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Stefano Iacobelli R.J.B. King Hans R. Lindner
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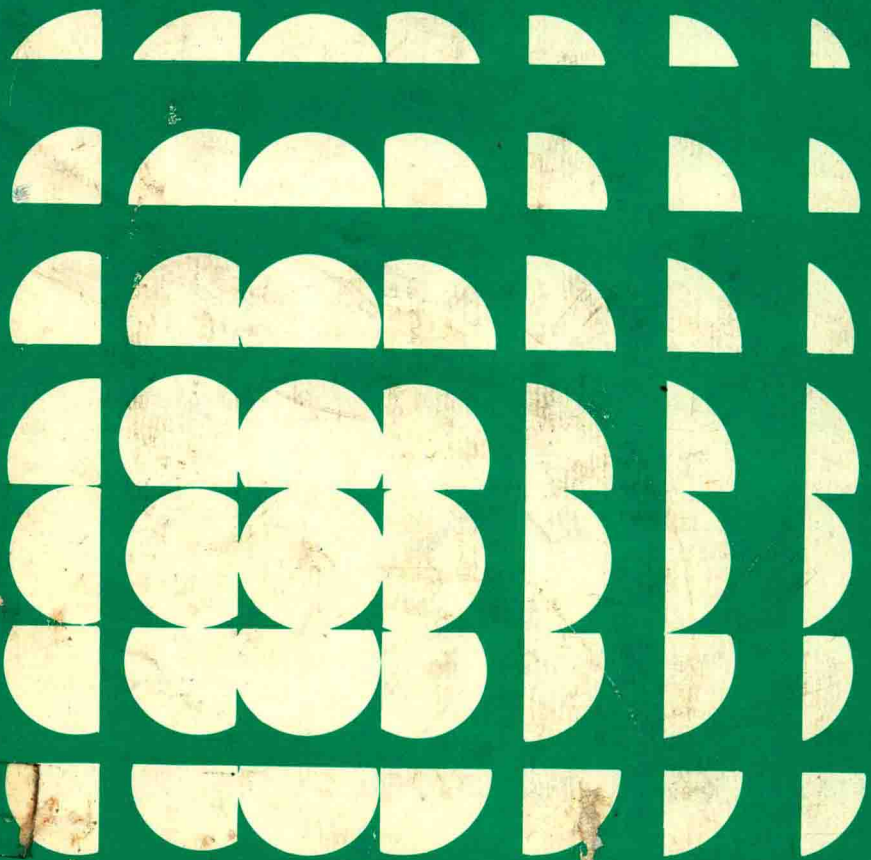

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Editors

Stefano Iacobelli
Laboratory of Molecular Endocrinology
S. Cuore Catholic University
Rome, Italy

R. J. B. King
Hormone Biochemistry Department
Imperial Cancer Research Fund
London, England

Hans R. Lindner
Department of Hormone Research
The Weizmann Institute of Science
Rehovot, Israel

Marc E. Lippman
Medicine Branch
National Cancer Institute
National Institutes of Health
Bethesda, Maryland, U.S.A.

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Hormone Action in Normal and Neoplastic Tissues: Sex Steroids

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Estrogen and Antiestrogen Effects on Thymidine Utilization by MCF-7 Human Breast Cancer Cells in Tissue Culture

Marc E. Lippman and Susan C. Aitken

Medicine Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

While incorporation of radioactive thymidine into acid-insoluble material is probably the most common method employed to estimate DNA synthesis, it may lead to erroneous conclusions. Conflicting data on the effects of estrogen on proliferation in MCF-7 cells made us wonder whether a more detailed analysis of precursor incorporation might elucidate some of the problems. A number of serious difficulties complicate the interpretation of incorporation data using this high specific activity trace.

First, the accurate determination of the true specific activity of labeled precursor in every experimental situation is critical.

Second, feedback (both positive and negative) by thymidine on a variety of key enzymatic steps in pyrimidine synthesis which affect its own utilization can occur. This is further complicated by the fact that intracellular-thymidine pools are relatively small. Consequently, addition of even small amounts of trace may seriously perturb the experimental system.

Third, deoxynucleotide pools may be compartmentalized intracellularly and differential incorporation of salvage and *de novo* derived thymidine may prevent legitimate projections to net DNA synthetic rates.

Fourth, and finally it is possible that metabolism of labeled precursor to products capable of eventual incorporation into material which is not DNA may occur.

The experiments summarized here are directed toward an accurate assessment of the role of the salvage pathway of pyrimidine synthesis and utilization in the response of the MCF-7 cell line to administration of estrogens and antiestrogens.

MATERIALS AND METHODS

MCF-7 cells, maintained in continuous tissue culture, were repeatedly shown to be free of mycoplasma contamination during this study. Conditions of culture have been previously described (1). Cells underwent two passages in IMEM

supplemented with 2.5% charcoal-treated calf serum and 10^{-7} moles/liter insulin prior to replicate plating in four or six well tissue culture dishes. When cells became subconfluent medium was replaced with IMEM containing 10^{-5} moles/liter phosphate and lacking asparagine. This reduction in phosphate content is necessary to obtain substantial incorporation of $[^{32}P]P_i$ into DNA. These experimental conditions do not affect growth curves or incorporation of radioactive precursors into macromolecular components of MCF-7 cells during the limited duration of these experiments (1). Four to twelve hr later medium was replaced with IMEM (10^{-5} moles/liter P_i), estradiol (5×10^{-9} moles/liter), or tamoxifen (2×10^{-6} moles/liter).

Before harvest, cells were incubated for 6 or 8 hr with $[^{32}P]P_i$ (1 μCi/ml) and/or [^{3}H]dThd (1–10 μCi/ml, 42–46 μCi/mmole) for a 2-hr period. The necessity for such a labeling procedure lies in the relatively long equilibration period required for $[^{32}P]P_i$ in this experimental system and the known perturbation of the system produced by radioactive thymidine. The details of any given experiment are described in Results.

An outline for the fractionation procedure for cell pellets following harvest can be found in Fig. 1. Detailed validation of fractionation and isolation methodologies will be found elsewhere (1).

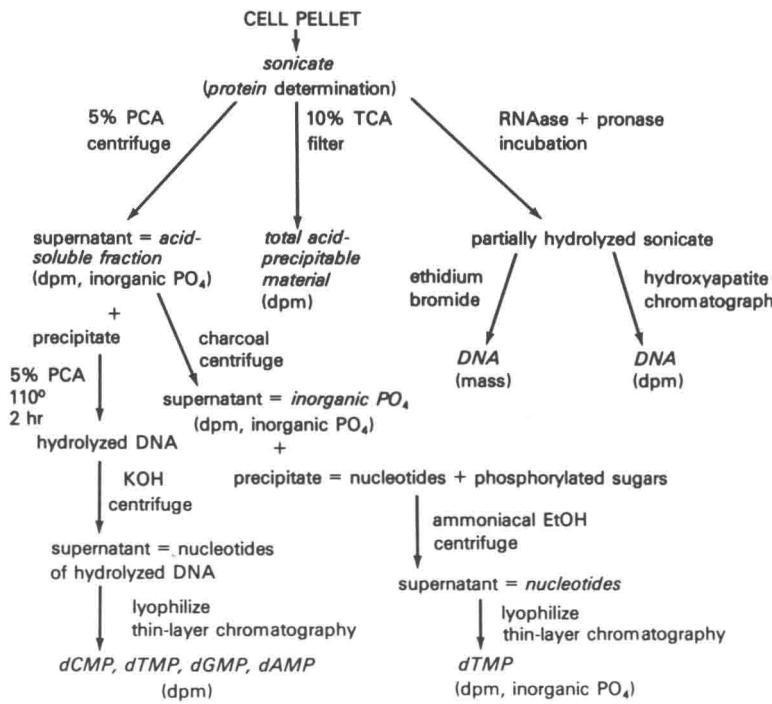

FIG. 1. Fractionation procedure used for examination of DNA synthesis in MCF-7 cells.

PARAMETERS

M	Size of intracellular pool
a	rate of entry of label into intracellular pool
b	rate of exit of label from intracellular pool

VARIABLES

L	dependent variable - amount of label in intracellular pool at any time t
t	independent variable - time after addition of label

EQUATIONS

$L(t) = M + (a-b)t - M(1+((a-b)/M)t)^{(-b/(a-b))}$	condition $a \neq b$
$L(t) = M(1-\exp(-at/M))$	condition $a = b$
$S(t) = L(t)/(M + (a-b)t)$	S = % of pool saturation

FIG. 2. Terms and equations related to kinetic analyses (MLAB) of incorporation data.

Kinetic analyses were based on the determination of radioactivity incorporated into acid-soluble fractions and DNA versus time. The coupling of such experiments with a modified isotope dilution technique (7) and subsequent analysis utilizing the NIH DEC-10 computer system and programming package MLAB (4) as previously described (1) permitted accurate measurement of thymidine pool size and analysis of thymidine kinetics. The parameters employed in the MLAB analysis are shown in Fig. 2. The precise details of such experiments will be presented in Results.

RESULTS

Three conditions are essential to the accurate use of thymidine in monitoring DNA synthetic rates: (a) exogenous dThd must be in equilibrium with intracellular precursor pools; (b) the true specific activity of thymidine in the system must be available; and (c) the perturbation of the system due to addition of trace must be appropriately controlled. A series of experiments relating to each of these conditions will be presented employing MCF-7 human breast cancer cells.

To establish the time required for equilibration of exogenous [^{3}H]dThd with precursor thymidine pools, the time course of incorporation of label into acid-soluble pools (Fig. 3A) and DNA (Fig. 3B) under varying experimental conditions was first examined. At time 0 [^{3}H]dThd was added to all wells and cells harvested at varying times thereafter. The time course of uptake of label into the acid-soluble pool of MCF-7 cells was between 1 and 60 min and is seen

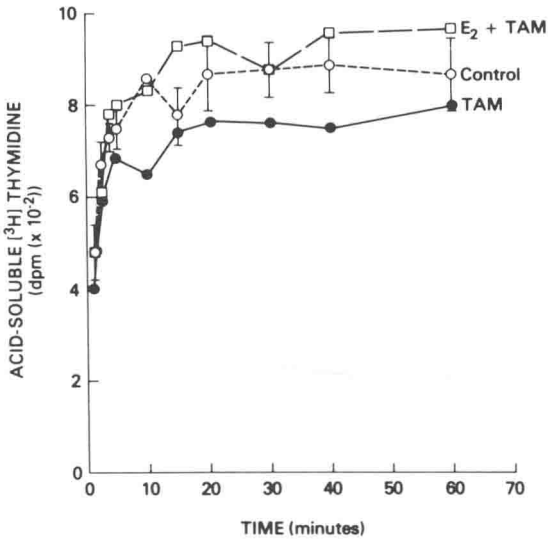

FIG. 3. A: Incorporation of ^3H dThd into the acid-soluble pool of MCF-7 cells. Cells were transferred to 10^{-5} moles/liter phosphate medium hormones (10^{-8} moles/liter estradiol, 10^{-8} moles/liter estradiol + 2×10^{-6} moles/liter tamoxifen) 32 hr prior to addition of [^3H]dThd (2 μCi/ml). Cells were harvested at the times indicated following addition of label. Values are the mean of two determinations and are normalized per unit protein. Standard deviations are presented only for control cells but did not exceed 10% for any group of samples.

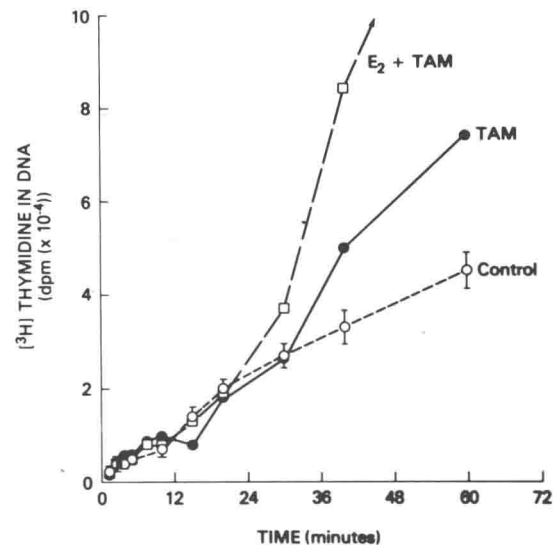

FIG. 3. B: Incorporation of [^3H]dThd into DNA of MCF-7 cells. Incorporation into DNA was determined by acid-precipitation on millipore filters. Values were normalized per unit protein and are presented as the mean of two determinations. Standard deviations are shown only for controls but were similar in all experimental groups.

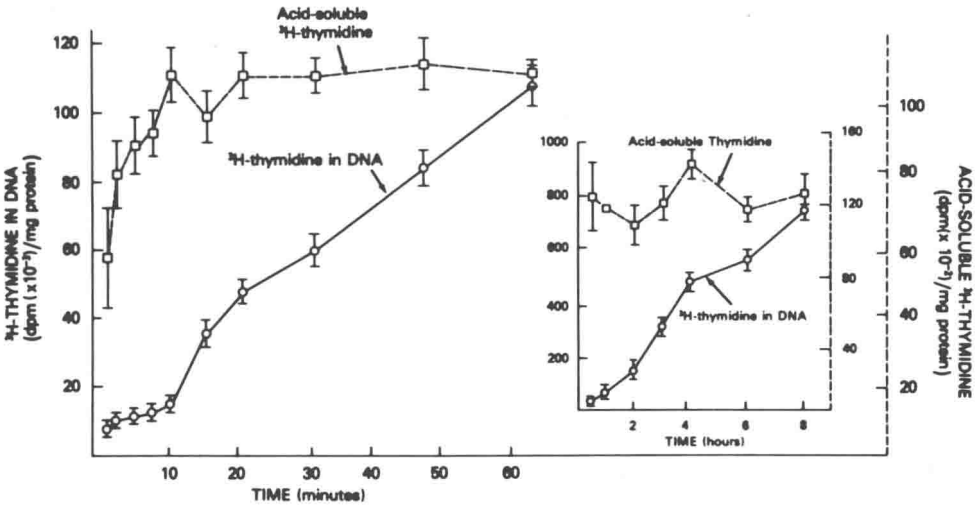

FIG. 3. C: Incorporation of [³H]dThd into DNA and acid-soluble pool of MCF-7 cells. Cells were treated as previously described but were placed in 10^{-5} moles/liter phosphate medium 24 hr prior to addition of [³H]dThd at time 0. Two separate experiments representing a short period of study (1–60 min and a longer time period (0.5–8 hr) are depicted. The latter is shown as an *inset.* In both experiments values are normalized per unit protein and are the average of three determinations ± 1 SD. DNA *(circles)* and acid-soluble *(squares)* fractions were isolated as previously described.

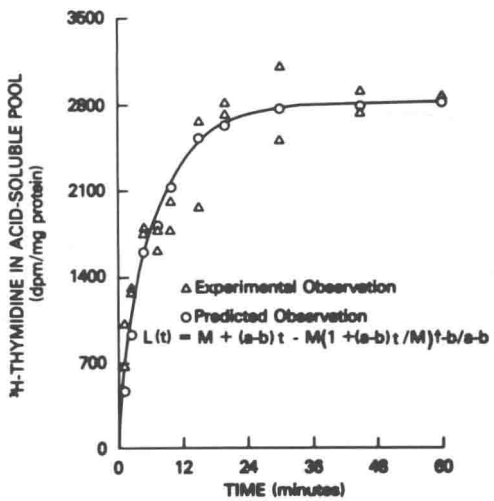

FIG. 3. D: Saturation curve for incorporation of [³H]dThd into acid-soluble pool of MCF-7 cells. Data obtained as described in Fig. 3A (dpm/mg protein) were fitted to the function $L(t) = M + (a - b)t - M(1 + (a - b)t/M)^{(-b/(a-b)}$ employing the computer programming package MLAB as previously described.

in Fig. 3A. Constant radioactivity was observed with 20 min of exposure to radioactive trace. No significant differences in the amount of label accumulating at equilibrium as a consequence of hormone treatment were detected. The incorporation of [^{3}H]dThd into partially purified DNA is presented in Fig. 3B. Incorporation became linear within 20 min, again suggesting that equilibration of the thymidine precursor pool for DNA synthesis had been attained. Figure 3B shows that both estrogen-treated and tamoxifen-treated cells displayed a higher rate of incorporation of dThd into DNA than did control cells. An explanation for this paradoxical observation following tamoxifen treatment—a condition long known to produce inhibition of thymidine incorporation (5,9)—is presented below.

The kinetics of incorporation of thymidine over a more extended time period are shown in Fig. 3C. Equilibrium conditions were maintained for the 8 hr of exposure to trace. Only the results observed in control cells are presented; however, a similar stability was evident in estrogen- and tamoxifen-treated MCF-7 cells. Figures 3A–C show that the 2-hr labeling period (which will be employed in subsequent experiments) satisfies the first condition of this study: equilibration of thymidine precursor pools within the time frame of a given experiment.

The next series of experiments to be discussed involve detailed kinetic analysis of thymidine pools employing three distinct analytic techniques: (a) MLAB (4); (b) linear regression analysis of incorporation into acid-soluble pools and DNA; and (c) a modified isotope dilution procedure (7). The MLAB computer programming package for graphic display and modeling of kinetic data and the equations developed by Cooper for two compartment systems (2) constitute the first approach to the complex issue of thymidine pools. MCF-7 cells are experimentally treated as previously described. The incorporation of label into the acid-soluble pool of control cells versus time (1–60 min) is fit to a function representing a two compartment system in which rate of entry and exit of label are approximately equal. Such a fit is shown in Fig. 3D for untreated MCF-7 cells. The excellent fit of data and function suggest that this program can yield significant information on the utilization of exogenous thymidine through the salvage pathway in MCF-7 cells. The parameters that can be defined by such computer assisted analysis (Fig. 2) include pool size (M) and rate of entry (a) and exit (b) of thymidine from the total acid-soluble pool.

Linear regression analysis can also be applied to incorporation data for label in acid-soluble pools and DNA versus time (Fig. 3A and 3B). The slopes of these lines (dpm/min) indicate the rate of incorporation of label. In the case of the acid-soluble pool, the initial velocity of the incorporation is of interest and only the earliest time points can be employed. In the case of incorporation into DNA, only the period of linearity at later time points is of interest.

However, neither MLAB analysis nor the linear regression analysis of incorporation data can establish pool parameters in terms of actual mass of thymidine in the absence of information on the effective specific activity of [^{3}H]dThd in (a) the extracellular environment and (b) the intracellular pool. In order to

investigate this question a modified isotope dilution technique (7) can be applied to the MCF-7 cell system. The basis of such an analysis is the ability of preexisting thymidine pools to compete with added labeled thymidine with respect to incorporation into a given end-product. Generally, three to four concentrations of thymidine are utilized in any given experiment. Cells are harvested after exogenous thymidine has equilibrated with intracellular precursor pools for DNA and the radioactivity in the acid-soluble pool and DNA is determined. Application of the equation presented in the legend of Table 1 to the acid-soluble pool provides an index of the extracellular competitive pool for thymidine incorporation. Applying this analysis to incorporation into DNA provides estimates of actual intracellular pool size. Table 1 demonstrates results obtained in control MCF-7 cells. These results suggest that substantial amounts of thymidine are available both extra- and intracellularly and that there is considerable variation in pool size over time in serum-free medium. The fact that addition of labeled thymidine in the range of 1 to 2 μCi ($2–5 \times 10^{-8}$ moles/liter) constitutes only 5 to 10% of total thymidine incorporated into intracellular pools and DNA suggests that [^{3}H]dThd incorporation is likely to provide highly inaccurate estimates of actual rates of DNA synthesis in the absence of information on the condition of said pools. It should be pointed out that IMEM contains no thymidine and that the charcoal-treated calf serum has negligible residual nucleoside.

Hormonal influences on thymidine pool kinetics were studied at 24, 36, and 48 hr after estrogen or tamoxifen treatment. These studies represent separate

TABLE 1. *Effective intracellular and extracellular pools of dThd*

Time (hr)	Extracellular pool (moles/liter)	Intracellular pool (moles/liter)	Protein (mg)
0	2.38×10^{-6}	6.30×10^{-8}	0.783
12	2.30×10^{-6}	1.67×10^{-7}	0.804
24	3.59×10^{-6}	6.38×10^{-7}	0.998
36	8.36×10^{-7}	7.12×10^{-7}	1.299
48	7.18×10^{-7}	5.67×10^{-7}	1.089

MCF-7 cells were replicately plated as previously described. Eight hours before time 0 cells were transferred to 10^{-5} moles/liter phosphate medium. Medium was again replaced 1 hr before time 0. [^{3}H]dThd (10 μCi/ml, 1 hr) was added with varying amounts of unlabeled dThd to achieve estimated extracellular molarities of 2×10^{-7}, 5×10^{-7}, or 10^{-6} moles/liter 1 hr before harvest. Cells were harvested at the times indicated (column 1) and aliquots were taken for determination of acid-soluble radioactivity (column 2), acid-precipitable radioactivity (column 3), and protein (column 4). Values for incorporation were normalized per unit protein. The effective pool size was determined according to Scudiero et al. (7) as follows:

T_1 = lower concentration of dThd
T_2 = Higher concentration of dThd
Q = ratio of label incorporated at concentration T_1 and T_2 (cpm 1/cpm 2)

Then P (effective pool size) + $(T_2 - (T_1 \times Q))/(Q - 1)$

experiments in which the incorporation of [^{3}H]dThd into the acid-soluble pool and DNA of MCF-7 cells were determined as a function of time of exposure to trace (1–60 min) and in which cells were additionally exposed to varying concentrations of dThd (2×10^{-8}, 5×10^{-8}, 10^{-6}, and 2×10^{-6} moles/liter) for a constant period of time (60 min). Coupling of experimental results employing MLAB, linear regression, and isotope dilution (described above) allows a rigorous analysis of thymidine pools and permits determination of whether extracellular salvage or intracellular dThd is primarily utilized in DNA synthesis. The parameters that can be estimated by this methodology are presented in Table 2. The contributions of extracellular salvage-derived thymidine to net DNA synthesis (b(s)/b) and to intracellular pools (a(s)/a) are of particular interest. These methods do not allow a breakdown of intracellular dThd into (thymidine derived from internal breakdown of DNA) or from the reduction and methylation of uridine derived from turnover of RNA. The term "salvage"

TABLE 2. *Significance and derivation of parameters utilized in analysis of thymidine pools in MCF-7 cells*

Parameter	Significance	Method of determination
1. M	Intracellular pool size (pmoles)	MLAB kinetic analysis [dpm/(spec. act. × ECDF × IDCF)]
2. L	[^{3}H]dThd component of intracellular pool (pmoles)	MLAB kinetic analysis [dpm/spec. act.]
3. a	Rate of entrance of dThd into intracellular pool (pmoles/min)	MLAB kinetic analysis [dpm/(spec. act. × ECDF × ICDF)]
4. a(s)	Rate of entrance of extracellular dThd into intracellular pool (pmoles/min)	Slope of line for incorporation of [^{3}H]dThd into acid-soluble pool [dpm/(spec. act. × ECDF)]
5. a(s)/a	Percent of dThd entering the intracellular pool from extracellular sources	Ratio of parameters 3 and 4
6. ECDF	Factor by which [^{3}H]dThd is diluted by extracellular dThd in medium	Method of Scudiero et al. (7) (isotope dilution)
7. b	Rate of exit of dThd from extracellular pool (pmoles/min)	MLAB kinetic analysis [dpm/(spec. act. × ECDF × ICDF)]
8. b(d)	Rate of incorporation of dThd into DNA (pmoles/min)	Slope of line for incorporation of ^{3}H dThd into DNA [(spec. act. × ECDF × IDCF)]
9. b(m)	Rate of loss of dThd from intracellular pool due to metabolism (pmoles/min)	b − b(d) (parameters 7 and 8)
10. b(s)	Rate of incorporation of extracellular dThd into DNA (pmoles/min)	Slope of line for incorporation of [^{3}H]dThd into DNA [dpm/(spec. act. × ECDF)]
11. b(s)/b(d)	Percent of dThd in DNA derived from extracellular sources	Ratio of parameters 10 and 8
12. ICDF	Factor by which [^{3}H]dThd is diluted by intracellular sources of dThd	Method of Scudiero et al. (7) (isotope dilution)