STUDIES ON THE DIVISION CYCLE

OF MAMMALIAN CELLS

V. Modifications of Time Parameters by

Different Steady-State Culture Conditions

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ABSTRACT

In cultures of murine neoplastic mast cells, the duration of different phases of the division cycle (G_1, S, G_2, and mitosis [M]) was determined under optimal and several well-defined suboptimal growth conditions. Two methods of evaluation were applied to the same culture system: first, the relative number of G_1, S, G_2, and M cells was determined by pulse labeling of samples with thymidine-^{3}H and subsequent radioautography in conjunction with a microfluorometric technique permitting rapid measurements of cellular DNA content; second, after pulse labeling with thymidine-^{3}H, the variations with time of the mitotic labeling index were analyzed. Suboptimal culture conditions were obtained by reducing the concentration of single essential medium components (leucine, glucose, or serum) or by the addition of specific metabolic inhibitors (actinomycin D, amethopterin). Growth-limiting culture conditions resulted in increased generation times. Even under control conditions, the cell number doubling time exceeded the generation time, and this difference was more pronounced in suboptimal media. Under most of the suboptimal conditions tested, the increase in generation time was attributable primarily to an extended duration of the G_1 phase. Under certain growth-limiting conditions, however, other phases were also prolonged. In addition, the variabilities of the generation time and of certain cell cycle phases were increased under suboptimal culture conditions. Results obtained by the two methods of evaluation were, in general, in good agreement with each other. Some differences were, however, observed and interpreted in terms of cell death and/or asymmetric frequency distributions of cell cycle parameters.

INTRODUCTION

A number of physical and chemical agents have been shown to interfere with the normal progression of cells through the division cycle. For instance, cell cycle characteristics may be modified under various suboptimal conditions sustaining proliferation of cell cultures at a reduced rate. Effects of different growth-limiting conditions have, however, not been compared in the same culture system. Furthermore, in previous studies of this type (1–6), cell cycle analysis was, with few exceptions, based on a single method of evaluation, and the cultures were not maintained under rigidly controlled steady-state conditions.

In the present communication, cell cycle char-

acteristics under optimal culture conditions are compared with those under a series of well-defined suboptimal conditions. Suboptimal culture media were obtained by reduction of the concentration of L-leucine, D-glucose, or serum, respectively, or by the addition of a specific metabolic inhibitor, such as actinomycin D or amethopterin. In order to avoid possible variations with time of cell cycle characteristics due to relatively infrequent dilution of cultures with fresh medium or to fluctuations in cell population density, cultures were maintained under steady-state conditions, and cell cycle analysis was carried out at a time when cell number doubling times remained constant at an elevated level imposed by the suboptimal growth conditions chosen.

In view of the possibility that a certain fraction of the cell population, particularly under suboptimal conditions, may not participate in continued proliferation, two methods of cell cycle analysis were applied: the variations with time of the mitotic labeling index after pulse labeling with thymidine-^{3}H were used to determine the generation time and the duration of various cell cycle phases of the cell population passing repeatedly through mitosis (7). In addition, by combining radioautography after thymidine-^{3}H pulse labeling with microphotometric determinations of cellular DNA content (8), relative numbers of cells in different cell cycle phases were obtained which were based on the entire cell population, including cells not participating in continued proliferation.

MATERIALS AND METHODS

Cell Line

Suspension cultures in vitro of a transplantable murine mast cell tumor (cell line P-815-X2) were used. From this cell line, which was obtained from the original P-815 tumor (9) by a selection process in vitro and two consecutive cloning procedures (10, 11), a new clonal subline (P-815X-d) was derived which is characterized by a near diploid chromosome number and a relative homogeneity of its cell population: 30–34 chromosomes/cell were found in 12% of cells, 35–39 chromosomes in 77% of cells, and 40–44 chromosomes in 11% of cells. In order to maintain these characteristics, the cells were kept frozen at −80°C in medium containing 10% dimethyl sulfoxide.

Culture Techniques

The general culture techniques have been previously described (10). After thawing a portion of the frozen cell suspension, the cells were cultured during several days in standard medium containing 10% dialyzed horse serum. This medium has been described as medium I (12). Subsequently the cells were suspended in the medium to be tested and incubated under steady-state culture conditions as reported recently (13). The rate of addition of fresh medium (2 ml/hr) corresponded to the volume of samples (2 ml) withdrawn every hour from the suspension culture, while the total volume of the cell suspension was adjusted to the rate of cell multiplication in order to keep the cell density (cell number per milliliter, as measured with a Coulter counter [Coulter Electronics Inc., Hialeah, Fla.]) nearly constant. In all experiments, cell density was between 2×10^5 and 5×10^5 cells/ml. Cell number doubling times were calculated from the rate of dilution of the culture and changes in cell density.

In order to produce specific metabolic deficiencies, experimental media were used which differed from the standard medium as follows: (a) limitation of L-leucine: 0.02 μmole/ml instead of 0.2 μmole/ml, (b) limitation of dialyzed serum: 1% instead of 10%, (c) limitation of glucose: 0.02 mg/ml instead of 2 mg/ml, (d) addition of actinomycin D at a final concentration of 5×10^{-7} mg/ml or 7×10^{-7} mg/ml, respectively, (e) addition of amethopterin at a final concentration of 2×10^{-5} μmole/ml in combination with hypoxanthine (0.03 μmole/ml) and glycine (0.1 μmole/ml). Under these conditions cell number doubling times (T_2) were prolonged as shown in Table I.

Procedure of Obtaining Samples for Cell Cycle Analysis

After an adaptation period which usually lasted 3–6 days after the onset of steady-state incubation, the cell number doubling time remained at a constant level. Subsequently samples A, B, and C were withdrawn for analysis according to the schedule illustrated in Fig. 1. In the second part of each experiment, the culture was concentrated by centrifugation and resuspension of cells in 10–15 ml of medium. This suspension was incubated during 20 min with 0.2 μCi/ml of thymidine-^{3}H (5 Ci/mmole, The Radiochemical Centre, Amersham, England). Subsequently the cell suspension was diluted to 30 ml with medium, centrifuged, and the cells were washed with medium and reincubated under steady-state conditions during 48 hr. The composition of the medium used for dilution of the culture after pulse labeling, for washing, and for reincubation of cells was identical to that used before pulse labeling except for the addition of unlabeled thymidine (0.01 μmole/ml). After reincubation of pulse-labeled cultures, samples D were withdrawn at intervals of 1 hr.
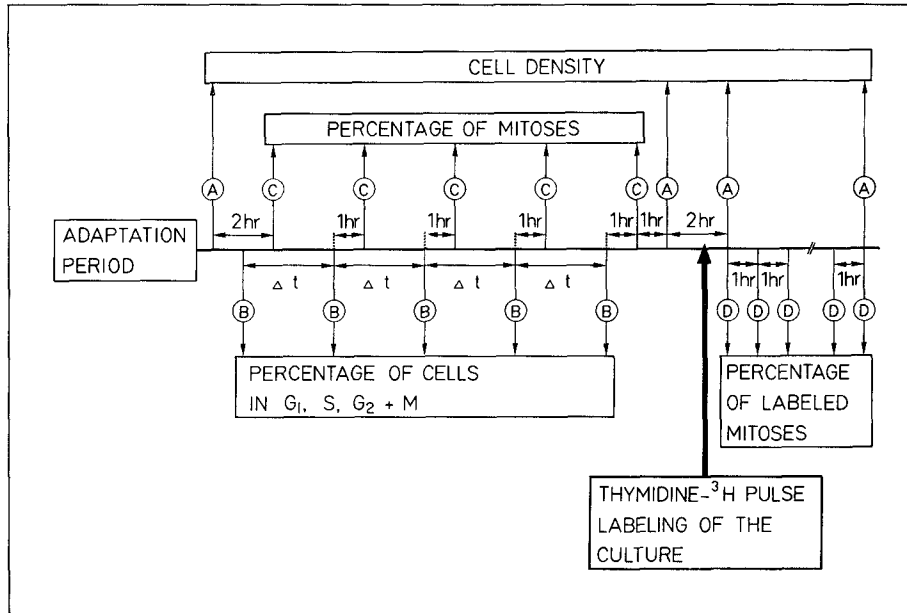
FIGURE 1 Procedure of obtaining samples from steady-state cultures for cell cycle analysis. Δt: 2 hr in control, 6 hr in limitation or inhibition experiments. Volumes of all samples A, B, C, and D were 2 ml each.

Processing of Samples B

Samples of 2 ml each were withdrawn from the cultures and incubated at 37°C during 20 min with thymidine-^{3}H (0.1 μCi/ml, 5 Ci/mmole). Immediately after this incubation period, the cells were fixed by mixing a portion of the suspension with an equal volume of ethanol–acetic acid–water (5:2:3, v/v). The cells were washed with this fixing solution; after 24 hr at 4°C, they were suspended in 70% ethanol, brought onto glass slides, air dried, and treated with a mixture of ethanol–acetic acid (3:1, v/v). Fixed smears were subjected to quantitative staining of DNA with the fluorochrome 2,5-bis-(4'-amino-phenyl-[1']) = 1,3,4-oxdiazole (BAO)[1] as described by Ruch (14). Hydrolysis was carried out in 1 N HCl at 60°C during 5 min. This was approximately half of the hydrolysis time required for maximum staining with the fluorochrome. The stained smears were processed for radioautography, using NTB-2 Kodak emulsion. After 14 days exposure at 4°C, the preparations were developed, fixed, and embedded with Fluormount (Edward Gurr Ltd., London).

Processing of Samples C and D

Cell suspensions of samples C were fixed and stained for determination of mitotic indices as previously described (15).

[1] *Abbreviations:* BAO, 2,5-bis-(4'-aminophenyl-[1']) = 1,3,4-oxdiazole; BUdR, 5-bromodeoxyuridine.

Smears were prepared of samples D as described for samples B, and stained by the Feulgen reaction. Hydrolysis time in 1 N HCl at 60°C was 12 min. Subsequently the smears were processed for radioautography as described for samples B.

Estimation of Radioautographic Background and Efficiency

The frequency distribution of grain numbers per cell was comparable in all preparations. Cells were considered to be labeled if covered by more than five grains. Fig. 2 indicates that under the conditions used, all cells incorporating thymidine-^{3}H were labeled after 6 min. A prolongation of the incubation time up to 20 min did not result in an increase of the proportion of labeled cells higher than expected from cellular kinetics, indicating that essentially all cells incorporating thymidine-^{3}H (S cells) were discerned in this system.

Determination of the Relative Number of G_1, S, G_2, and Mitotic (M) Cells and of Corresponding Time Parameters

The following three ratios were determined directly:
(a) percentage of mitotic (M) cells in samples C,
(b) percentage of labeled (S) cells in samples B, (c) ratio of the number of G_1 cells to the total number of unlabeled (G_1, G_2, M) cells in samples B. Cells were counted as mitoses from the appearance of distinctly
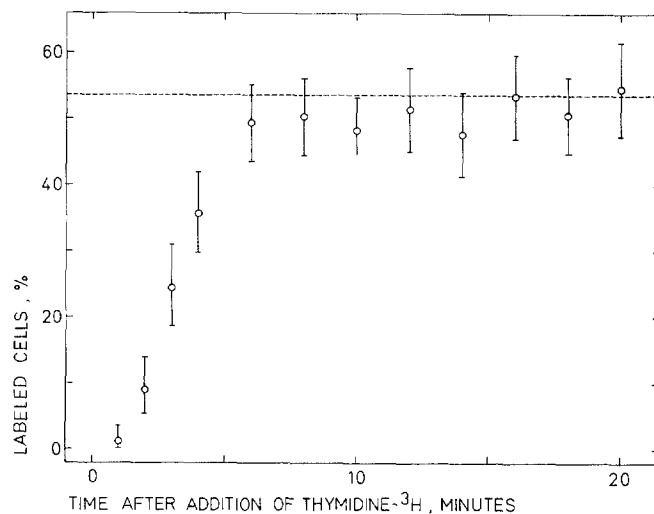
FIGURE 2 Labeling of cells as a function of time of incubation with thymidine-^{3}H. Cells were considered to be labeled if covered by more than five grains. Broken line: per cent labeled cells (mean of samples B from control experiments 1 and 2). Ranges represent 95% confidence limits.

visible chromosomes, through the entire metaphase and into anaphase, while mitotic cells after the onset of furrowing were excluded and counted as two G_1 cells. In radioautographs from samples B, unlabeled cells were classified according to their DNA content, which was measured by microfluorometry as described by Ruch (14). A typical frequency distribution of relative fluorescence intensity per unlabeled cell, as obtained from a culture under optimal conditions, is shown in Fig. 3. It is seen that with the aid of this rapid cytofluorometric method, G_1 cells could be distinguished from G_2 and M cells without difficulty. The duration of individual phases was obtained from the generation time as derived from the periodicity of the mitotic labeling index and the relative number of G_1, S, G_2, and M cells, with the assumption of an exponential frequency distribution $f(\tau)$ of cells with respect to their age (τ) in the cell cycle: $f(\tau) = e^{-k\tau}$, where $k = \ln 2/T$, T being the generation time. For these determinations the graphic method as proposed by Okada (16) was used. Corrections accounting for deviations from ideal pulse labeling will be discussed below. No corrections were, however, made for cell death and nonproliferating cells.

Evaluation of Variations with Time of the Mitotic Labeling Index

In order to confirm that steady-state conditions were maintained during the second part of the experiments, the percentage of labeled cells in samples D was determined and found to be constant during the observation period of approximately 30 hr. The
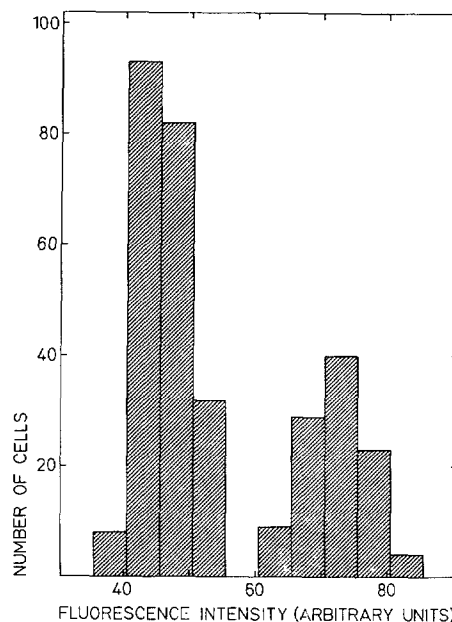

FIGURE 3 Frequency distribution of fluorescence intensity per unlabeled cell in a BAO-stained preparation. Results were obtained from a sample B of a control experiment.

percentage of labeled mitoses (more than five grains per mitotic figure) was determined in samples D which were obtained as shown in Fig. 1. As described by Quastler and Sherman (7), several cell cycle pa-

rameters may be derived from variations with time of the mitotic labeling index as illustrated in Fig. 4: the generation time (T_G) as the time interval between two successive ascending slopes; the duration of the G_2 phase and half the time required for mitosis $(t_{G_2+M/2})$ as the interval between labeling and the first ascending slope; the duration of the S phase (t_S) as the interval between the first ascending and the first descending slope; and the duration of the phases G_2, mitosis, and G_1 $(t_{G_2+M+G_1})$ as the interval between the first descending and second ascending slope. In our studies, these time intervals were measured between points of coincidence of the mitotic labeling index with the average percentage of labeled cells in samples D. In addition, differences in steepness of respective slopes permit an estimation of the variability of corresponding time parameters within the cell population; the steepness of the first ascending slope is determined by the duration of mitosis in addition to the variability of $t_{G_2+M/2}$ (and thus of t_{G_2+M}). In order to derive the duration of the G_1 phase (t_{G_1}) and t_{G_2+M} from the variations with time of the mitotic labeling index, t_M was estimated from the relative number of mitoses and T_G. Corrections accounting for nonideal pulse labeling will be discussed below.

RESULTS

Variations with Time of the Mitotic Labeling Index

Mitotic labeling indices, as determined in samples D obtained from pulse-labeled cultures under various conditions, are shown in Fig. 4. Corresponding numerical values for T_G and for the duration of cell cycle phases, after correction for nonideal pulse labeling (see below), are presented in Table III (columns D). Variations with time of the mitotic labeling index in cultures containing amethopterin could not be evaluated because labeled thymidine interfered with the effects of this inhibitor. Under control conditions, the percentage of labeled mitoses exhibits periodically occurring maxima and minima. The second and the third peak are similar to the first peak, which is characterized by a high degree of symmetry and by relatively steep slopes. These findings indicate a relatively small variability of all time parameters. T_G values obtained under growth-limiting conditions are prolonged and their variabilities are increased. These increases in the variability of T_G may be attributed primarily to increased variabilities of t_{G_1}. Reduction of the glucose concentration, however, also affected the variability of t_S,
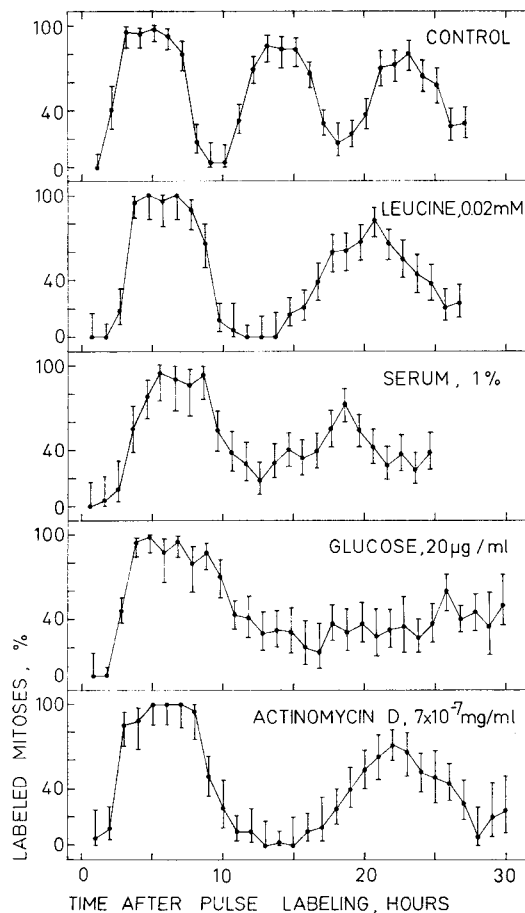

FIGURE 4 Time course of mitotic labeling index after pulse labeling with thymidine-^{3}H in cultures under optimal and various suboptimal growth conditions. Thymidine-^{3}H was added to cultures at zero time. Ranges represent 95% confidence limits.

and in medium containing 1% serum only, the variability of t_{G_2+M} was increased.

Generation Times and Cell Number Doubling Times

In the culture containing glucose at a reduced concentration, T_G could not be derived from mitotic labeling indices because of the absence of a second peak (Fig. 4). An alternative method of computing the generation time was, therefore, applied: T_g as presented in Table I was evaluated from the relative number of labeled cells in samples D and t_S as derived from the mitotic labeling index, by assuming a constant frequency distribu-

TABLE I

Generation Times and Cell Number Doubling Times under Various Culture Conditions

Experiment No.	T_2*	T_G‡		T_g§	f‖
	hr	hr		hr	
1 (control)	10.0	9.3	(8.8–9.9)	9.9	1.9
2 (control)	11.0	10.6	(9.7–11.3)	10.0	1.9
3 (leucine, 0.02 mM)	26.9	13.7	(13.0–14.6)	15.2	1.4
4 (serum, 1%)	17.4	12.5	(9.6–14.8)	16.8	1.6
5 (glucose, 20 mg/liter)	24.4			16.7	1.6
6 (actinomycin D, 5 × 10^{-7} mg/ml)	16.0	13.6	(12.4–)	17.5	1.8
7 (actinomycin D, 7 × 10^{-7} mg/ml)	27.9	16.3	(14.8–17.6)	19.7	1.5
8 (amethopterin, 2 × 10^{-5} mM)	20.8				

* T_2 , time required for doubling of cell number.

‡ T_G , time interval between two consecutive ascending slopes of the mitotic labeling index. In parentheses: intervals based upon 95% confidence limits of mitotic labeling indices as shown in Fig. 4.

§ T_g , derived from t_S and the relative number of labeled cells in samples D (see text).

‖ f, cell multiplication during one generation time T_G (or T_g , if T_G is unknown).

tion $f(\tau)$ of cells with respect to their age (τ) in the cell cycle: $T_g = t_S$/relative number of S cells.

The generation times T_G, as derived from the periodicity of the mitotic labeling index under various culture conditions, are summarized in Table I and compared with corresponding data for cell number doubling times (T_2) and for T_g. Under optimal culture conditions, T_2 exceeds T_G, and this difference is even more pronounced under all growth-limiting culture conditions tested. This is visualized also by the factor f which indicates the relative increase in cell number during one generation time T_G (or T_g , if T_G is not available). A certain proportion of the cell population did, therefore, not participate in continued cell proliferation. In control cultures, T_g is comparable to T_G, while the increase of t_g under limiting culture conditions is more pronounced than that of T_G.

Relative Numbers of Cells in Different Phases

The mitotic index in samples C and the relative number of G_1, S, and G_2 + M cells in samples B were determined in the first part of the experiments as described under Materials and Methods. Within individual experiments, the results obtained at the time intervals shown in Fig. 1 did not differ significantly from each other, indicating that a reasonable degree of asynchrony had been attained in the steady-state culture system used. Data from each experiment were, therefore,

pooled. The resulting distributions of cells with respect to different cell cycle phases are presented in Table II: with the exception of the effects of amethopterin, all growth-limiting culture conditions resulted in an increase of the relative number of G_1 cells.

Corrections Required for Comparison of Two Groups of Data

Based upon T_G (or T_g , if T_G was not available) and the relative number of G_1, S, G_2, and M cells, values for t_{G1}, t_S, t_{G2}, and t_M were estimated as described under Materials and Methods. The results are summarized in Table III (columns B and C) and compared with those derived from the periodicity of the mitotic labeling index (columns D). In the estimation of these data, the following three corrections were made: first, the availability of thymidine-^{3}H during the incubation time of 20 min results in underestimations of t_{G2+M} as derived from samples B and of t_{G1} as derived from samples D, with corresponding overestimations of t_S as derived from samples B and D. On the other hand, the time lag between addition of thymidine-^{3}H and labeling of cells was a few minutes only. The data presented in Table III are, therefore, corrected by 0.3 hr.

Second, in samples D a continued incorporation of label by cells entering DNA synthesis at the end of the 20 min incubation with thymidine-^{3}H should be considered, because these cells may have formed labeled thymine nucleotides at the

TABLE II

Relative Number of Cells in Different Phases (Per Cent of Total Cell Number and Estimations of 95% Confidence Limits) under Various Culture Conditions

Experiment No.	G_1 cells (samples B)	S cells (samples B)	S cells (samples D)	G_2 + M cells (samples B)	M cells (samples C)
	%	%	%	%	%
1 (control)	35.2 ± 1.8	50.6 ± 1.7	52.5 ± 3.8	14.2 ± 1.5	1.56 ± 0.13
2 (control)	32.5 ± 2.6	53.8 ± 2.0	59.0 ± 4.4	13.7 ± 2.3	2.08 ± 0.18
3 (leucine, 0.02 mM)	56.9 ± 2.1	35.9 ± 1.9	40.7 ± 4.4	7.2 ± 1.8	1.12 ± 0.11
4 (serum, 1%)	43.2 ± 2.3	41.8 ± 2.1	42.2 ± 4.2	15.0 ± 1.8	1.40 ± 0.12
5 (glucose, 20 mg/ liter)	48.1 ± 2.6	40.3 ± 2.1	46.8 ± 3.9	11.6 ± 2.1	1.36 ± 0.11
6 (actinomycin D, 5 × 10^{-7} mg/ml)	50.4 ± 3.0	37.1 ± 2.1	38.8 ± 4.0	12.5 ± 2.5	1.86 ± 0.17
7 (actinomycin D, 7 × 10^{-7} mg/ml)	68.5 ± 2.5	26.7 ± 2.3	36.5 ± 3.4	4.8 ± 1.4	1.21 ± 0.11
8 (amethopterin, 2 × 10^{-5} mM)	30.1 ± 2.1	61.1 ± 1.9		8.8 ± 1.5	1.20 ± 0.11

TABLE III

Estimated Durations of Cell Cycle Phases (t_{G_1}, t_S, t_{G_2+M}, t_M) as Obtained by Two Methods

Results based upon the generation time (T_G) and the relative number of cells in different phases (samples B and C) are compared with data derived from variations with time of the mitotic labeling index (samples D). Data are corrected for nonideal pulse labeling as discussed in the text.

Experiment No.	T_G	t_{G_1}		t_S		t_{G_2+M}		t_M
	D	B	D	B	D	B	D	C
	hr	hr		hr		hr		hr
1 (control)	9.3	2.6	2.8	4.7	4.5	2.0	2.0	0.2
2 (control)	10.6	2.6	3.1	5.7	5.4	2.3	2.1	0.3
3 (leucine, 0.02 mM)	13.7	6.5	5.7	5.5	5.2	1.7	2.8	0.3
4 (serum, 1%)	12.5	4.4	3.2	5.3	6.2	2.8	3.1	0.3
5 (glucose, 20 mg/liter)*	16.7	6.5	7.1	7.2	7.0	3.0	2.6	0.4
6 (actinomycin D, 5 × 10^{-7} mg/ml)	13.6	5.6	5.4	5.3	6.0	2.7	2.2	0.4
7 (actinomycin D, 7 × 10^{-7} mg/ml)	16.3	9.7	7.7	5.1	6.4	1.5	2.2	0.4

* Estimations based on the generation time T_g, because T_G is not available.

time of dilution with medium containing unlabeled thymidine. This would result in an overestimation of t_S and a corresponding underestimation of t_{G_1}. In fact, as shown in Table II, relative numbers of S cells in samples D exceeded those in samples B. Based on the average difference of 4%, the values of t_S and t_{G_1} were corrected accordingly. The corrections were 0.2–0.7 hr depending on the experiment.

Third, results obtained for t_{G_2+M} from mitotic labeling indices are overestimated due to manipulations required for labeling of cultures. In experiments with continuous thymidine-^{3}H labeling, i.e. without centrifugation of cells, the values obtained for t_{G_2+M} were shorter than those from

pulse-labeled cultures. In cultures under control conditions, this difference was 0.4 hr. In all experiments, values for t_{G_2+M} as derived from samples D were, therefore, corrected by 0.4 hr.

Duration of Individual Cell Cycle Phases

As seen in Table III, t_S and t_{G_2+M}, as derived from samples B, were affected to a relatively minor degree by the variations of culture conditions tested, and the increases in generation time in these suboptimal media are attributable primarily to increases in t_{G_1}. On the other hand, as seen in Table II, addition of amethopterin resulted in a

decrease of the relative number of G_1, G_2, and M cells. Based on the assumption of a generation time in the range of 10–20 hr, the values for t_{G1}, t_S, and t_{G2+M} in this experiment would be 2.3–4.7 hr, 6.4–12.8 hr, and 1.3–2.5 hr, respectively, suggesting that t_S is affected to a major extent by this inhibitor.

Data derived from the variations with time of the mitotic labeling index (Table III, columns D) are, in general, in good agreement with those derived from the relative number of cells in different cell cycle phases (columns B). In experiments 3 (leucine 0.02 mM) and 7 (actinomycin D 7 × 10^{-7} mg/ml), however, values for t_{G2+M} as obtained from samples B are considerably lower than corresponding values from samples D. Inverse differences were observed for t_{G1}. It is of interest that in these two experiments, values for t_{G2+M}, as derived from samples B, are even inferior to those of control cultures. In experiment 4 (serum 1%), t_{G1}, as based on data from samples B, exceeds that from samples D, while an inverse difference was obtained for t_S.

DISCUSSION

Characteristics of the Culture System

Addition of fresh medium to cell cultures at relatively infrequent intervals may result in a partial synchronization of cell proliferation (17). In order to avoid such synchronization effects in our experiments, the cultures were maintained under steady-state conditions. The results obtained indicate that after reaching a steady state, relative numbers of cells in different cell cycle phases did not undergo significant changes with time. The culture system used thus provided for satisfactory asynchrony of cultures.

In the studies reported in this communication, analysis of proliferation kinetics was carried out after cell number doubling times had adjusted to the particular suboptimal culture medium and remained constant at the new level. The possibility cannot be excluded that during the adaptation period cellular subpopulations might have been selected under the relatively unfavorable culture conditions. In view of the recent clonal origin of the cell line used and of the relatively short duration of the adaptation period, however, major changes of cellular characteristics due to selection processes appear rather unlikely.

As seen in Tables I–III, a small increase in the concentration of actinomycin D from 5 × 10^{-7} to 7 × 10^{-7} mg/ml resulted in markedly increased deviations of cell number doubling times and various cell cycle parameters from control values. With respect to the other suboptimal growth conditions tested, the studies were restricted to one concentration of the respective medium component. Results obtained with different suboptimal media may, therefore, be used for a qualitative comparison only. As further seen in Tables I–III, cell cycle parameters of the two control cultures were not identical. In particular, estimated values for T_G and t_S in these two cultures differed by approximately 1 hr. This may be attributed to a limited precision of the analysis or, more probably, to different lots of horse serum used in these two experiments. Variations in generation time of Chinese hamster and HeLa cells attributable to different lots of serum have been previously described (18, 19).

Relationships between Direct Biochemical Effects and Changes in Cell Cycle Characteristics

In order to provide for suboptimal culture conditions qualitatively different from each other, the culture medium was modified by reduction of the concentration of a certain essential component or by the addition of an inhibitor. The metabolic function of glucose and leucine as well as the mechanisms of action of actinomycin D and amethopterin are well known, and the immediate effects of modified culture media on cellular metabolism may, therefore, be expected to be fairly specific and to involve the production of metabolic energy or the synthesis of protein, RNA, or DNA, respectively. Actinomycin D, at the low concentration used, has been shown to interfere primarily with ribosomal RNA synthesis (20). On the other hand, after prolonged incubation secondary effects certainly do occur. For instance, inhibition of RNA synthesis (21) or DNA synthesis (22) in cultures deprived of one or all amino acids has been reported. Furthermore, inhibition of processes such as RNA or protein synthesis during one phase of the cell cycle may affect progression of cells through a later stage, e.g., the S phase (23, 24) or mitosis (25, 26). Under the conditions used in our experiments, the effects of inhibition of a biochemical reaction on progression of cells through various cell cycle

phases may, therefore, be rather indirect. On the other hand, some of the observed modifications of cell cycle characteristics differ markedly from each other and appear to reflect the particular suboptimal growth condition used. This supports the assumption of a causal relationship between direct metabolic effects of suboptimal culture media and observed modifications in the progression of cells through the division cycle.

Effects of Suboptimal Culture Conditions on Individual Cell Cycle Phases

As seen in Table III, under all growth-limiting culture conditions listed, a pronounced increase of t_{G1} was observed. The duration of the other phases was, however, also modified in certain suboptimal media. For instance, reduction of the glucose concentration to 20 mg/liter resulted in increases of t_S, t_{G2+M}, and t_M in addition to a prolonged t_{G1}. This indicates that all cell cycle parameters that were determined may be modified by appropriate culture conditions. It is of interest that cell cycle characteristics in medium containing 0.02 mM leucine were quite similar to those in the presence of actinomycin D.

Our findings that the G_1 phase is particularly sensitive to a variety of suboptimal culture conditions are, in general, in agreement with other reports. Whereas in HeLa cell cultures the most pronounced effect of low incubation temperatures was an increase of t_M (2), in human amnion cell cultures metaphase, and t_{G1} were most sensitive to this change in culture conditions (3). In L-5178Y murine leukemic cell cultures, lowered temperatures resulted primarily in increases of t_{G1} and t_S (4). Decreased pH values in the culture medium were found to result in an extended G_1 phase (1). In medium containing low concentrations of actinomycin D, the progression of L cells through G_1 and G_2 was impaired (6). A rather specific arrest of cultured Chinese hamster cells in the G_1 phase has recently been reported to occur after incubation in medium devoid of glutamine and isoleucine (27). In conclusion, modifications of cell cycle characteristics appear to depend on the type of the growth-limiting environment, on the time of incubation after transfer of cells to the suboptimal condition and on the cell line used. In the studies reported in the present communication, the effects of several different suboptimal

culture media were, therefore, compared using the same cell line and identical methods of culture and of cell cycle analysis. In addition, cultures were subjected to analysis after having attained steady-state proliferation kinetics.

Comparison of Data Obtained by Two Different Methods

As shown in Table III, time parameters derived from mitotic labeling indices were, in general, in good agreement with those derived from relative numbers of cells in different cell cycle phases. It should be noted, however, that these two groups of data are not entirely independent of each other: in calculating the duration of individual cell cycle phases from relative numbers of cells, also the generation time as derived from mitotic labeling indices was needed. In addition, t_M as derived from the generation time and the relative number of cells in mitosis was used to calculate t_{G2+M} and t_{G1} from mitotic labeling data.

On the other hand, the two methods of evaluation, although not completely independent, differ from each other with respect to the cell population included in the analysis: whereas mitotic labeling data exclusively refer to cells synthesizing DNA and subsequently entering mitosis, all cells in samples B were classified according to their position in the cell cycle, thus including cells not engaged in continued progression through the division cycle. It is, therefore, of interest that under certain growth-limiting culture conditions, particularly in experiments 3, 4, and 7, significant differences between results obtained by the two methods of evaluation were observed as mentioned under Results. These findings suggest that the exponential age distribution of cells, as defined under Materials and Methods, may be markedly modified in some suboptimal media. A fraction of the cell population appears, therefore, to be affected preferentially in certain phases of the cell cycle, resulting either in cell death and/or in a slower or completely arrested progression through the sensitive cell cycle phase. The assumption of cell death is supported by the observation of pycnosis of interphase nuclei and of mitotic figures under growth-limiting culture conditions. Modifications of proliferation kinetics by cell death have also been reported for a number of normal and simian virus (SV) 40-transformed cell lines (28). As seen in Table I, in the suboptimal

media used in experiments 3, 4, and 7, cell number doubling times markedly exceeded corresponding generation times. Such a difference was also observed in cell cultures incubated in media containing 5-bromodeoxyuridine (BUdR) at growth-limiting concentrations (5).

As seen in Fig. 4, in most experiments even the first peak of the mitotic labeling index did not reach 100%, indicating that some cells were proceeding very slowly through G_2 and/or mitosis. In fact, after continuous thymidine-^{3}H labeling of cultures under optimal conditions during 5 hr, the cell population still contained approximately 9% unlabeled G_1 cells and 1% unlabeled G_2 cells. Asymmetric frequency distributions of various cell cycle parameters may, therefore, contribute to the observed differences between generation time and cell number doubling time, as well as to the differences between results obtained by the two methods of evaluation.

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