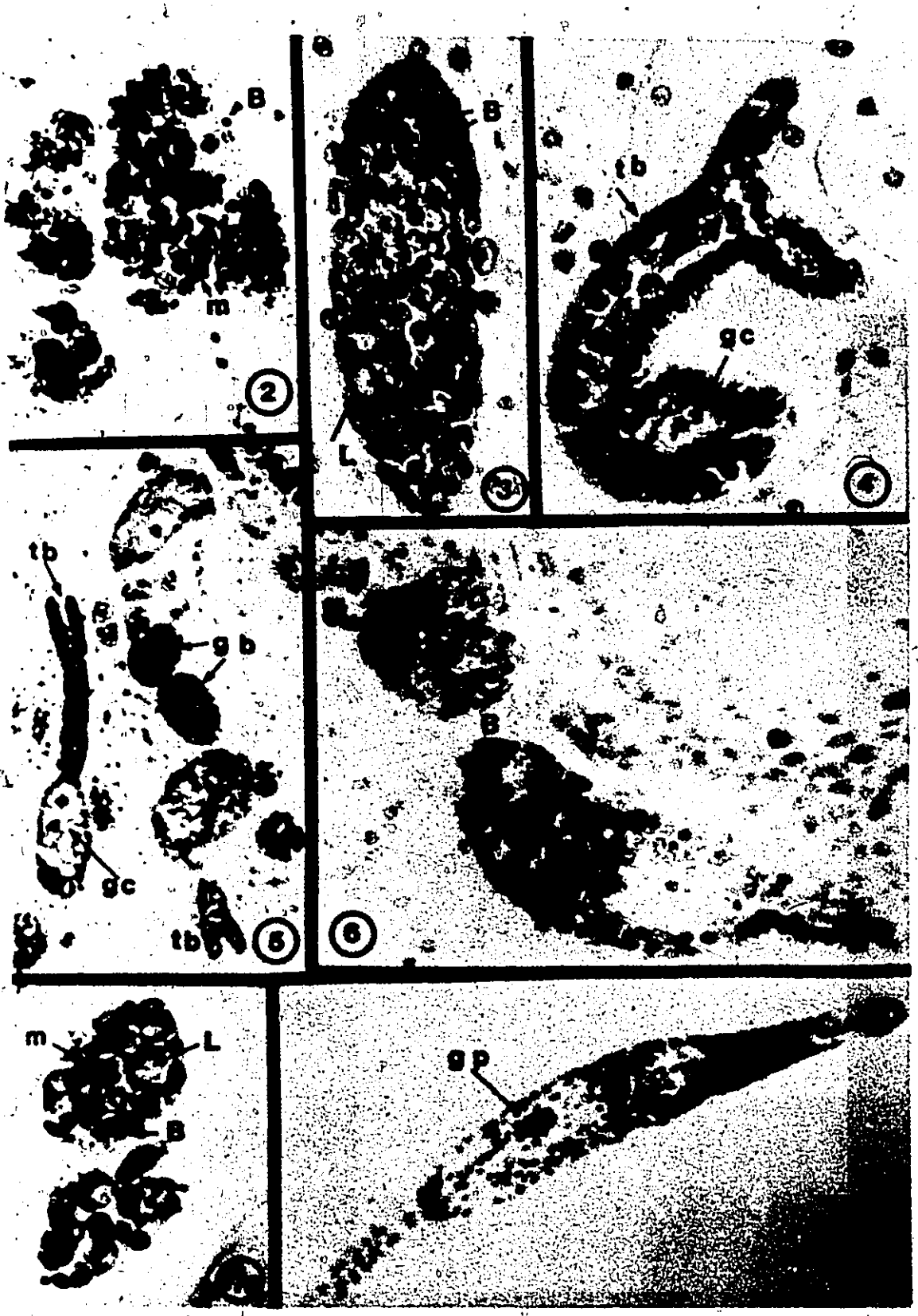
Figure 4. Cercarial larvae at the tail-bud stage of development. Note that the tail-bud (tb) is completely devoid of mitotic figures. The gland cell (gc) contains a large amount of cytoplasm; a faint outline of the nucleus is also present. Haematoxylin and eosin stained. Magnification X580.

Figure 5. Cercarial larvae at the tail-bud stage of development, and germinal balls consisting of less than 300 cells. In the cercarial larvae, mitotic figures and large-size nuclei are absent from the tail bud region (tb). Gland cells (gc) are present in one of the larvae. Note the absence of cytoplasm and the compacted arrangement of the nuclei in the two germinal balls (gb). Haematoxylin and eosin stained. Magnification X192.

Figure 6. Distribution of mitotic figures in two germinal balls consisting of 1100 cells. The lower germinal ball was sectioned longitudinally; the upper germinal ball was sectioned through the anterior portion of the body. Note that the mitotic figures are localized primarily in the anterior body region. A few mitotic figures are also found towards the posterior region of the body in the lower germinal ball. B = Bleb. Feulgen and Fast Green stained. Magnification X580.

Figure 7. Distribution of mitotic figures and large-sized interphase nuclei in two germinal balls consisting of less than 50 cells. M = Metaphase; B = Bleb; L = Large-size interphase nucleus. Haematoxylin and eosin stained. Magnification X640.

Figure 8. Head region of a mature T. ocellata cercaria. Note that the genital primordium (gb) is composed of small darkly-stained nuclei. Feulgen-stained. Magnification X192.


B
m
2
B
L
3
tb
gc
4
tb
gb
gc
tb
5
B
6
m
L
B
gp

4. DISCUSSION

In mitosis in eukaryotic cells there are four characteristic morphological stages: prophase, metaphase, anaphase and telophase. Non-mitotic cells generally lack sufficient variation in nuclear morphology to allow subdivision of interphase. Nevertheless, by defining the occurrence and the duration of the DNA synthetic period, interphase can be partitioned into three phases, that is, the DNA synthetic period, S; the presynthetic period, G_1; and the post-synthetic period, G_2 (Howard and Pelc, 1953; Quastler and Sherman, 1959; Wimber, 1960).

In the present study nuclei were found to vary in size, staining intensity and appearance of the chromatin. The phases of the cell cycle for proliferating cells of T. ocellata could be identified by comparing changes in nuclear morphology with changes in DNA content, nuclear area, and labelling patterns after exposure to ^{3}H-TdR. The following scheme is proposed. The Blebs are found in cells that are at the mitosis-G_1 transition point in the cell cycle, Small Dark nuclei (S.D.) in early G_1 cells, Medium Light nuclei (M.L.) in late G_1 cells, Medium Medium nuclei (M.M.) in S cells, and Medium Dark nuclei (M.D.) in G_2

cells. Large Light nuclei (L.L.) occur in late G_1 and early S cells and Large Medium nuclei (L.M.) in late S and G_2 cells (Fig. 34).

Nuclear morphologies similar to those described for T. ocellata have been observed in a number of cytological studies of the miracidial, germinal sac, that is the sporocyst and redial, and cercarial stages (Brooks, 1930; Chen, 1937; Rees, 1940; Pieper, 1953; Van der Woude, 1954; Cirordia, 1956; Guilford, 1958; Khalil and Cable, 1968; Voge and Seidel, 1972). Based on the descriptions given for the appearance of the chromatic material, the nuclei of these larval stages generally fall into three categories in which the chromatin may be either dispersed as a fine network or be condensed into knots along a fine network or appear as a few strands resembling coarsely arranged material. The three categories correspond to the observed chromatic patterns of the medium and large-sized nuclei found in T. ocellata. At present, however, the data are insufficient to suggest that the variations in the appearance of the chromatin which reflect the G_1, S and G_2 phases of the cell cycle in T. ocellata represent similar phases for other species of digenetic trematodes.

In T. ocellata, nuclei of two size classes designated as medium and large each have DNA contents ranging from 2C to 4C (Fig. 34). It may be that there are two discrete

populations of cell types proliferating during cercarial development; or, the cells may represent one proliferating population which tends toward a partial bimodality with respect to nuclear area during the late G_1, S and G_2 phases. The following considerations of the data obtained during the course of the present investigation tend to support the latter hypothesis:

(a) The size, appearance and staining intensity of the chromatin was extremely homogeneous in populations of cells in the mitotic to early G_1 phases of the cycle. In the absence of sufficient morphological variation all cells in mitosis and early G_1 probably represent one proliferating cell type.

(b) Analysis of the PLM data revealed that the proliferating cells were homogeneous for the duration of G_2 and mitosis; a situation which usually exists if there is only one proliferating cell population.

(c) In the seven germinal balls analyzed microspectrophotometrically, regions of overlap existed between the medium and large-sized nuclear classes. Overlap in area values would be expected to occur if the nuclei change continuously as the cells proceed through interphase.

Numerous workers (Brooks, 1930; Chen, 1937; Rees, 1940; Pieper, 1953; Van der Woude, 1954; Cirordia, 1956; Guilford, 1958; Khalil and Cable, 1968; Voge and Seidel, 1972)

distinguish between two proliferating populations of cells, during trematode larval development, on the basis of relative size and relative frequency. Generally, these studies do not include specific measurements of nuclear areas and the absolute frequencies of the nuclear types. Inspection of the drawings and photographs presented in these studies reveals basic similarities with the large and medium-sized nuclei found in T. ocellata. The observation of two sizes of nuclei during development has led to the theory that cells containing large-sized nuclei represent germ line cells and the more frequent cells with smaller-sized nuclei represent somatic line cells (Brooks, 1930; Cable, 1934; Ishii, 1934; Rees, 1940; Cort et al., 1948, 1954; Oliver and Mao, 1949; Pieper, 1953; Van der Woude, 1954; Cirordia, 1956; Cheng and James, 1960; James and Bowers, 1967b; Cheng and Bier, 1972). There are a number of uncertainties, which taken together, indicate that this theory must be reconsidered carefully and that it is not directly applicable to the developing T. ocellata cercariae.

No study has shown that the two distinctive cell lines are inheritable. That is, if the progeny of each cell line display a nuclear morphology which is characteristic of the parent cell. Notwithstanding the lack of direct evidence indicating maintenance of cell lineages, it may be granted that the cells containing the large-sized nuclei represent

germ line cells and that these cells form the cercarial genital primordium. However, in a fully developed cercaria of T. ocellata the genital primordium is composed of a mass of cells possessing small, darkly staining nuclei. Under the proposed theory, the cells which originally contain large-sized nuclei would undergo dramatic changes in nuclear morphology during the formation of the genital primordium. Presumably as their morphology changes the nuclei would transiently appear similar to the somatic nuclei. Such a sequential transformation makes it impossible to accurately trace the migration of the large-sized presumptive germ cells and determine if they do in fact give rise to the genital primordium. For this same reason, Van der Woude (1954) and Guilford (1958) were not able to determine if the cells located within the genital primordium were descendants of the somatic or germ line cells.

The use of nuclear size per se to define the germ line cells is questionable from another aspect. During the early stages of cercarial morphogenesis in T. ocellata, that is, when the germinal ball is composed of about 1100 cells, the proposed germ line cells are localized in the body region in which the gland cells eventually develop. Since both cell types contain large-sized nuclei the question arises as to whether or not gland cells originate from either somatic or germ line cells and whether gland cells may be confused

with germ line cells. Moreover, in the two largest germinal balls measured microspectrophotometrically about 13% of the large-sized nuclei were found to be polyploid. Cells with polyploid nuclei could represent the precursors of gland cells since: (a) high DNA contents are generally found in specialized cells of vertebrates (Swift, 1950) and invertebrates (Fallieri et al., 1969; Sin and Pasternak, 1970; Marshall et al., 1973), (b) the number of large-sized, polyploid nuclei parallels the number of gland cells reported for the cercariae of Trichobilharzia sp. (Talbot, 1936; Wu, 1953). Nuclear volume has generally been observed to be proportional to the quantity of nuclear DNA (Sparrow and Miksche, 1961; Laufer et al., 1967; Alfert and Das, 1969; Schreiber et al., 1969). If a similar relationship also applies to the nuclei in T. ocellata then the polyploid nuclei originated from medium-sized nuclei. That is, the large-sized, polyploid nuclei represent somatic line cells which have matured into gland cells.

Exceptions to the concept of nuclear size being directly proportional to the ploidy level have been noted (Schreiber and Angeletti, 1940; Schrader and Leuchtenberger, 1950; Swift, 1950; Baetche et al., 1967). Furthermore, in the proliferating cells of T. ocellata cells enter into DNA synthesis with at least a two and one-half fold difference in nuclear area and for some cells the area obtained at the

end of the G_1 phase probably remains unchanged as the cells pass through S and into G_2. In the absence of a direct relationship between nuclear volume and DNA content, cells containing polyploid DNA contents would represent germ line cells which have entered into the somatic cell line. Chen (1937), Van der Woude (1954), Guilford (1958) and others have also indicated that during cercarial development germ line cells can apparently enter into the somatic cell line and become specialized into somatic cell derivatives such as the oral sucker or pharynx.

Assuming that some cells containing large-sized nuclei differentiate into somatic components then these cells are not true germ line cells as defined in the classical sense. Thus, contrary to the germinal lineage hypothesis germ line determination in T. ocellata may not originate with the first cleavage of the progenitor cell. Perhaps cells which form the genital primordium arise during later stages of cercarial development, for example, in the cercarial larvae which are at the tail-bud stage.

It is obvious that nuclear size is not sufficient evidence to accurately identify either the germ or somatic cell lines. In a similar context there is a controversy whether one or more cell lines exist during the development of the tapeworm proglottid. Wikgren et al. (1967, 1971a,b) and Bolla and Roberts (1971) suggest that the primary

anlage is composed of a single cell line; whereas Douglas (1961a,b) proposes two inheritable cell lines. The need arises to use additional criteria to distinguish germ line cells from somatic line cells. In many organisms distinctive cytoplasmic features, for example RNA-rich granules, appear in the germ line cells (Chiquoine, 1953, 1954; Ullmann, 1965; Blackler, 1966; Wallace, 1968; Hubert, 1970a,b). The marked cells can then be traced up to the stage at which the gonads are formed. Such cytological markers were not observed in the cells containing large-sized nuclei of developing T. ocellata cercariae.

Nuclei, which resemble the Blebs found in the developing T. ocellata cercariae have also been observed in numerous other species of digenetic trematodes. Several interpretations of the role of the Bleb during larval development have been presented. Schubmann (1905), Goldschmidt (1905) and Tennent (1906) regarded the small intensely staining bodies to be lipid material. According to Brooks (1930) earlier workers had considered the Blebs to be either nucleoli, maturing snail eggs or artifacts such as dirt or undissolved stain. Woodhead (1931, 1954, 1957) considered the Blebs to be tail-less sperm; Cable (1934), Pieper (1953), Van der Woude (1954) and Guilford (1958) suggested that the Blebs were either pycnotic nuclei or represented the nuclei of cells that had just completed a mitotic division. Finally,

Jordan and Byrd (1967) and Byrd and Maples (1969) did not offer an explanation for the Blebs though they felt that the Blebs played an important role in the developmental cycle of trematodes.

Since the Blebs gave a positive Feulgen reaction they must contain chromatin, that is, they are of a nuclear origin. In the present study, Blebs are interpreted as representing cells at the mitosis-G_1 transition point; this is in agreement with the suggestion of Cable (1934), Pieper (1953), Van der Woude (1954) and Guilford (1958) that Blebs represent part of the cell cycle. It also seems reasonable to assume that the small intensely staining bodies, seen in numerous other species of digenetic trematodes, could represent cells at the mitosis-G_1 transition. One justification for this assumption, in addition to the morphological similarity, stems from the fact that the descriptions of the frequency and fate of the bodies follows a similar pattern during larval development as described for T. ocellata.

Cells in which the chromatin appeared as small intensely staining bodies have, in part, been used as cytological evidence by Bednarz (1962) and Khalil and Cable (1968) to suggest that reproduction in the larval stages of trematodes is by diploid parthenogenesis. The following considerations cast considerable doubt on applying this theory to developing T. ocellata cercariae.

The cells regarded as polar bodies by Bednarz and oögonia by Khalil and Cable are similar in size and appearance to what have been here interpreted as cells at a late stage of mitosis. The descriptions presented by Bednarz to account for polar body resorption and by Khalil and Cable to account for the oögonia transforming into oöcytes parallel the stages cells pass through in going from mitosis to G_1. Khalil and Cable indicated that the cells were capable of DNA synthesis and proliferation, as evidenced by their finding of labelled cells four to six hours after an exposure to ^{3}H-TdR. An analysis of the photometric data, in the present study, revealed that in the developing cercarial germinal balls, the Blebs have a constant DNA content and were 2C. Labelled Blebs were observed in the present study as early as five hours after exposing the cercarial larvae to ^{3}H-TdR. However, labelled Blebs were never observed at one and three hours. Khalil and Cable (1968) and Khalil (1968) did not indicate whether oögonia or other nuclear types labelled prior to four hours.

As support for their view that the Blebs represent either polar bodies or oögonia, these workers described what appeared to be tetrads at metaphase. The diploid chromosome number was estimated to be 12 to 14 for T. ocellata; these values compare with the number present in the somatic tissues of closely related species (Short, 1952a,b, 1955, 1957;

Short and Menzel, 1959, 1960). In all dividing cells examined during the course of the present investigation stages of meiosis, including the appearance of a tetrad showing 6 to 7 chromosomes, were never observed.

In the absence of any cytological evidence, Szidat (1962), James (1964), Cable (1965), James and Cable (1965) and James and Bowers (1967b) have also suggested that during larval development, the mode of reproduction is best regarded as a form of parthenogenesis of the type Suomalainen (1950) defined as apomictic or diploid parthenogenesis. Suomalainen lists the study by Cary (1909) on the germ cell cycle of Megalodiscus temperatus as one parasitic system which clearly demonstrates this form of reproduction. M. temperatus is the same species of trematode that Van der Woude later studied in detail (Van der Woude, 1951, 1954). She did not regard the small intensely staining bodies as representing the products of any meiotic division by rather, as previously discussed, as representing newly formed daughter cells arising after a mitotic division. In addition, Van der Woude was unable to find within the larval stages, cells in which the chromatic material resembled the meiotic chromosomes as described during adult gametogenesis.

Since meiotic reproduction does not occur during T. ocellata larval development the question still remains: what is the process of reproduction? Presently, a number

of investigators (Brooks, 1930; Cable, 1934; Rees, 1940; Cort et al., 1948. 1954; Pieper, 1953; Cirordia, 1956; Guilford, 1958) have interpreted the mitotic reproduction of the progenitive components as representing a form of polyembryony. Objections to the use of this term were raised in the INTRODUCTION. Moreover, the lack of any clear evidence to indicate a continuous germ cell lineage throughout cercarial morphogenesis have been discussed. At present, therefore, it is concluded that during T. ocellata cercarial development all proliferating cells divide mitotically and reproduction during larval development of the cercariae, and perhaps for the other stages as well, can be regarded simply as an asexual process.

In addition to such standard techniques as determining the MI and LI, changes in the frequency of each nuclear morphology class can be used to characterize the phases of the cell cycle within the proliferating cell population of developing T. ocellata cercariae. During cercarial development the MI, LI and the relative frequencies of the S.D. (G_1); M.M. (S); M.D. (G_2); L.L. (G_1-S); and L.M. (S-G_2) nuclear classes decreased whereas the frequency of the M.L. (G_1) class increased. In theory, if a cell population of proliferating cells is homogeneous for cell cycle parameters the number of cells in any one phase of the cycle is proportional to the duration of that phase relative

to the duration of the complete cell cycle (Quastler and Sherman, 1959). Since the relative frequencies of cells in mitosis, S and G_2 decreased in germinal balls of increasing total cell number, then the relative durations of these phases much also have decreased in a similar fashion. The estimates of relative duration may differ, however, from the actual duration of each phase if (a) during the growth of the cercarial larvae either the absolute durations of these phases shortened or total cycle time increased, and (b) the developing germinal balls accumulate non-dividing cells.

Partial evidence that the absolute duration of each phase and total cycle time of the proliferating cells remained constant during cercarial development was obtained from an analysis of the PLM data. The average time it takes proliferating cells to pass through the G_2 phase plus one-half of the mitotic phase was about four hours. A minimum estimate of G_2 phase was three hours. Since these two estimates differ by only one hour in all sizes of germinal balls examined it is concluded that the average duration of G_2 did not vary at different stages of cercarial development. Many of the germinal balls examined at the seven hour fixation had PLM values approaching 100 which also indicates that there is little variability in the duration of G_2 or mitosis.

The available evidence supports the suggestion that the apparent relative durations of the S, G_2 and mitotic phases in the cell cycle of proliferating cells are less than the actual relative durations since non-dividing (G_0) cells are indistinguishable from dividing cells, and since the non-dividing cells tend to increase the number of cells scored. Further the apparent relative duration of G_1 increases since the non-proliferating cells are held in this phase. The increase in the fraction of nuclei appearing as the M.L. (G_1) type indicates that this class of interphase nuclei probably represents non-dividing (G_0) cells. If this suggestion is correct, it would in turn imply that all M.L. nuclei are non-proliferative. The evidence indicates that the morphological sequence a proliferating cell passes through in the G_1 phases is: S.D. → M.L. → L.L. or M.M. Thus at least in the small germinal balls, M.L. nuclei are found in proliferating cells. Alternatively, the M.L. class of nuclei may include two physiologically different types, one type that is proliferative and another that is non-proliferative. These two physiologically different types of nuclei may always be present and during development an increasing proportion may appear as the latter type.

Prior to the 750-celled stage the absolute number of cells appearing in each phase of the cycle increased.

Between the 750 and 1100-celled stage the number of cells appearing in S, G_2 and mitosis decreased. These results indicate that the proliferative conditions found in balls consisting of 750 or fewer cells was apparently replaced by a non-proliferative condition in the larger germinal balls. Cells which were no longer cycling appeared as the M.L. non-proliferative type, since the absolute number of M.L. cells continued to increase. The switch does not affect all cells at the same time. Some cells continue to proliferate up to the 1800-1900 cell stage but as cell number increases the frequency of proliferative cells falls. The remaining proliferating fraction of cells with M.L. nuclei would probably contribute to this increase in growth. Studies on development in a wide variety of organisms have revealed that a minimum number of cells is necessary before differentiation can proceed (Brien, 1937; Muchmore, 1957; Grobstein, 1959; Holtfreter, 1965; Barth and Barth, 1968; Deuchar, 1969, 1970). Inasmuch as a dramatic drop in the numbers of proliferating cells was first recorded in the 750-celled germinal balls, 750 cells probably represents the critical mass size which must be attained before differentiation is initiated.

For many species of digenetic trematodes, the appearance of the tail-bud is used to mark the beginning of cellular differentiation (Brooks, 1930; Maldonado and

Malienzo, 1947; Van der Woude, 1954; Cheng, 1960; Cheng and James, 1960; Cheng and Bier, 1972). In T. ocellata the tail-bud was first observed in the germinal balls composed of about 1100 cells. Histological studies revealed that beginning with the appearance of a tail-bud subsequent cercarial development is characterized by extensive increases in the amount of cytoplasm surrounding many of the cells. These cells probably represent non-dividing cells that are engaged in the synthesis of cytoplasmic components which are necessary for the cells to acquire specialized features characteristic of a differentiated cell. For example, Cheng and Snyder (1962a,b), Cheng (1964), James and Bowers (1967a) and others have demonstrated that carbohydrates and lipids are metabolized and deposited in cells during cercarial morphogenesis beginning at about the stage when the tail-bud first appears. Further investigations will be required to determine the patterns of RNA and protein synthesis to examine the temporal patterns of cell division DNA, RNA and protein synthesis during cytodifferentiation of T. ocellata cercariae.

Accurate estimates for phase durations and total cycle time of a proliferating population of cells are normally derived from a PLM curve. A system in which the cells are partially synchronous and continue to label restricts the construction of a complete PLM curve. From partial

curves, however, estimates for the absolute duration of G_2 + M/2 lasting about four hours were obtained. These estimates, in turn, were used to convert the photometric data into phase durations. For germinal balls consisting of 513 or fewer cells the following estimates were obtained: t_C lasts 15.2 hours, in which t_{G_1} lasts 5.6 hours, t_S 4.8 hours, t_{G_2} 3.2 hours and t_M 1.6 hours. In the two largest germinal balls examined, the average estimates for t_C and t_{G_1} lasting 29.9 and 19.4 hours respectively, were approximately two to three times the values obtained for the smaller larvae. Inasmuch as the durations of the remaining phases remained unchanged, i.e. t_S lasts 5.6 hours, t_{G_2} 3.4 hours, t_M 1.5 hours, and these large germinal balls were at a stage of cercarial development when some cells have switched to become non-dividers, the duration of the G_1 phase also remained unchanged for the remaining proliferating cells. Thus the duration of t_C probably remains constant throughout cercarial development.

Numerous investigations have revealed that factors such as temperature, size and age of the snail host, availability of nutrients influence the rate of cercarial development (Cort et al., 1944, 1955; Kendall, 1949; Evans and Stirewalt, 1951; Pieper, 1953; Cheng and Snyder, 1962a; Dinnik and Dinnik, 1964; Lim and Lie, 1969; Ubelacker and Olsen, 1970a,b). For example, Pieper (1953) found that with

an increase in the water temperature from 21-24°C to 24-28°C, the time delay between miracidial infection and cercarial emergence from the snail host, was reduced from 55 to 21 days. The manner in which these factors influence the rates of cercarial development, for example, in terms of possible changes in the durations of the cell cycle phases described above, has yet to be determined.

SUMMARY

1. Variations in the morphology of interphase nuclei have been correlated with the cell's position in interphase.
2. The relative durations of S, G_2 and mitosis decrease with progressive differentiation of T. ocellata cercariae. The relative duration of G_1 increases with progressive development.
3. During cercarial development, the absolute numbers of cells appearing in all phases of the cycle increased until the 750-celled stage is reached. Thereafter cells continue to accumulate in the G_1 phase while the numbers of cells in S, G_2 and mitosis decrease. These changes may be due to the accumulation of non-dividing, 2C nuclei.
4. Beginning at about 1100-celled stage, which represents approximately one-half the total number of cells found in a mature cercariae, many of the cells acquire large amounts of cytoplasm. Accumulation of cytoplasm together with the accumulation of non-dividing cells indicate that many of the cells which cease to proliferate are differentiating.

5. The estimated values of the cell cycle parameters in developing T. ocellata cercariae are:

t_C = 15.2 hours

t_{G_1} = 5.6 hours

t_S = 4.8 hours

t_{G_2} = 3.2 hours

t_M = 1.6 hours

6. The cytological evidence revealed that all cell divisions are mitotic and taken together with the observed variations in nuclear morphology support the conclusion that only asexual reproduction occurs throughout all stages of T. ocellata cercarial development. A similar mode of reproduction probably occurs for all other larval stages of T. ocellata, and during larval development in numerous other species of digenetic trematodes.

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APPENDIX I

A germinal mass consisting of N total cells was scored and the frequency of mitotic nuclei was A (observed value). If a second germinal mass of similar size had been sampled what maximum value would its mitotic frequency, B (assumed value), have and still be considered to have been sampled from the same population as the first, i.e.,

	Mitosis	Interphase	Total
Sample No. I (observed)	A	N - A	N
Sample No. II (assumed)	B	N - B	N
Total	A + B	2N - A - B	2N

$$\text{Chi Square} = \sum_{i}^{4} \frac{(\text{observed value}_i - \text{expected value}_i)^2}{\text{expected value}_i}$$

Expected Values: number of mitotic nuclei $\frac{A + B}{2}$

number of interphase nuclei $\frac{2N - A - B}{2}$

At the 95% confidence interval, with one degree of freedom

$\chi^2 = 3.841 = C.$

Therefore $\chi^2 = C = \dfrac{[A - (\frac{A+B}{2})]^2}{(\frac{A+B}{2})} + \dfrac{[B - (\frac{A+B}{2})]^2}{(\frac{A+B}{2})} +$

$$\frac{[(N - A) - (\frac{2N - A - B}{2})]^2}{(\frac{2N - A - B}{2})} +$$

$$\frac{[(N - B) - (\frac{2N - A - B}{2})]^2}{(\frac{2N - A - B}{2})}$$

Solving the equation for B:

$$B^2(2N + C) + B[-2(AC - NC - 2AN)] + A(AC - 2NC + 2AN) = 0$$

Therefore $B = \dfrac{2(AC - NC - 2AN) \pm \sqrt{[4NAC(-8A+6N+C) - 4N(AC-NC-2AN)]}}{4N + 2C}$

B can have two values, these values correspond to the maximum and minimum range limits.

APPENDIX II

A. Graphical Analysis of a Theoretical Distribution

A histogram has been constructed for the frequency of 50 values derived from a theoretically-perfect Gaussian distribution (Fig. 35). The data can be transformed into a probability plot by ranking the individual values and plotting these values as cumulative percentages. For example, at the point x 16% of the sample is less than 15 units, i.e. there are 1 + 3 + 4 = 8 or 16% of the 50 values smaller than 15 units. When all the data is plotted, their values lie on a straight line. This line represents a normal distribution whose mean corresponds to the point M cutting the horizontal at 50%, i.e. 20 units. The standard deviation is estimated from the points which are cut off the straight line by the horizontals for 2.5% and 97.5% respectively. The values 10.5 and 29.5 represent 95% of the total data or ±2 standard deviations about the mean. Since the straight line represents a Gaussian continuous distribution, it is possible to transform this straight line into a unimodal curve which best fits the original data presented in the histogram. The height of

the Gaussian distribution, at successive intervals is derived from the equation $\frac{Nk}{\sigma} \phi(SD)$ where N = total number of values in the sample; k = interval of the histogram; σ = standard deviation of the sample; and $\phi(SD)$ is the height of the normal distribution at successive intervals. Successive intervals are calculated by adding or subtracting, from the mean of the sample, measurements of 0.5 (the estimated sample standard deviation).

B. Graphical Analysis of Three Overlapping Theoretical Distributions

A histogram consisting of two Gaussian distributions separated by a third, a square distribution, has been plotted (Fig. 36). The first Gaussian distribution consisted of 50 socres obtained from an idealized normal distribution with a mean and standard deviation of 20 and 4.75. The second consisted of 30 scores with a mean of 40 and standard deviation of 5.0. Overlapping each distribution are an additional 20 scores representing a square distribution.

The 100 scores can be transformed into a probability plot. The points do not fall on a straight line but on an S-shaped curve. The points of inflection were used as

estimates to segregate the scores into subpopulations. The first point of inflection occurred at 40%. Accordingly, the data was partitioned to include the first 40 values. Their percentage values were re-calculated using the equation $P_{x_i} = \frac{2i - 1}{2N} \times 100$.

x_i - ith ranked data value.

i = rank.

P_{x_i} = percentile of the ith data value.

N = subpopulation size.

These values gave a curve AB (Fig. 36). Similarly, partitioning of the data to include the first 50 and 60% of the total data yielded curves CD and EF. The best fit, in terms of a straight line, was obtained from the plot consisting of 50 scores. This is the best estimate for the size of this first subpopulation. The points begin to deviate from a straight line at 90% resulting from the overlap of the adjacent subpopulation. The straight line can be extended and from it estimates are derived for the subpopulation's mean and standard deviation. The estimates obtained were 20 and 4.75 respectively. A similar partitioning of the top 20, 30 and 40% of the data yielded curves GH, IJ and KL. From the straight line, derived from 30% of the data, the estimates for this second subpopulation's mean and standard deviation were 40 and 5.0 respectively.

The twenty percent of the data which is not included in either estimate constitutes the third subpopulation. Parameters of this population could be derived; but in the application of graphical method to the analysis of photometric data it is impossible to determine the type of distribution(s) contained within this portion of the data.

A comparison of the estimates derived from the graphical analysis technique with the original values used to generate the data are in exact agreement. It is therefore possible to use the probability plot as a means to derive estimates for the size, mean and standard deviations of 2C and 4C subpopulations.

FIGURE 35

Illustration of the graphical analysis technique. From a theoretically perfect Gaussian distribution 50 scores were selected and plotted as percent cumulative frequency (upper figure), and as a frequency distribution (lower figure). The area under the Gaussian distribution represents the distribution of the 50 scores.

X - point at which 15% of the scores are less than 15 units.

M - mean.

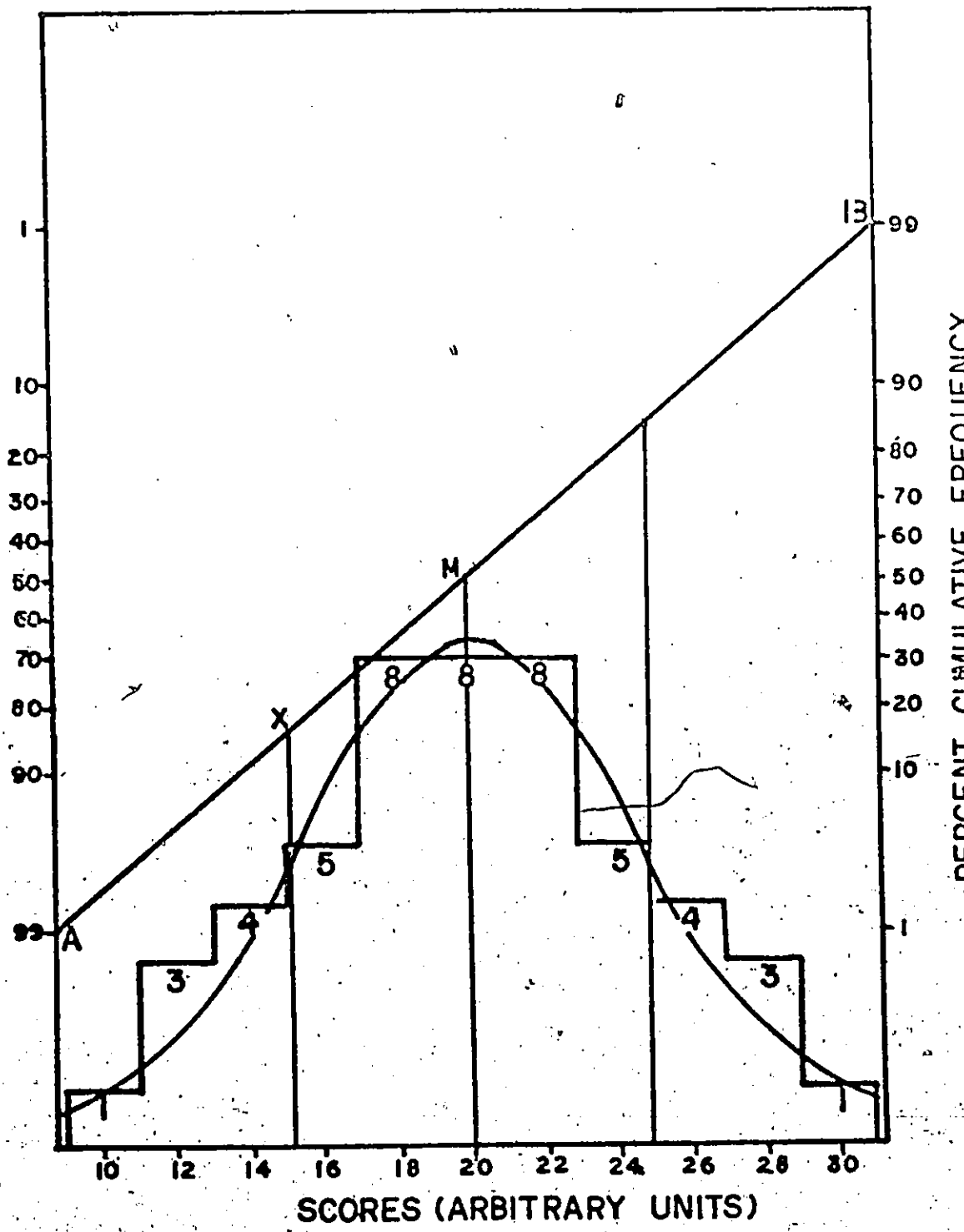
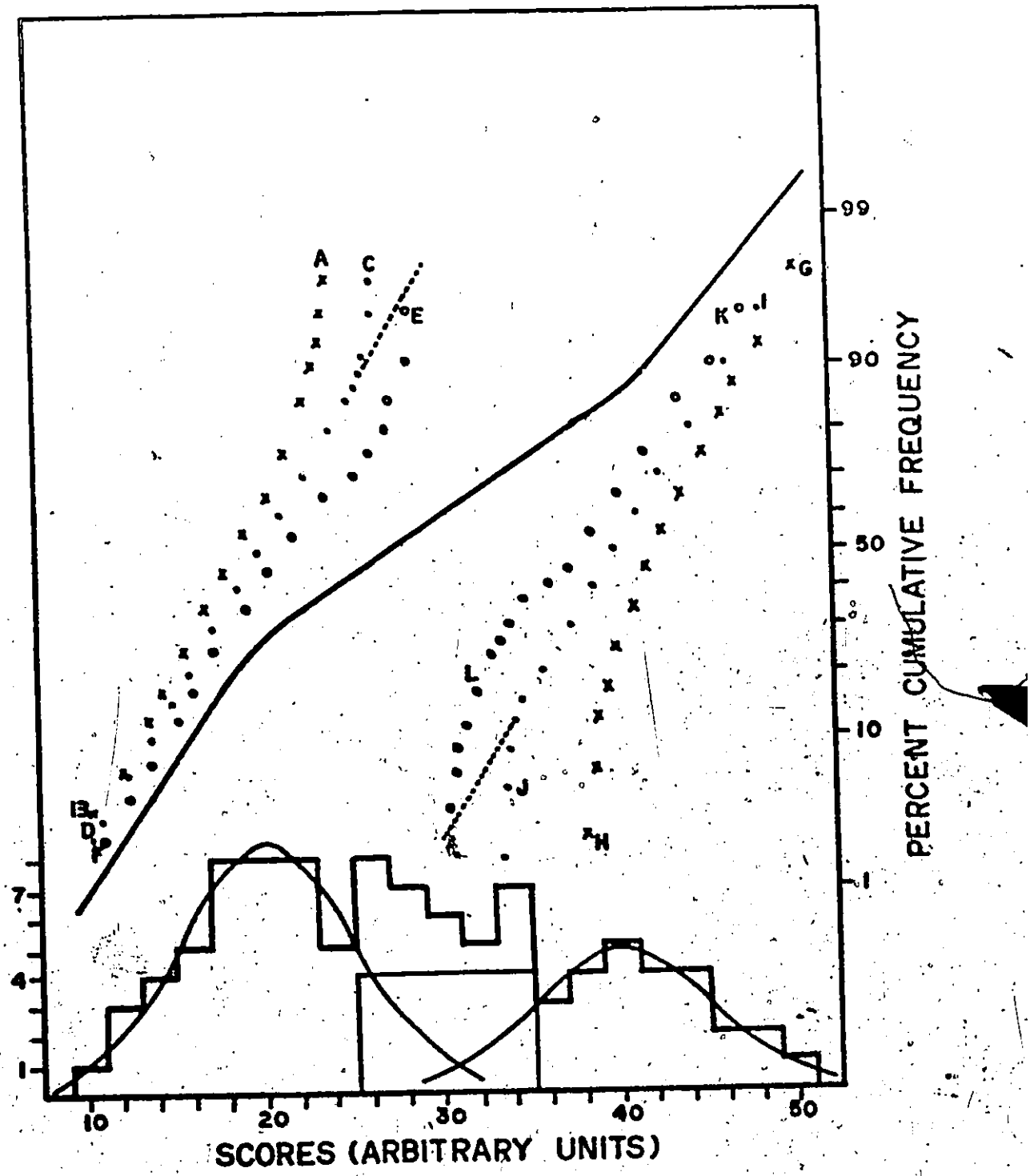

1
10
20
30
40
50
60
70
80
90
99
99
90
80
70
60
50
40
30
20
10
1
A
M
X
13
3
4
5
8
8
8
5
4
3
10
12
14
16
18
20
22
24
26
28
30
SCORES (ARBITRARY UNITS)
PERCENT CUMULATIVE FREQUENCY

FIGURE 36

Illustration of the graphical analysis technique. From two theoretically perfect Gaussian distributions separated by a square distribution, 100 scores were selected and plotted as percent cumulative frequency (upper figure) and as a frequency distribution (lower figure). The areas under the two Gaussian distributions and under the square distribution represent the values contained within the original data. See text for further details.


A
C
E
G
K
I
L
J
H
B
D
99
90
50
10
1
PERCENT CUMULATIVE FREQUENCY
7
4
1
10
20
30
40
50
SCORES (ARBITRARY UNITS)

APPENDIX III

Since $\frac{t_{G_2} + M/2}{t_C}$ is proportional to $\frac{\text{number of cells in } G_2 + M/2}{\text{total number of cells}}$

and $t_{G_2} + M/2 = 4.1$ hours (Table VI)

and fraction of cells in $G_2 + M/2 = \frac{25.0 + 9.4/2}{100} = 0.297$ (Table VIII)

therefore $t_C \approx \frac{4.1}{0.297} \approx 13.8$ hours.

Each phase of the cell cycle can be expressed in a similar fashion, e.g.

Since t_{G_1}/t_C is proportional to fraction of cells in G_1

and $t_C \approx 13.8$ hours

and fraction of cells in $G_1 = \frac{47}{100} = 0.47$ (Table VIII)

therefore $t_{G_1} \approx 0.47 \times 13.8 \approx 6.5$ hours.