Timing of the phases of the cell cycle with tritiated thymidine and Feulgen cytophotometry during the period of synchronous division in *Lymnaea*

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**SUMMARY**

The duration of the phases of the cell cycle during the 1-, the 2- and the 4-cell stage of the *Lymnaea* egg were determined with [$^3$H]thymidine and with Feulgen cytophotometry. The M, S and G$_2$ phases occupy 48, 27 and 25% of the first three cell cycles. A G$_1$ phase cannot be observed. Only from the 4-cell stage was [$^3$H]thymidine readily incorporated into DNA. The theory that an increase in respiration during the S phase of the 4-cell stage is connected with the energy requirements of DNA synthesis is discussed.

**INTRODUCTION**

In eggs of *Lymnaea stagnalis* it has been observed that cleavage delay and alterations of normal development, induced experimentally during the first cleavages, are related to periodically recurring cell activities (Geilenkirchen, 1964, 1966; Camey & Geilenkirchen, 1970; Labordus, 1970a, b, c; E. K. Boon-Niermeyer, personal communication). Depending on the kind of treatment, various sensitive periods have been demonstrated within each cleavage cycle. For the interpretation of the results information had to be obtained about recurring metabolic processes during the early period of development.

Reduplication of DNA in each cleavage cycle represents one of these processes. In the study of DNA metabolism the incorporation of [$^3$H]thymidine is frequently used as an indication for DNA synthesis. Another much-applied technique is the microphotometric determination of the Feulgen dye content of the nuclei. Results with both techniques will be dealt with in this paper.

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MATERIALS, METHODS AND RESULTS

Timing of the phases of the cell cycle using [3H]thymidine and autoradiography

Introduction

DNA synthesis can be demonstrated by the incorporation of labelled precursors. If the incorporation of thymidine (Tdr) is used as an indication of DNA synthesis, the following must be taken into consideration.

Tdr has to be converted successively into thymidine monophosphate (dTMP), thymidine diphosphate (dTDP) and thymidine triphosphate (dTTP) before it can be incorporated into DNA. This pathway is a minor route, it is a salvage pathway (Cleaver, 1967). The major route starts with the methylation of deoxyuridine monophosphate into dTMP (Friedkin, 1963). For this reason incorporation of Tdr can only be used as a reliable indicator of DNA synthesis if the enzymes for conversion of Tdr into dTTP are present. Even if the enzymes are present it must be realized (a) that exogenous Tdr may exert a feedback control upon the synthesis of thymidine nucleotides in the cell (Nemer, 1962), (b) that high concentrations of Tdr in the medium may block the cells from entering the DNA synthetic phase (Bootsma, Budke & Vos, 1964), and (c) that cells which fail to incorporate Tdr may replicate DNA (Stone & Prescott, 1964). Evidently, the rate of incorporation of Tdr does not necessarily reflect the rate of DNA synthesis, and lack of detectable incorporation does not necessarily indicate absence of DNA synthesis.

Materials and methods

Egg masses of Lymnaea stagnalis were obtained by spontaneous or stimulated oviposition. Stimulation was achieved by refreshing and aerating the water of the aquaria in which the adult snails were kept and raising the temperature to 25 °C (Raven & Bretschneider, 1942). The egg capsules were freed from an egg mass by cutting it open longitudinally and rolling the capsules over moistened filter paper. As the sticky mucus adheres to the paper the capsules become detached from each other. The eggs were stored in salt dishes with copper-free tap-water until used for experiments.

Within one and the same egg mass the divisions are not synchronous. Groups of almost synchronously dividing eggs were obtained by selecting eggs which started to divide within successive periods of 2 or 3 min. The first and last group of each egg mass were used to determine the average duration of the successive cleavage stages. The mean time-lapse between the first and last dividing group was about 30 min. The temperature at which the experiments were performed varied from 22 to 26 °C.

Autoradiography. The concentration of [3H]Tdr (Schwarz, specific activity 3 Ci/mM) in which the eggs were incubated was 25 μCi per ml tap-water. After incubation the eggs were decapsulated with watchmaker's forceps, fixed for at
least 1 h in alcohol 96 %, acetic acid 3:1, and washed in alcohol 70 % for one to several hours. Then the eggs were transferred to a 2 % agar solution at 60 °C in which they were oriented. After solidification, agar containing eggs was cut into small pieces which were dehydrated in an alcohol-amyl acetate series. After embedding in paraffin the blocks of agar were sectioned at 2 μm thickness. Sections were mounted on coated slides and covered with Kodak AR10 stripping film. In two experiments a number of slides were previously treated with DNase (Nutritional Biochemical Corporation) for 3 h at 37 °C. The enzyme was dissolved at a concentration of 0.1 μg/ml in a 0.05 M Tris-buffer (pH 7.4) with a final concentration of 0.003 M-MgCl₂. The slides were stored at 5 °C in light-tight boxes with silica gel. After an exposure time of 2–8 months the autoradiographs were developed in Kodak D-19b developer for 5 min at 18 °C, rinsed for ½ min in distilled water, fixed in acid fixative for 15 min and finally washed in filtered tap-water for 30 min. After drying, the autoradiographs were stained with methyl green–pyronine. A nucleus was scored as labelled if the total number of grains over the sections thereof was significantly higher than over comparable surfaces of the film adjacent to the egg.

**Cytophotometry.** At successive intervals eggs were decapsulated, fixed for 1 h in a mixture of alcohol 80 %, formol 40 %, acetic acid (85:5:10) or in alcohol 96 %, acetic acid (3:1), and subsequently washed in alcohol 96 % for about 24 h. With a small drop of alcohol the eggs were transferred to a clean slide. As long as the alcohol had not evaporated it remained possible to orient the eggs, usually with the vegetative pole facing the slide. Evaporation of the alcohol fixed the eggs firmly to the slide. Moreover, this procedure made the eggs shrink considerably, and this had the advantage of concentrating the Feulgen-positive material of the nuclei. Eggs of one lot were mounted on the same slide. Finally, the slides were dipped in a solution of 2 % agar at 60 °C and dried again. The agar film held the eggs to the slide, especially at the moment of transfer from hydrochloric acid to tap-water. Hydrolysis was performed in 5 N-HCl at 25 °C for ½ h. The eggs were washed for 30 min in stagnant tap-water and stained for 1 h with Schiff’s reagent, washed three times for 2–3 min in SO₂-water and finally in running tap-water for ½ h. As only the nuclei were stained it was difficult to localize the eggs. Therefore, the position of the eggs was marked with indian ink after passage through alcohol 96 % and before mounting. As soon as the alcohol had evaporated the eggs became visible and their position could be marked. After dehydration the slides were mounted in Caedax.

The absorption of light by individual nuclei was measured at 565 nm by means of a Zeiss microscope-photometer, similar to that described by MacGregor (1968). Either the method of Ris & Mirsky (1949) was used which in the aperture of the phototube was adjusted so as to contain the whole nucleus, or the method of Swift (1950) in which the area for measurement had a diameter of about 60 % of that of the nucleus.
Results

In this paper the 1-cell stage (first cleavage cycle, first cell cycle) of a developing egg of *Lymnaea* is defined as the period between the extrusion of the second polar body and the beginning of the first cleavage, the 2-cell stage is the interval between the beginnings of the first and second cleavages, the 4-cell stage is represented by the interval between the beginnings of the second and third cleavages (Fig. 1). At the third cleavage the first quartet of micrometers 1a–1d is formed. Subsequent cleavages are no longer synchronous, macro- and micromeres having their own cleavage rhythms.

![Diagram of cell cycles](image)

Fig. 1. The first three cell cycles of the *Lymnaea* egg. Each cycle represents the interval between the beginnings of two successive divisions.

In order to detect whether the egg capsules prevent the penetration of Tdr into the eggs, they were incubated either with or without the capsule during the 1-, the 2- or the 4-cell stage. Only in the autoradiographs of eggs incubated during the 4-cell stage could grains be observed over the nuclei. The number of grains in the group of eggs incubated within the capsule did not differ from that in the group of decapsulated eggs. Consequently it was decided to incubate the eggs within the capsule.

An increase in the incorporation of labelled Tdr from the 4-cell stage was substantiated by the results of eight experiments in which the reduplication time was studied. For each experiment only one egg mass was used, which was divided into synchronously dividing groups. The eggs were incubated at successive intervals during the 2- and the 4-cell stage. The duration of the corresponding stages within different egg masses was unequal. The average duration of the 2- and the 4-cell stage was $88 \pm 8$ and $86 \pm 8$ min, respectively. To summarize the results of the experiments, the actual incubation times have been transposed to relative times by conversion to a cell cycle of the average duration (Table 1). Mitotic stages observed at the end of the incubation periods are indicated in the same table. It must be realized that it was difficult to establish the transition from interphase to prophase and from telophase to interphase, because the staining intensity of the nuclei with methyl-green–pyronine was very weak.
Timing of the phases of the cell cycle in Lymnaea. I 355

Table 1. Incorporation of [3H]Tdr at the 2- and the 4-cell stages

(The average duration of these stages was 88 ± 8 and 86 ± 8 min, respectively. The actual incubation times have been transposed into relative times by conversion of the actual duration of a cell cycle into a cycle of the average duration.)

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From Table 1 it appears that in none of the eggs incubated during the 2-cell stage could an incorporation of \(^{3}H\)Tdr be detected. The first visible signs of mitosis were observed 51 min after the beginning of the 2-cell stage. The second cleavage started while the eggs were in late anaphase or early telophase. From 5 up to 11 min after the beginning of the 4-cell stage the eggs were in telophase; the first positive autoradiographs were already obtained from eggs fixed 6 min after the onset of cleavage. Thus a presynthetic or G\(_{1}\) phase must be absent. Subsequent groups with labelled nuclei could be obtained if the incubation started earlier than 29 min after beginning of division. From sections extracted with DNase prior to the application of the film, positive autoradiographs have not been obtained. Sections treated with buffer solution were labelled to the same extent as untreated controls. This proves that the incorporation of \(^{3}H\)Tdr indicates DNA synthesis. The most probable interval for the reduplication of DNA during the 4-cell stage is between 5 and 29 min; that is, between 7\% and 34\% of this stage. A postsynthetic or G\(_{2}\) phase of about 23 min (27\%) must be present as mitosis started at about 51 min.

Prior to the 4-cell stage no incorporation of \(^{3}H\)Tdr was detected. However, a very low rate of incorporation could not be excluded. Observation of a very low rate of incorporation requires a much longer exposure time of the autoradiographs. For this reason, in the following experiments eggs were incubated at different intervals during the 1- and the 2-cell stage. Experimental conditions were the same as in the preceding experiments, but the average exposure time of 2 months was prolonged to 8 months. With this method positive autoradiographs were obtained during both stages. As the eggs had not been synchronized the reduplication time must be deduced in the following way. The average duration of both the 1- and the 2-cell stage was 85 min. Assuming that the duration of mitosis was the same as at subsequent stages, the eggs must have been in prophase at about 50 min after the second maturation division and 50 min after first cleavage, respectively. As in the preceding experiments, eggs fixed in prophase were unlabelled. Since the incubation time was 20 min it can be concluded that the reduplication must have been completed in the first 30 min of both stages. Eggs with telophase nuclei fixed at the onset of the 2-cell stage appeared to be labelled; as the incubation was started during the preceding meta- or anaphase, this implies the absence of a G\(_{1}\) phase.

**Timing of the phases of the cell cycle with Feulgen-stained nuclei**

*Introduction*

The first attempts to demonstrate the presence of DNA in interphase nuclei of eggs of *Lymnaea* during early cleavage stages by means of the Feulgen reaction have been negative (Raven, 1946; Minganti, 1950). Negative results have been reported also for eggs of sea urchins (Marshak & Marshak, 1953; Immers, 1957).

The proportionality between dye content and amount of DNA depends, among other things, upon fixation and hydrolysis. Hydrolysis of DNA with
Timing of the phases of the cell cycle in Lymnaea. I

Hydrochloric acid is an essential condition for the Feulgen reaction; it releases purine bases and transforms DNA into apurinic acid. Depurination can be performed in a wide range of temperatures. At high temperatures, however, apurinic acid may be solubilized or extracted, thus reducing the amount of Feulgen-positive material. If hydrolysis is performed at lower temperatures and even if it is continued for several hours, the intensity of the Feulgen stain remains undiminished (Jordanov, 1963; Decosse & Aiello, 1966; Böhm, 1968). For this reason, it was decided to reinvestigate the relationships in Lymnaea. Hydrolysis was performed at 25 °C in 5 N-HCl. Nuclear staining was still further intensified by shrinking the eggs, thus concentrating the Feulgen-positive material.

Results

Timing of mitotic stages. It was difficult to synchronize the eggs before first cleavage. The only reference point for synchronization was the extrusion of the polar bodies and the duration of the mitotic cycle preceding first cleavage has not been studied extensively. One series of observations has been performed.

After the extrusion of the second polar body the female chromosomes started to swell into karyomeres. These chromosomal vesicles gradually coalesced and formed the female pronucleus, which was irregular and usually ring-shaped. Simultaneously, the sperm head was transformed into the regularly shaped and homogeneously stained male pronucleus. The pronuclei became closely apposed but did not fuse (Raven, 1945). Mitosis started 53 min after the extrusion of the second polar body. First cleavage was reached after 90 min.

At the 2- and the 4-cell stage the mitotic cycle could be studied in detail. Eggs were synchronized at the beginning of a cleavage, fixed at regular intervals, Feulgen-stained and analysed for the duration of the different mitotic stages.

The cells were considered to be in prophase as soon as the first indication of a linear organization of the chromosome material could be distinguished. At the end of prophase the nuclear membrane disappeared. Prometaphase represented the movement of the chromosomes towards the equator of the spindle. During metaphase the chromosomes were localized in the equatorial plane. At anaphase the sister chromatids separated and moved towards the opposite poles of the spindle. At the end of chromosome movement telophase is reached.

The results for the 2- and the 4-cell stage are shown in Figs. 2 and 3, respectively. The results of different experiments have been summarized in the same way as mentioned before (Table 1). The duration of the 2- and the 4-cell stage was 84 ± 6 and 82 ± 8 min, respectively. In both stages mitosis started approximately at 50 min (60 % of the cycle).

Timing of the period of DNA synthesis by means of cytophotometry of Feulgen-stained nuclei. Because of the lack of an easily visible reference point, synchronization before first cleavage was difficult. Besides, the female pronuclei were too irregular to be measured with the applied techniques. Swift & Kleinfeld (1953)
reported the same difficulty for eggs of the grasshopper *Melanoplus differentialis.* Finally, the polar bodies were usually situated in the light path of the pronuclei, especially of the female pronucleus. For these reasons, time and duration of the first S phase was partly determined in one experiment only. Up to 30 min after the extrusion of the second polar body the male pronucleus could be measured separately. The results (Fig. 4) demonstrate that Feulgen stain of the male pronucleus did not increase during the first 12 min. It seems reasonable to assume that DNA synthesis in the male pronucleus would have been completed about 37 min after the extrusion of the second polar body. If the same is applicable to the female pronucleus, it may be concluded that a G$_1$ phase has been very short or even absent, and that the pronuclei must have passed a G$_2$ phase from 37 to 56 min after completion of maturation.

![Fig. 2. Duration of mitotic stages during the 2-cell stage. At short intervals after the onset of the 2-cell stage about 10 eggs were fixed. Abscissa: time scale; ordinate: % of nuclei in telophase (●), interphase (×), prophase (○), prometaphase (+), metaphase (□), anaphase (△).](image)

The pattern of DNA synthesis during the 2- and the 4-cell stage is shown in Fig. 5 and Fig. 6, respectively. At early telophase the measured values were smaller than the theoretically expected diploid amount. Discrepancies between the real dye content and the measured absorption could be expected during mitosis when the stain is not uniformly distributed (Swift & Kleinfeld, 1953). At late telophase the chromosomes swell into karyomeres resulting in a more homogeneous distribution of the Feulgen dye. In this way proportionality is
Timing of the phases of the cell cycle in Lymnaea. I

Fig. 3. Duration of mitotic stages during the 4-cell stage. At short intervals after the onset of the 4-cell stage about 10 eggs were fixed. Abscissa: time scale; ordinate: % of nuclei in telophase (●), interphase (×), prophase (○), prometaphase (+), metaphase (□), anaphase (△).

Fig. 4. Reduplication of DNA in the male pronucleus. The amount of DNA is expressed in multiples of the relative amount of DNA present in the sperm head, x (a haploid nucleus). Means and standard errors of determinations of Feulgen-dye content in one experiment. Each point represents 2–7 nuclei.
restored. Consequently, at the earliest telophase stages it was not possible to distinguish between swelling of the chromosomes and the beginning of DNA synthesis. However, it seems reasonable to assume that reduplication started

![Graph showing DNA reduplication at the 2-cell stage.](image)

**Fig. 5.** Reduplication of DNA at the 2-cell stage. Amount of DNA expressed in multiples of the relative amount of DNA, \( x \), present in a haploid nucleus (sperm head). Means and standard errors of the means of five experiments. Each point represents approximately 25 eggs.

![Graph showing DNA reduplication at the 4-cell stage.](image)

**Fig. 6.** Reduplication of DNA at the 4-cell stage. Amount of DNA expressed in multiples of the relative amount of DNA, \( x \), present in a haploid nucleus (sperm head). Means and standard errors of the means of five experiments. Each point represents approximately 25 eggs.
Timing of the phases of the cell cycle in Lymnaea. I

6 min after the beginning of cleavage when the blastomeres were in mid-telophase. At that moment the absorption reached the diploid value. Reduplication was completed after 30 min. It may be concluded that at the 2- and the 4-cell stage the blastomeres did not pass through a G₁ phase. As mitosis started at about 48 min (Figs. 2 and 3) the nuclei passed through a G₂ phase of 18 min. For the 4-cell stage these results are confirmed by the autoradiographic data listed in Table 1.

DISCUSSION

The cell cycle of a differentiated cell can be divided into four phases: mitosis (M phase), followed by a postmitotic-presynthetic phase (G₁ phase), the period in which DNA is reduplicated (S phase) and the interval between the completion of DNA synthesis and the onset of mitosis (G₂ phase) (Howard & Pelc, 1953; Stevens, Daoust & Leblond, 1953). It is characteristic of embryonic cells that they do not pass through a G₁ phase. Reduplication of DNA starting at telophase has been reported for eggs of sea urchins: Arbacia punctulata (Black, Baptist & Piland, 1967), Paracentrotus lividus (Pasteels & Lison, 1951; Nemere, 1962), Strongylocentrotus purpuratus (Hinegardner, Rao & Feldman, 1964; Brookbank, 1970), of the sand dollars Dendraster excentricus (Brookbank, 1970) and Echinarachnius parma (Young, Hendler & Karnowsky, 1969), of the phasmid Clitumnus extradentus (Bergerard, 1955), of the brine shrimp Artemia salina (Fautrez & Fautrez-Firlefyn, 1953) and for the 2-cell stage in the amphibian Xenopus laevis (Graham & Morgan, 1966). Apparently, the same holds true for Lymnaea as during the first three cleavage cycles a G₁ phase could not be demonstrated.

After the second maturation division the two pronuclei pass separately through the first S phase. Synthesis of DNA before nuclear fusion has been reported similarly for the frog Xenopus (Graham, 1966), the sea urchin Paracentrotus lividus (Anderson, 1969), the sand dollar Echinarachnius parma (Simmel & Karnowsky, 1961), the cricket Melanoplus differentialis (Swift & Kleinfeld, 1953), the caddis-worm Sabellaria (Pasteels & Lison, 1951) and the rat and mouse (Alfert, 1950; Dalcq & Pasteels, 1955; Sirlin & Edwards, 1959). In Lymnaea fertilization takes place in the spermoviduct while the eggs are in the first meiotic metaphase (Bretscheider, 1948). Maturation is completed about 300 min later. During this period the sperm head remains localized beneath the cell surface. Only after the end of the maturation divisions does it move towards the centre of the egg, start to swell and finally become associated with the female pronucleus. Karyogamy does not take place (Raven, 1945). Completion of the second maturation division apparently triggers migration and swelling of the male pronucleus and the beginning of DNA synthesis.

The difference between the rate of incorporation of exogenous Tdr before and after second cleavage needs further comment. Reduplication of DNA and synthesis of associated histone are energy-requiring processes. For this reason
several authors postulated a relationship between reduplication of nuclear material and consumption of oxygen (Zeuthen, 1951; Comita & Whiteley, 1953; Holter & Zeuthen, 1957; Whiteley & Baltzer, 1958). A relation between the incorporation of Tdr into DNA and the consumption of oxygen has been demonstrated for HeLa cells by Robbins & Morril (1969).

The uptake of oxygen by individual eggs of *Lymnaea* during the 2- and the 4-cell stage has been measured by Geilenkirchen by means of the Cartesian diver method (1961). In both stages the onset of mitosis coincides with a peak in the uptake of oxygen. However, only at the 4-cell stage a superimposed peak coincides exactly with the incorporation of [³H]Tdr into DNA.

The coincidence of an increased respiratory rate and an increased incorporation of exogenous Tdr into DNA observed at the 4-cell stage might indicate a relation between DNA synthesis and oxygen consumption. If a causal relationship exists between these two processes, the number of oxygen molecules represented by the peak superimposed on the basic pattern of the respiratory rate must be of the same order of magnitude as the number of oxygen molecules necessary for the production of high-energy-rich ATP molecules associated with the reduplication of DNA and the synthesis of nucleohistone. This amount can only be partly calculated. The insertion of each nucleoside into a strand of DNA requires three molecules of ATP. In deoxyribonucleohistone three or four amino acids are present per nucleotide (Bloch, 1963). One peptide bond is formed at the expense of three energy-rich phosphate bonds (Lehninger, 1965). The DNA content of a sperm cell of *Lymnaea* is $2 \times 10^{-12}$ g (R. T. Hinegardner, personal communication; J. J. Haaijman, unpublished results). If it is assumed that the average molecular weight of the nucleotides is 333, then $0.72 \times 10^{10}$ nucleotides are present in a diploid nucleus and three to four times as many amino acids. Insertion of these nucleotides requires at least $2.16 \times 10^{10}$ ATP molecules, the formation of peptide bonds ultimately requires about $7.56 \times 10^{10}$ ATP molecules. Consequently, to insert the theoretical amount of nucleotides and amino acids into deoxyribonucleohistone at the 4-cell stage the embryo requires approximately $38.64 \times 10^{10}$ molecules of ATP. This corresponds to about $6 \times 10^{10}$ molecules of oxygen, for the oxidation of one molecule of glucose with six molecules of oxygen with an efficiency of 42% yields 38 molecules of ATP (Lehninger, 1965). It can further be calculated from Geilenkirchen's data (1961) that the respiratory peak superimposed on the basic pattern of respiration during DNA synthesis at the 4-cell stage represents approximately $38 \times 10^{10}$ molecules of oxygen. So, if it is correct to relate this increased oxygen uptake with reduplication of DNA, a number of other energy-requiring processes must also be involved. One of these processes might be the activation of enzymes associated with DNA synthesis. In *Escherichia coli* the phosphorylation of Tdr into dTMP requires two molecules of ATP; one molecule as a phosphate donor and one for the activation of Tdr kinase, i.e. to increase the affinity of the enzyme for its substrate (Okazaki & Kornberg, 1964). Another energy-requiring
process might be the synthesis of template RNA for the production of nuclear protein. During the S phase of HeLa cells a class of small polyribosomes appears (Robbins & Borun, 1967). In sea-urchin eggs light polyribosomes seem to be engaged in the production of histones (Kedes, Gross, Cognetti & Hunter, 1969; Nemer & Lindsay, 1969). Therefore it is not unlikely that the nucleus produces messenger RNA for the synthesis of histone. At the moment, however, there is no experimental evidence that in *Lymnaea* histones as well as the required templates are produced during the DNA synthetic phase.

During the S phase of the 2-cell stage no change in the basic pattern of oxygen consumption has been observed (Geilenkirchen, 1961). During the same stage [³H]Tdr is only sparingly incorporated into DNA. This might be explained by the existence of a pool of phosphorylated precursors already present in the unfertilized egg. The presence of a pool of acid-soluble deoxyribonucleotide precursors in the unfertilized egg has been demonstrated in a number of organisms (Grant, 1965). A supply of phosphorylated precursors will reduce the amount of ATP molecules necessary for DNA synthesis, and concomitantly the increase in the respiratory rate will be less pronounced or even absent. Another possible explanation might be a difference in the permeability of the egg to the label. However, in a separate series of experiments (to be published elsewhere), no difference in the uptake could be observed.

Summarizing, it may be concluded that it is hard to substantiate or to refute the theory that an increase in respiration during the S phase of the 4-cell stage is connected with the energy requirements of DNA synthesis.

**RÉSUMÉ**

*Détermination des phases du cycle cellulaire par la thymidine tritiée et Feulgen cytophotométrie pendant la période de division synchrone chez Lymnaea*

La durée de la mitose et de la réduplication de l'ADN a été déterminée par l'incorporation de la [³H]thymidine et par la détermination photométrique de la teneur relative en ADN dans les stades à 1, 2 et 4 cellules du développement de l'œuf de la limnée.

Jusqu'au stade à 8 blastomères la mitose prend 48 %, la réduplication de l'ADN 27 % et la phase G₂ 25 % de la durée du cycle. Les cellules ne passent pas par une phase G₁.

L'incorporation de la thymidine exogène est largement augmentée au stade à 4 blastomères, ce qui coïncide avec une élévation de la respiration pendant la réduplication de l'ADN. Au stade à 2 cellules, quand l'incorporation est plus modérée, la respiration basale n'est pas changée.

La supposition est discutée que l'élévation de la respiration pendant la phase S soit liée avec la réduplication du désoxyribonucléohistone.

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REFERENCES


Timing of the phases of the cell cycle in Lymnaea. I


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