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STUDY OF CELL KINETICS BY COMPUTER-ANALYZED FLOW CYTOMETRIC HISTOGRAMS

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Abstract—A mathematical procedure for analyzing the cell proliferation kinetics from DNA content histograms, measured by flow cytometry, is presented. This procedure is based on a cell cycle model which uses a continuity equation for the S-phase transit. Four tumor cell lines in culture were studied. A human melanoma line (JR01) was examined in exponential growth. For another human melanoma (M14) and a human astrocytoma (DF) lines, a sequence of flow cytometric histograms with the corresponding growth curve was processed, determining the rate of DNA synthesis and the S-phase cell influx. The same kinetic parameters were evaluated also in a murine tumor line (3LL C108) treated with an antineoplastic drug (ICRF 159).

1. INTRODUCTION

Since the introduction of automatic flow cytometry [1] in the study of cell population kinetics, several methods have been developed in order to extract relevant proliferative parameters from the measured DNA fluorescence distributions [2]. Due to the relevance of the DNA synthesis in the replicative mechanism of the cell, some methods allow to reconstruct the pattern of the DNA synthesis rate of cells exponentially growing [3–8]. Other methods interpret time-sequences of DNA flow cytometric (FCM) histograms in terms of multicompartment models of the cell cycle [9–11].

In the present paper we describe a method for analyzing cell population kinetics also in perturbed growth condition. This method uses a time-sequence of FCM histograms and the measured population sizes, in order to obtain estimates of the cell distribution in the cycle phases, of the DNA synthesis rate, and of the S-phase influx. The procedure is based on a cell population model proposed in Ref. [12]. In the present work we report the results obtained by analyzing the proliferative characteristics of four *in vitro* tumor cell lines in different growth conditions: exponential growth (lines JR01 and DF), saturating growth (lines DF and M14), and perturbed growth (line 3LL C108).

2. THEORY

The population model proposed in Ref. [12] is structured in terms of the cell DNA content, and assumes no intrapopulation variability of the DNA synthesis rate and no cell loss. The model equations are

$$\frac{\mathrm{d}}{\mathrm{d}t}N_1 = -f(t) + 2g(t) \tag{1}$$

$$\frac{\partial}{\partial t}n(x,t) + \frac{\partial}{\partial x}[v(x,t)n(x,t)] = 0, \quad 1 < x < 2,$$
$$v(1,t)n(1,t) = f(t) \tag{2}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}N_2 = v(2,t)n(2,t) - g(t), \tag{3}$$

where: $N_1(t)$ and $N_2(t)$ denote the number of cells with DNA content at t equal to 1 and 2, respectively; $n(x,t)$ is the cell density in S with respect to the DNA content x; $v(x,t)$ is the DNA

synthesis rate at t of a cell with DNA context x; $f(t)$ is the S-phase influx; $g(t)$ is the mitotic rate. In the absence of cell loss $g(t)$ is equal to dN/dt, where $N(t)$ is the number of cells in the population at time t. We note that the use of the continuity equation (2) for representing the progression of cells along the S phase and for estimating the rate of DNA synthesis, was firstly proposed in Ref. [3] and more recently in Refs [6, 13].

From the model equations the following expressions for $v(x, t)$ and $f(t)$ can be obtained:

$$v(x,t) = \frac{1}{N(t)\tilde{n}(x,t)} \frac{d}{dt}\left[N(t)\left(2 - \theta_1(t) - \int_1^x \tilde{n}(z,t)\,dz\right)\right] \tag{4}$$

$$f(t) = \frac{d}{dt}\left[N(t)(2 - \theta_1(t))\right], \tag{5}$$

where $\theta_1(t)$ is the G1/G0 fraction and $\tilde{n}(x, t) = n(x, t)/N(t)$ is the DNA distribution in S. It has been found convenient [7] to express $\tilde{n}(x, t)$ in terms of θ_1 and of a parametrizing function $w(x, t)$, $1 < x < 2$, according to

$$\tilde{n}(x,t) = \frac{2 - \theta_1(t)}{w(x,t)} \exp\left[-\int_1^x \frac{dz}{w(z,t)}\right]. \tag{6}$$

With this parametrization for $\tilde{n}(x, t)$, equation (4) can be rewritten as

$$v(x,t) = w(x,t) \frac{d}{dt}\left[\ln[N(t)(2 - \theta_1(t))] - \int_1^x \frac{dz}{w(z,t)}\right]. \tag{7}$$

The rate of DNA synthesis for a population in balanced exponential growth (the rate and the DNA distribution are time-independent in this growth condition) becomes simply

$$v(x) = \alpha w(x), \tag{8}$$

where $\alpha = d \ln N(t)/dt$ is the population growth rate. In Refs [7, 8] a computational algorithm was proposed which extracts from a FCM histogram a piecewise constant approximation of w, and reconstructs the DNA distribution (see next section).

In an arbitrary growth condition, given a sequence of FCM histograms, equation (7) allows to compute the time integral of $v(x, t)/w(x, t)$ over the interval $[t_i, t_{i+1}]$ between two successive histograms. By approximating $v(x, t)$ as a time-invariant $v_i(x)$ in this interval, and evaluating the integral

$$\int_{t_i}^{t_{i+1}} dt/w(x,t)$$

by the trapezoidal rule, the following expression can be obtained:

$$v_i(x) = \frac{\ln \dfrac{N(t_{i+1})}{N(t_i)} + \ln \dfrac{2 - \theta_1(t_{i+1})}{2 - \theta_1(t_i)} + \displaystyle\int_1^x \left(\frac{1}{w(z,t_i)} - \frac{1}{w(z,t_{i+1})}\right) dz}{\dfrac{t_{i+1} - t_i}{2}\left(\dfrac{1}{w(x,t_i)} + \dfrac{1}{w(x,t_{i+1})}\right)}. \tag{9}$$

The average S-phase influx in $[t_i, t_{i+1}]$ turns to be

$$f_i = [N(t_{i+1})(2 - \theta_1(t_{i+1})) - N(t_i)(2 - \theta_1(t_i))]/(t_{i+1} - t_i). \tag{10}$$

These quantities allow to compute in the same interval the S-phase transit time

$$T_S = \int_1^2 dx/v_i(x),$$

and a rough estimate of the mean residence time in G1/G0 according to

$$T_{G1/G0} = \frac{N(t_i)\theta_1(t_i) + N(t_{i+1})\theta_1(t_{i+1})}{2f_i}.$$

Note that in balanced exponential growth it is $T_{G1/G0} = \theta_1/\alpha(2 - \theta_1)$ [8].

It appears that, by means of equations (9) and (10), it could be possible to determine $v_i(x)$ and f_i from two successive histograms of the sequence and from the corresponding cell counts.

Unfortunately, the errors affecting usually the measurement of the population size cause large deviations on the estimates of both the DNA synthesis rate and the S-phase influx. This is not surprising since equations (9) and (10) contain a finite-difference approximation of the derivative of $N(t)$. On the contrary, it has been found that the errors on θ_1 and w do not affect severely the above estimates in the majority of cases [14].

Two different approaches, based on the consideration of the whole FCM sequence and the related growth curve, seem to be feasible in order to obtain filtered values of $N(t_i)$: (a) a convenient law for fitting the growth curve (e.g. logistic, polynomial); (b) a nonparametric estimate of $N(t_i)$ obtained by a regularization procedure. Our approach, that moves along the line (b), will be outlined in the next section.

3. COMPUTER ANALYSIS

3.1. Single FCM histogram

The DNA distribution over the cell population is related to the distribution $v(\xi)$ of the DNA-dye complex fluorescence ξ, measured with the flow cytometer, by the equation [2]

$$v(\xi) = \theta_1 K(\xi, 1) + \int_1^2 K(\xi, x)\tilde{n}(x)\,dx + \theta_2 K(\xi, 2), \tag{11}$$

where θ_2 is the G2M fraction, and $K(\xi, x)$ is the kernel that expresses the fluorescence dispersion.

On the basis of equations (6) and (11) an algorithm has been implemented which, from a FCM histogram, yields least-square estimates of: (i) the G1/G0 fraction θ_1; (ii) the values $w_1, \ldots, w_M$ of a piecewise constant approximation of the function $w(x)$ that parametrizes $\tilde{n}(x)$; (iii) the parameters of a suitable analytical form of $K(\xi, x)$. $K(\xi, x)$ was chosen as

$$K(\xi, x) = \frac{1}{\sqrt{2\pi}\sigma(x)} \exp\left[-\frac{1}{2}\left(\frac{\xi - m(x)}{\sigma(x)}\right)^2\right].$$

The mean fluorescence of a cell with DNA content x, $m(x)$, and the standard deviation $\sigma(x)$ were assumed to be

$$m(x) = \xi_{G1} + (\xi_{G2} - \xi_{G1})(x - 1)$$
$$\sigma(x) = cx^b$$

where ξ_{G1} and ξ_{G2} are the mean fluorescences of G1/G0 and G2M cells respectively.

The main steps of the algorithm can be outlined as follows.

1. Least square estimates of ξ_{G1} and c are obtained on the G1/G0 peak truncated at $3\sigma(1)$ on the r.h.s.
2. Least square estimates of ξ_{G2} and b are similarly obtained on the G2M peak truncated at $3\sigma(2)$ on the l.h.s.
3. Keeping ξ_{G1}, ξ_{G2}, c and b fixed, the least square estimates of $\theta_1, w_1, \ldots, w_M$ are obtained. The program increases M up to a predetermined value M_{max} (usually 6–8 for histograms with $10^4 - 3 \times 10^4$ cells distributed in 60–70 channels, and with CV of G1/G0 peak in the range 2–4%) at which the procedure stops.

At the end of the procedure, the DNA distribution in S is reconstructed by means of equation (6), and the G2M fraction is computed. In this way the S-phase DNA distribution $\tilde{n}(x)$ is approximated by a piecewise exponential function over M_{max} equal compartments. If the FCM histogram is measured in balanced exponential growth and the population growth rate α is known, the rate of DNA synthesis can be computed from equation (8). The algorithm was tested on simulated and experimental histograms in Refs [7, 8].

3.2. Time-sequence of FCM histograms

In non-exponential growth conditions of the cell population, the recovery of kinetic parameters by means of equations (9) and (10) requires a preliminary filtering of the growth curve as stated in the previous section.

Let us consider a sequence of FCM histograms and the related population size measurements at the times $t_1, \ldots, t_n$. Let $y_i = \ln N(t_i)$ and let z_i be the logarithm of the measured population size at t_i. We assume $z_i = y_i + \epsilon_i$, where ϵ_i is an uncorrelated zero-mean noise with variance σ_i^2. Let $\Delta_i = y_i - y_{i-1}$, $i = 2, \ldots, n$, and $\Delta_1 = y_1$. The estimate of $N(t_i)$, $i = 1, \ldots, n$, is obtained by minimizing with respect to $\Delta_1, \ldots, \Delta_n$ the following index:

$$J = \sum_{i=1}^{n} \left(z_i - \sum_{j=1}^{i} \Delta_j \right)^2 \frac{1}{\sigma_i^2} + \lambda \sum_{i=1}^{n-2} \int_1^2 (v_{i+1}(x) - v_i(x))^2 \, dx, \tag{12}$$

where λ is a regularization parameter. This estimate depends critically on the choice of λ. For very small λ the estimated $N(t_i)$ tend to reproduce the measured values, with possible large deviations on the reconstructed DNA synthesis rate patterns. For very large λ fictitious values of $N(t_i)$ are obtained, causing a compression of the actual changes of the rate along the sequence.

In order to determine an optimal value of the regularization parameter λ, we adopted the criterion of minimizing the expected value of the squared estimation error $\hat{y}_\lambda - y^*$, where y^* and $\hat{y}_\lambda$ are the vectors of the true and estimated values, respectively. It can be seen from the structure of index (12) and from expression (9) that the estimate $\hat{y}_\lambda$ has the form $\hat{y}_\lambda = Q_\lambda z + p_\lambda$, where z is the vector of the z_i [14]. Q_λ and p_λ are a matrix and a vector respectively, that depend both on λ and on the DNA distributions reconstructed from the histograms of the sequence. Thus the mean square error turns out to be

$$\varphi = [(Q_\lambda y^* + p_\lambda) - y^*]^T [(Q_\lambda y^* + p_\lambda) - y^*] + \text{trace}(Q_\lambda \Psi Q_\lambda^T), \tag{13}$$

where Ψ is the covariance matrix of the noise vector ϵ. Since the vector y^* of the true values is unknown, we minimized with respect to λ an unbiased estimate of φ given by

$$\tilde{\varphi} = [(Q_\lambda z + p_\lambda) - z]^T [(Q_\lambda z + p_\lambda) - z] + \text{trace}((2Q_\lambda - I)\Psi). \tag{14}$$

This expression was obtained by replacing y^* with z in equation (13) and subtracting the bias.

In summary, the main steps of the procedure for the analysis of a FCM sequence are the following.

1. All the histograms of the sequence are analyzed, as previously described, estimating θ_1 and $w(x)$ for each histogram.
2. Matrix Q_λ and vector p_λ are constructed, and the index (14) is minimized finding the optimal value $\tilde{\lambda}$.
3. The estimates of the DNA synthesis rate and of the S-phase influx are computed by equations (9) and (10), using $\hat{y}_{\tilde{\lambda}}$.

A complete description of this procedure, accompanied by tests on simulated data, appeared in Ref. [14].

4. APPLICATIONS

The rate of DNA synthesis was obtained for four cell lines in culture. The DF and JR01 lines were originally established at Regina Elena Institute in Rome. The DF line derives from a biopsy of a patient bearing an astrocytoma. The JR01 line originates from a metastatic nodule of a patient affected by a malignant melanoma. Both lines grow continuously in RPMI medium supplemented with 10% FCS. The passages 60th and 16th for the two lines respectively were used. The M14 line, derived from a human metastatic melanoma nodule and established at the University of California at Los Angeles, is maintained in RPMI 1640 medium supplemented with 10% FCS. The passage 125th was used. The fourth line was a variant of the murine 3LL line, denoted as C108, growing in Waymouth's medium supplemented with 15% FCS at the passage 218th.

The growth of the three human lines was monitored by measuring the number of cells and the DNA content histograms at different times up to the 8th day of growth. The DNA content was measured by a FACS 420 flow cytometer on cells treated with RNA-ase (50 U/ml) and stained with propidium iodide (50 μg/ml). The 3LL C108 line in the exponential growth was treated with ICRF 159 (150 μg/ml) for 1, 3 and 6 h. The number and the DNA content of the cells, stained with the same protocol, were measured at time 0 (control) and at the above times.

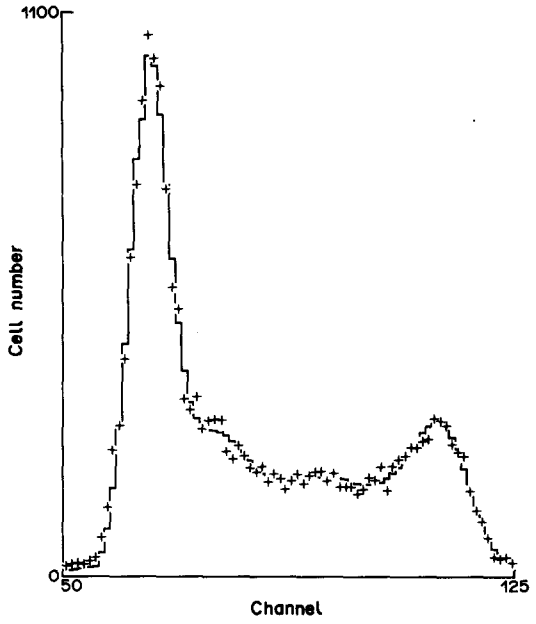

Fig. 1. FCM histogram of the DNA content of JR01 cells at the 6th day of growth. +: experimental points. ——: fitting fluorescence distribution.

The FCM histograms of the JR01 and DF cells in exponential growth were analyzed by the computational algorithm described in Section 3.1. As an example, Fig. 1 shows the experimental histogram of JR01 cells at the 6th day together with the fitting fluorescence distribution. The growth rate α was estimated from the measured growth curves, obtaining 0.031 h^{-1} for JR01 and 0.023 h^{-1} for DF cells. For both lines, we derived the cycle phase fractions, the DNA synthetic rate using equation (8), and the duration of the S and G1/G0 phases. Figures 2 and 3 show the patterns of the rate of DNA synthesis for the two lines; the durations of the S and G1/G0 phases are reported in the figure legends. As it can be seen, for each line the pattern does not change appreciably in the exponential growth. Moreover, the pattern does not differ substantially between the two lines, and the same is true for the duration of the synthetic period. On the contrary, the duration of the cycle (as indicated by the value of the growth rate α) and of the G1/G0 phase are longer in the DF line.

For the DF and M14 lines a complete sequence of histograms was also considered. In order to evaluate the DNA synthesis rate according to equation (9), the histograms were analyzed, and the

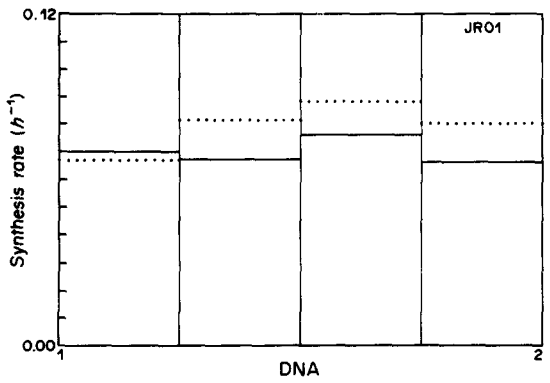

Fig. 2. Pattern of DNA synthesis rate vs DNA content, estimated in 4 S-phase compartments, for JR01 cells in exponential growth at 4th (——) and 6th day (·····) after seeding. Estimated S-phase transit time: 14.3 h (4th day), 12.8 h (6th day). G1/G0 residence time: 6.7 h.

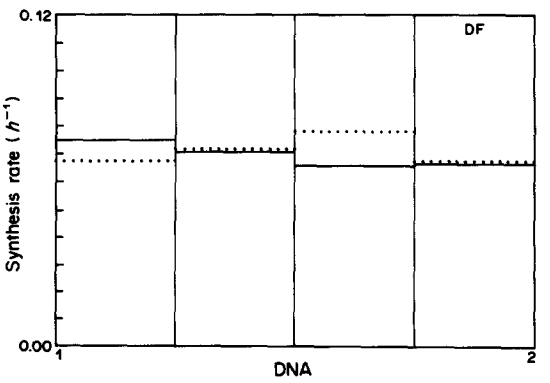

Fig. 3. Pattern of DNA synthesis rate vs DNA content, estimated in 4 S-phase compartments, for DF cells in exponential growth at 3rd (——) and 4th (·····) day after seeding. Estimated S-phase transit time: 14.4 h (3rd day), 14.1 h (4th day). G1/G0 residence time; 14.6 h.

Table 1. Population size, fraction of cells in cycle phases, G1/G0 and *S* transit times, estimated at different growth stages of DF line

Days	Population size (measured) 10^5 cells/ml	Population size (estimated) 10^5 cells/ml	G1/G0 (%)	*S* (%)	G2M (%)	$T_{G1/G0}$ (h)	T_S (h)
3	0.47	0.45	50.4	42.2	7.4		
						15.1	14.5
4	0.69	0.79	50.4	41.5	8.1		
						16.7	14.8
6	2.70	2.25	50.3	40.4	9.3		
						38.6	15.5
7	3.20	3.48	71.0	21.3	7.7		

Table 2. Population size, fraction of cells in cycle phases, G1/G0 and *S* transit times, estimated at different growth stages of M14 line

Days	Population size (measured) 10^5 cells/ml	Population size (estimated) 10^5 cells/ml	G1/G0 (%)	*S* (%)	G2M (%)	$T_{G1/G0}$ (h)	T_S (h)
4	0.70	0.75	43.5	46.3	10.2		
						7.5	11.5
4.6	1.19	1.17	37.8	52.6	9.6		
						8.3	12.0
5	1.59	1.58	39.4	47.8	12.8		
						10.0	12.2
5.6	2.63	2.40	44.7	42.9	12.4		
						12.8	12.7
6	3.42	3.15	49.2	40.3	10.5		
						21.1	12.8
6.6	3.96	4.47	61.7	29.7	8.6		

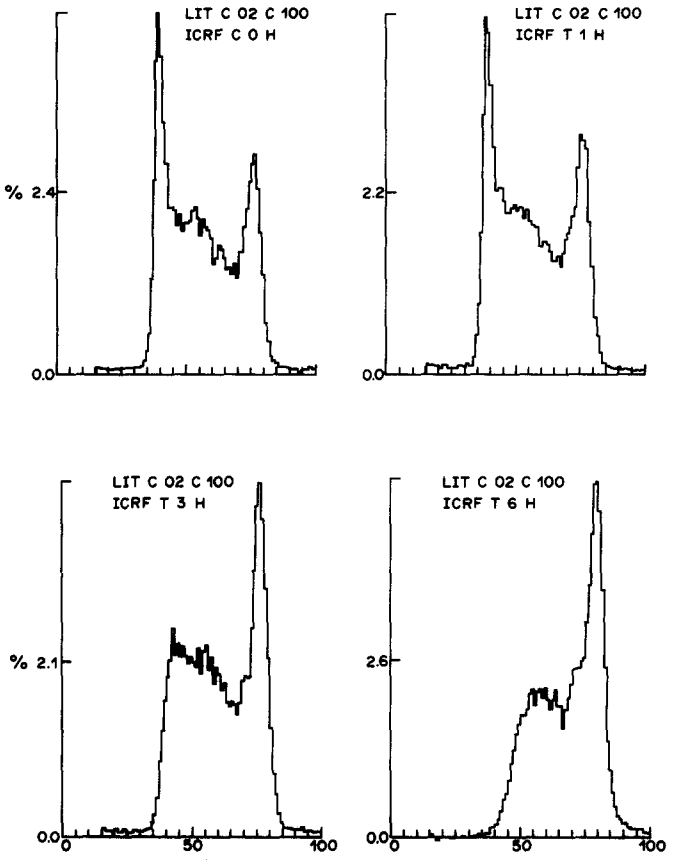

Fig. 4. FCM histograms of 3LL C108 cells at time 0 (control), and at 1, 3 and 6 h of treatment with ICRF 159.

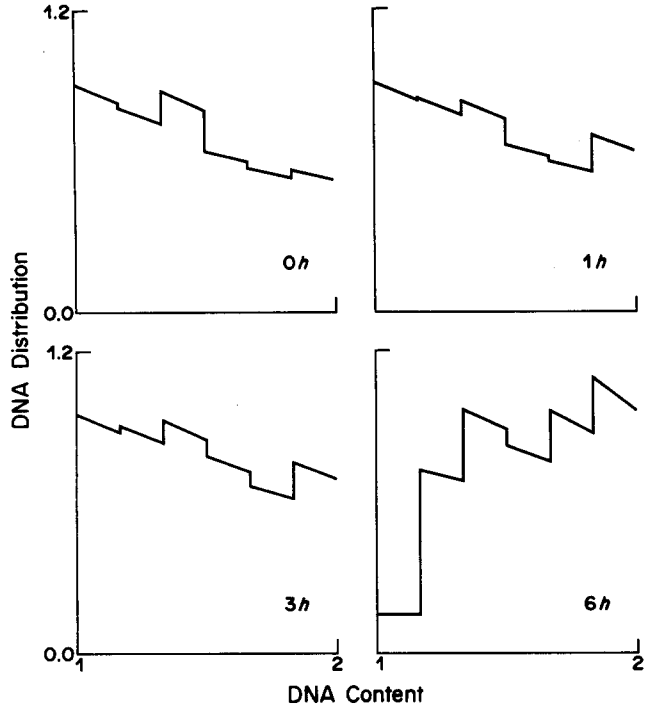

Fig. 5. DNA distributions in S-phase, estimated in six compartments, for 3LL C108 cells at the times indicated.

Table 3. Population size, fraction of cells in cycle phases and S-phase transit time estimated at different times of treatment of 3LL C108 cells with ICRF 159

h	Population size (measured) 10^4 cells/ml	Population size (estimated) 10^4 cells/ml	G1/G0 (%)	S (%)	G2M (%)	T_S (h)
0	4.9	5.1	15.6	70.2	14.2	
						12.5
1	5.2	5.2	14.2	72.9	19.9	
						12.2
3	5.1	5.3	1.1	79.3	19.6	
						12.9
6	6.1	5.6	0	75.0	25.0	

filtering procedure described in Section 3.2 was applied to the measured growth curve. Tables 1 and 2 give the relevant kinetic parameters for the DF and M14 line, respectively. It can be seen that the approach to the plateau proceeds via an increased G1/G0 fraction with a corresponding increase of the G1/G0 transit time, whereas the S phase duration does not change appreciably.

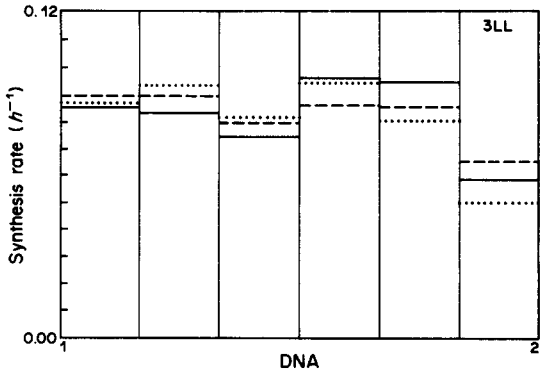

Fig. 6. Pattern of DNA synthesis rate vs DNA content, estimated in 6 S-phase compartments, for the 3LL C108 line treated with ICRF 159. Rates estimated in the following time intervals: 0–1 h (——), 1–3 h (– – –), 3–6 h (· · · ·).

In Fig. 4 the sequence of four histograms of the treated 3LL C108 cells are displayed, showing an accumulation of cells in the G2M phase of the cycle. The corresponding estimated DNA distributions in S-phase are shown in Fig. 5. The first three distributions are substantially unchanged, suggesting that the S-phase influx is exponentially decreasing [7]. The fourth distribution shows the starting of S-phase depletion. Table 3 summarizes the results of the analysis of the whole sequence. As expected from the mechanism of action of the drug [15], the G1/G0 phase is progressively depleted, with a corresponding increment of the G2 phase. The duration of DNA synthesis is substantially unchanged, as well as the pattern of the DNA synthesis rate (Fig. 6).

5. CONCLUDING REMARKS

The proposed mathematical procedure appears to be able to give realistic estimates of some relevant characteristics of cell populations growing *in vitro* in different biological situations. Cell cycle related mechanisms, as well as the response to drug treatment, are to some extent clarified by the analysis method outlined in this paper.

The results reported indicate that for the two tumor cell lines studied up to the saturation, the elongation of the G1/G0 residence time is the main event in the transition to the plateau phase. The time duration of the synthetic period appears on the contrary a constant characteristic of the cells.

The DNA measurement and the related mathematical analysis allowed us to recognize a constant duration of the S phase even in the case of a cell population treated with the ICRF 159 drug. This drug seems to affect only the G2M phase of 3LL C108 cells.

This kind of analysis could help in devising pharmacological experiments using cell cultures, and in properly timing the administration of cell cycle specific drugs. However, the applicability of the method of analysis in its present form is restricted to those experiments in which the condition of negligible cell loss, assumed in the underlying population model, is verified.

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