Table II. Growth of *Caenorhabditis elegans* in suspension culture

Time (days)	1 l culture		10 l culture	
	Population number (nematodes/ml)	pH of medium	Population number (nematodes/ml)	pH of medium
0	3,750	5.20	8,800	5.51
2	7,900	5.29	15,000	5.49
4	18,500	5.60	26,000	5,61
7	76,000	6.50	90,000	6.07

medium consisting of 3% gistex, 3% soy-peptone, 1% dextrose, 1% bacto-casitone and 500 µg/ml haemoglobin (Merck, Federal Republic of Germany) was chosen for large scale cultivation of *Caenorhabditis elegans*.

BUECHER and HANSEN[9] first reported on the mass culturing of axenic nematodes in deep medium using continuous aeration. Cultures were performed in glass washing bottles. A 500 ml culture of *C. elegans* in a medium containing 3% yeast extract, 3% soy-peptone, 0.7% dextrose, MEM vitamins® 100 × solution (Grand Island Biological Co., Grand Island, New York) and HLE reportedly yielded a nematode population of over 100,000 nematodes per ml and a wet weight of 5 g of nematode tissue. The large scale cultivation technique reported here is applicable to quantities up to 10 l and over. It combines the effect of constant aeration and stirring in glass ware (spinner flasks) that has originally been designed for the axenic and semi-continuous culture of suspended cells.

A typical experiment is set up as follows. Nematodes are grown up in 100 ml of medium divided over five 250 ml screw-capped Erlenmeyer flasks. When the total yield is at least 2×10^6 nematodes, the cultures are transferred into a small spinner flask containing 1 l of culture medium including 1 ml of antifoam emulsion M-30 (Serva, Federal Republic of Germany). Antibiotics are routinely added to prevent contamination: penicillin G at 1,000 U/ml, streptomycin at 1 mg/ml and fungizone (amphotericin B) at 10 µg/ml. Sufficient air flow is bubbled through the medium, which is mixed slowly by a magnetically driven stirrer mechanism. The air is sterilized first by passage through a 1 inch in-line filter holder equipped with a 0.22 µm pore size membrane. When the population is near its maximum, i.e. when at the end of the exponential growth phase the population growth rate at first slows down, it is transferred into fresh medium in a 10 l capacity spinner flask and grown up until harvest. As with thin film cultures, the pH of the medium rises constantly with increasing nematode number and thus must be frequently checked. Any rise above pH 6.5 is readily followed by subsequent death of a significant part of the nematode population. Fatal rise of the pH can be overcome by the use of moderately acid medium (e.g. pH 5.2 with acetic acid) and by acidifying as needed, or by passage of the air through an acetic acid solution prior to bubbling through the medium.

Population growth of *C. elegans* and coincident changes of the pH of the medium are represented in Table II. It was not attempted to correct for the rise in pH in the 1 l culture; but in the 10 l culture the air was bubbled first through a 25% solution of acetic acid. The total number of nematodes obtained from the 10 l culture was near 1 billion. These were harvested and washed 3 times in 45% (w/w) sucrose solution yielding a wet weight of almost 40 g of very clean nematode tissue. This was obtained in less than 6 weeks, starting from some 50 worms.

This is the first report, to my knowledge, of the cultivation of nematodes in conditions and with yields that are remarkably comparable with those reported for cells and bacteria. There is probably no restriction to further cultivation of suitable free-living nematodes in large capacity fermentators.

[9] E. J. BUECHER and E. L. HANSEN, J. Nematol. *3*, 199 (1971).
[10] W. R. LOWER, E. L. HANSEN and E. A. YARWOOD, J. exp. Zool. *161*, 29 (1966).

Continuous Labelling Method for Autoradiographic Analysis of Cell Cycle Parameters in Steady State Cell Systems[1]

D. GERECKE, A. JEGSEN and R. GROSS

Medizinische Universitätsklinik Köln, Joseph-Stelzmann-Strasse 9, D–5 Köln 41 (German Federal Republic, BRD), 3 November 1975.

Summary. Under certain conditions, continuous availability of ^{3}H-thymidine in steady-state cell systems results in a linear increase of the fraction of labelled cells, the equation of which can be used to determine cell cycle parameters of the system investigated.

For analysis of cell cycle parameters in cell renewal systems, a method introduced by QUASTLER and SHERMAN[2] is usually employed. The method is based on flash labelling of DNA-synthesizing cells by ^{3}H-thymidine (^{3}H-TdR) and subsequent autoradiographic observation of the percentage of labelled mitoses (PLM-method). If wavelike oscillations of labelled mitoses are observed, the experiment gives information about the duration of all phases of the cell cycle. However, if clearcut second waves of labelled mitoses are not obtained, due to strong variations in cell cycle parameters, the available information may be limited to the premitotic gap (t_{g_2}), the mitotic time (t_m) and DNA-synthesis time (t_s), while the cell cycle time (t_c) cannot be measured. Serious problems may arise from early reutilization of ^{3}H-TdR labelled DNA in the cell system under investigation, as demonstrated for granulocytopoiesis[3]. In this situation, a cohort of flash-labelled cells cannot be produced, waves of labelled mitoses are not observed and the method fails to give any results. In order

[1] This research was supported by the Deutsche Forschungsgemeinschaft.
[2] H. QUASTLER and F. G. SHERMAN, Expl Cell Res. *17*, 420 (1959).
[3] D. GERECKE and R. GROSS, Blut *31*, 43 (1975).

to overcome these limitations of the PLM-method, an alternative approach for the determination of cell cycle parameters in steady-state cell systems employing *continuous* labelling with ^{3}H-TdR was theoretically developed and subsequently verified by experiment in mouse granulocytopoiesis.

Theory. Cell renewal systems can be subdivided into a proliferating cell compartment containing all cells in the cell cycle, and a non-proliferating cell compartment containing the cells which have irreversibly left the cycling stage. An influx from morphologically unidentified stem cells into the recognizable proliferating compartment may exist (Figure 1). For the following calculations, 6 assumptions are made: a) the system is in a steady-state, b) a possible influx into the proliferating cell compartment is small compared with the efflux from that compartment, c) the system does not contain a significant proportion of G_0-cells, d) the transit time through the non-dividing cell pool has a defined minimum value, which exceeds the cycle time of the proliferating cells, e) cell death does not occur within the compartments, f) cell cycle time parameters show no variations.

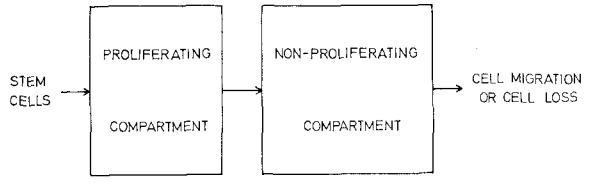

Fig. 1. Flow diagram of a cell renewal system.

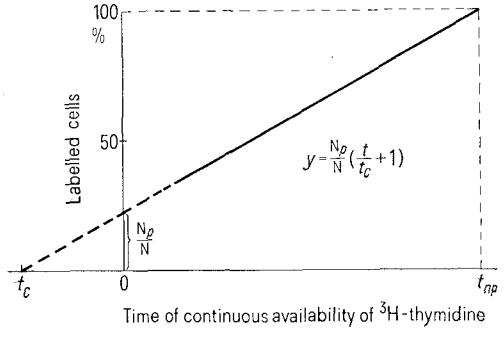

Fig. 2. Theoretical linear increase of the fraction of labelled cells in a steady state cell system during continuous availability of ^{3}H-TdR (for explanation of symbols see text).

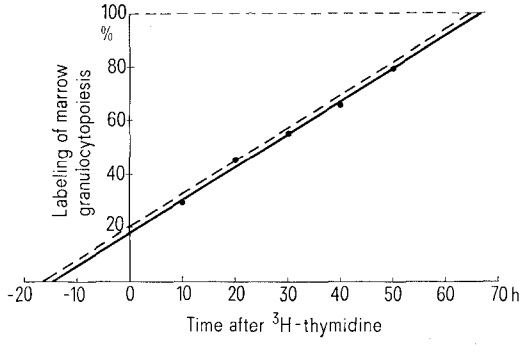

Fig. 3. Labelling of bone marrow granulocytopoiesis in the mouse during continuous availability of ^{3}H-TdR. Each experimental point represents the mean value of 6 animals.

The following symbols are used:

- N, total number of cells within the cell system.
- N_p, number of cells in the proliferating compartment.
- N_{np}, number of cells in the non-proliferating compartment.
- F, flow rate of cells from proliferating to non-proliferating compartment (cells/unit time).
- t_s, DNA synthesis time.
- t_{g2}, premitotic gap.
- t_m, mitotic time.
- t_c, cell cycle time.
- t_{np}, transit time through non-dividing cell pool.
- y, fraction of labelled cells within the cell system.
- t, running time of continuous availability of ^{3}H-TdR.

If continuous availability of ^{3}H-TdR is provided to the proliferating cell pool starting at $t = 0$, all proliferating cells will be labelled after t_c–t_s. As long as the transit time through the non-proliferating compartment is considerably longer than t_c–t_s, the further increase of the fraction of labelled cells within the whole cell system will be linear until labelled cells start to leave the non-proliferating pool. For the time interval defined by t_c–$t_s \leq t \leq t_{np}$, the linear equation describing the fractional increase of labelled cells during continuous availability of ^{3}H-TdR will be derived. In order to simplify the calculation, it is assumed that $t_{g_2}+t_m = 0$, which means that the flow of labelled cells into the non-proliferating cell compartment starts immediately as soon as ^{3}H-TdR becomes available.

The general expression for a linear function with the slope m and going through point $P_1(t_1; y_1)$ is given by

$$y - y_1 = m \cdot (t - t_1) \qquad \text{(I)}$$

In our example, the slope m is given by the fractional increase of labelled cells F/N within the cell system. Complete labelling will be achieved at $t = t_{np}$, so that $t_1 = t_{np}$, and $y_1 = 1$. Entering these expressions into equation I, we obtain

$$y - 1 = \frac{F}{N} \cdot (t - t_{np})$$

and

$$y = \frac{F}{N} \cdot t + 1 - \frac{F}{N} \cdot t_{np} \qquad \text{(II)}$$

The flux F of cells in the system can be expressed either by the output of the proliferating compartment $F = N_p/t_c$ or by the flow of cells through the nonproliferating compartment $F = N_{np}/t_{np}$. In substituting F in equation II by these terms, we arrive at

$$y = \frac{N_p \cdot t}{N \cdot t_c} + 1 - \frac{N_{np}}{N}$$

and

$$y = \frac{N_p \cdot t}{N \cdot t_c} + \frac{N_p}{N}$$

or

$$y = \frac{N_p}{N} \cdot \left(\frac{t}{t_c} + 1\right) \qquad \text{(III)}$$

Equation III describes the linear increase of the fraction of labelled cells for a cell renewal system in steady state during continuous availability of ^{3}H-TdR for the time interval t_c–$t_s \leq t \leq t_{np}$. Discussion of this linear function shows that it has interesting intersections with the abscissa and the ordinate. Thus, for

$t = 0$ it follows that $y = \frac{N_p}{N}$

and for

$y = 0$ it follows that $t = -t_c$.

In other words, the fraction of proliferating cells within the total cell system can be obtained by extrapolation of the linear function to the ordinate; and the cell cycle time can be estimated by extrapolation to the abscissa. Furthermore, as stated above, for $t = t_{np}$, the fraction of labelled cells is equal to 1. Thus, the intersection of the linear function with the 100% level will yield t_{np}. These properties of equation III are shown graphically in Figure 2. Since, in continuous labelling experiments, the entrance of labelled cells into the non-proliferating compartment does not start immediately at $t = 0$ as assumed above, but only after $t_{g_2}+t_m$, the linear function with the properties of equation III can be obtained by shifting all experimental data by the value of $t_{g_2}+t_m$ to the left.

For the sake of simplicity, in the above calculations variations of cell cycle parameters were disregarded. Considering such variations, equation III remains valid for the observation period $(t_c - t_s)_{max} \leq t \leq t_{np\,min}$, if t_c is replaced by the arithmetic mean of t_c. Accordingly, the intersections of the equation will then yield the mean values of the cell cycle parameters.

Experimental results. The theory outlined above was applied to mouse granulocytopoiesis. ♂ Balb/c mice (24–28 g) were injected with ^{3}H-TdR (1 µCi/g body weight) at 8-h intervals to ensure that all cells produced within the experimental period were labelled. Previous experiments had shown that significant numbers of labelled granulocytes do not appear in the circulation up to 50 h after the first injection of ^{3}H-TdR (= minimum transit time through the non-dividing marrow pool). Hence, animals were killed at 10, 20, 30, 40 and 50 h, femoral marrow was removed and processed for autoradiography. The percentage of labelled granulocytic marrow cells was scored after adequate exposure time and Giemsa staining by evaluating 500 cells irrespective of their stage of maturation.

The results obtained are shown in Figure 3. Each point on the graph represents the mean value of 6 animals. A linear increase of the percentage of labelled granulocytic marrow cells was observed between 10 and 50 h, confirming the theory developed above. Extrapolation of the experimental curve to the abscissa and to the 100% level gave values for t_c of 14 h and for t_{np} of 66 h, respectively. The relative size of the proliferating pool, obtained from the ordinate at $t = 0$, was 18%. From the initial part of a PLM-experiment, $t_{g_2}+t_m$ for marrow granulocytopoiesis was estimated to be 2 h. In correcting for $t_{g_2}+t_m$ in the way described above, the final estimates were $t_c = 16$ h, $t_{np} = 64$ h and relative size of proliferating cell pool = 20% (dotted line, Figure 3).

Discussion. The method introduced here for determination of various kinetic parameters of proliferating cell systems is generally applicable under steady state conditions as long as the minimum transit time through the non-proliferating cell compartment considerably exceeds the value of $t_c - t_s$. Compared to the PLM-method, this alternative approach shows a number of advantages. Because of the linear relationship described by equation III, only 2 samples taken from the cell system under investigation during continuous availability of ^{3}H-TdR are needed to obtain estimates for the cycle time, the proliferating fraction and the transit time through the non-proliferating pool. If, in addition, the percentage of labelled cells 1 h after injection of ^{3}H-TdR is determined (flash labelling index), calculation of DNA synthesis time is also possible. Thus, 3 biopsies may give as much information as a complete PLM experiment requiring between 10 and 20 tissue samples. Moreover, in some sit uations, where PLM experiments fail to give clearcut results, the continuous labelling method remains applicable. Whenever second waves of labelled mitoses are not observed after flash labelling, it seems worthwile to investigate the cell system in question under continuous availability of ^{3}H-TdR in order to find out whether a linear increase of the percentage of labelled cells can be demonstrated, which allows application of the theory developed above. Early reutilization of labelled DNA, a process which may lead to a failure of PLM experiments, does not interfere with the continuous labelling method. Thus, in mouse granulocytopoiesis, a plateau curve of labelled mitoses at 100% was observed after a single injection of ^{3}H-TdR due to tracer reutilization, and information about cell cycle parameters was restricted to $t_{g_2}+t_m$ in that experiment[4]. By a continuous labelling experiment, however, it was possible to obtain information about the cell cycle time of the proliferating cells and the proliferating cell fraction in this renewal system, as demonstrated in the experimental part of this communication.

[4] D. GERECKE, unpublished observation.

CURSUS

Italy
ISPRA-Courses on Urban Air Quality Management

in Ispra (Varese), 6–8 October 1976

The course will treat the following items in detail:
Siting and land use: Pollutant monitoring and mapping; admittance of future emitters; urban planning.
Control systems: Pollutant level forecast by models; episode control; warning and alarm systems; emission-reduction strategies. *Social aspects:* Injuries and damages; air quality standards; emission reduction: cost-benefit; arrangements for emergency; legal and administrative aspects.
Practical experiences.
Course coordinator: K. H. Müller, Joint Research Centre, Ispra Establishment.
For information please contact: Secretariat Ispra-Courses, Centro Euratom, I–21020 Ispra, Varese, Italy. Phone 0332/780131, int. 219; Telex 38042–38058.