# DETERMINATION OF THE TIME OF POPULATION DOUBLING IN MONOLAYER CULTURES

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The time of population doubling is an important index of the rate of growth of a cell population in vitro, giving an idea of the rate of protein synthesis by the cells of this population (the time of population doubling must be differentiated from the regeneration time, i.e., the mean duration of mitosis, measured by means of a motion picture investigation [8, 9, 11]).

Of the existing methods of calculating the time of doubling of a cell population in monolayer, and also in suspension cultures, the following may be mentioned: counting the number of cell nuclei or cells at definite time intervals [7, 15] and calculating the rate of growth of the cell population by determining the protein content [10, 12]. When the first method is used, the time of population doubling may be determined graphically [17] or analytically [7]. In both cases the method of counting the cell nuclei is used [14], 15].

Counting the nuclei by this method is complicated, and during the statistical analysis the existence of a statistical optimum [2] is not taken into consideration, with the result that regression analysis and the method of least squares have been used for calculating the population doubling time [8, 17]. This makes the determination of the population doubling time using counting of the nuclei (or cells) much more complicated and puts it out of reach of a large proportion of experimental workers.

The object of the present investigation was to determine the population doubling time by the use of the statistical optimum existing in the counting chamber when used for counting suspensions of uniform independent particles [2].

Cell lines HeLa and DAPT [1] were grown at 36° in Carrel's flasks 5 cm in diameter on medium No. 199 with the addition of 20% ox serum. The medium was changed every 5 days. The HeLa cells were detached with a 0.02% solution of versene, and the DAPT cells with a 0.5% solution of trypsin. In these conditions the initial cell suspensions were homogeneous, like the suspensions used for counting, and the cells showed little sign of forming conglomerates. A suspension of intact cells was used for counting, which was done in a Goryaev's chamber [3]. The portion of the counting chamber chosen as conventional counting unit was one-fifth of it (45 large squares), which is convenient from the point of view of counting practice. The initial concentration of cells in both cases was approximately $10^5$/ml. Hence there were about 20 cells to one-fifth of the Goryaev's chamber.

The cell suspension was poured into each flask in a volume of 5 ml. The same volume of medium was added during changing. The cells were counted on alternate days in a trypsinized or versene-treated suspension obtained by mixing the contents of two flasks. Because of the sharp increase in the number of cells in the flask in the process of growth, the volume of trypsin or versene added to the flask during counting varied from 5 to 20 ml. Counting took place until the number of cells in the flasks stopped increasing.

The limits of the statistical optimum and, hence, the closeness of $\bar{x}$ and m* were obtained by determining the degree to which the equation

$$(\sigma_{p.d.} / \sqrt{\bar{x}}) = 1.00 \qquad (1)$$

*Symbols used in this paper: $\sigma_{p.d.}$ is the standard deviation of Poisson distribution; $\bar{x}$ the sample mean number of cells (measured density of suspension); m the mean number of cells for the aggregate (true density of suspension); n the number of tests; t the time of population doubling; T the duration of logarithmic phase of growth; $N_1$ and $N_2$ the number of cells at the beginning and end of the logarithmic phase of growth.

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TABLE 1. Determination of the Optimal Number of Counts Required to Give a Value of the Ratio $\sigma_{p.d.}/(\bar{x})^{1/2}$ close to 1.00 when Using $^1/_5$ of the Goryaev as the Conventional Counting Unit (results of 10 measurements).

| No. of counts | No. of cells in chambers (minim. and maxim.) | Mean value of ratio $\frac{\sigma_{p.d.}}{\sqrt{\bar{x}}}$ | Deviation of mean value of ratio $\frac{\sigma_{p.d.}}{\sqrt{\bar{x}}}$ from 1.00 | Maximal deviation of value of ratio $\frac{\sigma_{p.d.}}{\sqrt{\bar{x}}}$ from 1.00 in individual exp. |
|---|---|---|---|---|
| 2 | 288—315 | 0,71 | 0,29 | 0,87 |
| 3 | 280—355 | 1,06 | 0,06 | 0,89 |
| 4 | 280—335 | 1,16 | 0,16 | 0,93 |
| 5 | 100—315 | 1,10 | 0,10 | 0,67 |
| 10 | 176—315 | 0,92 | 0,08 | 0,31 |
| 20 | 85—315 | 1,01 | 0,01 | 0,33 |

TABLE 2. Determination of Optimal Number of Cells to be Counted to Give a Value of the Ratio $\sigma_{p.d.}/(\bar{x})^{1/2}$ close to 1.00 when 10 Counts Were Made (results of 10 measurements)

| Total No. of cells in chambers (minim. and maxim.) | Mean value of ratio $\frac{\sigma_{p.d.}}{\sqrt{\bar{x}}}$ | Deviation of mean value of ratio $\frac{\sigma_{p.d.}}{\sqrt{\bar{x}}}$ from 1.00 | Maximal deviation of value of ratio $\frac{\sigma_{p.d.}}{\sqrt{\bar{x}}}$ from 1.00 in individual experiments |
|---|---|---|---|
| 43—80 | 0,73 | 0,27 | 0,55 |
| 89—110 | 0,98 | 0,02 | 0,33 |
| 176—315 | 0,92 | 0,08 | 0,31 |

was satisfied by the number of areas of the counting chamber taken as the conventional unit of counting (the number of counts) and the total number of cells which must be counted during these counting procedures. Equation (1) is a property of Poisson's law, governing the distribution of cells in a counting chamber [5, 6]. Satisfaction of the equation during the counting of cells in a counting chamber has been demonstrated by many investigators [2, 4, 5, 6]. The method used to establish the limits of the statistical optimum is shown clearly in Tables 1 and 2. The method has been described in detail in an earlier paper [2].

It is clear from Tables 1 and 2 that the optimal number of counts for obtaining agreement with Eq. (1) was 10, and the optimal total number of cells which had to be counted during this number of counting operations was about 200 (about 100 per chamber). Taking these data into consideration, the mean values of 10 measurements of the number of cells in one-fifth of the Goryaev's chamber, and not the individual results of measurement of the number of cells, was calculated. In the statistical analysis a 1% level of significance was adopted.

The population doubling time was determined analytically and graphically. For the analytical method the formula suggested by Bryant and co-workers [7] was used:

$$t = \frac{T}{3{,}32\,(\lg N_2 - \lg N_1)}. \qquad (2)$$

The duration of the logarithmic phase of growth for the HeLa cells was taken as 11 days, and for DAPT cells 9 days.

When the graphic method was used, the logarithms of the mean numbers of cells corresponding to $^1/_5$ of the Goryaev's chamber were plotted along the axis of ordinates, and the number of days along the axis of abscissas. The population doubling time was determined from the formula:

$$t = (\log 2/\tan \alpha) \qquad (3)$$

where $\alpha$ is the angle between the experimental straight line and the axis of abscissas. A similar formula was used by Yamada and Takano [17]. The value of tan $\alpha$ was determined directly from the graph.

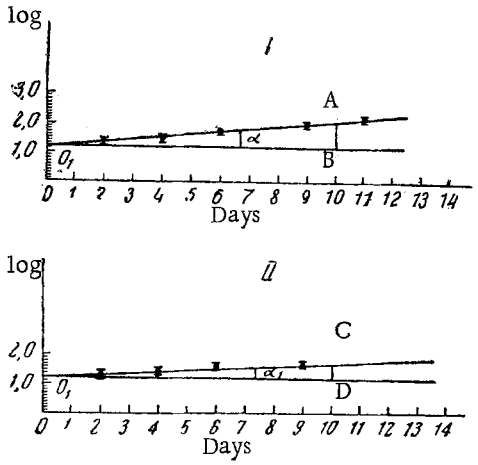
With the analytical method of calculation the population doubling time of the HeLa and DAPT cultures was 3.31 days ($\approx$ 79.4 h) and 6.02 days ($\approx$ 144.5 h), respectively.

Since each value was the mean of 10 measurements, the logarithms of these values showed hardly any scatter when plotted on the graphs (see figure). The almost complete absence of scatter of the experimental points on the graph made it easy to draw a straight line of approximation through them, in good agreement with the straight line obtained by the method of least squares, without using the equation based on the method of least squares, which would have greatly complicated the method of calculation.

As the figure shows:

$$\tan \alpha = (AB/O_1B) = 0.09, \qquad \tan \alpha_1 = (CD/O_1D) = 0.05.$$

According to Eq. (3), $t_{HeLa} \approx 3.34$ days ($\approx 80.2$ h) and $t_{DAPT} \approx 6.02$ days ($\approx 144.5$ h).




Graph of rate of growth of cell lines HeLa (I) and DAPT (II). Along the axis of ordinates – logarithms of mean values of 10 measurements of the number of cells corresponding to $\frac{1}{5}$ of a Goryaev's chamber. The horizontal lines at the experimental points denote the logarithms of the upper and lower 99% confidence intervals, $\alpha$ and $\alpha_1$ are the angles between the experimental straight lines and the axes of abscissas, and AB and CD are the perpendiculars drawn to the axes of abscissas (for convenience of calculation of the tangents of the angles $\alpha$ and $\alpha_1$ points were chosen along the axes of abscissas corresponding to 10 days). Remainder of explanation in text.

By the use of the proposed method of determining the population doubling time of cells growing in monolayer cultures, the influence of one of the most variable factors, largely responsible for the scatter of the experimental points, can be removed. This factor is the error of random distribution of the cells in the counting chamber [6], the importance of which is obvious from an examination of the results obtained by Yamada and Takano. Removal of the influence of the error of random distribution of the cells in the counting chamber is possible because of the statistical optimum, the use of which ensures that the experimental conditions correspond to some of the main properties of Poisson's distribution: 1) m is the only parameter determining the whole form of the distribution [5]; 2) the approximation to the theoretical formula is increased with an increase in n [16].

In addition, if the statistical optimum is used, there is no need to count such a large number of cells, and it becomes possible in practice to operate with mean values, which is difficult to do when other previously recommended counting methods are used [14, 15]. Further investigations are necessary to determine the type of distribution of the frequencies of the population doubling time.

During repeated experiments the variations can be expected which will arise as a result of even slight changes in the denominator in Eqs. (2) and (3). Since in experimental conditions counting takes place on alternate days, it is difficult to detect the boundary between the lag phase and the logarithmic phase of growth. To make this detection possible, counting must be carried out more frequently in the first days of cultivation.

By using the method suggested by the author, the standardization of the media and sera used for cultivation can be verified and the effect of various chamical and biological factors on the rate of growth of cells in vitro can be studied.*

## SUMMARY

The purpose of investigation was to determine the time of cell population doubling in monolayer cultures in account with the statistical optimum existing during calculation of homogeneous and independent particles in a counting chamber. The use of the statistical optimum enables operation with the mean numbers of cells per section of the counting chamber chosen as the conventional calculation unit, which accounts for correspondence under experimental conditions with some basic characteristics of Poisson's distribution and eliminates the influence of the error of random distribution of cells in the counting chamber on the variation of experimental data. In cultivation of HeLa and DAPT cells in Carrels's flasks with a diameter of 5 cm at 36°C on medium 199 with an addition of 20% bovine serum (original concentration $10^5$ ml, suspension volume 5 ml, change of medium once in every five days) the time of HeLa cell population doubling, calculated by the analytical and graphic methods, was equal to 79.4 and 80.2 respectively, and that for DAPT cells to 144.4 and 144.5 h. The proposed method for determination of the time of population doubling may be of importance for control of the standards of media and sera used for cultivation and for investigation of the influence of various chemical and biological factors on the speed of cell growth in vitro.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of the first issue of this year.

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