APPENDIX I

Determination of doubling time

During the exponential growth phase, the number of the cells at any time *t, N(t)* depends on the initial number of cells, N_o , the number of new cells formed by division, *V(t)* and the number of cells *Z(t)* that died or detached from monolayer during this time. The increase in cell population due to cell division is commonly characterized by the parameter ‘doubling time’ t_d . This parameter can be easily determined in the ‘ideal case’ *i.e.* when *Z(t)* = 0.

1. Ideal case: Z(t)=0.

The rate of cell multiplication r_m may be determined from the following equation:

$$r_m = \frac{dN}{dt} = \alpha N \tag{1}$$

where ″ is a constant.

The differential equation (1) can be solved by integrating:

$$\int_0^t \frac{dN}{N} = \alpha \int_0^t dt \tag{2}$$

then:

$$\log N - \log N_0 = \alpha t \tag{3}$$

or:

$$N = N_0 \times 10^{at} \qquad (4)$$

where a = " log e . 0.4343 ". A time *t* in which the cell population doubles in its number is called 'doubling time', t_d. Therefore by replacing it in Eq. (4) we get:

$$2N_0 = N_0 \times 10^{a\, t_d} \qquad (5)$$

hence :

$$t_d = \frac{\log 2}{a} \qquad (6)$$

then Eq. (4) assumes the form:

$$N = N_0 \times 10^{\frac{t \log 2}{t_d}} = N_0 \times 2^{\frac{t}{t_d}} \qquad (7)$$

or:

$$\log N = \frac{\log 2}{t_d}\, t + \log N_0 \qquad (8)$$

From equation (8) t_d can be determined. log *N(t)* can be plotted vs time *t*

$$\log N(t) = (b \pm \Delta b) + (a \pm \Delta a) \times t \qquad (9)$$

where *b, a* - parameters of the linear fragment of growth curve (see Figure A1.1);) *b* and) *a* denote the standard deviation of *b* and *a*, respectively. The initial number of cells N_0 is given by experimental data or can be calculated as the intersect:

$$N_0 = 10^b \quad , \qquad \Delta N_0 = N_0 \times \Delta b \times \frac{1}{\log e} = N_0 \times \Delta b \times 2.303 \qquad (10)$$

and the doubling time is calculated from the slope of the straight line according to Eq. (6):

$$t_d = \frac{\log 2}{a} = \frac{0.301}{a} \quad , \quad \Delta t_d = t_d^2 * \frac{\Delta a}{\log 2} = 3.322 * t_d^2 * \Delta a \qquad (11)$$

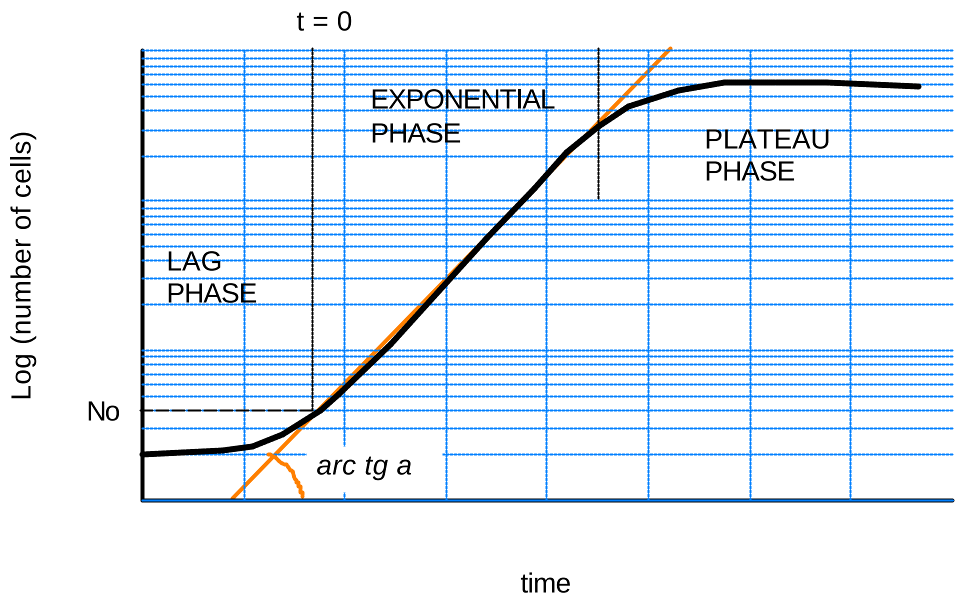

FIGURE A1.1. Growth curve of cells cultured without passaging.

2. Determination of t_d when Z(t)>0.

In the real case, the death and detachment from monolayer of the part of cell population leads to decrease of the value of *N(t)*, therefore artificially increasing doubling time of cell culture. This means that Eq. 8 cannot be used to determine the actual doubling time, being an average of only viable cells.

A working assumption can be made that the (experimentally determinable) number of dead and detached cells still ‘proliferates’ contributing to monolayer count, and therefore the doubling time of the culture independent of cell loss was determined by means of the method of successive approximations.

Let :

V - the number of viable cells,

Z - the sum of dead and non-adherent cells.

At the zero level of approximation we assume that the population of $Z = Z^o$ = const. Then total hypothetical number of cells is:

$$T^0 = N + Z^0 \qquad (12)$$

The zeroth approximated value of doubling time t_d^o may be determined from the linear regression $\log T^o$ vs time (see 'ideal case') and serves to the next step-approximation:

$$T^1 = N + Z^1 \times 2^{\frac{t}{t_d^0}} \qquad (13)$$

Doubling time t_d^1 determined from the linear regression $\log T^1$ vs time may be used in the second step calculation:

$$T^2 = N + Z^2 \times 2^{\frac{t}{t_d^1}} \qquad (14)$$

etc. The further approximations were made until differences in two subsequent t_d values obtained were negligible ( ) t_d = ± 0.001 h).