EFFECTIVE SPECIFIC ACTIVITY DILUTION IN LABELED DNA OF CULTURED MAMMALIAN CELLS (L5178Y)

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ABSTRACT Upon labeling the DNA of cultured mammalian cells with radioactive thymidine it was found that there was a large reduction in the specific activity of radioactive thymidine in DNA when this specific activity was compared to the specific activity of the exogenous supply. This reduction in specific activity may be determined once the effective dilution factor for thymidine in mammalian cells is known. The average effective dilution factor was found to be 2×10^{-4} M for L5178Y cells in log growth.

INTRODUCTION

When studying the incorporation of radioactive thymidine or thymidine analogues (e.g. 5′-bromodeoxyuridine) into mammalian cell DNA, it is desirable to insure that the precursors have been incorporated uniformly into DNA. Uniform labeling means that on the average the specific activity of newly replicated DNA is a constant throughout the labeling period. Lack of uniform labeling may result because of: (*a*) the degradation of thymidine (1, 2, 3); (*b*) the toxicity of high concentrations of thymidine (4, 5, 6); and (*c*) radioactive toxicity of labeled thymidine (6, 7). While attempting to achieve uniform labeling (7) it became essential to evaluate the contribution of endogenous cellular pathways to the freely mixing pool of acid soluble derivatives which includes thymidine monophosphate (TMP), thymidine diphosphate (TDP), thymidine triphosphate (TTP), and thymidine (TdR). In this report a method is given to estimate the relative contribution of cell metabolism to the "thymidine pool" in terms of what is called the "effective dilution factor." A knowledge of this factor enables one to plan mammalian cell experiments in which a certain specific activity of newly replicated DNA is necessary.

MATERIALS AND METHODS

Suspension cultures of mouse leukemia, L5178Y, were used in all experiments (9, 10, 11). The cells were cultured in Fischer's medium (Grand Island Biological Company, Grand

Island, New York) containing 10% horse serum. The doubling time of the cell population varied between 10 and 11 hr. Thymidine-^{3}H of known concentration and of known specific activity was added to exponentially growing cell populations (20,000 cells/ml–400,000 cells/ml). It has been shown by Morris and Fischer (4, 5) that high concentrations of thymidine (over 10^{-5} M) interfere with cell growth. Therefore, when a high concentration of thymidine was used, deoxycytidine in a concentration equal to $\frac{1}{10}$ of that of the thymidine was also added to minimize the toxicity (4, 5, 16). In experiments involving long incubation periods, this attention to thymidine toxicity was found to be essential. During incubation with thymidine-^{3}H, the cell number was checked periodically using a Coulter Counter Model A (Coulter Electronics, Inc., Hialeah, Fla.) to make certain the cell population was indeed in the exponential growth phase. After incubation, the cells were harvested and then washed with 10% cold trichloroacetic acid (TCA) (4°C) five times to remove the acid-soluble fraction of thymidine-^{3}H. The cells were then heated with 5% TCA in a boiling water bath for 15 min, cooled and sedimented at 5,000 rpm for 5 min. The supernatant liquid was removed and divided into two fractions. One fraction was used to estimate the DNA concentration by means of the diphenylamine reaction (12). $\frac{2}{5}$ of a milliliter of the other fraction was added to 15 ml of Bray's solution (13) and 1 ml of water, and counted in a liquid scintillation counter. The double channel ratio method (14) combined with quenching standards was used to estimate the number of disintegrations per 0.2 ml/min.

RESULTS AND DISCUSSION

Fig. 1 represents one of several long-term experiments. The cell suspension was incubated with 10^{-4} M thymidine containing 1 μc/ml of thymidine-^{3}H and 10^{-5} M deoxycytidine. The upper figure (lower curve) shows that the cell number increased exponentially with time during the entire incubation period. The lower figure shows that the specific activity expressed per unit newly replicated fraction of DNA-thymidine was constant throughout the incubation period. When the specific activity per unit newly replicated fraction of DNA-thymidine was shown to be constant, the preparation was said to be "uniformly labeled."

In both short- and long-term experiments, once the uniformity of the labeling was established, the effective thymidine dilution factor is calculated as follows:

Let A = specific activity of newly synthesized DNA.

B = specific activity of intracellular thymidine-^{3}H.

C = specific activity of exogenous thymidine-^{3}H.

D = concentration of exogenous thymidine.

E = the effective dilution factor in M.

Then, $A = B = (C)(D)/(D + E)$ and therefore

$$E = D\,(C - A)/A.$$

The results of six experiments in which the effective thymidine dilution factor has

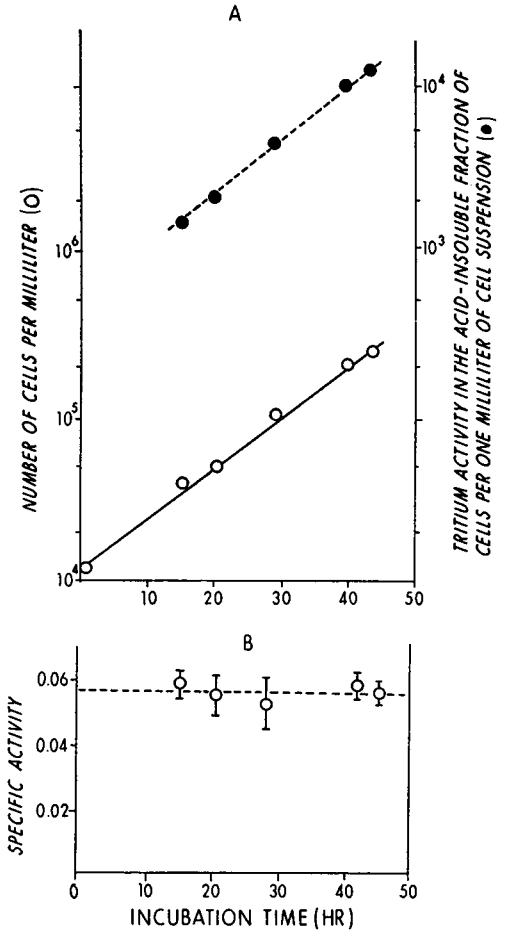

FIGURE 1 Uniform labeling of cellular DNA with thymidine-^{3}H. The cell suspension was incubated with 10^{-4} M thymidine containing 1 μc/ml of thymidine-^{3}H and 10^{-5} M deoxycytidine. The upper figure (*A*) shows the relationship of the incubation time with the cell number (solid line with open circles) and the tritium activity in the acid-insoluble fraction (dotted line with solid circles) in 1 ml of cell suspension. Lower figure (*B*) shows the relationship of the incubation time to the specific activity per newly replicated fraction of DNA.

been calculated are given in Table I. It is important to note that the experiments were performed under a large variety of conditions. The incubation times varied from 3.0 to 50 hr; the concentration of thymidine varied from 7.5×10^{-7} to 10^{-4} M; and the activity of the labeling solution from 0.05 to 2 μc/ml. The average dilution of the radioactive thymidine in all the experiments was 2.0×10^{-4} M. The variation in the dilution factor appears to vary slightly as a function of the concentration, however, the change is only about a factor of four while the concentration varies by more than two orders of magnitude.

It is important to note that the effective thymidine dilution factor is not necessarily a measure of the actual thymidine pool in the cell but merely a useful guide in the design of experiments in which an estimate of the DNA specific activity after certain labeling conditions is necessary. The actual "pool size" (8) in the cell is a function of the degradation rate of thymidine (1, 2, 3), possible preferential use of

TABLE I

ESTIMATION OF EFFECTIVE THYMIDINE DILUTION FACTOR

Exp. no.	Max incubation time	Type of radioactive isotope	Concn of exogenous thymidine	Concn of radioactive thymidine	Specific activity per newly replicated DNA*	Effective dilution factor
	hr		*M*	*μc/ml*		*M*
224	50	thymidine-^{3}H	10^{-4}	2	1.35×10^{-1}	3.0×10^{-4}
VI	3.5	thymidine-^{14}C	5.0×10^{-5}	0.17	3.1×10^{-2}	0.98×10^{-4}
183	10	thymidine-^{3}H	2.0×10^{-6}	1	2.7×10^{-1}	0.98×10^{-4}
147	10	thymidine-^{3}H	7.5×10^{-7}	0.05	1.1×10^{-2}	0.67×10^{-4}
266	50	thymidine-^{3}H	10^{-4}	1	5.5×10^{-2}	3.8×10^{-4}
V	3.5	thymidine-^{3}H	3.1×10^{-5}	0.63	4.2×10^{-2}	3.7×10^{-4}
III	3.0	thymidine-^{3}H	4.6×10^{-6}	0.43	1.2×10^{-1}	0.88×10^{-4}
					Avg	2.0×10^{-4}

* The specific activity was expressed as dpm per 1.18×10^{-11} g DNA (an average DNA content of a cell). The specific activity in μc/mM can be obtained by multiplying these by a conversion factor of 4.7×10^{6}.

the deoxyuridine monophosphate → TMP pathway over the TdR → TMP pathway, and possible other factors such as the relationship of the vicinity of the exogenous pool of TdR to the sites of DNA synthesis.

It is interesting that Cleaver (15) has recently estimated the thymidine pool size to be around 10^{-4} M/cell and 10^{-3} M/nucleus, based on radioautographic data interpreted in terms of the Quastler (8) model. It is clear, therefore, that in the design of mammalian cell experiments in which DNA is labeled with radioactive thymidine or thymidine analogues one must take care to consider the very large contribution of endogenous thymidine pathways.

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