Hydra: Research Methods

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Chapter 21

Cell Cycle Analysis of Hydra Cells

Charles N. David

PURPOSE

To measure kinetics of cell cycle and differentiation kinetics, and nuclear DNA content.

INTRODUCTION

Knowledge of the cell cycle kinetics and differentiation kinetics of all hydra cell types is essential to understanding the contribution of each cell type to tissue level development. Obtaining such information has been greatly facilitated in recent years by techniques for labeling hydra with [^{3}H]thymidine (Chapter 25), analysis of cells by maceration (Chapter 20), and microfluorometric techniques for determination of nuclear DNA content. Using these techniques it has been possible to determine the cell cycle kinetics of epithelial cells, interstitial stem cells, and proliferating nematoblasts (David and Campbell, 1972; Campbell and David, 1974) and the differentiation kinetics of nerve cells and stenotele, desmoneme, and isorhiza nematocytes (David and Gierer, 1974).

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MATERIALS

[^{3}H]Thymidine (20–100 μCi/ml; 30 mCi/mM); 2,5-bis(4′-aminophenyl-1′)-1,3,4-oxdiazole (BAO) (Fluka, A.G., Buchs, Switzerland); $K_2S_2O_5$; Kodak AR10 Stripping Film or Kodak NTB Autoradiographic Liquid emulsion.

PROCEDURES

Doubling Time of Populations of Cells

Basic information about the doubling time of a cell population is obtained from growth curves of hydra cultures by analyzing the increase in number of the various cell types. Prepare replicate dishes with 10 hydra each and feed all of them daily being careful not to lose any animals or detached buds. On various days, macerate (Chapter 20) the entire contents of a dish and count the number of cells of a given cell type. Cell numbers of hydra fed daily should increase exponentially; the doubling time of the cell population can be read directly from a semilog plot of cell number versus time. If the entire cell population is dividing mitotically (proliferative fraction = 1.0), then the population doubling time equals the average cell cycle time. If the proliferative fraction is less than 1.0, then the population doubling time will be longer than the cell cycle time.

Cell Cycle Time and Proliferative Fraction

Estimate both cell cycle time and proliferative fraction from the kinetics with which a cell population becomes labeled when continuously exposed to [^{3}H]thymidine. To label hydra continuously with [^{3}H]thymidine, inject animals with 0.1 μl of isotope (20–100 μCi/ml) every 8–12 hr. (Each injection results in a pulse label. However, since the S phase of hydra cells is about 12 hr, repeated pulsing with [^{3}H]thymidine at intervals shorter than 12 hr assures that every cycling cell picks up the label at some point in its S phase). Macerate samples of labeled hydra at various times during the course of the labeling experiment and determine by autoradiography the labeling index (labeled cells/total cells) of the cell type being investigated. Expose autoradiograms of hydra labeled with [^{3}H]thymidine at a concentration of 20–100 μCi/ml for 2 weeks.

An example of a continuous labeling experiment for epithelial cells is shown in Fig. 1. The initial [^{3}H]thymidine pulse labels about 20% of epithelial cells, and the labeling index increased monotonically to 95% by 3 days. The results indicate that 95% of epithelial cells cycled through S phase

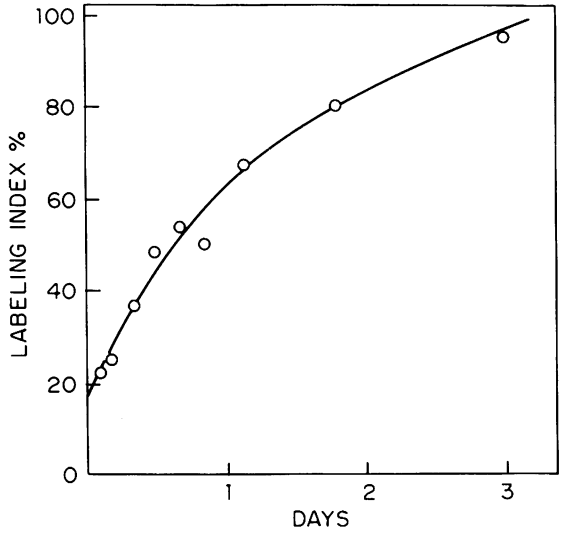

FIGURE 1. Increase in the labeling index of epithelial cells in hydra "continuously" exposed to [^{3}H]thymidine.

in 3 days, and thus the proliferative fraction is at least 0.95. The length of time required to label the entire proliferative fraction of a population is equal to the length of the cell cycle minus the S phase. From the data in Fig. 1, the total epithelial cell cycle is estimated to be 72 hr + S phase, or 84 hr assuming an S phase of 12 hr (see below).

S Phase

Estimate the duration of S phase by using a double labeling technique in which a population of cells in S phase is first labeled with [^{3}H]thymidine and then, after an interval, relabeled with [^{14}C]thymidine (Wimber and Quastler, 1963). Pulse labeling with [^{3}H]thymidine identifies a population of cells in S phase. With time this population proceeds through S phase and enters G2. The fraction of S-phase cells which have moved into G2 after a given interval can be determined by relabeling with ^{14}C; cells in G2 do not take up the [^{14}C]thymidine. The time required for all ^{3}H-labeled cells to move into G2 and therefore not to be labeled by a second ^{14}C pulse is a measure of the length of S phase.

To determine S phase by this procedure, label several groups of 5–10 hydra with [^{3}H]thymidine. Immediately relabel one group with [^{14}C] thymidine; relabel the other groups with [^{14}C]thymidine after 5, 10, 15, and

20 hr, respectively. Macerate each group about 30 min after the [^{14}C] thymidine pulse. Count the percentage of cells labeled only with ^{3}H in autoradiograms prepared with Kodak stripping film. (Cells labeled with ^{3}H and ^{14}C can be distinguished from each other in autoradiograms because ^{14}C decays are more energetic and penetrate the film whereas ^{3}H decays are less energetic and only expose the film immediately above the nucleus of a labeled cell).

A simpler version of the double labeling procedure can be carried out by using only one interval between ^{3}H and ^{14}C. Cells which leave the S phase between the two isotope pulses will be labeled with only [^{3}H]thymidine. The ratio of cells labeled solely with ^{3}H to all cells labeled with ^{14}C increases as the interval between pulses increases. For any given interval between pulses (t) the following relationship holds:

$$^{3}H/^{14}C = t/S$$

where S is the length of S phase, ^{3}H is the fraction of cells labeled solely with ^{3}H, and ^{14}C is the fraction of cells labeled with ^{14}C alone or with ^{14}C and ^{3}H.

G2 Phase

Estimate the duration of G2 by the method of labeled mitoses (Quastler and Sherman, 1959). In most cases mitotic figures in epithelial cells, gland cells, and interstitial cells can be identified in macerations of hydra tissue. However, to obtain data on nests of mitotic interstitial cells (nematoblasts) it is necessary to use histological sections since nests of mitotic interstitial cells are fragile and break up into single cells upon maceration.

To analyze labeled mitoses, pulse label several groups of 5–10 hydra with [^{3}H]thymidine. Macerate (or fix and section) one group of hydra at various times after the pulse. The choice of the time to take those samples depends on the cell type being analyzed: epithelial and gland cells have G2 periods of from 24 to 72 hr and samples can be taken every 8 hr; interstitial stem cells and nests of nematoblasts have G2 periods of from 4 to 24 hr and samples must be taken every 2–3 hr. Mitotic figures can be identified in maceration preparations using phase optics. Staining those cells with Feulgen reagent, however, makes mitotic figures more conspicuous and facilitates scoring. Map mitotic figures on the slides and then cover them with autoradiography film. Expose the slides for 2 weeks, develop them, and determine which mitotic figures are labeled. Plot the fraction of labeled mitoses versus time.

Examples of labeled mitoses curves for nests of two and four interstitial cells are shown in Fig. 2. for synchronous cell populations, the labeling index

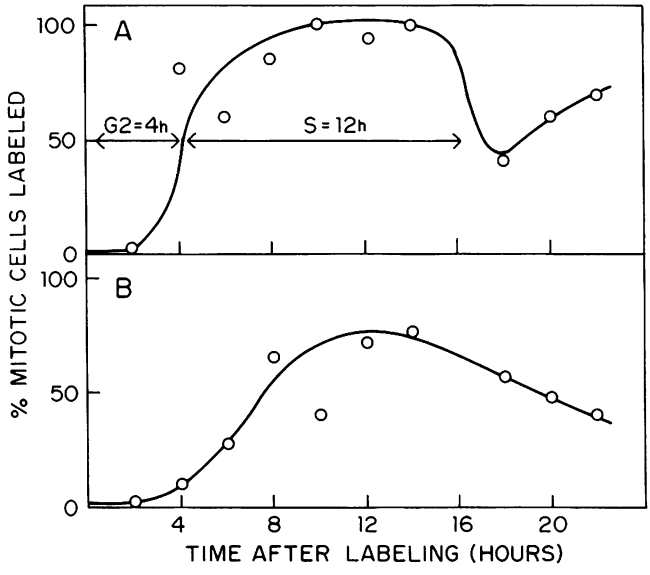

FIGURE 2. Percentage of mitotic figures labeled following pulse of [^{3}H]thymidine. (A) Nests of four interstitial cells; (B) nests of two interstitial cells.

for mitoses rises rapidly from 0 to 100% at a time when the block of cells labeled in S phase has progressed through G2 and entered mitosis. The labeling index remains 100% for a period corresponding to the duration of S and then decreases to 0%. When the block of labeled cells again moves through mitosis, the index again rises to 100%. Natural cell populations are not normally so synchronous because of variations in the speed with which individual cells progress through the cycle. Nevertheless, nests of four interstitial cells yield a fairly square wave of labeled mitoses from which it is possible to estimate G2 = 4 hr and S = 12 hr (Fig. 2A). By comparison, nests of two interstitial cells yield a broad curve which never reaches 100% labeled mitoses (Fig. 2B). Such behavior indicates *variability* in the length of G2 that is significantly greater than the length of S phase (12 hr). Such variability in G2 is a characteristic feature of hydra cells.

Duration of Mitosis

Estimate the duration of mitosis (t_m) in exponentially growing population from the relationship: $t_m = (MI \times T)/2$, where MI is the mitotic index and T the population doubling time (Cleaver, 1967).

Nuclear DNA Content

Measurements of nuclear DNA content on individual cells provide useful additional information about the distribution of cells in various phases of the cell cycle. Cells in G1 have 2C, cells in S have 2C–4C, and cells in G2 have 4C nuclear DNA content, respectively. Measurements of nuclear DNA content are made by staining macerations with a fluorescent Feulgen reagent (Ruch, 1966) and determing the fluorescence intensity of individual nuclei. From the frequency distribution of cells with DNA contents between 2C and 4C, it is possible to estimate the proportion of cells in G1, S, and G2. Proliferating epithelial cells, interstitial stem cells, and nematoblasts have S or G2 DNA contents; virtually none of these cells have G1 DNA contents. By comparison, differentiating nerve cells and nematocytes have G1 DNA contents.

To determine nuclear DNA contents, prepare macerations as described in Chapter 20. Wash the slides briefly and dip them in 1 *N* HCl at 60°C for 10 minutes. Rinse slides in water and stain them for 2 hr with a solution containing 0.01% BAO and 0.5% $K_2S_2O_5$ in 0.05 *M* HCl. Wash slides three times in freshly prepared solution of 0.5% $K_2S_2O_5$ in 0.05 *M* HCl. Determine the fluorescence intensity of individual nuclei using a fluorescence microscope equipped with a photomultiplier.

Differentiation Kinetics and Turnover of Time of Nonproliferating Differentiated Cell Populations

Nerves and nematocytes are nonproliferating differentiated cells derived from stem cells and have G1 nuclear DNA content, indicating they differentiate following a terminal mitosis. To determine the time course of their differentiation and the rate (cells per hydra per day) at which these cells are being produced by hydra tissue, pulse label several groups of hydra with [^{3}H]thymidine and sample macerations of the animals at various times after labeling. Following autoradiography, score the labeling index of nerve cells or nematocytes. The lag time between [^{3}H]thymidine labeling and the first appearance of labeled nerve cells or nematocytes is a measure of the time from the end of the last S phase of the precursor to the appearance of the differentiated cells. For nerve cells it is commonly 12–24 hr; for stenotele nematocytes, 72 hr; for desmoneme and isorhiza nematocytes, 50 hr.

To determine the rate of differentiation of nerve cells and nematocytes, label groups of hydra continuously with [^{3}H]thymidine and sample animals at various times after the start of labeling. The continuous labeling procedure labels the entire precursor cell population and thus after a period of time all newly differentiated nerves and nematocytes will be labeled. From the

increase in labeled cells per unit time, estimate the rate of differentiation in cells per hydra per day.

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