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THE TEMPORAL RELATIONSHIP BETWEEN DEOXYRIBONUCLEIC ACID SYNTHESIS AND CELL DIVISION IN SYNCHRONOUS CULTURES OF THE COLONIAL GREEN ALGA, EUDORINA ELEGANS

by

KENNETH ALLAN LEE

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Examining Committee:

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Author: _____

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(date)

ABSTRACT

The temporal sequence of DNA synthesis, karyokinesis, and cell division was studied in synchronous cultures of Eudorina elegans. Cell division synchrony was induced under culture conditions of intermittent illumination and periodic dilutions to a constant coenobial concentration. During each 24 hour synchronous life cycle 95 percent of the gonidia underwent 4 or 5 successive nuclear divisions within 3 hours followed by daughter coenobia release over $1\frac{1}{2}$ to $2\frac{1}{2}$ hour period. E. elegans was found to be inefficient to incorporating the exogenous tritium labelled DNA precursors, thymine, thymidine, uracil, urdine, and adenine into DNA moieties. The uptake of the tritiated precursors did not reflect the timing and kinetics of nuclear DNA synthesis. Colourimetric determinations of extracted DNA revealed that DNA synthesis appeared to be confined to the period of gonidial cleavage. Microspectrophotometric quantitization of feulgen - DNA content per nucleus strongly suggested that during cell division one round of DNA replication preceded each of the four to five successive mitotic events. Each DNA replication required an average of 13 minutes followed by a mitosis completed in about 2 minutes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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CHAPTER I

INTRODUCTION - GENERAL

Deoxyribonucleic acid (DNA) synthesis in normal, actively dividing eukaryotic cells occupies a restricted portion of interphase, designated the S period which is temporally separated from cell division, M, by the pre and post DNA synthetic periods known as G_1 and G_2. (Howard and Pelc,1953). This subdivision of the cell cycle into G_1, S, G_2, and M reflects the importance of the nuclear events since these subsections are defined by the state of the genetic material rather than the overall condition of the cell. Moreover, all other growth activities are ultimately geared directly or indirectly to the accomplishment of the reproductive nuclear processes. The primary aim of this study is to determine the interrelationship of two of these subsections, S and M, with respect to time in the colonial alga, Eudorina elegans.

Eudorina is a colonial, motile green alga belonging to the family Volvocaceae. Each colony or coenobium is composed of 16- or 32-biflagellated cells arranged peripherally in spheroidal double-layered gelatinous envelope. The cells, equal in size, measure 12 to 20μ in diameter; the colonies ranging from 80 to 160μ in diameter (Goldstein,1964). All cells in the colony are reproductive, each mature cell or gonidium undergoing four rapid sucessive cell divisions resulting in the formation of a 16-celled curved plate or

plakea. To complete daughter colony formation the cup-shaped plakea undergoes inversion. In this way each of the 16 cells of the maternal coenobium produces a new 16-celled daughter coenobium. Therefore, from the original 16-celled gonidium, 256 daughter cells are produced by 64 successive mitotic divisions. The cluster of 16 daughter coenobia enclosed by the maternal gelatinous envelope, designated a mulberry, soon breaks up releasing the progeny coenobia.

Under less than optimal growth conditions, the four mitotic divisions each mature cell undergoes occupy a total period of one hour within a life cell cycle time of five days or 120 h. (Rayns and Godward, 1965). The 120 h period is divided into a pre-mitotic period of 72 h and a mitotic interval and a post-mitotic period of 48 h during which cytoplasmic cleavage and plakeal inversion occurs. The series of four mitoses, each requiring 15 min , follow in rapid succession with an insignificant amount of time spent in interphase. These calculations (Rayns and Godward, 1965) are supported by observations of living cells of Eudorina sp. (Cave and Pocock, 1951) indicating that the time between the disappearance of the nucleolus and appearance of two daughter nucleoli is ten min. They noted that the nucleolus disperses at late prohase and reforms at telophase as is observed in higher plants.

The implication of the above observations is that the replication and segregation of the genetic material in Eudorina are not necessarily alternate events in the cell cycle. The uncoupling of the alternate occurrence of DNA replication and cell division could be responsible

for the limitation of the number of division events, and thus the maintenance of the coenobial nature of Eudorina. Since investigations of the driving forces for the continuation of the cell cycle, of the regulation of cell growth and of how the asexual reproduction of cells is controlled hinges primarily on the analysis of the regulation of DNA replication and segregation, any variance from the norm aid in the understanding of the chain of cause and effect that correlates cell functions for balanced growth and reproduction. The possibility that total DNA replication might occur during the long pre-mitotic period to be followed by multiple segregations reducing the DNA levels prompted the present study.

The progressive accumulation of nuclei with higher DNA contents formed by stepwise doubling of DNA without intervening reduction by mitosis has been noted for Tetrahymena, after heat shock induced synchrony (Jeffery et al.,1970). In Chlamydomonas reinhardtii the amount of DNA synthesized was not proportional to the degree of cell division within a single synchronous cycle under conditions of limiting light intensity (Kates et al.,1968). DNA replication may occur twice while only one nuclear division followed. As a result diploid vegetative cells were produced. During the subsequent light-dark cycle these cells divided without DNA synthesis yielding two cells with the normal 1C DNA complement. Four polyploid species of Chlamydomonas have been reported in which the production of daughter cells by amitosis was accompanied by a sequential reduction in chromosome number (Buffaloe, 1958). The existence of polyploid forms or of alternations of diploid

and haploid generations is also suggested for the colonial Volvocaceae (Cave and Pocock,1951). This variation in the genetic constituion suggested for some green algae indicates that the initiation of DNA replication and the process of cell division may be controlled by different mechanisms.

A survey of the literature of the mode of DNA synthesis in the unicellular green algae revealed the concensus that a round of DNA replication precedes each subsequent mitotic event. In synchronized Chlorella cultures four DNA replications and nuclear divisions alternated at equal intervals during an 8 h period (Wanka and Mulders,1967). Although earlier reports suggested that sufficient DNA synthesis for several divisions is completed well before the first division in Chlamydomonas (Bernstein,1963; Jacobson and Lee,1967), more recent evidence indicates that "when vegetative cells divide into four cells it appears likely that a round of DNA replication precedes each of the two consecutive nuclear divisions" (Kates et al.,1968). Nuclear DNA synthesis in synchronized cultures occurs in a diphasic manner over a five hour period, with the first doubling of nuclei commencing after the first doubling of DNA and the second nuclear division lagging behind the second doubling of DNA. In the colonial green flagellates, Volvox carteri (Yates and Kochert,1972) and V. aureus (Tucker and Daren,1972) nucleic acid synthesis is concomitant with increasing cell number during gonidial cleavage.

Although it is known that DNA replication does not occur during the interdivisional interphase for some unicellular and colonial green

algae, the precise interrelationship of mitosis, nuclear division, cytokinesis and DNA replication, with respects to timing, kenetics, interdependencies, and developmental stages, is not known for Eudorina. Based on the assumptions of Rayns and Godward (1965) that intermitotic interphases are very short or absent in Eudorina, it was decided that the temporal sequence of the cell divisional processes and DNA replication should be more closely studied. This study, then, was initiated with the purpose of verifying the cytological observations of Rayns and Godward and of discovering whether or not there is an uncoupling of the alternate occurrence of DNA synthesis and cell division in Eudorina. Hopefully, this study will lead the way towards the elucidation of the regulatory processes governing limited cell division in the colonial flagellates.

The information obtained in this study is presented in the following chapters (2 to 5) in individual manuscript format. Chapters 2, 4 and 5 have been submitted for publication, and chapter 3 is presented in the same format in order to maintain uniformity. A general bibliography as well as concluding chapter (6) have been added.

CHAPTER II

INTRODUCTION - THE INDUCTION OF SYNCHRONOUS CELL DIVISION IN *EUDORINA ELEGANS*

In *Eudorina elegans* Rayns and Godward (1965) reported that during vegetative growth each cell of a coenobium (colony) was capable of dividing 4 or 5 times within one hour. They noted a prolonged "interphase" period of about 5 days separating the blocks of rapid division sequences. In this same study it was noted that each division process took about 10 min with the interphase separating each division being insignificant. This essential lack of interphase in some Volvocales had been seen previously (Cave and Pocock,1951).

These observations appear to require either extremely rapid DNA replication or unique pattern of replication prior to the sequence of divisions. The latter is plausible since a breakdown of the normal relationship between DNA replication and nuclear division has been shown for heat synchronized *Tetrahymena* (Jeffery *et al.*,1970). In order to clarify the problem in *Eudorina*, more detailed analysis of the timing of cellular events was undertaken. A reasonable first step appeared to be an attempt to synchronize the organism in order to facilitate chemical and cytological examination of the cell cycle parameters.

This report outlines the development of procedures for, and evaluation of, synchronous growth in *Eudorina elegans*. As an initial result, a more precise estimate of the time intervals involved during the division processes was obtained.

MATERIALS AND METHODS

The Organism

An axenic culture of Eudorina elegans strain 1193 (originally isolated by Goldstein (1964) and designated 56 f) obtained from the Culture Collection of Algae, Indiana University (Starr,1964) was used in the experiments to be described. Stock cultures of the alga were maintained and periodically cloned as described by Wentworth (1970).

Media

The liquid media for growth of the alga were normally a modified Bristol's salts medium (BM) (Cain,1965) or complex enriched medium (BC) (Kemp and Wentworth,1971). Other media used during the course of this study included Bold's Basic Medium (BBM) (Cain,1965),Volvox medium (VM) (Starr,1971) and High Salt Medium (HSM) (Sueoka,1960). Additional modifications of the culture conditions will be introduced in the text where applicable. The liquid media were solidified as desired with 1.5% or 0.6% Difco Bacto Agar for plates or top agar respectively. All media were sterilized by autoclaving at 15 psi.

Standard Growth Conditions

Small liquid cultures were grown in 50 ml of medium in 200-250 ml erlenmeyer flasks. Facilitation of gas exchange and maintenance of the coenobia in suspension were achieved by continuous shaking of the cultures at the rate of 80-90 oscillations/min on a shaking platform. Normally, cultures were grown at 32 $\pm$ 1C (occasionally 22 $\pm$ 1C) under banks of 40-watt cool white fluorescent lights. This overhead illumination provided an intensity of about 10^4 ergs $cm^{-2}sec^{-1}$ and was automatically controlled by a time clock to give 16 h light and 8 h dark per 24 h period.

Large (4 l) liquid cultures were grown in glass cylinders. The algae were kept in suspension by means of a magnetic spin bar and by bubbling 3% or 5% CO_2 in air through the culture. Samples were removed by closing the air escape vents and forcing the algal containing medium through a sampling tube.

Viable Organism Counts

Titres of viable organisms were determined by mixing appropriately diluted aliquots with 2.5 ml of molten (45 C) top agar and pouring the mixture over BC agar plates. After the top agar had solidified, the plates were inverted and incubated under the standard light:dark (16:8) cycle at 32 C. The colonies on duplicate plates, visible after 5 or 6 days of growth, were averaged and the titre calculated. The use of a Coulter counter, haemocytometer, or turbidity measurements at 560 nm were found to be unsatisfactory for the present study.

Microscopic Studies

Aliquots of culture of Eudorina were concentrated by centrifugation, and resuspended in 3:1 (alcohol:acetic acid) or 3% glutaraldehyde as fixatives at room temperature and left overnight. The fixed organisms were washed thoroughly in 1.7×10^{-3} M phosphate buffer, and resuspended for 20 min in 3.5 N HCl at 37 C (Fand,1972). Following hydrolysis, and washing in 1 N HCl and water, samples were squashed between a microscope slide and cover glass in order to separate the cells of the coenobium and spread the cells of the developing embryos. The cover glass was removed after freezing the preparation, the material brought to room temperature, and stained in Schiff's reagent (Deitch,1966). Following bleaching in bisulphite solution, the preparation was

dehydrated and mounted in Permount.

Living organisms were observed passing through the sequential cell divisions in organisms squashed between a slide and cover glass. Considerable pressure was required to immobilize and to spread an organism sufficiently for observation, which distorted, at least, the timing of the division sequences.

RESULTS

Previous studies of growth of Eudorina elegans showed that a generation time (i.e. arbitrarily taken as the time necessary for a 16X increase in organisms) of about 24 h was possible (Wentworth,1970). Growth was in the enriched BC medium at 32 C, with a light:dark regime of 16 h light and 8 h dark per 24 h period. Light intensity was about 10^4 ergs $cm^{-2}s^{-1}$. Conditions resulting in synchronous growth of a variety of photoautotrophic organisms suggested that synchronous growth of Eudorina would probably require light:dark cycles in non-enriched minimal media (see Carroll et al.,1970), and that divisions would be restricted to the dark cycle. A wide variety of growth conditions were employed in an attempt to find culture conditions capable of supporting daily (circadian) cycles of growth in a minimal medium. These involved the use of the different minimal media, BM, BBM, HSM and VM as well as different light:dark regimes per 24 h period, temperatures (20 C to 38 C), and light intensities ($7.5x10^3$ to $2.6x10^4$ ergs $cm^{-2}s^{-1}$). Aeration of the culture with air containing 3 or 5% CO_2 at different flow rates was another variable tried in order to obtain a greater increment of organisms per 24 h cycle. Aeration with gas containing added CO_2 was not used with the inadequately buffered Volvox

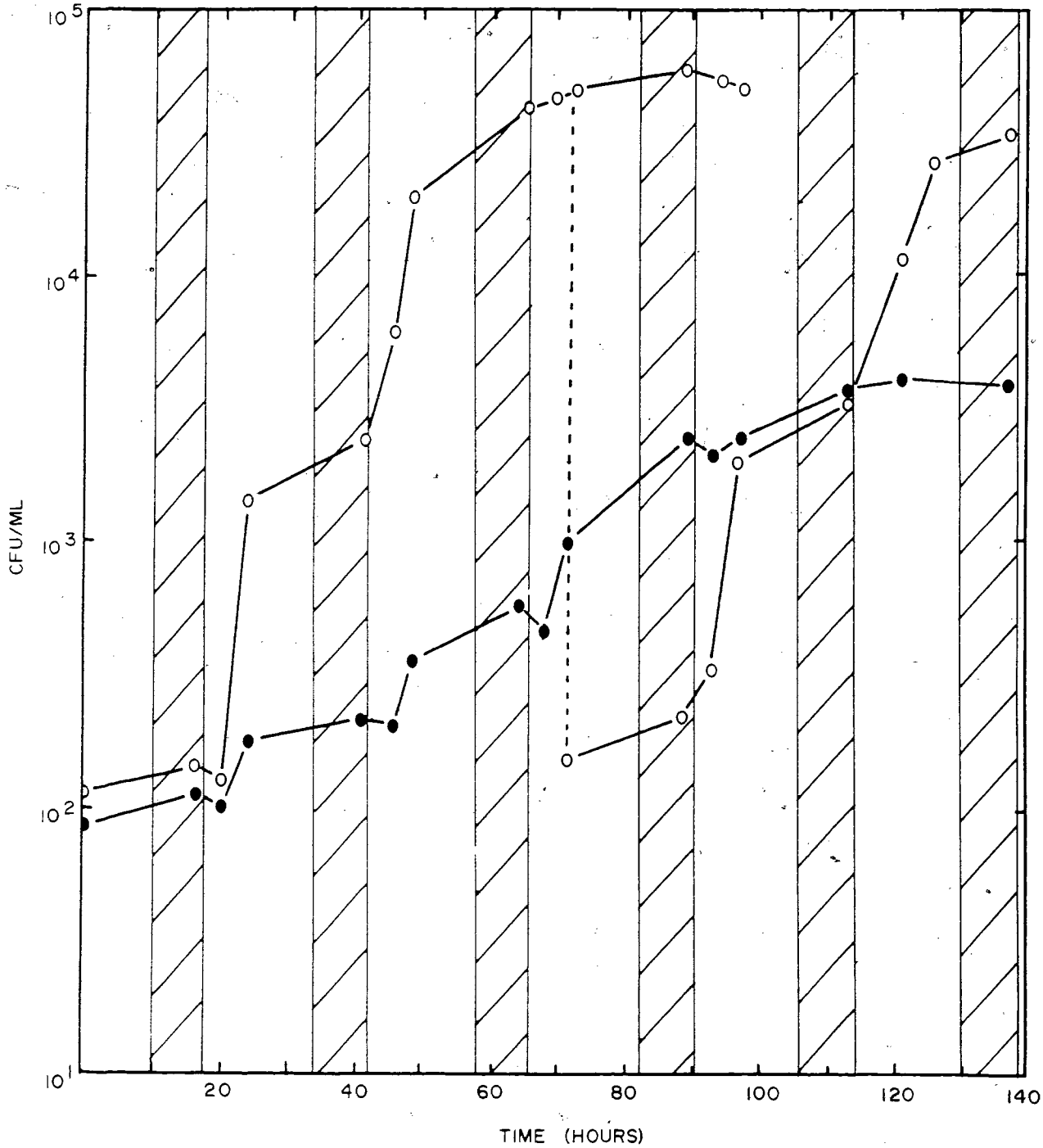
medium. These attempts failed to produce conditions which promoted a significant proportion of the population to divide during a 24 h period.

The results obtained with the BM medium were typical. The population grown in BM medium (fig. II-1) was capable of periodic release of organisms during the light period. The observed doubling of the number of organisms could be achieved by about 5% of the population releasing daughter coenobia. An apparent adaptation to growth in BM occured in (fig. II-1) since the third cycle had a greater growth increment when compared to the first and second cycles. However, this enhanced production of daughter organisms was not consistently maintained nor enhanced when the population was subcultured into fresh BM medium. The adapation was probably due to utilization of organic compounds released by either growing or autolysing cells.

An additional factor was observed during a series of experiments designed to determine the effect of different concentrations of inorganic supplements on the growth of Eudorina. Doubling the amount of Ca^{++} from 2.5×10^{-3} M to 5×10^{-3} M appeared to facilitate release of the daughter coenobia from the parental envelope. Consequently, the time required for this release was reduced. Since no adverse effects were seen, the higher Ca^{++} level was routinely incorporated into the medium.

Some enhancement in the number of coenobia capable of producing daughter organisms per growth cycle was observed by the addition of acetate to the minimal media. However, acetate concentrations much above 0.05% (6×10^{-3}M) were lethal. This is in contrast to the effect of acetate on growth of Chlamydomonas, where 0.2% (2.4×10^{-2}M) sodium acetate is routinely employed (Senger and Bishop,1969).

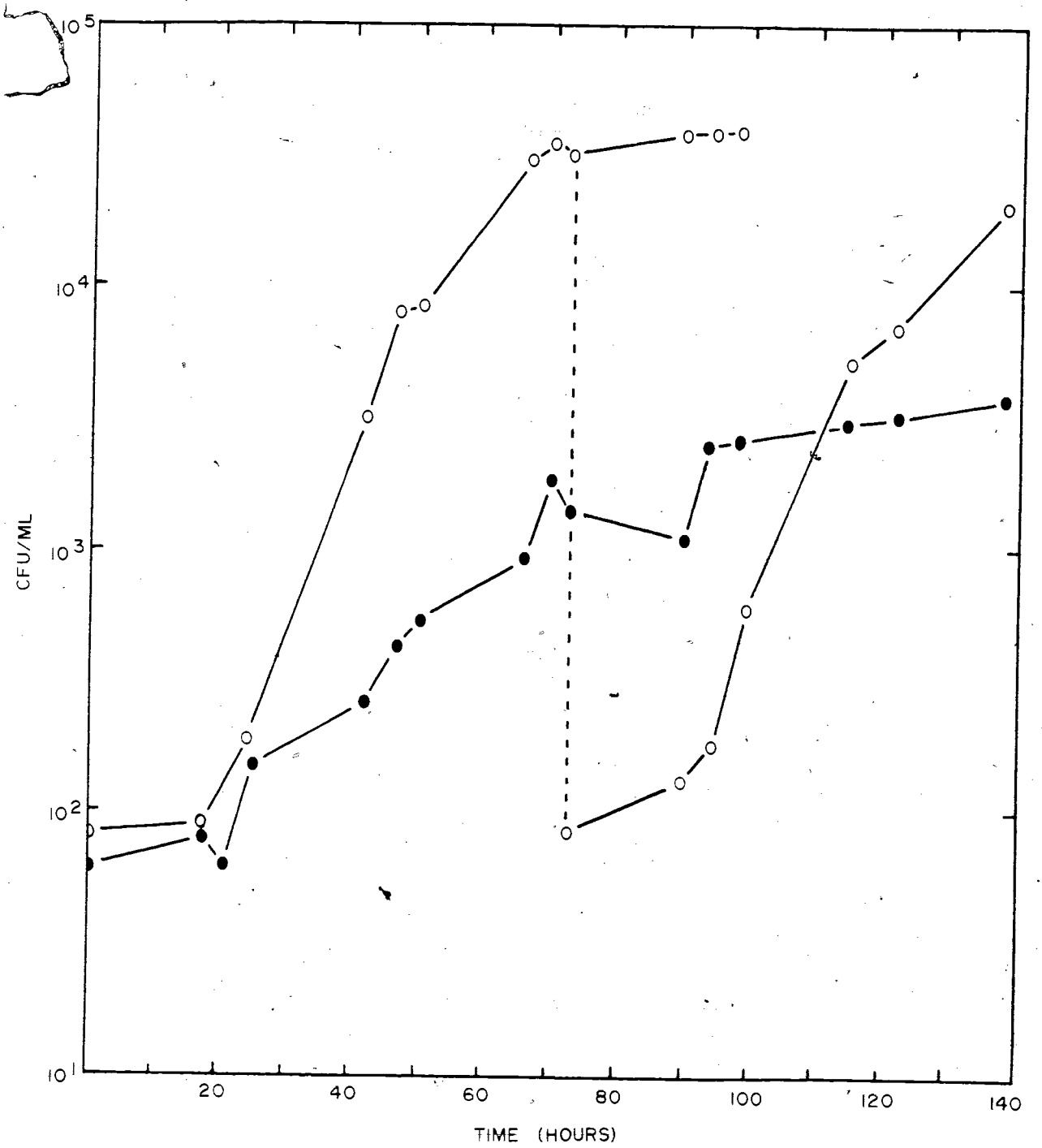
Figure II-1. Synchronous growth of E. elegans 1193 on a 16 h:8 h light:dark cycle at 32 C. Cultures were grown in complete medium (BC) (o) or minimal medium (BM) (•). Dilution from the early stationary phase culture to fresh BC medium is indicated by the dashed line.


10^5
10^4
10^3
10^2
10^1
CFU/ML
20
40
60
80
100
120
140
TIME (HOURS)

During routine growth of Eudorina in BC medium a major portion of the population occasionally released daughter coenobia over a 4 to 8 h period during the light cycle. Such synchronized release had also been observed when different light regimes were tried (cf. Fig. II-2), and an appropriately primed population in BC medium could begin division in the dark cycle. Synchronous release, as depicted in Figures II-1 and II-2, was initially not consistent, nor was the pattern easily maintained. Consequently, studies to improve the sucess rate were undertaken with attention being focused on the unique conditions of growth in the enriched BC medium and the occurrence of divisions during the light period.

A series of observations led to the development of consistent conditions maximizing the number of organisms which would divide and release daughter organisms during each growth cycle. The maximum population density which could be achieved in culture was about 10^5/ml, and as a result the amount of growth in a 24 h period depended on the initial coenobial concentration (Fig. II-3). A high initial concentration of organisms not only reduced the growth increment but also reduced synchrony. A young, dilute, well lighted, and well nourished culture produced a majority of 32-celled coenobia and relatively few 16-celled coenobia. The maximum increase expected upon dilution of such a culture would be 32X (Fig. II-4). Insufficient dilution not only reduced synchrony and the expected increase in daughter coenobia but also reduced the number of cells per daughter coenobium to 16. This reduction was reflected in the next cycle of growth (Fig. II-4).

Figure II-2. Growth of *E. elegans* 1193 in continuous light at 32 C. Cultures were grown in either complete medium (BC) (o) or minimal medium (BM) (•). Dilution from the early stationary phase culture to fresh BC medium is indicated by the dashed line.


10^5
10^4
10^3
10^2
10^1
CFU/ML
20
40
60
80
100
120
140
TIME (HOURS)
O-O-O
O-O

Synchronized growth of a variety of organisms has been achieved by initiating a culture with a uniform sub-population (Tamiya et al.,1961). Attempts to enrich populations of Eudorina for uniform sub-populations by differential centrifugation or natural sedimentation of the coenobia were unsuccessful. Centrifugation resulted in sedimentation of essentially all the coenobia, regardless of the speed or duration of the centrifugation. Also, a uniform correlation between the coenobial size and the rate of their settling through a column of water was not observed.

A large proportion of a coenobial population could be induced to divide synchronously upon regrowth in BC following cold shocks at 4 C for 24 h, or starvation in phosphate buffer for 15-20 h. It is probable that several alternating cycles of starvation or cold and regrowth would synchronize the majority of the population. However, these experiments were not carried out since a more convenient and presumably less traumatic method to obtain synchronously dividing populations was developed.

When an asynchronous culture was grown to stationary phase, the population became quite uniform. The coenobial size was small, characteristic of newly released organisms. Dilution of this population to about 100 organisms per ml, 8 to 12 h before the onset of the dark period, resulted in synchronous release of daughter coenobia soon after the next light period began. As depicted in Fig. II-1, the synchronous release was repeated during the next light period. The increments usually obtained from such population ranged from 16X to 25X suggesting the stationary population contained either a majority of 16-celled

Figure II-3. The effect of initial population density on synchronous growth of E. elegans 1193. Aliquots of the same washed culture (o) were transferred to fresh BC medium and grown at 32 C on a 16:8 light: dark cycle. The lights were off from hour 8 until hour 16 and from hour 32 until hour 40. The filled symbols (•) represent the growth of a culture started from a culture that had completed a previous synchronous increase at a low organism density (cf. Fig. 4).

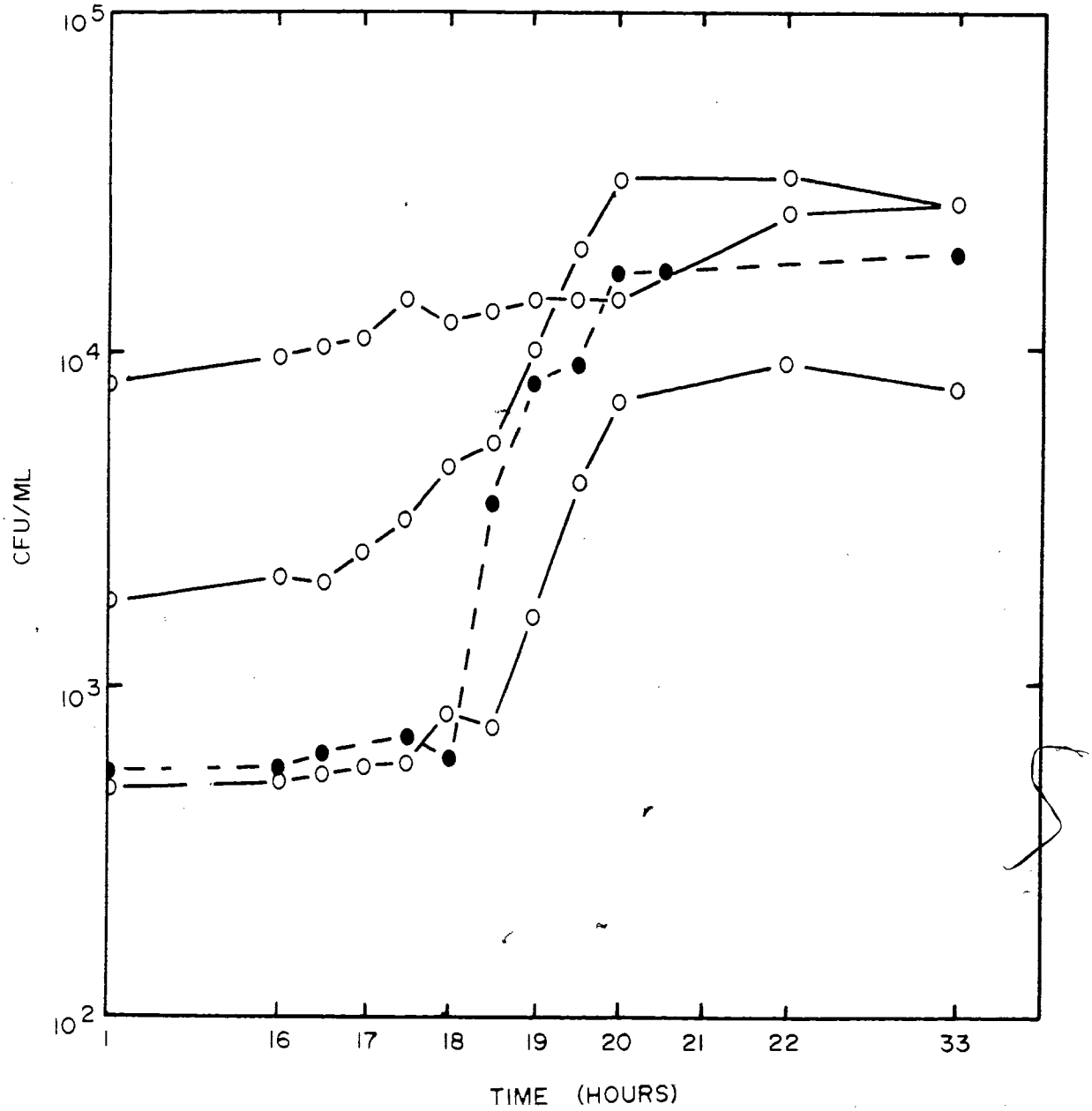
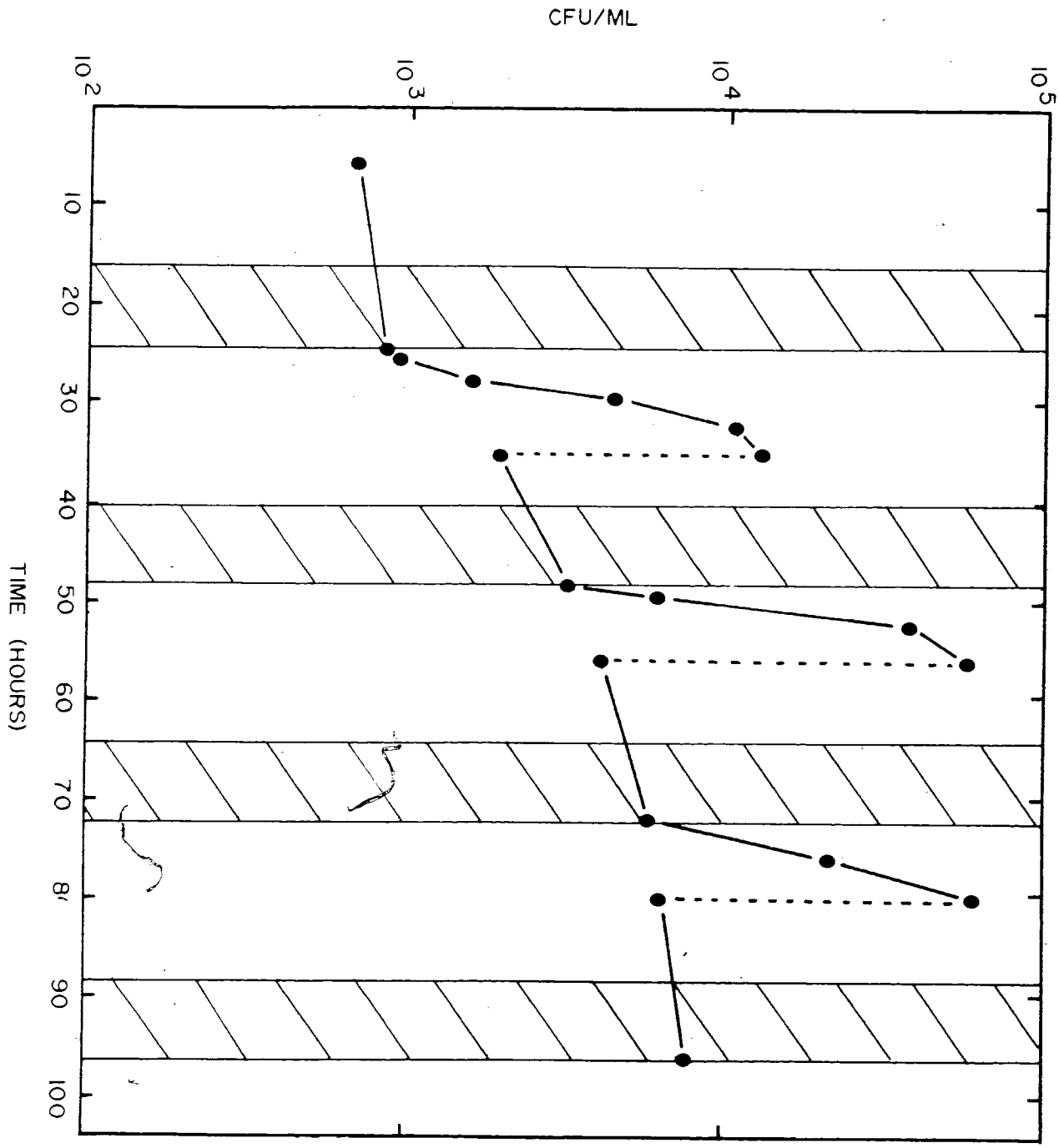

10^5
10^4
10^3
10^2
CFU/ML
1
16
17
18
19
20
21
22
33
TIME (HOURS)

Figure II-4. The effect of population density on growth increase of E. elegans 1193 during subsequent cycles. A synchronous culture maintained at low density was diluted into fresh BC medium and incubated at 32 C on a 16:8 light:dark cycle. Daily dilution of the culture maintained synchronous growth. The increases during each cycle, corrected by adding the increase due to late division, were 24X, 25X, and 12X respectively.


CFU/ML
10^2
10^3
10^4
10^5
TIME (HOURS)
10
20
30
40
50
60
70
80
90
100

organisms or various proportions of 16- and 32-celled organisms. Dilution of the culture after the second cycle maintained the synchronous pattern (Fig. II-1). Although these dilutions were necessary to maintain the concentration of organisms within the range for exponential growth, the light cycles were critical for the maintenance of the above synchrony of the system (Fig. II-2). In this case, the same population used to initiate the culture grown on the L:D cycle (Fig. II-1) was diluted and grown in continuous light. The initial lag before resumption of growth was still evident, but the subsequent growth was exponential for about 40 h. When this culture was diluted after reaching the early stationary phase, the same lag followed by an exponential phase growth pattern was repeated (Fig. II-2). If dilution occurred during the exponential phase, the lag phase no longer occurred and growth continued exponentially.

Maintenance of synchronous growth usually involved daily dilution, following release of the daughter coenobia, to a starting density of from $5X10^2$ to 10^3 coenobia per ml. This usually involved about a 25X dilution. When a synchronously growing culture was lost, a new one could be initated from a stationary phase culture. Microscopic examination of the stationary phase culture ensured that the population was uniform, and that it had not begun senescent decline. This decline was recognized by the presence of abnormal coenobia fragmentation.

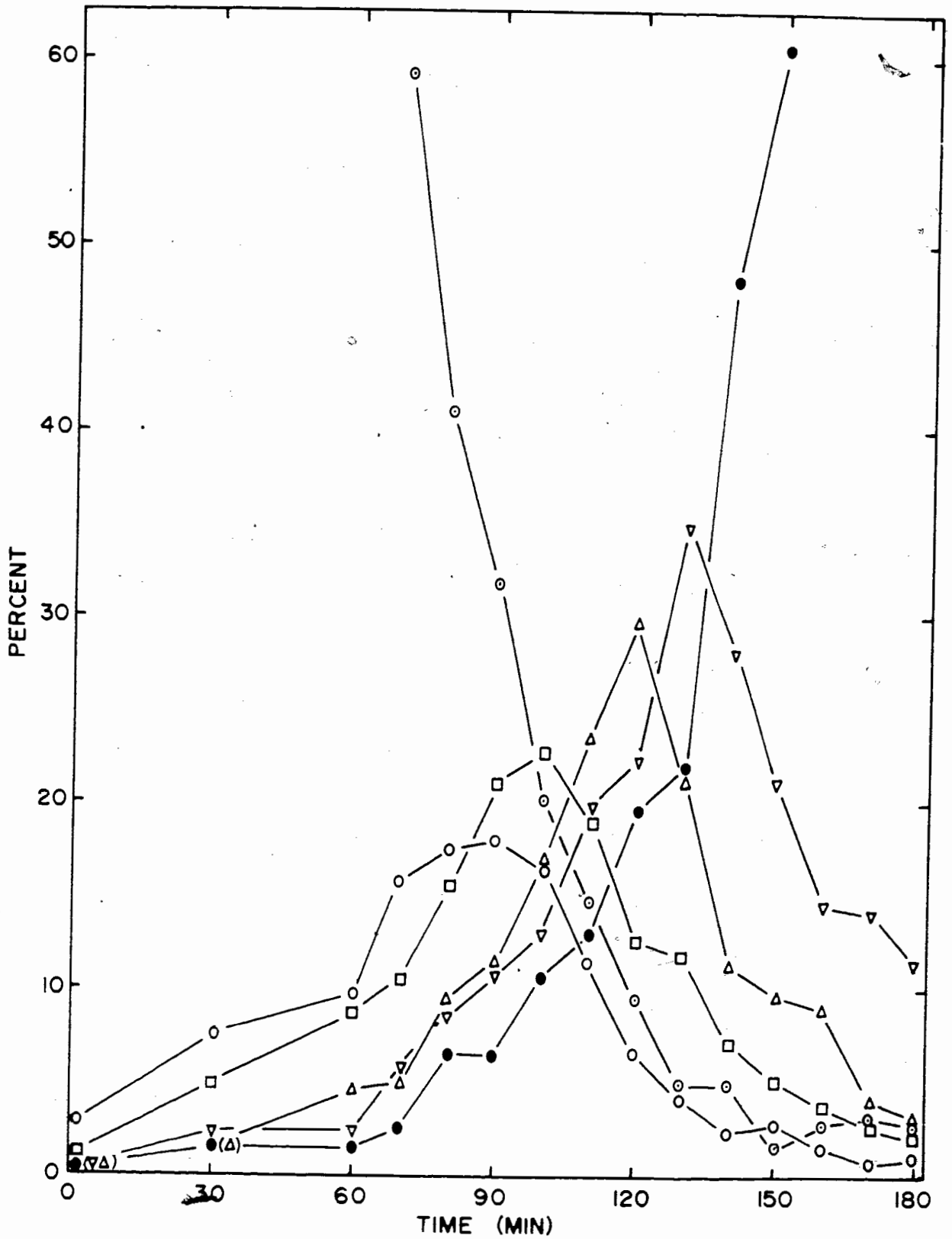
Evaluation of Synchrony

As noted in the previous section, the initial criterion for synchrony was the periodic release of daughter coenobia over a brief period every 24 h. A more detailed examination of the nature and timing

of nuclear and cell division was carried out on populations synchronized by dilution and growth in BC medium on a light:dark (16:8) cycle. The number of nuclei per developing daughter colony was counted as a function of time in aliquots taken at intervals from a synchronous population. The frequency distribution is presented in Fig. II-5. In this population, about 80% of the cells divided to form 32-celled daughter coenobia, the remainder forming 16-celled coenobia. The culture could be divided into two principal cell populations; about 25% started divisions during the first hour (Fig. II-3), while 65% started during the second hour. Less than 5% began the sequence in the third hour. Very few cells divided before the major segments of the populations, and about 2% of the population remained undivided after the third hour.

The overlapping of the distributions of the various nuclear classes reflected the asynchrony of the population, as well as the inherent gradient of development existing within each organism involved in vegetative replication. The progression of the division numbers where the 2-nucleate daughter coenobium became 4-nucleate, then 8-nucleate, and so on, suggested that the frequency data could be replotted as a progression of sequential events. To do this the number of 32-nucleate daughter coenobia present at each time was adjusted by adding the number of 16-nucleate embryos present in the same sample. The 16-nucleate embryos were similarly "corrected" by adding the 8-nucleate embryos seen in the appropiate sample to the "corrected" 32-nucleate coenobia. This procedure was repeated until finally the corrected 2-nucleate stage was obtained. These transformed data are presented as Figure II-6.

Figure II-5. Frequency distribution of nuclear numbers in developing daughter colonies of a synchronous population of E. elegans 1193. The nuclear numbers are: mononucleate premitotic (⊙), 2 nucleate (○), 4 nucleate (□), 8 nucleate (△), 16 nucleate (▽), and 32 nucleate (●).


60
50
40
30
20
10
0
PERCENT
0
30
60
90
120
150
180
TIME (MIN)
(▽△)
(△)

The regression lines were calculated for the linear portions of the curves, and from the slopes of these lines the X-intercepts were determined. In this population of Eudorina the treatment of the data introduced a slight error because not all the 16-celled daughter coenobia became 32-celled. Since it appeared to be a constant error for all values no correction was attempted.

Division Intervals

An estimate of the duration of each nuclear division (i.e. interphase to interphase) was made by the following methods. First, the interval can be obtained directly from the differences in the values of the X-intercepts of the regression lines calculated from the progression of the nuclear number classes (Table II-1). Second, the areas under the frequency distribution curves represent the relative duration of each stage. Therefore, each area as a fraction of the total of the areas was determined by weighing the curves of each nuclear number class. Each area divided into the total average time for the division sequence gave an estimate of the time required for each division. The time from the disappearance of 50% of the mononucleated cells to the appearance of 50% of the 32-nucleated daughter coenobia gave a value for the interval from the end of the first mitosis to the end of the fifth mitosis. Inspection of Figure II-5 showed this time to be 62 min. The duration of the calculated intervals between successive divisions is given in Table II-2. A third measure of the intervals was obtained by direct observation of the time between cytokinetic events occurring in the development of the individual daughter coenobia. This assumed that karyokinesis and cytokinesis were coupled in this rapidly dividing

Figure II-6. Progression of mitotic events replotted from Figure 5 as described in the text. The nuclear numbers are: 2 nucleate (o), 4 nucleate (□), 8 nucleate (△), 16 nucleate (▽), and 32 nucleate(●). The dashed lines represent the regression lines determined from the increasing portions of each curve.

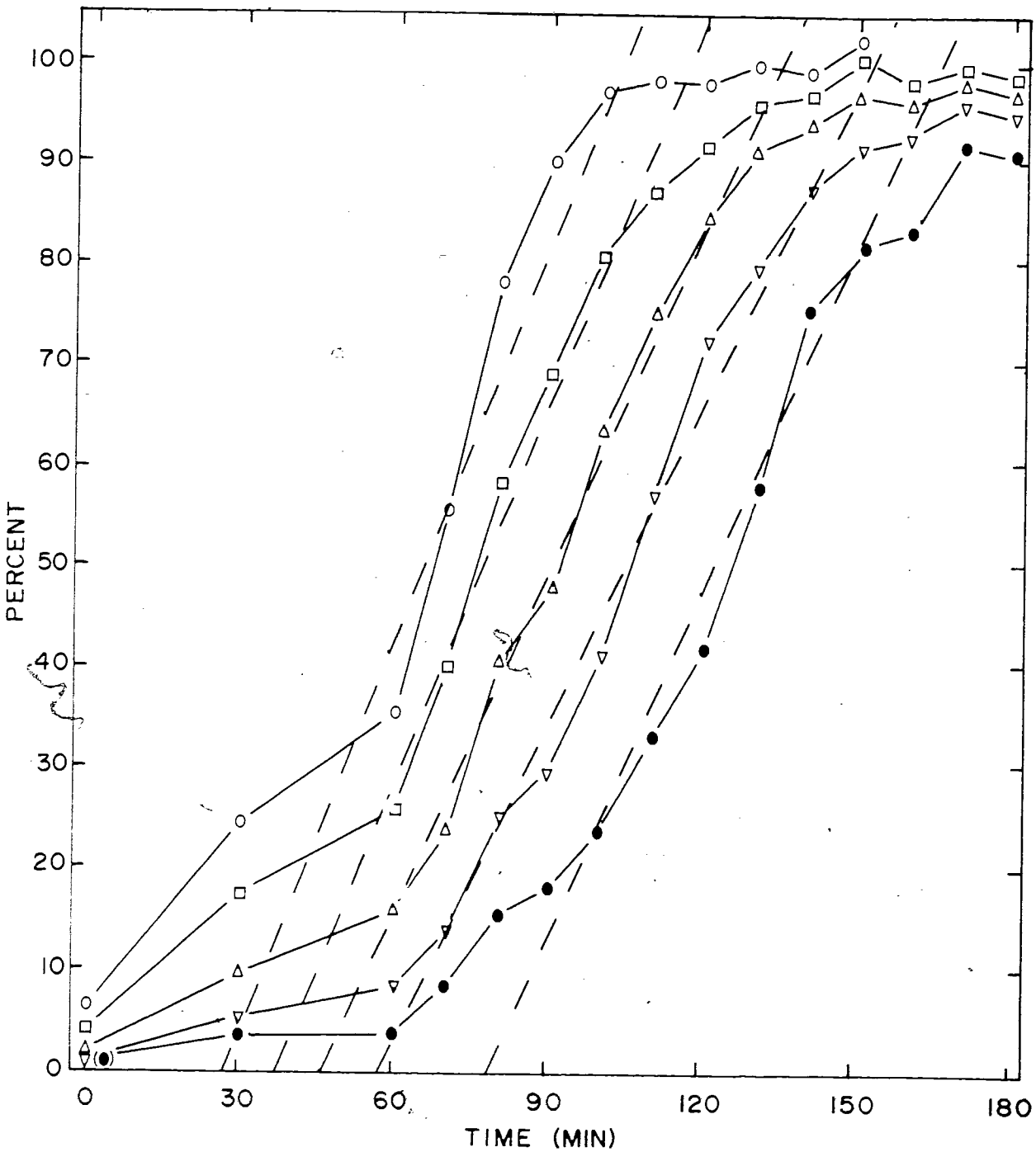

PERCENT
TIME (MIN)
0
10
20
30
40
50
60
70
80
90
100
0
30
60
90
120
150
180

TABLE II-1 The interval between successive nuclear and cell divisions in Eudorina calculated from the data in Figs. II-6 and II-8.

Interval Nuclear (cell) Number	Duration (Min) Nuclear	Cell
2 - 4	11	13
4 - 8	9	12
8 - 16	11	8
16 - 32	21	25
Total	52	58

The valves were obtained from Figs. 6 and 8 as the time separating the extrapolation of the regression line for each stage to the X axis (frequency = 0). The valves are given to the nearest minute.

TABLE II-2 The interval between successive nuclear divisions in *Eudorina* calculated from the frequency distribution curves in Figs. II-5 and II-7.

Interval (Nuclear Numbers)	Relative Area		Time (Min)
2 - 4	1.0		13
4 - 8	1.198		15
8 - 16	1.223		16
16 - 32	1.381		18
		Total	62

The values were obtained by weighing the frequency curves for each class and dividing the total interval (62 min) required for the disappearance of 50% of the mononucleate cells and the appearance of 50% of the 32 nucleate daughter coenobia.

system. In order to keep a given cell and its derivatives under observation the parent coenobium had to be compressed between a cover glass and the slide. Division did continue under these conditions, but was slowed down and required about twice as long to complete the processes as the time obtained by the previous methods (Table II-3).

Assuming a uniform interval separating divisions, an average interval was obtained by dividing the observed interval of 62 min by the number of divisions, i.e. 4, occurring in the interval. An average time of 15.5 min separated divisions.

Light microscopic examination of Feulgen stained cells suggested that cytokinesis does follow almost immediately after each nuclear division. The information concerning the number of cells per developing coenobium, derived from the same population used to examine nuclear numbers, is presented in Figures II-7 and II-8. As expected these curves depicting cell numbers were very similar to those for nuclear number per developing coenobium. The time between cytokinetic events was usually 3 to 5 min longer than the interval for corresponding nuclear events. The discrepancy of times at the 8 to 16-celled stage may be due to a reduction in 16-celled organisms caused by their release as mature coenobia.

Mitotic Index

The number of nuclei engaged in division at each stage in the developing coenobia was quite uniform and averaged 12.4% (Table II-4). Although the nuclear divisions within each developing embryo were synchronous the developing embryos within a single coenobium were not synchronous due to a staggering of the entry of the cells into division.

TABLE II-3 The time required for successive cell divisions obtained from direct observation of gonidial cleavage.

Stage	No. of embryos, observed	Time(Min)± S.E.
2 - 4	17	22.9 ± 2.2
4 - 8	14	28 ± 1.8
8 - 16	9	26.7 ± 1.7
16 - 32	2	32 ± 1.6

Figure II-7. Frequency distribution of cell number in developing daughter colonies of a synchronous population of E. elegans 1193. Symbols are the same as in Figure 5.

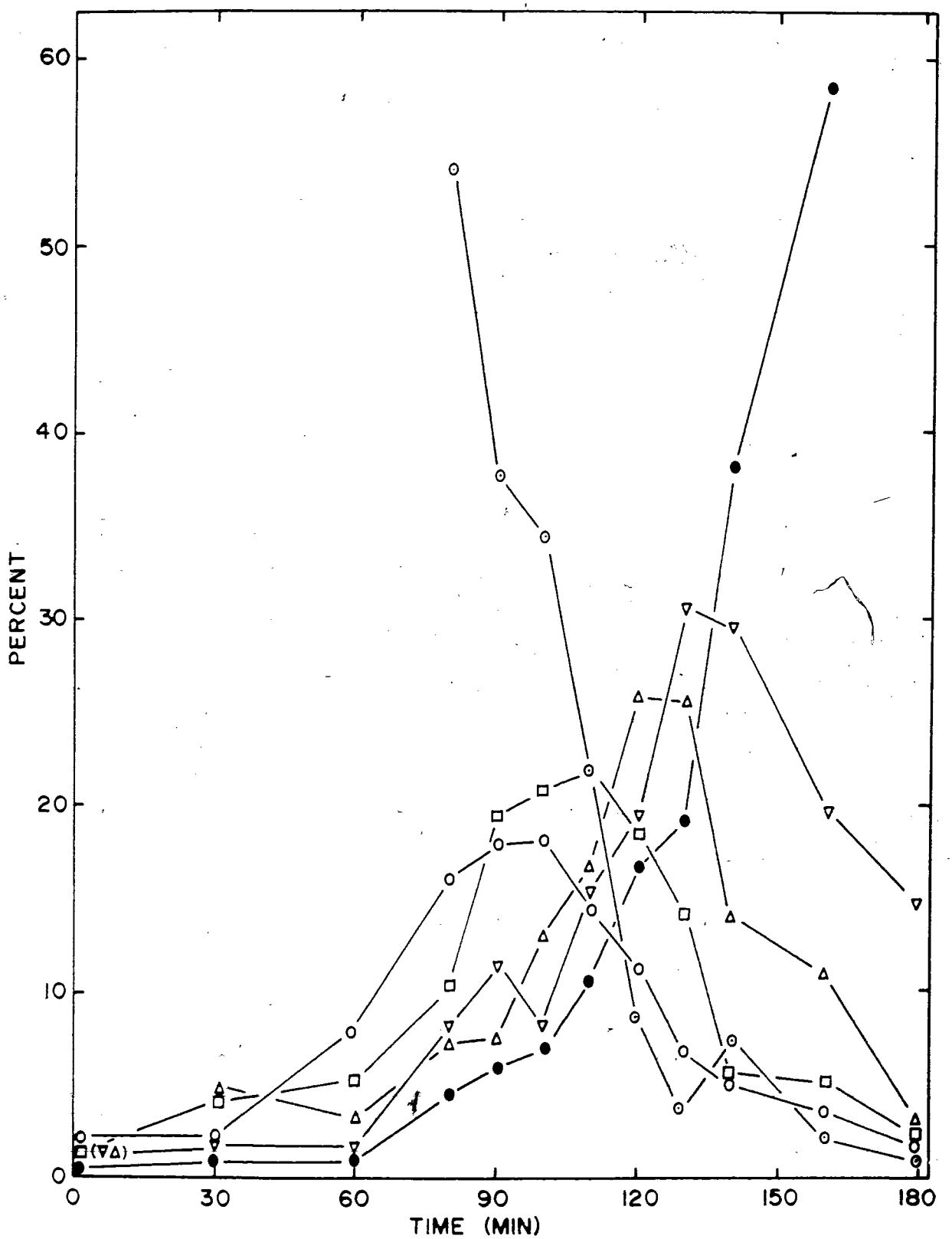

60
50
40
30
20
10
0
PERCENT
0
30
60
90
120
150
180
TIME (MIN)
(▽△)

Figure II-8. Progression of cell division events replotted from Figure 7 as described in the text. Symbols are the same as in Figure 6.

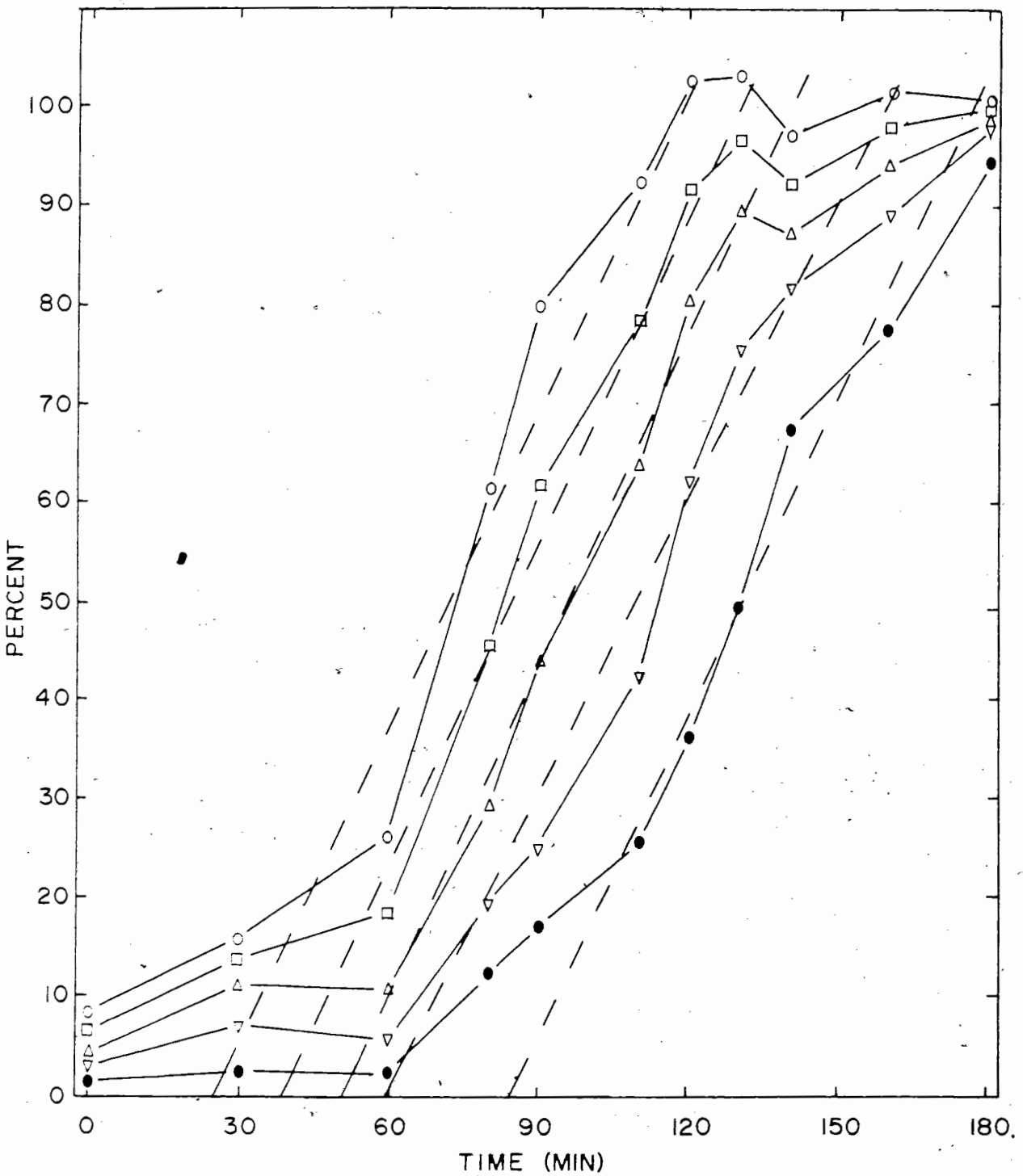

PERCENT
TIME (MIN)
0
10
20
30
40
50
60
70
80
90
100
0
30
60
90
120
150
180.

TABLE II-4 The percentage of cells engaged in mitotia of the 2, 4, 8 and 16 celled stages of development.

Cell Stage	% Mitotic Figures
2	13.6
4	11.9
8	13.4
16	11.7
Average	12.4

An aliquot of a synchronous culture was fixed, hydrolysed and stained by the Feulgen procedure. About 100 developing daughter coenobia at each stage were scored.

Release of the daughter coenobia from the parental envelope usually did not occur until development of all the coenobia within the envelope was complete.

DISCUSSION

Populations of Eudorina elegans 1193 grown at 32 C can be synchronized by light-dark cycles (16:8) provided an enriched medium supports growth and the pre-division population density is maintained below about 10^3 organisms per ml. Under these conditions cell division usually occurs during the light phase.

The establishment of procedures to synchronize Eudorina is viewed as a technique permitting studies of the relationship between DNA synthesis and cell division. Therefore, a systematic study into the enviromental factors which may regulate the induction of cell division was not undertaken. However, several observations, both casual and systematic, give some insight into factors which influence the degree of synchrony as well as the time during the light:dark cycle where divisions occur. These will be discussed briefly because of their potential usefulness in any subsequent studies on synchrony and development in Eudorina.

Synchronization of many photoautrotrophic organisms is believed to occur by limiting the growth in the light to a degree compatible with maintenance of growth and predivision development only (Bernstein, 1960;Schmidt,1961). The organisms that reach this level of development then divide during the ensuing dark period. Growth during the dark period is discouraged by culturing the organisms on a minimal medium. Repetition of the photoautotrophic growth periods and the

dark, nongrowth (but dividing) periods eventually entrains the population to a relatively high degree of synchrony.

However, Eudorina must be grown mixotrophically in order to obtain a significant proportion of the population capable of undergoing division during a single 24 h growth cycle. In addition, cell division usually occurs during the light period. Synchronization of Chlorella using mixotrophic conditions has been reported (Senger and Bishop,1969). Although the addition of 0.5% glucose allowed cell material production to continue during the dark period, cell division still occurred in the dark. This suggested that Chlorella is dependent on light for sufficient growth to support cell division.

The necessity of a regular photoperiod for the maintenance of synchrony in Eudorina is evident, but the exact role of the light: dark cycle in synchrony induction is unclear. Perhaps the light period is required to supplement the energy requirements of the cells and to reduce the organism's generation time to within a circadian period. The fact that synchronized cell divisions can be initiated using either continuous light or light:dark regimes suggests that timing of events in Eudorina may not be triggered by the light:dark shifts. A constant time period between cell divisions and the onset of the light period was reported for Chlorella (Tamiya et al.,1961). Its absence in Eudorina suggests that Eudorina lacks a specific pacemaker (Zeitgeber). Consequently, Eudorina may respond more readily to relatively minor changes in the culture enviroment. The ability of an organism not to divide or of an individual cell within a coenobium to adjust the number of nuclear divisions to 4 under

conditions of slight crowding or to 5 when population densities are low is perhaps one such response. These adjustments may enhance synchrony since the mitotic gradients which exist within each coenobium may be dampened by this capacity of each cell to independently complete either 4 or 5 mitotic divisions. It is also possible that all the cells of organisms starting division later than the majority of the population may be stimulated to complete only 4 divisions. For these organisms, daughter coenobial release would coincide more closely with the bulk of the population. Specific information regarding this latter possibility was not sought and it remains speculation.

EVALUATION OF SYNCHRONY

Since this work reports the first systematic attempt to synchronize Eudorina, an evaluation of the success will be compared to an "ideally" synchronized culture. The criteria used are those of (Senger and Bishop,1969). (1) All cells in the population should divide within one generation time, (2) the number of mitotic products should be constant for each cell in the population, (3) optimal growth conditions resulting in the shortest generation time should be achieved and maintained, (4) the synchronizing procedure should cause a minimum amount of change in the physiological state of the cells, and (5) the duration of the burst of cell division should be as short as possible.

In Eudorina usually less than 3% of the cells do not divide within one growth cycle. However, the number of mitotic products varies, being either 16 or 32. The growth conditions appear to be near optimal since attempts to further enhance the growth rate were unsuccessful. In addition, a comparison of the growth rate of E. elegans 1193 with

those tabulated for other green algae (Hoogenhaut and Amesz,1965) reveals that very few algae achieve the growth rate found in the present study. The major aligning force for synchrony in Eudorina appears to be alternating light:dark cycles. The physiological changes were considered to be minimal because the growth medium is enriched, and therefore the metabolic deprivation encountered upon shift to the dark is reduced. The synchronized population requires about 2.5 h to complete cell division. Therefore it is considered that Eudorina elegans 1193 approaches the ideal for a synchronized system.

The synchronization index (SI) formulated by Scherbaum (1962;1964) is a means of comparing the degree of synchrony achieved in different systems.

$$SI = 1 - \frac{t + \tilde{\tau}(2-n)}{1.12\tilde{\tau}}$$

where: n = fraction of cells dividing synchronously + 1, t = duration of the spread of division events, and $\tilde{\tau}$ = gt = generation time during normal logarithmic growth.

In Eudorina the period over which nuclear division occurred for about 97% of the population is 2.5 hours. A value for n = 1.97 (0.97 + 1) is typical and $\tilde{\tau}$ = 24 h is regularly achieved. The calculation SI value = 0.88. The mitotic gradients found in Eudorina can contribute as much as 0.75 h to the spread of division events, a feature which reduces the SI values from 0.91 to 0.88. However, the SI value of 0.88 compares favourably with tabulated values ranging from 0.20 to 0.80 (Scherbaum,1962), while a SI value of 0.83 reported for Chlamydomonas moewusii was considered "perhaps the highest of any species to date" (Bernstein,1960). A SI value of 0.63 has been obtained

for E. elegans (Rayns and Godward,1965), but the credibility of the value may be questioned since they report a generation time of 5 days and maximum values for cell division of 10% per day.

On the basis of the results reported and discussed here the present system probably represents one of the best examples of induced synchrony exhibited by any organism.

Mitotic Time

The mitotic time is the time required by one nucleus to complete the kinetic processes of division (prophase to telophase). Since E. elegans undergoes a typical mitosis (Cave and Pocock; Goldstein,1964), a mitotic time can be calculated from the proportion of nuclei in division compared to the proportion in interphase, given that the time interval between divisions is known. The proportion of nuclei in division in a synchronous population of Eudorina is 12.4%, and an average time interval between divisions is 15.5 min. The calculated time for mitosis is a rapid 2 min with an interphase period of about 13 min. This value differs appreciably from the mitotic time of 10 min obtained by Rayns and Godward (1965). They made the assumption that "the series of mitoses follow in rapid succession, any time spent in interphase being insignificant compared with the mitotic time". The studies reported here show that about 20% of the time utilized by Eudorina for the division sequence involves the kinetic aspects of the cell cycle, the remainder is the interphase period. These data do not provide evidence on the temporal relationship of DNA replication and the growth cycle. However, sufficient time appears to exist for replication of DNA to immediately precede each nuclear division.

CHAPTER III

INTRODUCTION - ISOTOPIC LABELLING OF NUCLEIC ACIDS IN E. ELEGANS

The problems involved in specifically labelling the DNA of the algae are well known (Wanka et al.,1964; Sheridan and Steffensen,1965). The DNA precursors thymine or thymidine are incorporated into nuclear DNA at very low efficiencies. This low level incorporation into nuclear DNA appears to result from the failure of the exogenously supplied compounds to be phosphorylated in vivo. The salvage phosphorylating enzyme, thymidine kinase, was not detected when it was specifically looked for in Chlorella (Wanka et al.,1964). However, thymidine labelling of chloroplast DNA has been demonstrated in some marine algae (Sheridan and Steffensen,1965) and in Chlamydomonas (Swinton and Hanawalt,1972). In Eudorina an uncharacterized minor fraction was labelled with thymidine which yielded thymine dimers upon ultraviolet irradiation (Kemp et al.,1972). They suggested the fraction might be chloroplast DNA, although no evidence is given. Labelling of the nucleic acids of Chlorella by exogenously supplied uracil or uridine has been achieved (Wanka et al.,1970). Adenine was effective in labelling the nucleic acids of Chlamydomonas (Chiang,1968).

The present study examined the ability of Eudorina to incorporate exogenous nucleic acid precursors into DNA and RNA. Although low levels of incorporation were detected, no correlation existed between the period of nuclear DNA and the uptake of radioactivity.

MATERIALS AND METHODS

Eudorina elegans 1193 (Indiana University Culture Collection of Algae)

was used for these studies and was usually grown as liquid suspension cultures in either enriched (BC) medium or minimal (BM) medium (Kemp and Wentworth,1971) at 32 C. The standard light intensity of 100 ergs $mm^{-2}s^{-1}$ was used during the 16 h light phase which alternated with an 8 h dark phase. Synchronous cultures could be obtained under these conditions (Chapter II).

Radioisotopes: Tritium labelled compounds were obtained from Amersham-Searle and the manufacturers specifications of specific activity and purity were used. The compounds were: thymine (^{3}H-T), 23.6 Ci/mM; thymidine (^{3}H-TdR), 27.8 Ci/mM; adenine (^{3}H-A), 15 Ci/mM; uracil (^{3}H-U), 25.8 Ci/mM.

Incorporation studies: Cultures of E. elegans were usually grown for 2 days in 0.1 to 2.0 uCi/ml added to the BC or BM media. Uptake of the isotopically labelled compounds was examined by removing aliquots of the culture at intervals following the addition of the compound.

Extraction of labelled material from Eudorina. Labelled cultures were harvested by centrifugation, washed 3 times with Na-phosphate buffer (1.7×10^{-3}M, pH7) before extracting the cells by a modified Smillie and Krotkov (1960) procedure. The following steps were carried out at 4 C. The culture was washed 2 times with absolute methanol, once with 5% TCA, and once with 95% ethanol. The residue was extracted for 2-3 min with boiling ethanol:ether (2:1). Radioactivity detected in a second ethanol:ether extraction was usually at a background level. RNA was removed by 4 hydrolyses in 0.2 N KOH at 70 C for 20 min. The material was cooled, acidified, and washed with cold 95% ethanol after each KOH extraction. The radioactivity was at background levels in

the fourth extract.

DNA was extracted from the pellet by hydrolysis with 5 or 10% TCA or 5% PCA for 15 min at 90 C. This extraction was repeated 3 times reducing the radioactivity in the final extract to background levels. The residue remaining was considered to be the protein fraction.

Chromatographic procedures. Paper chromatography was used to examine the fidelity of the isotopic incorporation. The RNA and DNA samples were first lyophilized then redissolved in 80% formic acid and hydrolyzed at 175 C for 30 min in sealed glass tubes. The protein fraction was dissolved directly in the formic acid and hydrolyzed. The hydrolyzed material was taken to dryness under a stream of nitrogen, resuspended in a minimal volume of 0.1N HCl and spotted on Whatman 3MM chromatography paper. Descending chromatograms were developed in butanol:acetic acid: water (80:12:30v/v) (Smith,1963) for 18-22 h at room temperature.

Radioactivity determinations. The dried chromatograms were cut into 0.5 cm segments and eluted in 0.5 ml of 0.1N HCl before adding 10 ml of Dioxane based scintillation fluid. The samples were counted using a Packard TriCarb liquid scintillation counter.

Radioactivity measurements of liquid extracts of labelled material were performed by adding aliquots directly to the scintillation fluid. The protein residue was first solubilized with Soluene 100.

Chemical estimation of nucleic acids and protein. DNA was estimated by the diphenylamine procedure (Burton,1956), RNA by the orcinol reaction (Markham,1955), and protein by the method of Lowry et al., (1951).

RESULTS

Isotopically labelled adenine, thymine, thymidine, and uracil were incorporated to a limited extent into the nucleic acids of Eudorina (Table III-1). Attempts were made to increase the efficiency of incorporation. These included nutrient reduction or starvation prior to addition of the labelled precursor, increasing the concentration of the labelled material, inclusion of adenosine in the incubation medium (Lark, 1960; Klenow, 1962), and attempted isolation of a thymine-requiring mutant using the folic acid antagonist, amethopterin, as the selecting agent (Wachman et al., 1962). None of these procedures were successful.

Despite the low levels of labelling found in Eudorina (i.e. less than 1% of the amount expected if incorporation proceeded to equilibrium) the measurement of the uptake of adenine and thymidine during synchronous growth was attempted (Fig. III-1). The isotope was present throughout the synchronous cycle and samples were removed at intervals for analysis of the radioactivity incorporated into the DNA and RNA fractions. Adenine incorporation was maximal in both RNA and DNA within 8 h or less from the time of isotope addition. The low level of thymidine uptake occurred mainly during 2-6 h period immediately following the addition of the isotope (Fig. III-2). The addition of the isotope during either the light or dark periods of the daily cycle did not influence the pattern of uptake (Fig. III-2). Therefore, the period of precursor uptake did not correspond with the period of synchronous synthesis of DNA.

From these results the question of incorporation vs non-specific binding became important. Previous studies had shown that thymine was extensively metabolized in Eudorina while thymidine could be recovered

TABLE III-1 The extent of incorporation of labelled nucleic acid precursors into acid precipitable material.

PRECURSOR	DPM/CFU	
	RNA	DNA
THYMINE	-	0.05-0.22
THYMIDINE	-	0.008-0.12
ADENINE	11.3	0.45
URACIL	0.06	0.012

Figure III-1. The uptake of labelled adenine into RNA (▽) and into DNA (▼) and of labelled thymidine into DNA (●) during synchronous growth. The increase in CFU (○) and relative increase of DNA (×) are also shown. The shaded bar indicates the dark period of the 16:8 light:dark cycle.

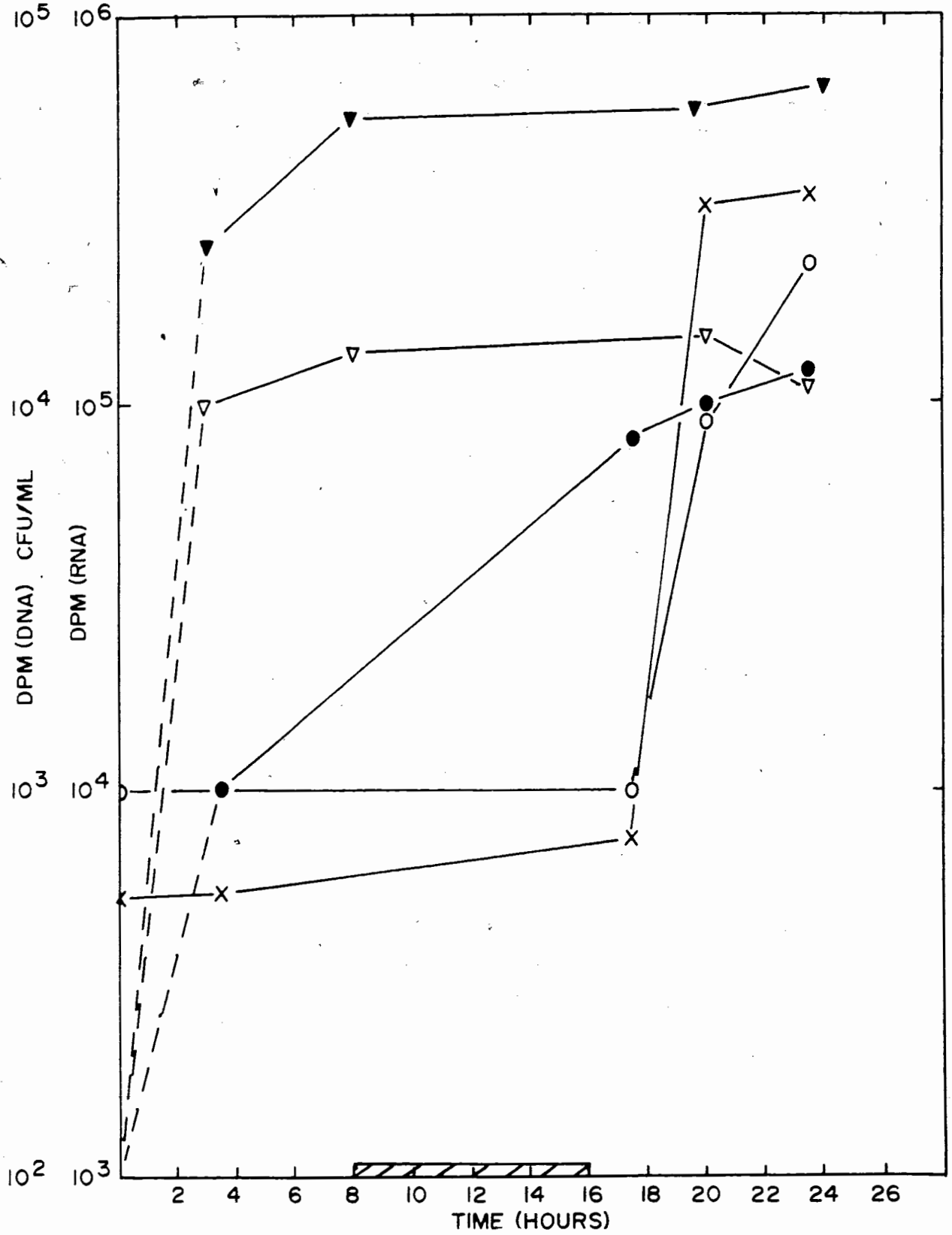
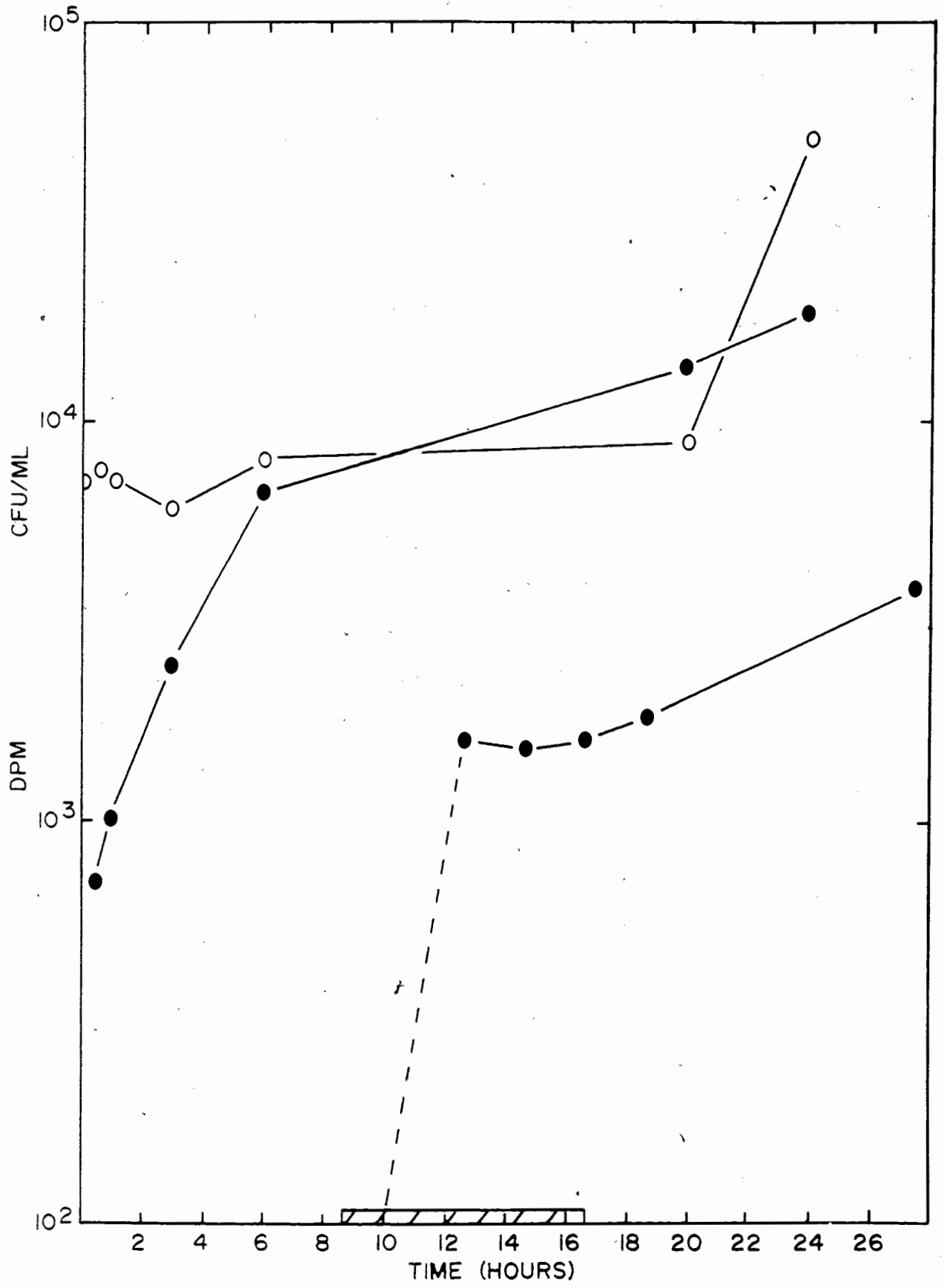

10^5
10^6
10^4
10^5
10^3
10^4
10^2
10^3
DPM (DNA) CFU/ML
DPM (RNA)
2
4
6
8
10
12
14
16
18
20
22
24
26
TIME (HOURS)

Figure III-2. The uptake of labelled thymidine into DNA following the addition of the precursor during the light period (•) and during the dark period (○). The corresponding increase in CFU (○) of the synchronized culture is shown.


10^5
10^4
10^3
10^2
CFU/ML
DPM
2
4
6
8
10
12
14
16
18
20
22
24
26
TIME (HOURS)

(as thymine) in formic acid digests of the KOH insoluble material (Kemp et al.,1972). Uracil and adenine were shown to be useful for nucleic acid labelling in Chlorella (Wanka et al.,1970) and Chlamydomonas (Chiang,1968) respectively. Therefore it was decided to examine the fidelity of the labelling obtained using uracil and adenine in Eudorina.

Eudorina was grown for several days in the presence of ^{3}H-uracil or ^{3}H-adenine before being collected, washed, and extracted as described in Materials and Methods. Chromatography of formic acid hydrolysates of the RNA, DNA, and protein fractions gave the radioactive profiles presented in Figs. III-3 and III-4. For both compounds the principal peak of radioactivity in the RNA sample corresponded to the relative mobility of the compound used to label the algae.

In the uracil labelled material the RNA sample (Fig. III-3) also had significant activity associated with Rf values higher than uracil. The DNA sample not only had activity in the region corresponding to uracil, but also had peaks with lower (0.17) and higher (0.6) Rf values. The protein fraction was contaminated by uracil and had additional activity running as a broad smear ahead of the uracil peak.

A major peak corresponding to adenine was removed in chromatograms of hydrolysates of the RNA and DNA fractions from ^{3}H-adenine labelled Eudorina (Fig. III-4). A second peak (Rf 0.14) was also present in these chromatograms. Chromatograms of the protein fraction were characteristically a smear of radioactivity extending from Rf 0.1 to 0.9.

A series of experiments involving examination of thymidine uptake into DNA fractions by heat killed, acid killed, and hydroxyurea inhibited organisms was carried out. The results were inconclusive but the recovery

Figure III-3. Chromatographic analysis of formic acid hydrolysates of RNA (o), DNA (•), and protein (Δ) fractions from a culture grown in the presence of labelled uracil.

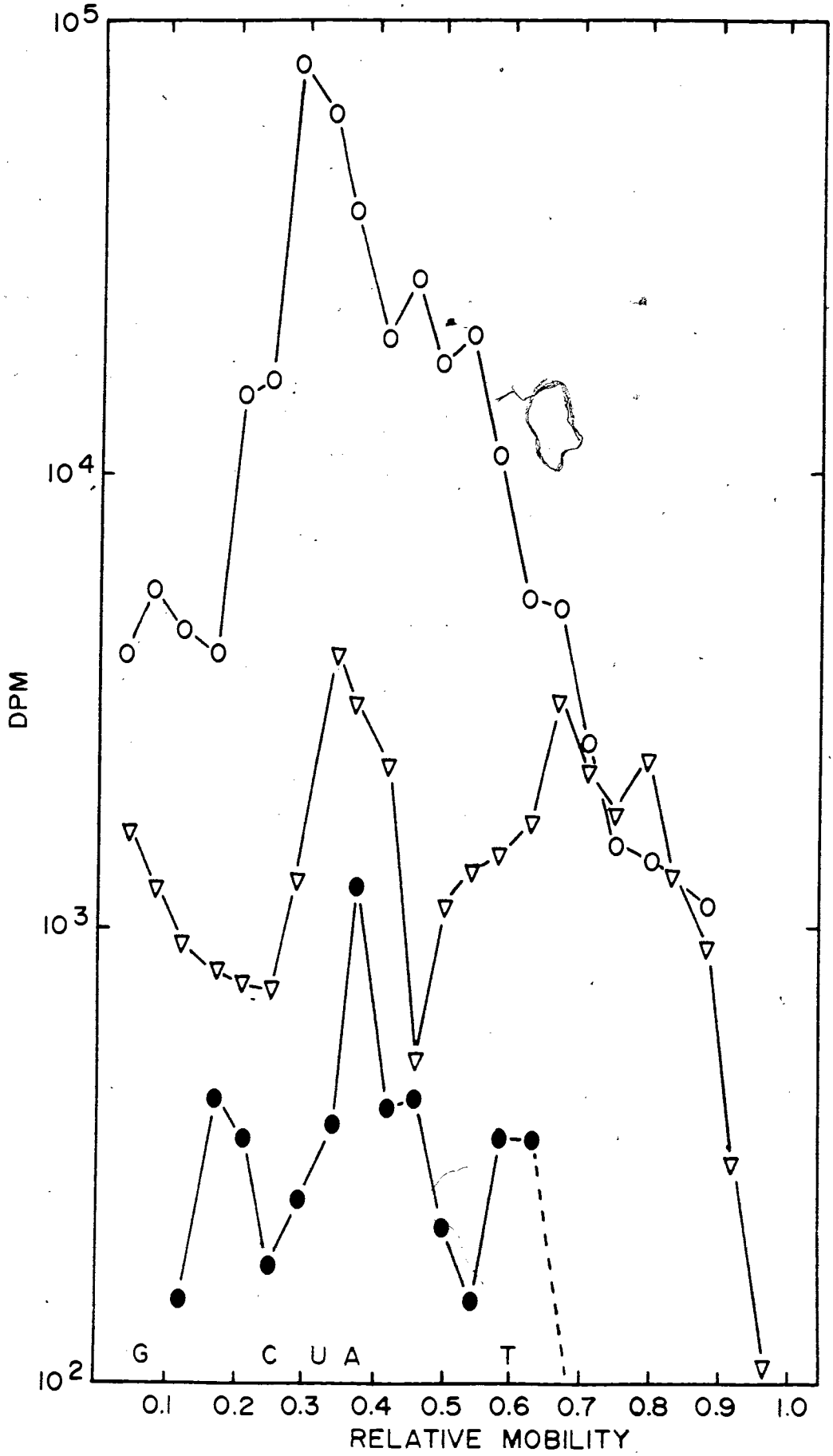
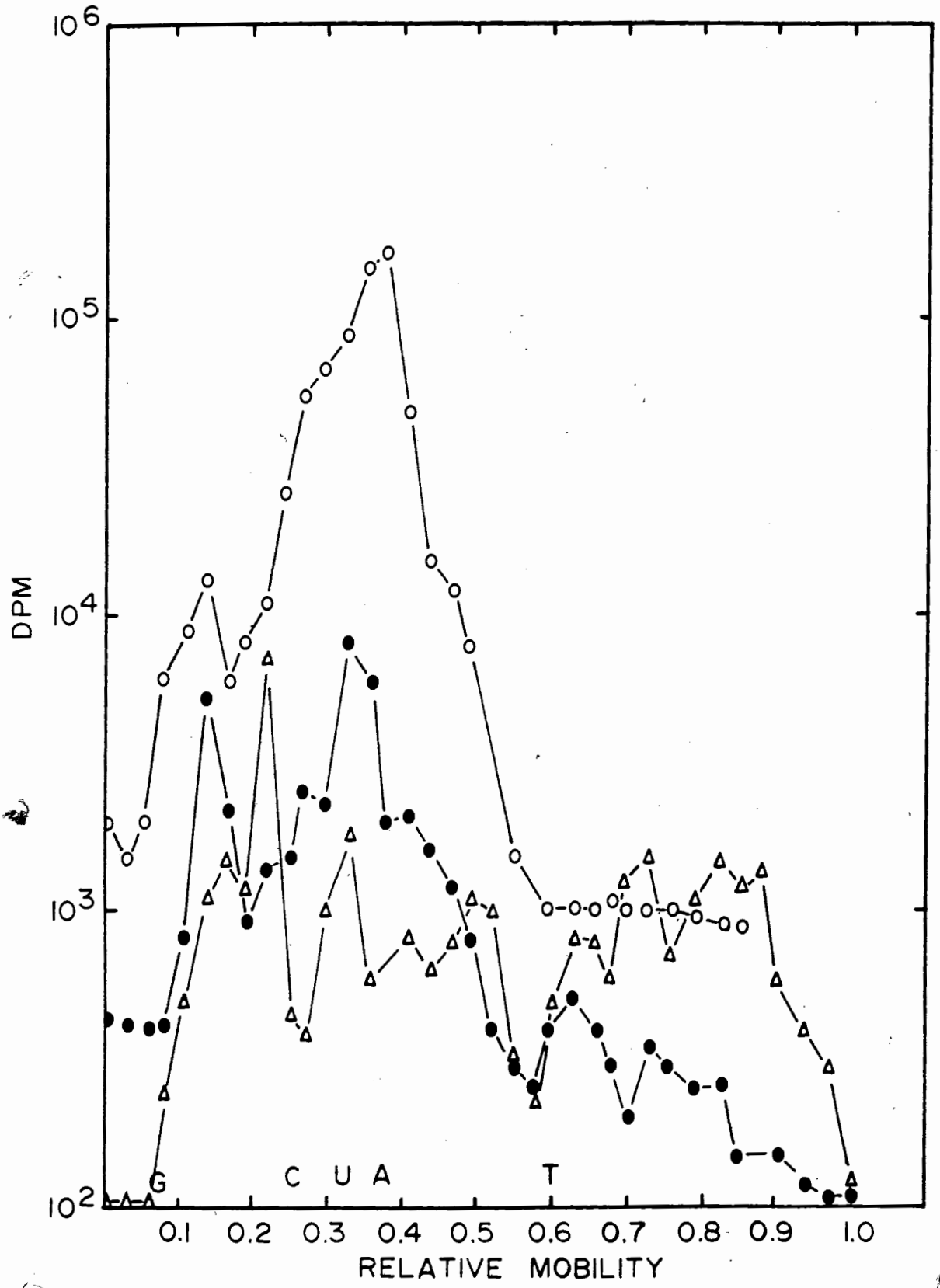

10^5
10^4
10^3
10^2
DPM
G
C
U
A
T
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
RELATIVE MOBILITY

Figure III-4. Chromatographic analysis of formic acid hydrolysates of RNA (o), DNA (•), and protein (Δ) fractions from a culture grown in the presence of labelled adenine.


10^6
10^5
10^4
10^3
10^2
DPM
G
C U A
T
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
RELATIVE MOBILITY

of label (^{3}H-TdR) suggested that non-specific trapping could occur.

Radioautographic examination of sections of Eudorina following labelling with either ^{3}H-TdR, ^{3}H-T, or ^{3}H-A showed a random, low distribution of exposed silver grains over the cells. If non-specific trapping of the label could account for some of the uptake observed, the radioautographs revealed that the gelatinous sheath surrounding the cells and the colony was not involved.

DISCUSSION

Various attempts to increase the amount of incorporation of nucleic acid bases and nucleosides into macromolecular components of Eudorina were unsuccessful. Perhaps the simplest explanation is that general, efficient salvage pathways may not exist in Eudorina. This was previously suggested to be the case in some marine algal nuclear systems (Steffensen and Sheridan,1965). The failure to detect the salvage enzyme thymidine kinase in Chlorella (Wanka et al.,1964) supports this suggestion.

Many of the experiments reported in this chapter are incomplete. However, the low level of uptake into the macromolecular fraction and the uncertainty regarding the purity of the fractions shows this approach to be impractical. Speculation regarding metabolic DNA in algae (Iwamura,1965), or non-specific binding of the labelled compounds to carbohydrates (Counts and Flamm,1966) or protein (Morley and Kingdom, 1972), are also unwarranted in the present case. Instead, in subsequent chapters, colourimetric and microspectrophotometric estimations of DNA replication will be presented.

CHAPTER IV

INTRODUCTION - CHEMICAL ESTIMATIONS OF DNA CHANGES DURING SYNCHRONOUS GROWTH OF *EUDORINA ELEGANS*

The development of methods to obtain synchronous growth of *Eudorina elegans* (Chapter II) affords the opportunity to examine the time course of DNA synthesis accompanying the rapid sequence of cell divisions in this organism. Each cell of a 16- or 32-celled coenobium divides 4 or 5 times within a total time of 1 h. The plakeal (flat-plate) stages formed invert, and are released as daughter (16- or 32-celled) coenobia. This process requires 2 to 4 hours in a synchronous population and is followed by an "interphase" of about 20-22 h. The timing of events suggests the possibility that total DNA replication could occur during the "interphase" to be followed by the division processes, reducing the DNA levels. The lack of precise, temporal alteration of DNA replication and cell division has been noted for *Tetrahymena*, after heat shock induced synchrony (Jeffery et al,1970). Polyploid cells have been reported in several species of *Chlamydomonas* and the production of daughter cells was accompanied by a reduction in chromosome number (Buffaloe,1958). However, sequential alternations of DNA synthesis and nuclear divisions were found in *Chlorella* (John *et al.*,1972) and have been suggested to occur in *Chlamydomonas reinhardii* (Kates *et al.*, 1968). That the interdivisional interphase is not the period of DNA replication has been found for *Volvox carteri* (Yates and Kochert,1972) and *V. aureus* (Tucker and Darden,1972). DNA replication is restricted to the period of gonidial cleavage in these colonial organisms.

The present study reveals that DNA replication is concomitant with the mitotic sequences. In addition, standard nucleic acid extraction procedures (Smillie and Krotkov,1960) followed by the DNA estimation by diphenylamine are not suitable for studies with Eudorina. Instead, the indole procedure (Hubbard et al.,1972) for DNA estimation as well as milder extraction procedures are necessary.

MATERIALS and METHODS

Cultures of Eudorina elegans 1193 (Culture Collections of Algae, Indiana University) were grown in an enriched medium consisting of a mixture of Bristol's salt solution (Cain,1965) and a modified Euglena medium (8:2 v/v) (Kemp and Wentworth,1971). Samples of the algae were harvested at intervals from 4 litre cultures growing synchronously on a 16h:8h light:dark regime at 32 C and concentrated by centrifugation at 4 C.

Nucleic acids were extracted from the material using a modification of the procedure of Smillie and Krotkov (1960). The cold (4 C) methanol extraction was repeated 3X, followed by a cold 5% perchloric acid (PCA) extraction. The residue was washed with cold 95% ethanol and extracted 3X in boiling ethanol-ether (2:1). After washing the residue in cold 5% PCA, nucleic acids were usually extracted with 5% PCA at either 90 C or 70 C for varying times. This extraction step was repeated and the supernatants were combined and analysed for RNA and DNA. The final residue was dissolved by heating in 2 N NaOH, neutralized with HCl and analysed for protein.

Protein was estimated by the Lowry method (Lowry et al.,1951) with bovine serum albumin as the standard, and RNA by the orcinol

reaction (Markham,1955) using adenosine at the standard. DNA was determined by either the diphenylamine reaction (Burton,1956) or the indole test (Hubbard et al.,1972). Polymerized calf thymus DNA was the standard for the DNA determinations. Standard errors calculated for replicate samples of the chemical tests were less than 5%.

Nuclei were observed in samples fixed in 3:1 (alcohol:acetic acid), squasned, hydrolyzed in 3.5 N HCl at 37 C (Fand,1970), and stained in Schiff's reagent (Deitch,1966). The nuclei in about 1,000 organisms were counted for each sample time.

RESULTS

Synchronously growing cultures of E. elegans were examined for changes in DNA content using the standard method of Smillie and Krotkov (1960) where the nucleic acid extraction step used 5% PCA at 90 C for 15 min (Fig.IV-1) and DNA estimation was by the diphenylamine reaction. During the first synchronous period of division DNA replication followed the cell divisions. In addition, DNA replication as determined by this procedure was biphasic. The second period of replication came after the release of the daughter organisms. A 30 fold increase in the content of both RNA and protein also occured at this time (see Fig.IV-4). During the second synchronous cycle of cell divisions the degree of synchrony was not as good as in the first cycle, due to insufficient dilution of the culture (Kemp and Lee,1975). However, DNA replication lagged behind cell divisions in this case as well.

In order to determine the reason for this biologically peculiar situation, the release of the chromagenic substances during distinct phases of the cycle was examined. Extraction of nucleic acids from a

non-dividing population was carried out at either 70 C or 90 C for different times and the chromogens released analysed by both the diphenylamine and indole tests (Fig. IV-2). In the two populations represented in Fig. IV-2, maximum release of the chromogenic substances at 70 C was reached after a 10 min hydrolysis period. The DNA values estimated by the indole procedure were consistantly higher than the estimates found using the diphenylamine test. Also, the chromogen reacting in the indole test was unchanged at 70 C for at least 150 min, whereas the material reacting in the diphenylamine test was progressively masked or destroyed after 20 min at 70 C in 5% PCA. Hydrolysis at 90 C gave maximal values at 5 min followed by an immediate decline of the diphenylamine reactive material. The destruction of the indole chromogenic substances at hydrolysis temperatures above 80 C as reported for model systems (Hubbard,1972) also occured in cell hydrolysates of Eudorina. This was seen as a reduction in the maximum DNA values obtained and the progressive loss of material detected by the indole test when hydrolysis was at 90 C (Fig. IV-2).

The same pattern was found when dividing populations were examined (Fig. IV-3), except that hydrolysis times to obtain maximal extraction were generally longer. The minimum time for maximal extraction of chromogenic material was also variable when different populations of organisms in division were hydrolyzed at 70 C. Continued hydrolysis beyond this minimum caused a gradual reduction in the diphenylamine reaction, but the indole response reached a maximum and remained constant for at least 150 min.

These data indicated that the chromogens reactive in the diphenylamine

Figure IV-1. Relative increase in nuclear number (o), colony forming units (viable daughter coenobia) (Δ), and DNA (•) in synchronous populations of Eudorina elegans. DNA was estimated by the diphenylamine reaction following hydrolysis of sample in 5% PCA at 90 C for 15 min.

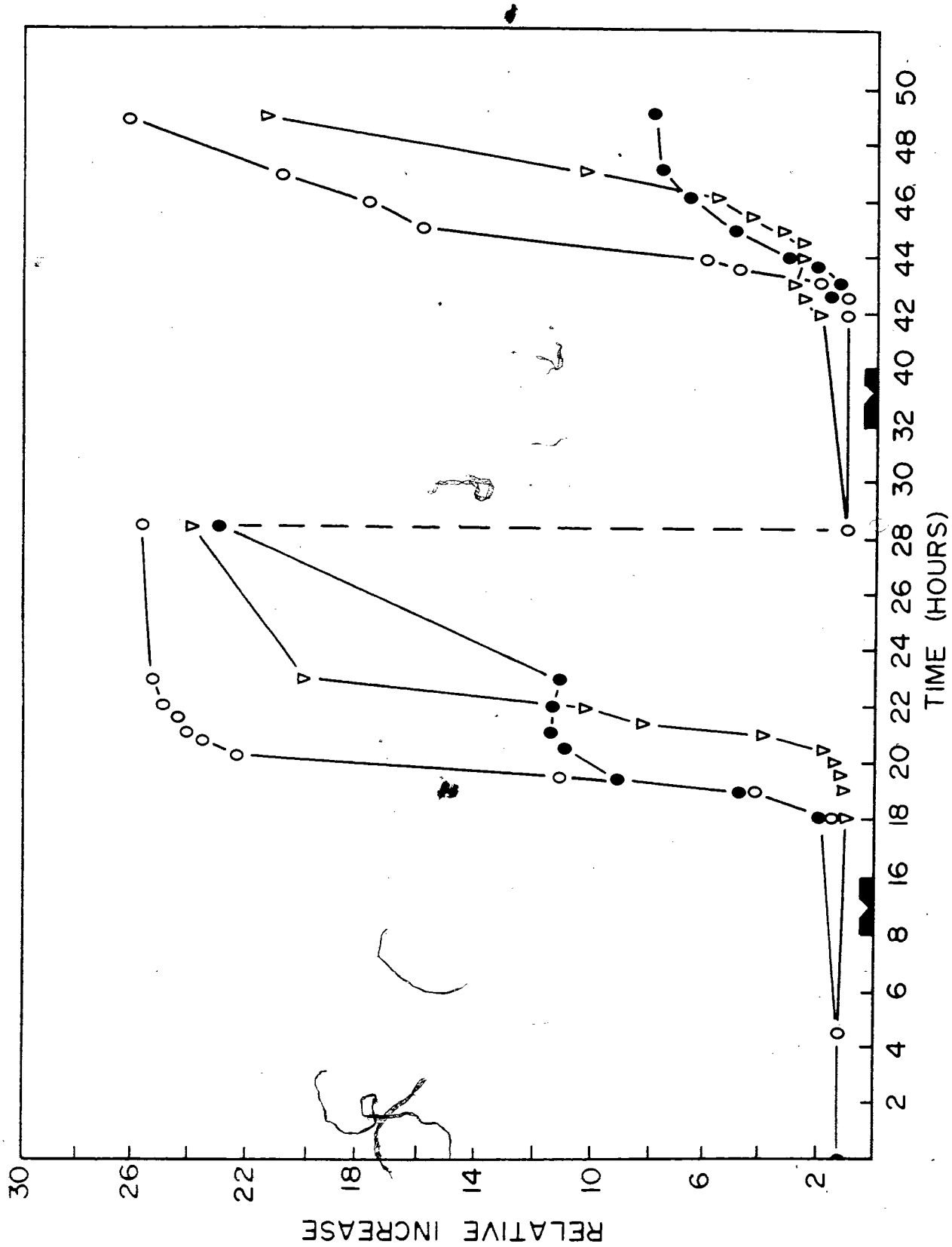
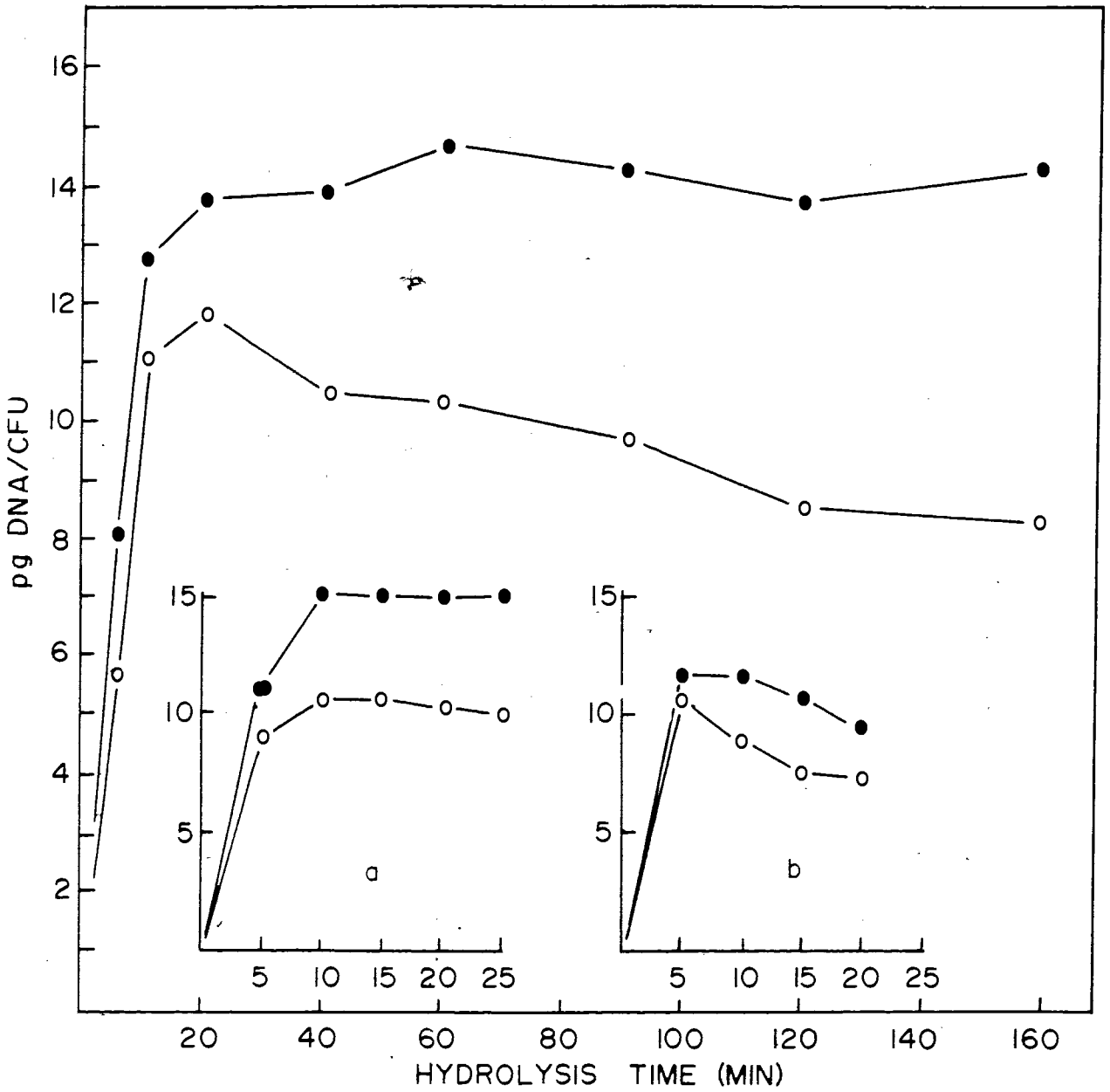

RELATIVE INCREASE
30
26
22
18
14
10
6
2
TIME (HOURS)
2
4
6
8
16
18
20
22
24
26
28
30
32
40
42
44
46
48
50

Figure IV-2. Diphenylamine (o) and indole (•) estimates of DNA content in a nondividing population of Eudorina following different hydrolysis times in 5% PCA. The main figure shows extended hydrolysis times at 70 C. Short hydrolysis times are shown in inserts a (70 C) and b (90 C) for a different population.


pg DNA/CFU
16
14
12
10
8
6
4
2
20
40
60
80
100
120
140
160
HYDROLYSIS TIME (MIN)
15
10
5
a
5
10
15
20
25
15
10
5
b
5
10
15
20
25

and indole reactions were too unstable, or were interfered with, when hydrolysis in 5% PCA was carried out at 90 C. Although more stable at 70 C the diphenylamine reaction still indicated losses and subsequent reductions in the specific chromogens. On the other hand, as in the non-dividing populations, the chromogens involved in the indole reaction from dividing organisms were stable at 70 C for extended periods.

Synchronous populations of Eudorina were re-examined utilizing the indole procedure to anaylze the DNA chromogens extracted at 70 C for 60 min (Fig. IV-4). Changes in RNA, protein, and nuclear number were also determined at intervals during synchronous growth.

The major increase in DNA and the increase in daughter cells occurred almost coincidentally over a 3 h period. These increases started at the beginning of the light period and both had total increments of about 29 times. The 4 fold increase which occurred in the interval between zero time and the beginning of the next light phase may reflect asynchrony in the system, or may correspond to organellar DNA synthesis.

Proteins and RNA showed a gradual increase in amounts per cell from the time of inoculation until the release of the daughter coenobia. A more rapid rate of synthesis then occurred during the next four hours resulting in a doubling of the amounts of RNA and protein present at the time of daughter coenobial release. The total increment of RNA was 25 times and of proteins, 27 times. This is reasonable agreement with the increase in cell number. The increase in the number of viable organisms in this culture was 14X.

Figure IV-3. Diphenylamine (o) and indole (•) estimates of DNA content in dividing population of Eudorina following different hydrolysis times in 5% PCA. Main figure shows estended hydrolysis times at 70 C. Short hydrolysis times, carried out using a different population, are shown in the insert. Diphenylamine estimates following 90 C hydrolysis are represented by the dotted line.

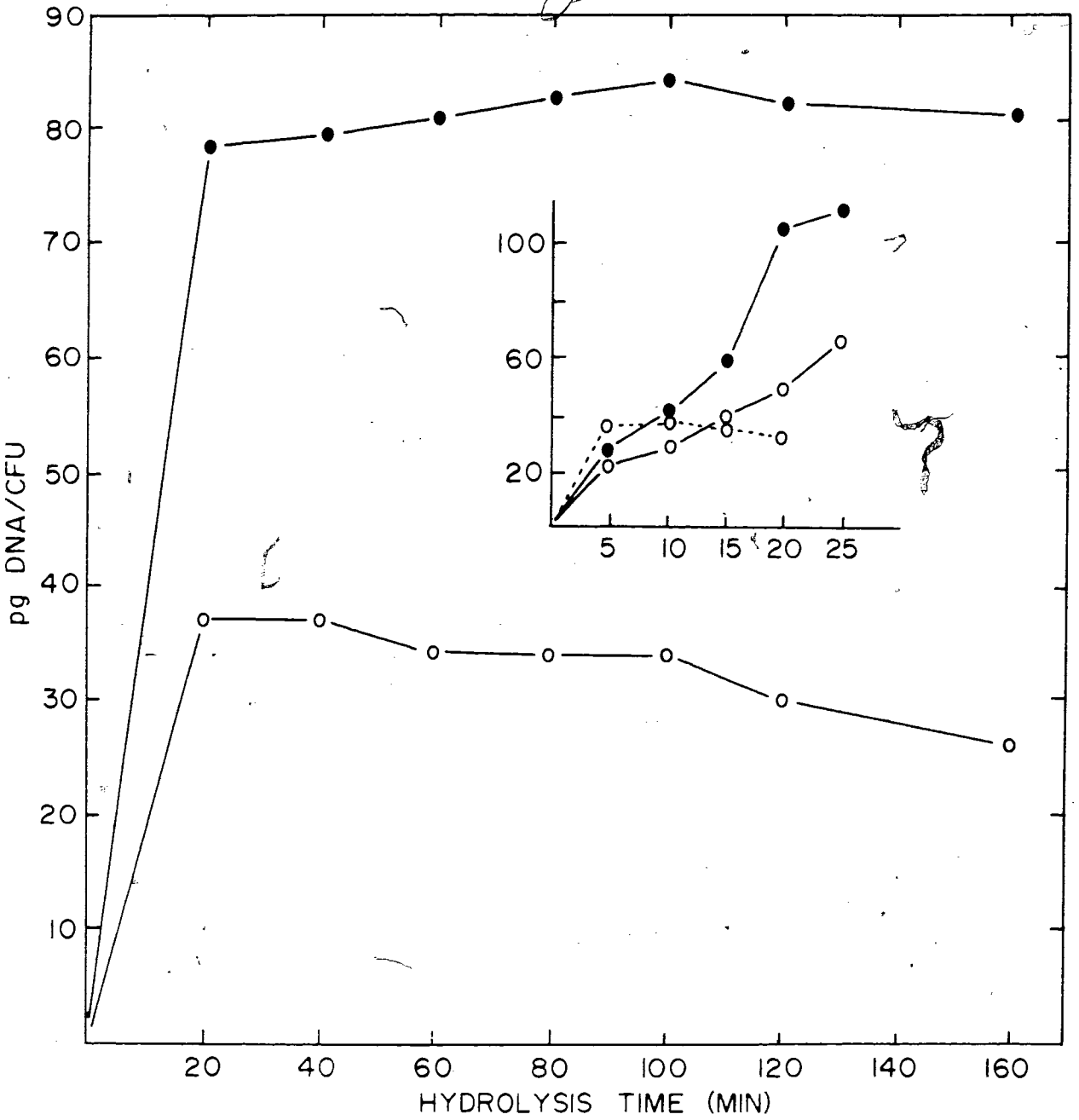
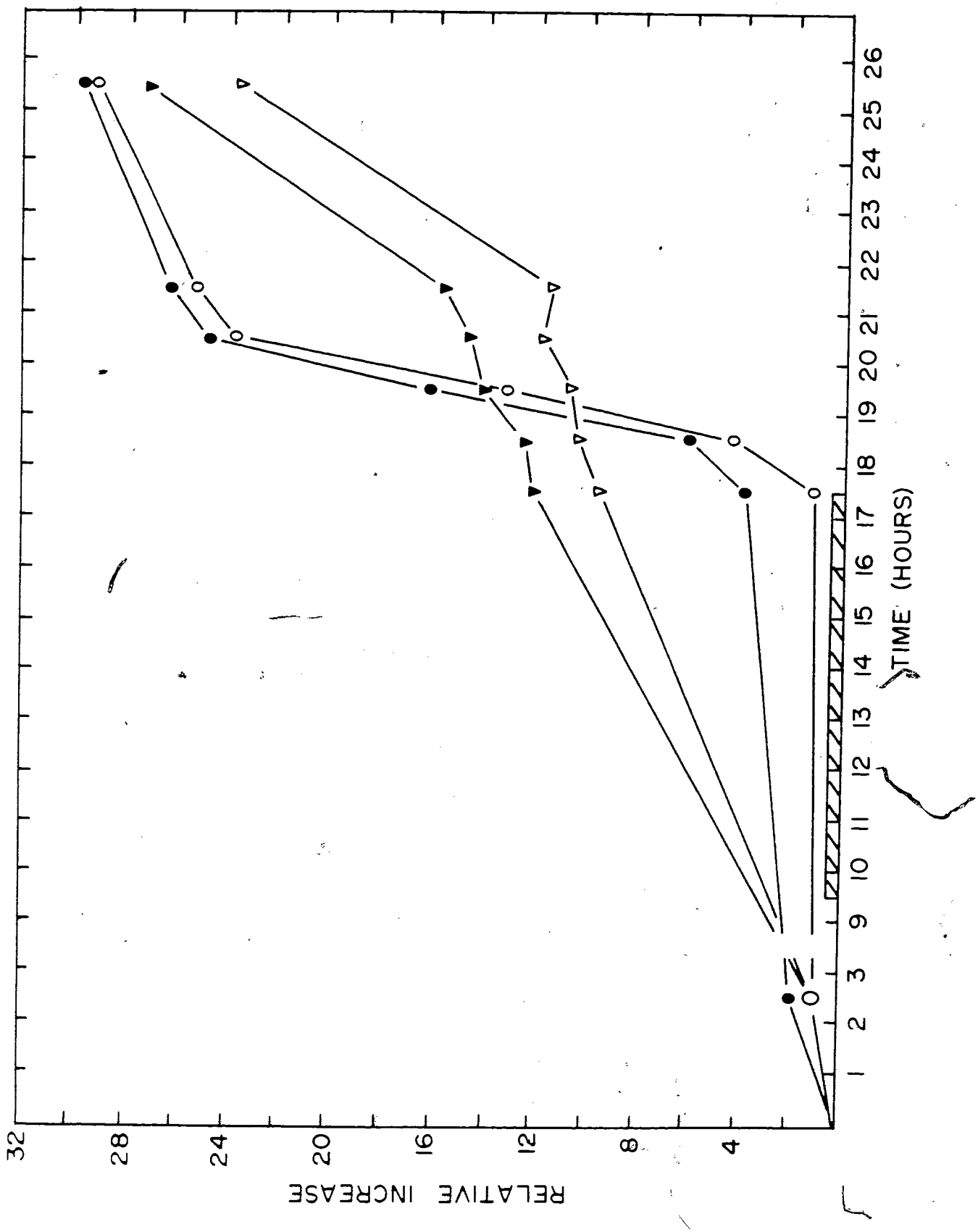

90
80
70
60
50
40
30
20
10
pg DNA/CFU
20
40
60
80
100
120
140
160
HYDROLYSIS TIME (MIN)
100
60
20
5
10
15
20
25

Figure IV-4. Relative increase in DNA (•) and nuclear numbers (o) in a synchronous population of Eudorina. Hydrolysis was in 5% PCA at 70 C for 60 min and DNA estimates by the indole procedure. Relative RNA (▽) and protein (▼) increase are also shown for a synchronous Eudorina population.


RELATIVE INCREASE
32
28
24
20
16
12
8
4
TIME (HOURS)
1
2
3
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26

DISCUSSION

The increment observed in viable organisms during a synchronous cycle of Eudorina elegans is dependent upon the average number of cells per organism produced in the previous cycle. A synchronous population is usually obtained by dilutions of a stationary culture in which most organisms are 16 celled. Since each cell gives rise by division to a daughter organism, the increase in viable organisms is about 16. However, the fresh medium and the diluted condition inducing synchronous growth allows a significant proportion of the daughter organisms to have 32 cells, i.e. to have undergone 5 divisions. The amount of DNA increase expected in these cases will approach 32 times. In practice, the increase is about 25 to 30 times since not all cells divide 5 times, but stop after 4 divisions (Chapter 2).

The problem in the present study has been to obtain estimates of the amount and timing of the DNA increases. It is clear that the substrates reacting with diphenylamine (purine deoxyribose) and indole (5' deoxyribose monophosphate) are destroyed by a hot (90 C) acid extraction (see Webb and Lundstrom,1965; Hubbard et al.,1972). Although hydrolsis in 5% PCA at 70 C appears to liberate an apporiate amount of indole reactive material, the measurable diphenylamine reactive material is low. The reason for this is unclear, but the discrepancy appears to be stage dependent. In non-dividing populations the maximal content measured by the diphenylamine reaction is about 83% of that measured by the indole reaction. When dividing populations are examined, the diphenylamine reaction gives a maximal DNA estimate which is only about one-half of the value obtained by the indole procedure.

Therefore, any DNA increment in Eudorina associated with, and measured during, cell divisions will be underestimated by the diphenylamine reaction. This reduction in DNA estimates during the growth cycle results in the measured increment lagging behind the real increment in DNA amounts. Higher and presumably more accurate estimates of DNA values are again obtained with diphenylamine after completion of the division sequences and release of the daughter organisms (Fig. IV-1).

Another problem is that the optimal extraction conditions obtained for a non-dividing population are not optimal for dividing populations. Circadian fluctuations in the diphenylamine- and Feulgen- DNA values found for peripheral leukocytes (Fontaine and Swartz,1972) were considered to be due to conformational changes in the leukocyte chromatin. Growth cycle dependent changes in chromatin structure may also account for the changes in DNA extractability in Eudorina. In any case, the stability of the material reacting with the indole test when hydrolysis is carried out at 70 C seems to allow maximal extraction at all stages. The minor fluctuations in extractability during the progress of a population through division are also eliminated by using the longer hydrolysis time at 70 C.

Utilizing the indole reaction to estimate material extracted from samples taken from synchronous population of E. elegans 1193 the amount of DNA prior to replication is about 0.5×10^{-12} g per nucleus. In addition, the replication period is coincident with the interval of nuclear and cell division. The interval separating the increase in DNA and the increase in nuclear number is in the order of 2-3 min. These data are all consistant with the interpretation that each nuclear division

is preceded by a round DNA replication. However, due to the overlapping of the division processes within the population, cycles of DNA replication not separated by cell divisions cannot be ruled out entirely by this study.

The data from the present study not only illustrate that DNA synthesis is confined to the period when active nuclear division occurs, but also that 4.5 DNA doublings occur within 3 h. The average time per DNA doubling of 40 min is shorter than durations reported for other synchronized algal systems. For example, the four successive doublings of DNA in Chlorella pyrenoidsa, strain 211-8b each required 110 min (Wanka and Mulders,1967), while strain 211-8p required 150 min (McCullough and Conn,1972). Chlamydomonas reinhardti cultured on a light:dark (16:8) cycle doubled its DNA content in an average time of 77 min (Kates et al.,1968). When grown under the more rigorous conditions of a 12 h:12 h light:dark cycle, an average DNA doubling time of 60 min was achieved (Lien and Knutsen,1973). Information from other studies with Eudorina suggests that a round of DNA replication in an individual cell may be as short as 10-15 min (Chapters II and V).

CHAPTER V

INTRODUCTION - MICROSPECTROPHOTOMETRIC ANAYLSIS OF DNA REPLICATION IN EUDORINA ELEGANS

In Eudorina elegans the vegetative cycle involves a rapid sequence of 4 or 5 mitotic divisions in each cell of the parent coenobium. This sequence, which occurs every 24 h, requires about 1 h (Rayns and Godward, 1965), with each 2 or 3 min division separated by an interphase of about 10 min (Chapter II). We have recently established that DNA replication occurs coincidentally with the cleavage processes (Chapter IV), a situation analogous to that reported for Volvox carteri (Yates and Kochert, 1972) and V. aureus (Tucker and Darden, 1972).

However, a mitotic gradient within each organism, coupled with the multiple, rapid sequence of divisions did not allow a precise relationship between DNA replication and cell division to be determined by chemical analysis of synchronous populations (Chapter IV). Also, since the uptake of radioactive precursors, i.e. thymidine, into DNA in Eudorina is very low (Hemp et al., 1972), and perhaps not localized in the nucleus (Swinton and Hanawalt, 1973), different approaches are required to examine the timing of DNA replication in colonial systems. Quantitative cytochemistry was chosen, although the small nuclear size and low DNA content per nucleus presented difficulties. Recent improvements in the application of the Feulgen procedure (Fand, 1970) and the mechanization of the quantitative evaluation of the stained material enabled us to determine the DNA replication precedes each

nuclear division. The entire 10 min interphase appears to be devoted to this replication process.

MATERIALS and METHODS

Squash preparations of live cells from synchronous cultures of E. elegans were fixed in 3:1 (alcohol: acetic acid) for 30 min following removal of the cover glass after freezing the preparation. The fixed cells were hydrated, hydrolysed in 3.5 N HCl at 37 C (Fand,1970), and stained in Schiff's reagent (Deitch,1966). After bleaching in sulphite solution, they were dehydrated and mounted in glycerine (RI=1.515).

Absorption by the bound dye was measured at 570 nm using a Carl Zeiss Scanning Microphotometer (SMP) equipped with a precision scanning stage (Zimmer,1970). The SMP had an Ultrafluor 100X oil immersion objective (NA=1.25), an aperture of 1μ and used a scanning step size of 1μ.

Instruction to the microphotometer and digital analysis of the microscope image was performed by a PDP-12A digital computer using a programme designated APAMOS (Automatic photometric analysis of microscopic objects by scanning) which is similar to the original programme TICAS (Taxonomic intracellular analytical system) (Wied et al.,1970). Visual checks and elimination of extraneous items in the scanned area could be made during the display of the measured points on an accompanying CRT display unit.

Interphase nuclei were scanned from all stages of daughter coenobial development. Several groups of nuclei, each derived from a single parental cell, were measured to establish that these nuclei were proceeding through the division sequences in synchrony. Once this was

found to be the case, subsequent measurements were done on single nuclei as representative of the stage of development of the particular embryo. Although cytokinesis follows each nuclear division (Chapter II) the stages were called, for convenience 1N(PM), 2N, 4N, 8N, 32N, and 1N, to designate late premitotic, the products of 1, 2, 3, 4 or 5 successive nuclear divisions, and the nuclei of newly released organisms, respectively. Chemical analysis had shown that the 32N and 1N populations should represent the 1C (non-replicated) class for DNA values (Chapter IV).

RESULTS

In order to optimize the amount of dye bound by the nuclei of E. elegans, the time of hydrolysis in 3.5 N HCl at 37 C and the amount of time the hydrolyzed material was incubated in the Schiff's reagent was varied. The optimum time for hydrolysis was 20 min and for staining a minimum of 60 min was required (Fig. V-1). Unhydrolyzed, stained material as well as hydrolyzed, unstained material do not have sufficient absorption at 570 nm to influence the detection system. Examples of a matrix of extinction values obtained as a print-out of the CRT display of microspectrophotometric scans of individual cells or developing embryos are presented as figures V-2a, V-2b, V-2c, V-2d, V-2e, V-2f, V-2g. The nuclear orientation within the squashed preparations of developing embryos as well as the individually measured absorption values (X100) of the Feulgen stained interphase nuclei are evident. Since the step size of the individual scan points was 1μ, the relative nuclear sizes (stained areas) could be obtained directly from the scan patterns. Note that the scans are of a single cell or the products of a single cell, developing as one of the 16 or 32 celled daughter organisms.

Figure V-1. The effect of hydrolysis and staining times on the amount of measurable dye by nuclei in non-dividing populations of Eudorina.

a. Staining time in Schiff's reagent with a hydrolysis time of 25 min.

b. Hydrolysis time in 3.5 HCl at 37 C using a staining time of 2 h.

The bound dye was measured at 570 nm and is presented an average absorbance units (AU) ± SE per nucleus.

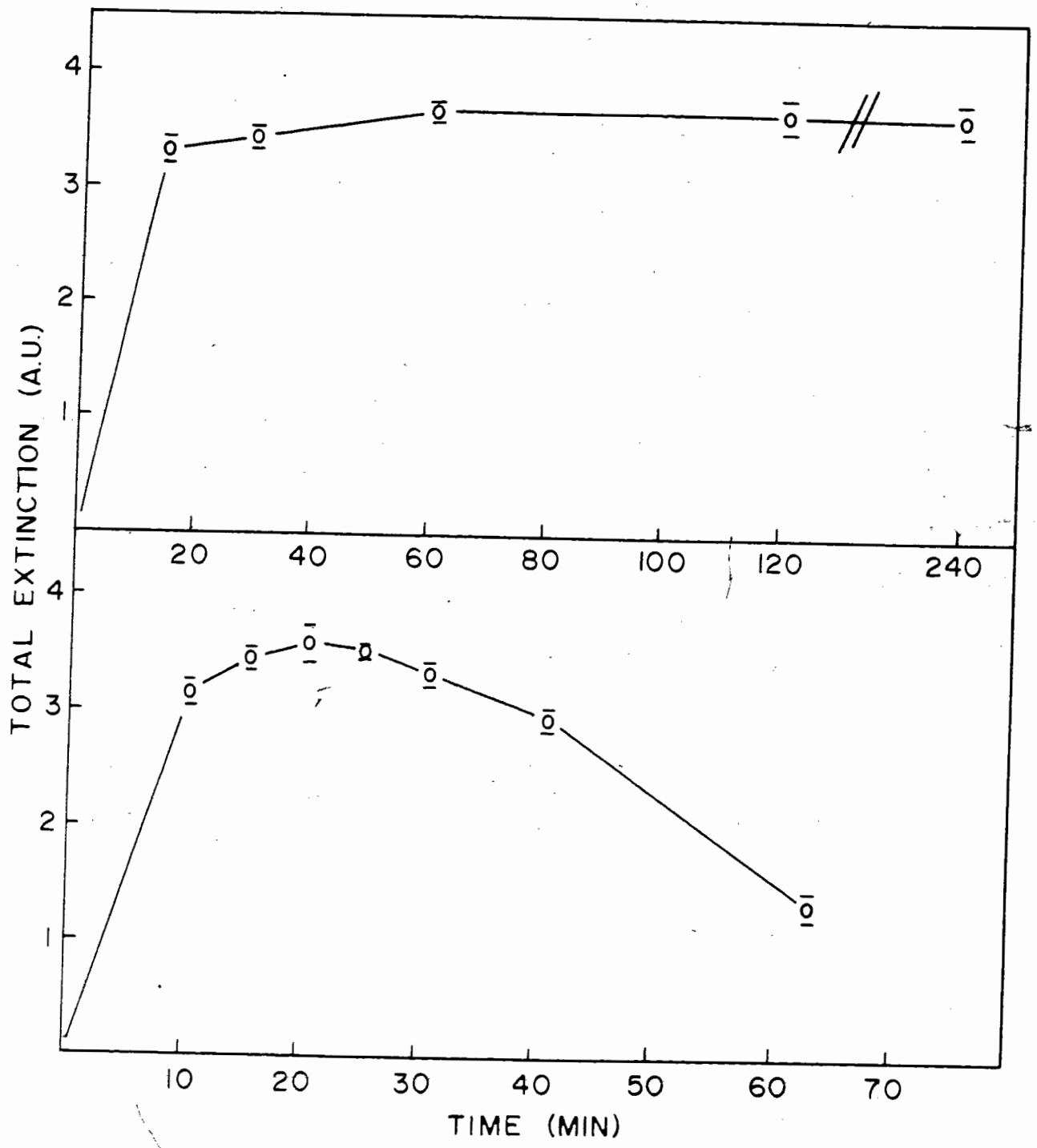

TOTAL EXTINCTION (A.U.)
4
3
2
1
20
40
60
80
100
120
240
4
3
2
1
10
20
30
40
50
60
70
TIME (MIN)

Figure V-2a. A matrix of extinction values (x100) of a scan Feulgen stained premitotic nucleus (INPM) of Eudorina. The unstained (i.e. undetected) area witnin the nucleus is the nucleolus.

8 13 12 13 9

7 7 8 6 10 12 11 9

9 7 9 14 15

10 9 13 13 6

7 8 7 6 9 15 15 10

6 7 7 8 6 7 7 15 18 7

7 11 10 10 9 8 10 11 9

7 12 10 8 8 9

7 11 8 8

Figure V-2b. A matrix of extenction values (x100) of a scan of a Feulgen stained 2-nucleate (2N) developing coenobium of Eudorina.

7 6

8 10 12 10

8 10 13 16 14 11 7

7 12 14 16 16 15 13 8

6 11 13 15 11 13 14 9

7 10 10 9 8 9 11 12

9 9 7 7 6 8 7

6 7 6 6 6

6 6

6 6

6 6 7 9 8 6

7 6 6 8 11 10 9 7

6 7 6 6 7 10 10 9 7

8 8 8 8 6 9 11 9 8

7 9 10 9 8 8 11 9 6

7 11 10 9 11 11 10 7

9 11 12 12 12 8 6

7 9 9 7

Figure V-2c. A matrix of extinction values (x100) of a scan of a Feulgen stained 4-nucleate (4N) developing coenobium of Eudorina.

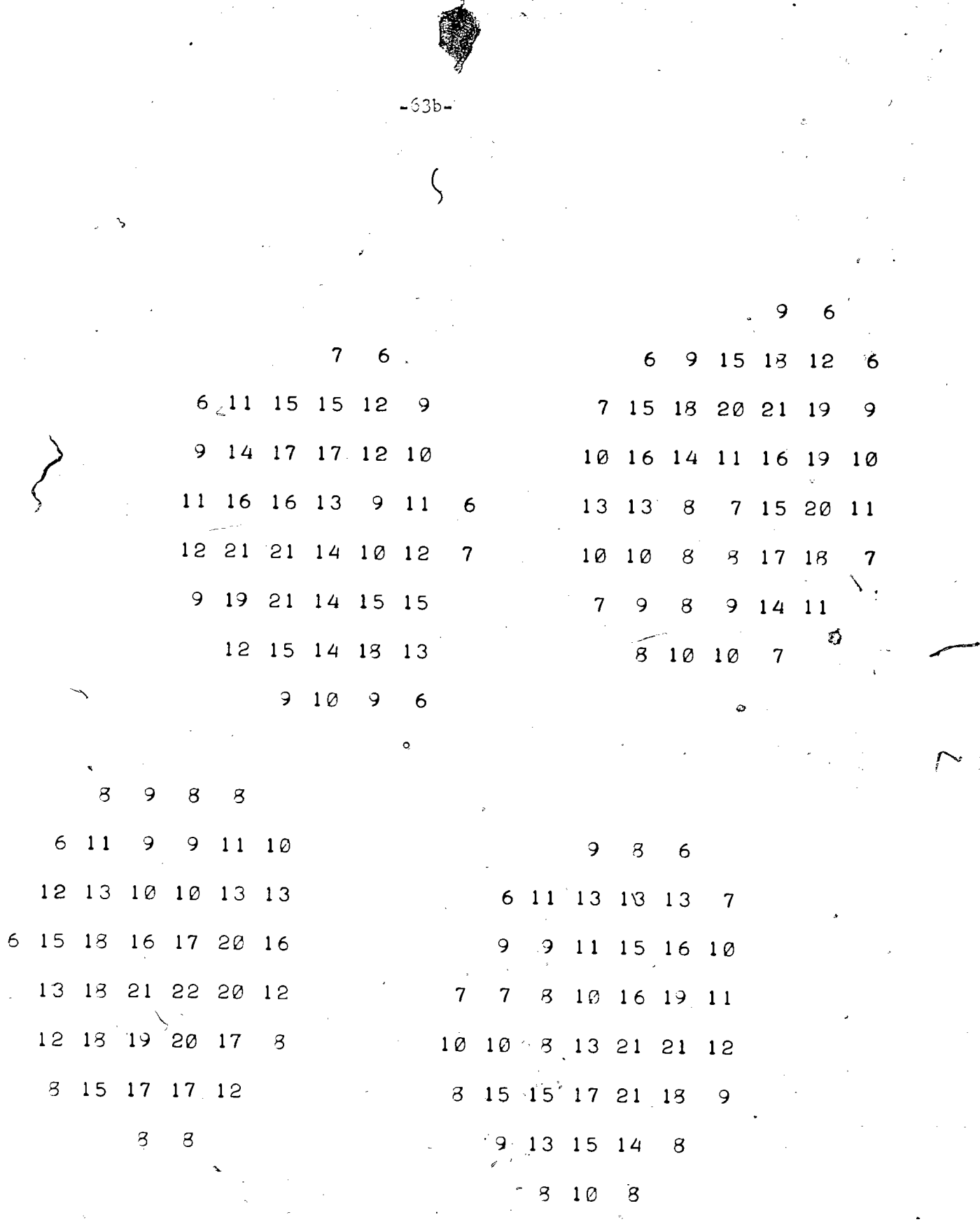

9 6
6 9 15 18 12 6
7 15 18 20 21 19 9
10 16 14 11 16 19 10
13 13 8 7 15 20 11
10 10 8 8 17 18 7
7 9 8 9 14 11
8 10 10 7
7 6
6 11 15 15 12 9
9 14 17 17 12 10
11 16 16 13 9 11 6
12 21 21 14 10 12 7
9 19 21 14 15 15
12 15 14 18 13
9 10 9 6
8 9 8 8
6 11 9 9 11 10
12 13 10 10 13 13
6 15 18 16 17 20 16
13 18 21 22 20 12
12 18 19 20 17 8
8 15 17 17 12
8 8
9 8 6
6 11 13 13 13 7
9 9 11 15 16 10
7 7 8 10 16 19 11
10 10 8 13 21 21 12
8 15 15 17 21 18 9
9 13 15 14 8
8 10 8

Figure V-2d. A matrix of extinction values (x100) of a scan of a Feulgen stained 8-mucleate (8N) developing coenobium of Eudorina.

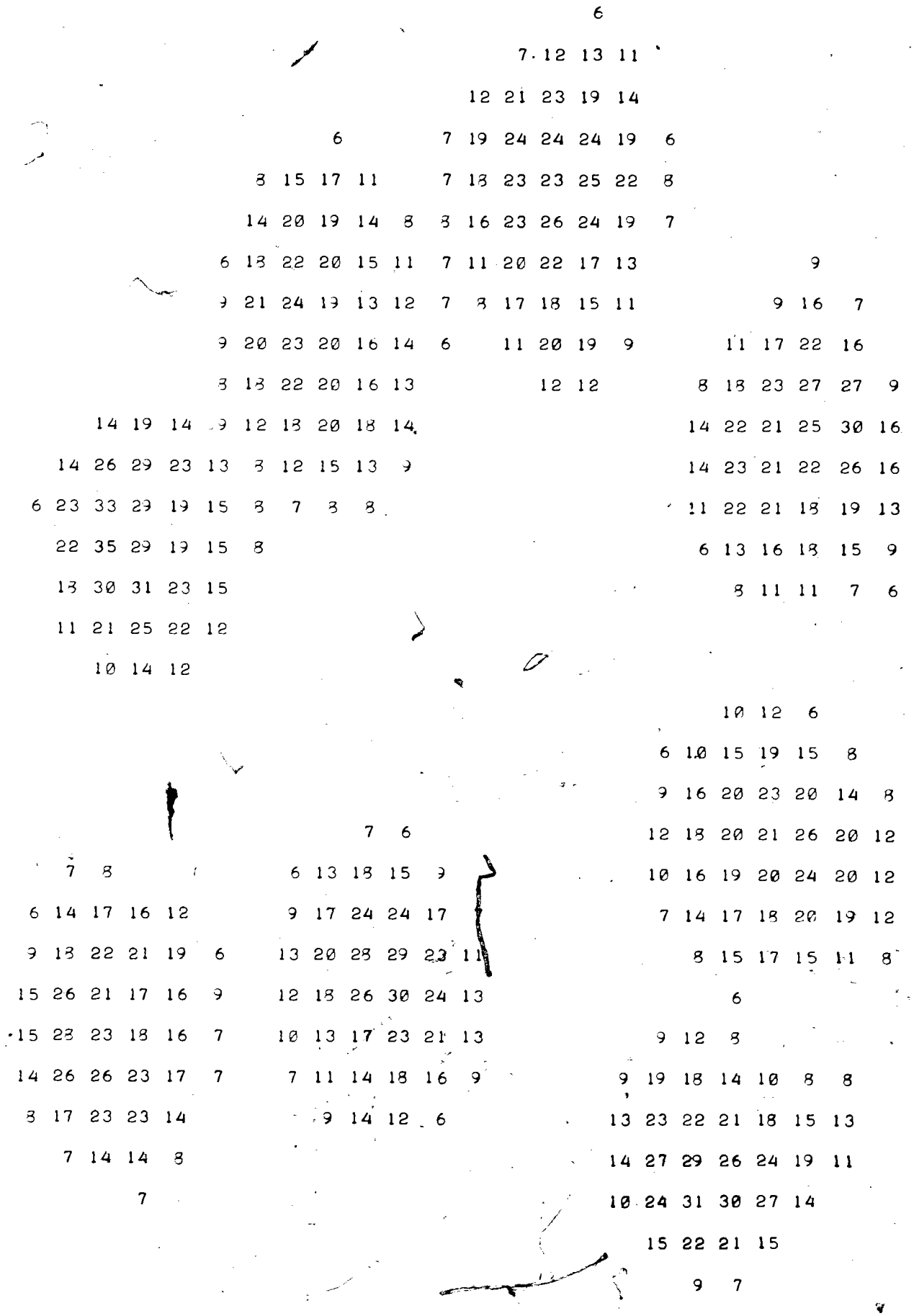


Figure V-2e. A matrix of extinction values (x100) of a scan of a Feulgen stained 16-nucleate (16N) developing coenobium of Eudorina.

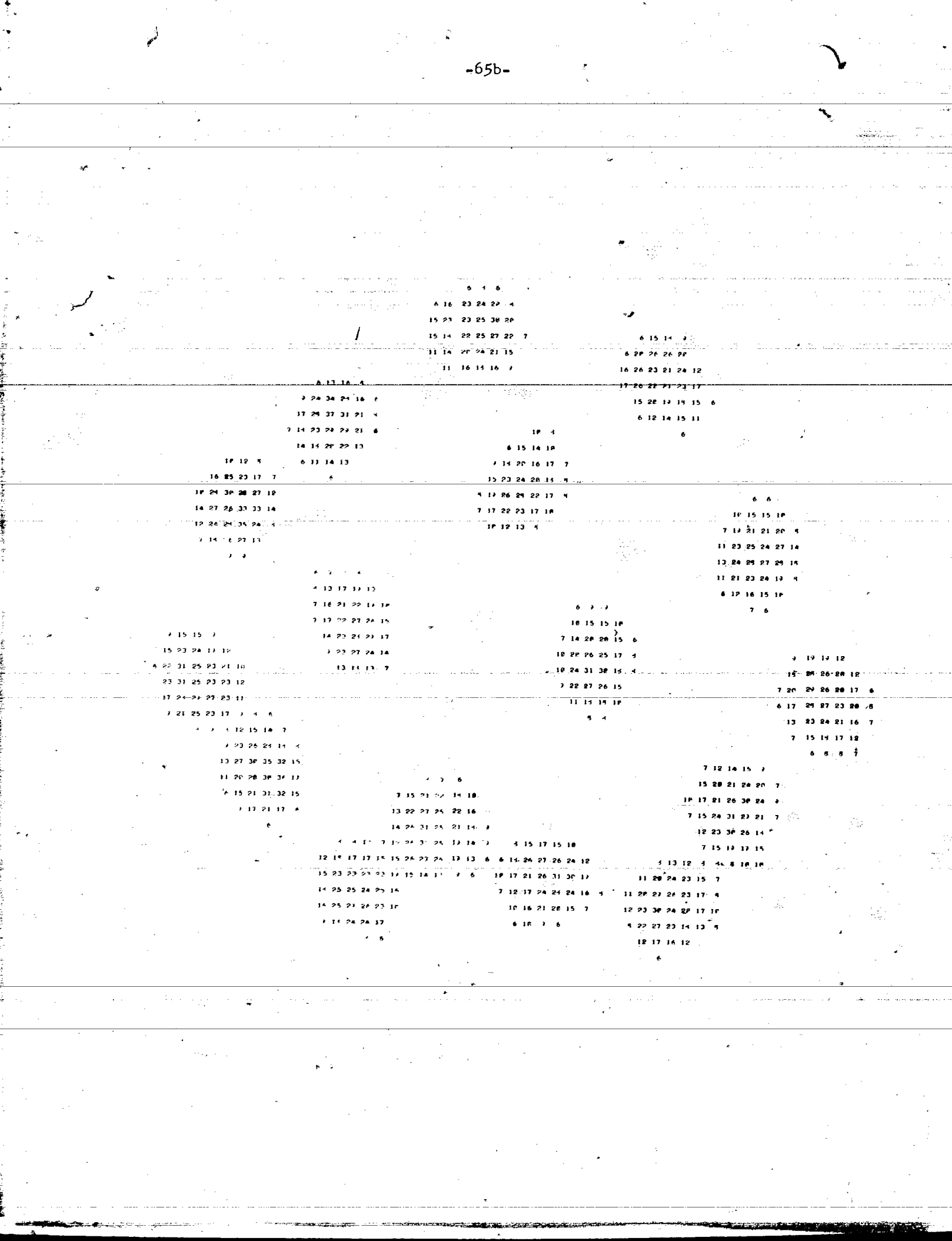


Figure V-2f. A matrix of extinction values (x100) of a scan of a Feulgen stained 32-nucleate (32N) final mitotic embryo.

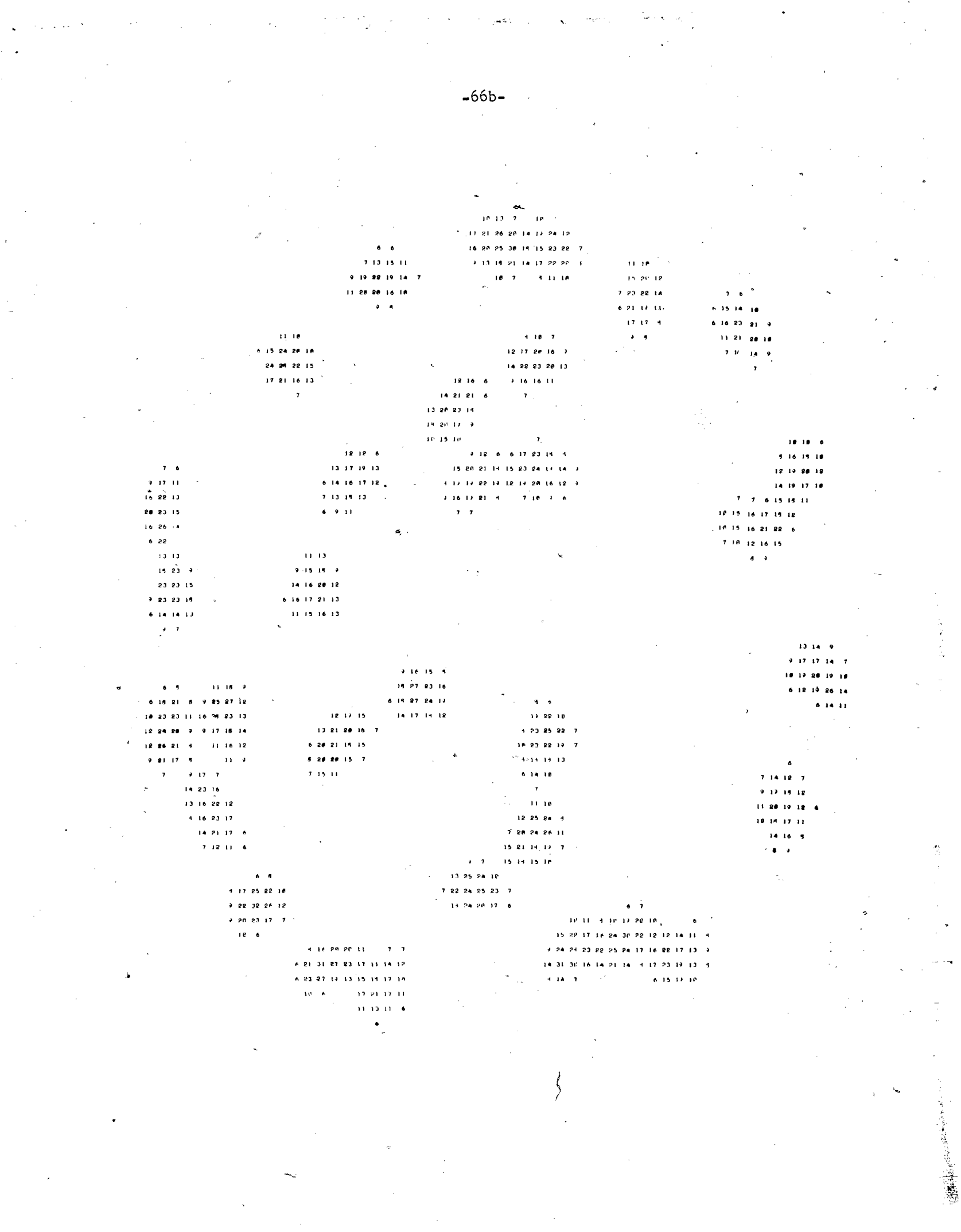


Figure V-2g. A matrix of extinction values (x100) of a scan of a Feulgen stained postmitotic nucleus (IN) of Eudorina 3 h after the completion of the mitotic events.

	8	14	13	9	6
9	17	19	17	16	11
11	16	16	14	16	13
6	14	20	17	14	9
	9	18	20	13	

Internal consistency of the scans was checked by repeated scanning of the same group of nuclei. The mean values of these repeat scans were not statistically significantly different.

As a result of the developmental gradient found in Eudorina, several stages coexist for a time within the same organism. Therefore, nuclear scans of embryos following different numbers of divisions were obtained from within the same organism. Differences introduced by preparative procedures were minimized in these circumstances. A maximum lag time of 45 min between the division of the penultimate cell and the entry of the ultimate cell of a coenobium into division was observed.

The cells of the mature coenobium (1NPM) have large, diffuse, and consequently faintly stained nuclei. An area within the nucleus not stained by the Schiff's reagent, presumably the nucleolus, was often seen (Fig V-2a). When the cell divided, the resulting 2-celled stage also possessed a definite nuclear area but the nucleolar region was difficult to detect. However, the nucleolus was present at this and at subsequent interphase stages and showed up clearly in material stained with Azure blue (see also Cave and Pocock,1951). As the number of divisions increased the nuclear region remained distinct in each division product but the area of each nucleus decreased (Fig V-3). The stained nuclear area after the 5th nuclear division was about one-third of the area of the premitotic nuclei. It was assumed that the absorbing area of the cell at 570 nm was equivalent to the nuclear area of the cell (cf. Gottlieb-Rosenkrantz and O'Brien,1971), and therefore the total estinction values could be directly compared.

Newly released organisms have been shown previously (Chapter IV) not to enter DNA synthesis for about 20 hours. Thus, the extinction value of 3.77 ± 0.03 obtained from 107 individual nuclei at this stage was considered to be the 1C value (Table V-1, Fig. V-4). When developing embryos were examined at various stages, a range from 1C to 2C values was obtained. Values approaching 3C or 4C or beyond were never encountered (Table V-1, Fig V-4). The frequency distribution patterns of the total extinction values were reasonably symmetrical. An exception was the 16 N class, where a minor peak near the 1C value was evident. Statistical analysis (F-test) comparing the mean values of the seven classes of measurements (representing immediate pre-division, the products of 1, 2, 3, 4, and 5 divisions, and newly released organisms) showed significant differences. However, these data analysed by the Student-Newman-Keul multirange test (Sokal and Rohlf,1969), revealed that statistically similar groups were present (cf. Fig. V-4).

The mitoses occurring within a single embryo were synchronous (Goldstein,1964; Lee and Kemp, unpbl.) and followed each synchronous sequence of DNA synthesis, as observed in 2N, 4N, 8N, and 16N nuclei.

DISCUSSION

Microspectrophotometric examination of Feulgen-DNA content at various stages during vegetative development of Eudorina elegans established that DNA synthesis precedes each nuclear division. Therefore, schemes involving total DNA synthesis prior to all or some of the divisions are excluded, since such schemes would require total extinction values in excess of the 2C values observed. The skewed nature of the frequency distribution patterns of total extinction

Figure V-3. Decrease of the nuclear area ± SE as the nuclear number increases in the developing coenobia of Eudorina. The nuclear area corresponds to the stained region by the SPM.

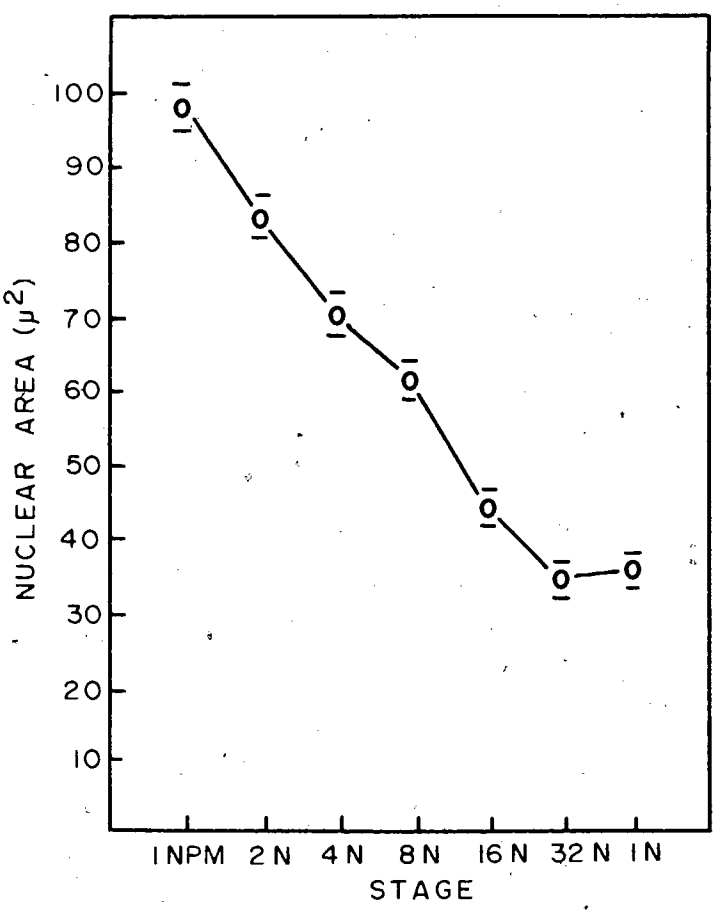
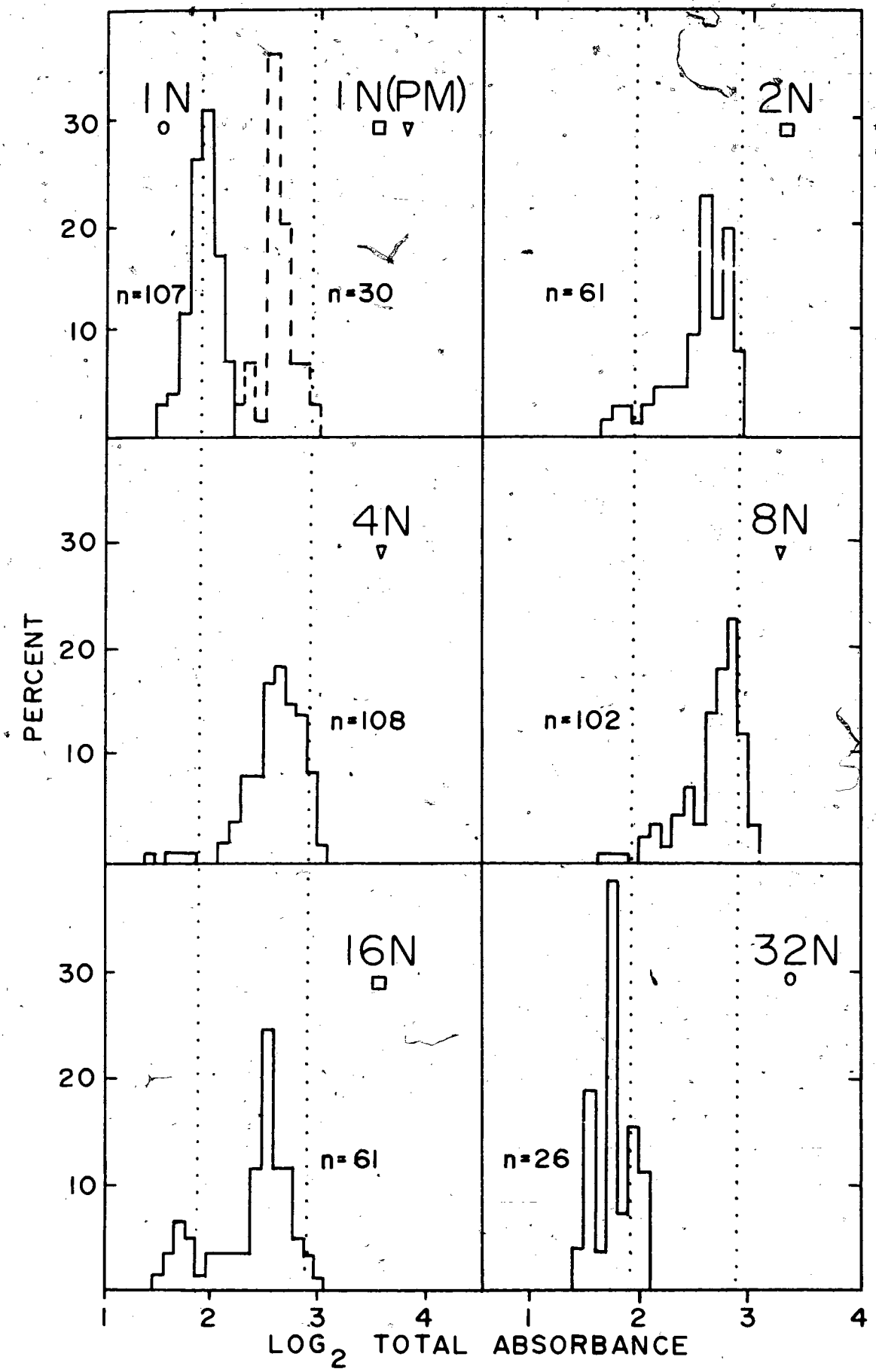

NUCLEAR AREA (μ^2)
100
90
80
70
60
50
40
30
20
10
1NPM
2N
4N
8N
16N
32N
1N
STAGE

Table V-1 Area and total extinction of Feulgen stained nuclei of developing Eudorina elegans.

STAGE	Number	Area (nm^3) + SE	Mean extinction (ME) + SE	Log_2 ME + SE
1N(PM)	30	97.8±2.3	6.00±0.12	2.58±0.27
2N	61	82.5±2.1	5.69±0.14	2.51±0.77
4N	108	69.7±1.2	6.20±0.10	2.63±0.56
8N	102	61.2±1.9	6.46±0.13	2.69±0.44
16N	61	43.6±1.01	5.59±0.19	2.48±0.60
32N	26	33.6±1.06	3.50±0.12	1.80±0.26
1N	107	35.0±0.48	3.77±0.03	1.91±0.18

Figure V-4. Frequency distribution histograms of the relative DNA content, as $\log_2$ total absorbance, of developing coenobia of Eudorina elegans. The dotted lines represent the 1C and 2C values. The nuclear classes designated by the same symbol represent satistically indistinguishable classes.


1N
1N(PM)
2N
4N
8N
16N
32N
n=107
n=30
n=61
n=108
n=102
n=61
n=26
PERCENT
10
20
30
1
2
3
4
LOG2 TOTAL ABSORBANCE

values towards the 2C value indicates that a G1 period between the divisions within a sequence is insignificant. In addition, the lack of a definite subpopulation exhibiting 2C values suggests that division occurs immediately following a round of DNA replication. Thus a G2 period is also absent. The presence of two populations of nuclei in the 16 N class does not mean that a G1 exists uniquely for this stage, but rather that a fraction of the organisms cease dividing while many proceed to the 32 N class. This interpretation is supported by actual counts of the progeny Eudorina, where both 16- and 32-celled organisms are seen. The general conclusion is that DNA replication occupies essentially all of the interdivision period for divisions two through four or five.

The diffuse nature of the 1N(PM) nucleus makes estimates of DNA levels inaccurate because of the low stain density. However, scans of 1N(PM) nuclei in coenobia containing cells already in division suggest DNA replication is in progress. Chemical estimates of DNA amounts also indicates that this replication must occur just prior to the division itself (Chapter IV). We conclude that the first cycle of DNA replication probably has similar parameters as the subsequent sequences.

The statistical differences detected between the nuclear and number classes confirm the 32-celled pre- or immediate post-release coenobia as unique and presumably, as the 1C class. The fluctuations in the amount of Feulgen-DNA measured for the nominally homogeneous 1C classes (1N ~~and 32N~~) demonstrates the small measurement errors resulting from preparative and instrumental variations. Such minor errors are magnified in Eudorina as a result of the small nuclear size and low

DNA content.

Average values approaching the 2C class, obtained in the developing embryos, emphasize the rapidity of the initiation of DNA synthesis following mitosis. The differences within the groups of developing embryos are not unexpected and are believed to indicate different extents of DNA synthesis. Some fluctuations due to measurements error may be superimposed on the real values due to the low amounts of bound dye. On the other hand, the differences between the groups of developing embryos could reflect slight differences in the rates of DNA synthesis in the organisms of different nuclear number.

Relative nuclear size decreased in a linear manner with each nuclear division. The final nuclear area is about one-third of the initial value, suggesting that nuclear growth occurs during the development of a new organism. In Chlamydomonas reinhardii, the nuclear envelope remained intact during division, expanding and infolding over the daughter nuclei (Johnson and Porter,1968). This process was not fully examined in Eudorina, but the increase in total nuclear size per embryo is consistent with a similar expansion of the nuclear envelope occurring in Eudorina.

The amount of DNA which must be replicated per nucleus in the 10-15 min interval available is estimated by the indole procedure to be about 5×10^{-13} g (Chapter IV). Assuming that Eudorina replicates DNA at rates similar to those reported for eukaryotes (cf. Taylor,1968), i.e. 2 μm per min, then about 8000 initiation sites could be present. However, no information of DNA replication rates is available for algae, and it is possible that the unique cell cycles encountered are coupled with unique patterna and/or rates of DNA replication.

CHAPTER VI

DISCUSSION - GENERAL

The primary aim of the present investigation was the elucidation of the temporal sequence of DNA synthesis, nuclear division and cytokinesis in the life stages of the colonial green alga, Eudorina elegans. During the growth cycle of 5 days, each cell undergoes 4 or 5 karyokinetic events in a period of 1 h to produce 16 or 32 daughter cells, respectively (Rayns and Godwards, 1956). This unique observation suggested the possibility of unusual sequencing of DNA replication and segregation. These could involve multiple rounds of DNA replication prior to multiple rounds of cell division instead of the normal alternation of replication and segregation.

This study has revealed that in vegetative cells of Eudorina, like other organisms, a round of DNA duplication preceded each nuclear division. However, unlike other plant and animal cells, DNA replication, nuclear and somatic divisions alternate consecutively 4 times in the course of 1 h followed by an intervening growth period of 23 h. Further investigations to provide evidence to explain the grouping of 4 or 5 divisions rather than the normal regular alternations of growth and division over the entire life cycle would be of interest. A reduction of the expenditure of time and energy by the cells during division and a better utilization of the photo-synthetic period could be taken into consideration.

This study does support the finding of Rayns and Godward (1965) in that a mature vegetative cell can divide into 16 or 32 daughter cells in

about 1 h . However, the mitotic time of 3 min determined in this study differs from the mitotic time of 10 to 15 min calculated by Rayns and Godward and the 10 min interval between the disappearance and appearance of the nucleolus of mitotic cells observed by Cave and Pocock (1963). These differences may be attributed to the use of different strains of Eudorina and/or the enhanced growth rates used in the present study. Moreover, while Ryans and Godward assumed that little or no time was spent in interphase, it has been shown that a significant amount of time (10 min) is spent in interphase as compared to mitosis. One must speculate that Eudorina does possess a very efficient mechanism for DNA duplication. Based on the estimated rate of DNA helix replication for eukaryotic organisms, on the length of the synthetic period, and on the amount of DNA, about 8000 seperate intrachromosomal initiation sites must exist for DNA synthesis. This speculation assumes that the estimated rate of DNA replication, the current concepts of chromosome structure and replication, and simultaneous replication of all initiation points are applicable for Eudorina.

The growth period of 23 h can be considered an extended G_1 period. After the first doubling of DNA a G_2 period was not detectable nor was there any detectable G_1 and G_2 periods between each of the following 3 or 4 S periods. This observation would suggest that under conditions of rapid cell division G_1 and G_2 are expendable. However, even in their absence, the events associated with G_1 and G_2 may still be present, only temporally displaced in the cell cycle.

The rapid progression of the mature gonidia through cell division indicates that there is little time to correct any errors made by the

cell during replication and division. Thus Eudorina is capable of interrelating and coordinating a variety of functional capacities and is able to ascertain specific information concerning the amount of structural elements existing to ensure the fidelity of multiple rounds of cell division. The integrity of such a complex system may be examined by the selective application of conditions that alter those critical functions necessary for the initiation, continuation, and/or completion of cell division. Any perturbations to the system would be manifested by abnormal cellular events. This approach, although only superficially discussed, may provide further insight into those components of the cycle regulating cell reproduction.

The attainment of cell division synchrony during this study has provided the opportunity to investigate the progression of nucleic acid synthesis in the life cycle of Eudorina and will prove to be very useful in future investigations. The unified progression of cells through their life cycle amplifies the cellular processes of a single cell at any given time. Thus the sequence and regulation of gene action in development and differentiation can be determined with greater ease. Not only will synchronized cultures provide the tool for the analysis of any cell parameter but the study of the causal factors responsible for the aligment of individual cell cycles may give an indication of conditions necessary for cell reproduction. This study has provided one of the best synchronized systems available: (1) the degree of cell cycle aligment is excellent; (2) cultures are easily established and maintained over an indefinite number of cycles; (3) physiological shocks which would impose unnatural patterns of cellular consituent

accumlations are not employed; and (4) the organism is not unicellular but multicellular. As far as I am aware this is the first report of synchronous growth of a colonial organism.

In Eudorina synchronous cellular events are exhibited at three levels. Firstly, all the cells of a culture can be induced to divide together. Secondly, each of the 16 or 32 cells of coenobium divides almost in unison within the same coenobium, showing only a very shallow mitotic and cleavage gradient. Thirdly, a very tight synchrony exists for each stage of nuclear and cytoplasmic division within each developing embryo. Although it remains to be shown, it is expected that these cells undergoing synchronous reproduction are morphologically and psysiologically similar at any given time. Also considering the multicellular nature of Eudorina, the present system is readily available for the study of intercellular interactions. Since this coenobial organism represents an evolutionary attempt at multicellular organization future research in this direction could prove promising.

The aim of most current work on cell reproduction is the determination of the molecular events that underlie each cycle of division (G_1, S, G_2, M) with the hope of finding the cause and effect relationships that coordinate the entire cycle. It is hoped that this study has laid a solid foundation for research, using Eudorina as an experimental system for such studies.

BIBLIOGRAPHY

Bernstein,E. 1960. Synchronous division in Chlamydomonas moewusii. Science 131: 1528-29.

Bernstein,E. 1964. Physiology of an obligate photoautotroph (Chlamydomonas moewusii) I. Characteristics of Synchronously and randomly reproducing cells and hypothesis to explain their population curves. J. Protozool. 11 (1): 56-74

Buffaloe, N.D. 1958. A comparative cytological study of four species of Chlamydomonas. Bull. Torrey Bot. Club. 85: 157-178.

Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62: 315-323.

Cain, Brother, J. 1965. Nitrogen utilization in 38 fresh water Chlamydomonad algae. Can. J. Bot. 43: 1367-78.

Carroll, J.W., Thomas, J., Dunaway, C. and O'Kelley, J.C. 1970. Light induced synchronization of algal species that divide preferentially in darkness. Photochem. Photobiol. 12: 91-8.

Cave, M.S. and Pocock, M.A. 1951. Karyological studies in the Volvocaceae. Amer. J. Bot. 38: 800-11.

Chiang, K.S. 1968. Physical conservation of parental cytoplasmic DNA through meiosis in Chlamydomonas reinhardi. Biochem. 60: 194-200.

Counts, W.B. and W.G. Flam. 1966. An artifact associated with the incorporation of thymine into DNA preparations. Biochem. Biophys. Acta 114: 628-630.

Deitch, A.D. 1966. Cytophotometry of nucleic acids. In Weid, G.L. (Ed.), Introduction to Quantitive Cytochemistry, Academic Press, N.Y. 327-54.

Fand, S.B. 1972. Enviromental conditions for optimal Feulgen hyrolysis. In Weid, G.L. and Bahr, G.F. (Ed.), Introduction to Quantitative Cytochemistry II, Academic Press, N.Y., 211-222.

Fontaine, J.C. and Swartz, F.J. 1972. Fluctuations of Feulgen- and diphenylamine-DNA in peripheral leukocytes. J.Cell Bio. 80: 281-290.

Giles, K.W. and A. Myers. 1956. Improved Diphenylamine Method for the estimation of deosyribonucleic acid. Nature 206: 93.

Goldstein, M. 1964. Speciation and mating behavior in Eudorina. J. Protozool. 11, 317-344

Gottlieb-Rosenbrantz, P. and O'Brien, R. 1971. A cytophotometric study of the deoxyribonucleic acid-feulgen dye content and area of human granulocytes and lymphocytes. J. Histochem. Cytochem. 19, 232-243.

Hoogenhaut, H. and Amesz, J. 1965. Growth rates of photosynthetic mocroorganisms in laboratory cultures. Arch. Mikrobiol. 50: 10-25.

Howard, A. and S.R. Pelc. 1953. Synthesis of deoxyribonuleic acid in normal and irradiated cells and its relationship to chromosome breakage. Heredity 6 (suppl.): 261-274.

Hubbard, R.W., Matthew, W.T. and Moulton, D.W. 1972. Factors influencing the determination of DNA with indole. II. Recovery of hydrolyzed DNA from protein precipitates. Anal. Biochem. 46: 461-472.

Iwamura, T. 1965. Change of nucleic acid content in Chlorella cells during the course of their life-cycle. J. Biochem. 42: 575-589.

Jacobson, B.S. and T.C. Lee. 1967. Macromolecular synthesis and delayed mitotic death due to radiation and drugs in Chlamydomonas and Chlorella. Rad. Res. 31: 368-381.

Jeffery, W.R., Stuart, K.D. and Frankel, J. 1970. The relationship between deoxyribonucleic acid replication and cell division in heat-synchronized Tetrahymena. J. Cell Bio. 46: 533-543.

John, P.C.L., McCullough, W., Atkinson, A.W. Jr., Forde, B.S., and Gunning, B.E.S. 1972. The cell cycle in Chlorella. In Balls , M. and Billett, F.S. (Eds.), The Cell Cycle in Development and Differentiation, Cambridge University Press, N.Y., 61-76.

Johnson, U.G. and Porter, K.R. 1968. Fine structure of cell division in Chlamydomonas reinhardi. J. Cell Biol. 38, 403-425.

Kates, J.R., Chiang, K.S. and Jones, R.F. 1968. Studies on DNA replication during synchronous vegetative growth and gametic differentistion in Chlamydomonas reinhardtii. Expl. Cell Res. 49: 121-135.

Kemp, C.L. and Lee, K.A. 1975. Synchronous growth in the colonial alga, Eudorina elegans (submitted for publication).

Kemp, C.L., Tsao, and G. Thorson. 1972. Ultraviolet light induced thymine dimers and repair processes in the alga Eudorina elegans. Can. J. Microbiol. 18: 1809-1815.

Kemp, C.L. and Wentworth, J.W. 1971. UV radiation studies on the colonial alga Eudorina elegans. Can. J. Microbiol. 17: 1417-24.

Klenow, H. 1962. Further studies of the effect of deoxyadenosine on the accumulation of deoxyadenosine triphosphate and inhibition of Deoxyribonucleic acid synthesis in Ehrlich ascites tumor cells in vitro. Biochem. Biophs. Acta 61:885-896.

Lee, K.A. and Kemp, C.L. 1975. Chemical estimations of DNA changes during synchronous growth of Eudorina. (Submitted for publication).

Lee, K.A. and Kemp, C.L. 1975. Microspectrophotometric analysis of DNA replication in Eudorina elegans. (Submitted for publication).

Lien, T. and Knutsen, G. 1973. Phosphate as a control factor in cell division or Chlamydomonas reinhardti, studied in synchronous cultures. Exptl. Cell Res. 78: 79-88.

Lowry, O.H. Rosenbrough, N.J., A.L. and Randall, R.J. 1951. Protein measurements with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Markham, R. 1955. Nucleic acids, their components and related compounds. In Paeck, K. and Tracey, M.V. (Ed.), Modern Methods of Plant Analysis, vol. IV Springer-Verlag, Berlin, 246-303.

McCullough, W. and John, P.C.L. 1972. A temporal control of the de novo synthesis of isocitrate lyase during the cell cycle of the eucaryote Chlorella pyrenoidsa. Biochem. Biophys. Acta 296: 287-296.

Morley, C.G.D. and H.S. Kingdom. 1972. Use of ^{3}H-thymidine for measurement of DNA synthesis in rat liver - a warning. Anal. Biochem. 45: 298-305.

Rayns, D.G. and Godward, M.B.E. 1965. A quantitative study of mitosis in Eudorina elegans in culture. J. Exptl. Bot. 16: 569-80.

Scherbaum, O.H. 1962. A comparison of synchronized cell division in protozoa. J. Protozool. 9: 61-4.

Scherbaum, O.H. 1964. Comparison of synchronous and synchronized cell division. Exptl. Cell Res. 33: 89-98.

Schmidt, R.R. 1961. Nitrogen and phosphorous metabolism during synchronous growth of Chlorella pyrenoidosa. Exptl. Cell Res. 23: 209-17.

Senger, H. and Bishop, N.J. 1969. Light-dependent formation of nucleic acids and its relation to the induction of synchronous cell division in Chlorella. In Padilla, G.M., Whitson, G.L., and Cameron, I.L. (Ed.), The Cell Cycle: Gene-Enzyme Interactions, Academic Press, N.Y., 179-202.

Sheridan, W.F. and D.M. Steffensen. 1965. Incorporation of ^{3}H-thymidine into chloroplast DNA of marine alga. J.Cell Biol. 25: 619-626.

Smillie, R.M. and Krotkov, G. 1960. The estimation of nucleic acids in some algae and higher plants. Can. J. Bot. 38: 31-49.

Smith, K.C. 1963. Photochemical reactions of thymine, uracil, uridine, cytosine, and bromouracil in frozen solutions and in dried films. Photochem. and Photobio. 2: 503-517.

Sokal,R.R. and Rohlf, F.J. 1969. Biometry, W.H. Freeman, San Francisco.

Starr, R.C. 1964. The culture collection of algae at Indiana University. Amer. J. Bot. 9: 1015-44.

Starr, R.C. 1971. The culture collection of algae at Indiana University - Additions to the collection July 1966 - July 1971. J. Phycol. 7: 350-62.

Steffensen, D.M. and W.F. Sheridan. 1965. Incorporation of ^{3}H-thymidine into chloroplast DNA of marine algae. J. Cell. Biol. 25: 619-620.

Sueoka, N. 1960. Mitotic replication of deoxyribonucleic acid in Chlamydomonas reinhardi. Proc. Natl. Acad. Sci. 46: 83-91.

Swinton, D.C. and P.C. Hanawalt. 1972. In vivo specific labeling of Chlamydomonas chloroplast DNA. J.Cell Biol. 54: 592-597.

Tamiya, H., Morimura, Y., Yokota, M. and Kunieda, R. 1961 Mode of nuclear division in synthronous cultures of Chlorella: Comparison of various methods of synchronization. Plant and Cell Physical. 2: 383-403.

Tamiya, H. 1966. Synchronous cultures of algae. Ann Rev. Plant Physical. 17: 1-26

Taylor, J.H. 1968. Rates of chain growth and units of replication in DNA of mammalian chromosomes. J. Molec. Biol. 31; 579-594.

Tucker, R.H. and Darden, W.H. 1972. Nucleic acid synthesis during the vegetative life cycle of Volvox aureus M5. Arch. Mikcobiol. 84: 87-94.

Wachsman, J.T., S. Kemp and L. Hogg. 1964. Thymine less death in Bacillus metaterium. J. Bacter. 87 (5): 1079-1087.

Wanka, F., H.F.P. Joosten and W.J. de Grip. 1970. Composition and synthesis of DNA in synchronously frowing cells of Chlorella pyrenoidsa. Arch. Mikrobiol. 75: 25-36.

Wanka, F. and Mulders, P.F.M. 1967. The effect of light on DNA synthesis and related processes in synchronous cultures of Chlorella. Arch. Mikrobiol. 58: 257-269.

Wanka, F., I.K. Vasil and H. Stern. 1964. Thymidine kinase: The dissociability and its bearing on the enzyme activity in plant material. Biochem. Biophys. Acta 85: 50-59.

Webb, J.M. and Lundstrom, H.V. 1965. Acid solubilization of animal tissue nucleic acids as related to their extraction and estimation. Arch. Biochem. Biophys. 112: 273-281.

Weid, G.L., Bahr, G. and Bartels, P. 1970. Automatic analysis of cell images by TICAS. In Weid, G.L. and Bahr, G. (Eds.), Automated Cell Indentification and Cell Sorting. Academic Press, N.Y., 195-356.

Wentworth, J.W. 1970. A preliminary investigation of growth parameters, of ultra-violet light-and drug-sensitivity and of mutant isolation of the colonial green alga, Eudorina elegans. Ehrenberg. M. Sc. Thesis, Simon Fraser University, Burnaby, Canada.

Yates, J.E. and Kochert, G. 1972. Patterns of RNA and DNA synthesis in synchronized cultures of Volvox carteri. J. Phycol. 8 (suppl): 16.

Zimmer, H.G. 1970. Automatic analysis of microscopic images. Zeiss Information 74, 126-132.

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