Biochemical Pharmacology, Vol. 34, No. 13, pp. 2319–2324, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

RAPID DETERMINATION OF THYMIDYLATE SYNTHASE ACTIVITY AND ITS INHIBITION IN INTACT L1210 LEUKEMIA CELLS *IN VITRO*

JACK C. YALOWICH* and THOMAS I. KALMAN†

Departments of Biochemical Pharmacology and Medicinal Chemistry, State University of New York at Buffalo, Buffalo, NY 14260, U.S.A.

(*Received* 27 *August* 1984; *accepted* 2 *October* 1984)

Abstract—A rapid and convenient tritium release assay for measuring thymidylate (dTMP) synthase activity and its inhibition within intact mammalian cells is described in detail. Short-term incubation of murine leukemia L1210 cells with an appropriately labeled substrate precursor, either deoxyuridine ([5-^{3}H]dUrd) or deoxycytidine ([5-^{3}H]dCyd), allowed for: (1) uptake and intracellular conversion to the substrate deoxyuridylate ([5-^{3}H]dUMP); and (2) the obligatory displacement of tritium from [5-^{3}H]-dUMP during the dTMP synthase catalyzed reaction. Tritium released into the aqueous environment was quantitated after a quick one-step separation of tritated H_2O from other radiolabeled materials and cell debris. The amount of tritium released was evaluated as a function of a number of variables, including the concentration of labeled substrate precursors, cell number, and incubation time. Tritium from [5-^{3}H]dCyd was released significantly faster than from [5-^{3}H]dUrd under a variety of conditions. Both 5-fluorodeoxyuridine (1 μM) and methotrexate (10 μM), which effectively block intracellular dTMP synthesis, completely inhibited the release of tritium from either [5-^{3}H]dCyd or [5-^{3}H]dUrd demonstrating that the release of tritium is mediated exclusively by the dTMP synthase catalyzed reaction. In addition, there was a good correlation between tritium release, cellular uptake, and incorporation of [2-^{14}C]dUrd into DNA. The inhibitory effects of antifolates such as methotrexate were independent of the type of labeled precursor used. In contrast, preferential interference with the release of tritium from [5-^{3}H]-dCyd by dCyd derivatives and from [5-^{3}H]dUrd by dUrd derivatives was observed, suggesting that competition for uptake and/or phosphorylation may contribute to the overall effects of certain nucleoside analogues on cellular dTMP synthase activity measured using the tritium release assay.

Inhibition of DNA-thymine biosynthesis significantly contributes to the cytotoxicity of many clinically useful antimetabolites, e.g. 5-fluoropyrimidines and antifolates, and involves interference with the activity of thymidylate (dTMP) synthase (5,10-methylenetetrahydrofolate: dUMP *C*-methyltransferase, EC 2.1.1.45), the key enzyme of this biosynthetic pathway. Measurement of these drug effects at the cellular level most frequently relies on incorporation of radiolabeled nucleosides into DNA. For example, the incorporation of dUrd‖ alone [1, 2] or in comparison with dThd incorporation [3, 4] has often been utilized, as well as the so-called dUrd suppression test [5–8] which measures the degree of inhibition of radiolabeled dThd incorporation by large concentrations of exogenously added dUrd. These assay systems measure dTMP synthase activity indirectly and may be misleading since macromolecular synthesis is required to observe effects at a previous enzymatic step. In addition, exogenous dUrd may compete with radiolabeled dThd for phosphorylation by dThd kinase [9]. Furthermore, it was reported [10] that blockade of dTMP biosynthesis is accompanied by enhanced dUrd/dThd catabolism via dThd phosphorylase which may also affect accurate measurement of dTMP synthase activity using incorporation techniques.

A more direct assay of dTMP synthase is based on the release of tritium from the specifically labeled substrate, [5-^{3}H]dUMP, during the enzyme catalyzed reaction. This tritium release assay [11–16] was described originally by Lomax and Greenberg [12, 13] and Roberts [11] and has been further developed for use in bacterial cells [17]. We have reported previously [18–23] the successful adaptation of this tritium release assay for the measurement of dTMP synthase activity in intact mammalian cells (see Fig. 1). This intact cell assay permits a direct and rapid assessment of enzyme activity and its inhibition by cytotoxic agents, with the added advantage over cell-free enzyme systems that there is a preservation of the integrity of the cell-membrane, multi-enzyme complexes, and the network of metabolic pathways and their control within the cell. In this report we describe in detail the various conditions for the

* Present address: Clinical Pharmacokinetic and Pharmacodynamics Laboratory, Pharmaceutical Division, St. Jude Children's Research Hospital, Memphis, TN 38101.

† Address all correspondence to: Dr. Thomas I. Kalman, State University of New York at Buffalo, 457 Cooke Hall, Buffalo, NY 14260, U.S.A.

‖ Abbreviations: dCyd, 2′-deoxycytidine; 5-FdUrd, 5-fluoro-2′-deoxyuridine; dThd, thymidine; dUrd, 2′-deoxyuridine; MTX, methotrexate; and TCA, trichloroacetic acid.

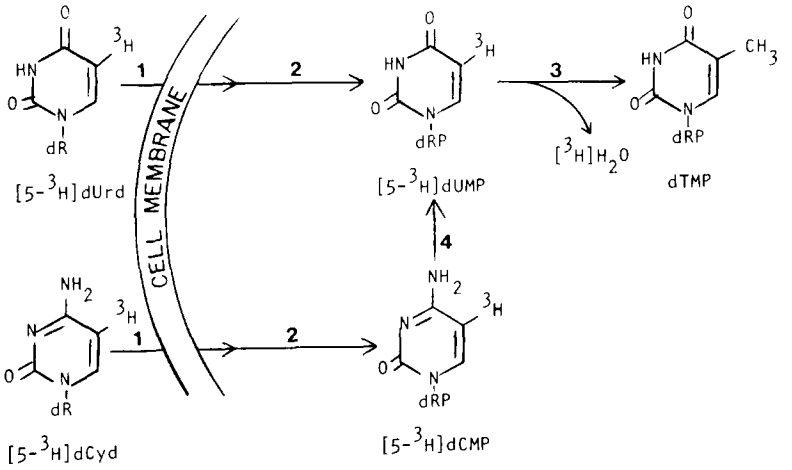

Fig. 1. Biochemical events involved in the intact cell tritium release assay. (1) Cellular uptake of the radiolabeled nucleosides, [5-^{3}H]dUrd or [5-^{3}H]dCyd. (2) Intracellular phosphorylation to the corresponding 5′-monophosphates. (3) Displacement of tritium from [5-^{3}H]dUMP during the dTMP synthase catalyzed reaction. The displaced tritium is released into the aqueous milieu and can be counted as tritiated water (^3H_2O). When [5-^{3}H]dCyd is used, the deamination (4) of [5-^{3}H]dCMP is also required for [5-^{3}H]dUMP formation.

measurement of dTMP synthase activity and its inhibition in intact murine leukemia L1210 cells.

MATERIALS AND METHODS

[5-^{3}H]dUrd (sp. act. 10–15 Ci/mmole) was purchased from ICN (Irvine, CA); [5-^{3}H]dCyd (5 Ci/mmole) and [2-^{14}C]dUrd (51 mCi/mmole) were from Schwarz-Mann (Orangeburg, NY). [5-^{3}H]dUrd and [5-^{3}H]dCyd were routinely purified in 1 mCi batches by preparative TLC on Eastman Quanta-Gram cellulose plates (PQ2) using the solvent mixture *n*-butanol–acetic acid–water (5:2:3, by vol.). Aliquots of the radiolabeled material (3–6 nmoles) were added to vials, dried and stored in a dessicator under vacuum at 4°. Immediately before use, the purified [5-^{3}H]dUrd and [5-^{3}H]dCyd were adjusted to 10^{-5} M with aqueous solutions of unlabeled dUrd and dCyd respectively. 5-Fluoro-2′-deoxyuridine was obtained from Hoffmann-LaRoche (Nutley, NJ). Methotrexate was provided by the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Samples of 5-ethynyl-dUrd were gifts from Drs. M. Bobek and M. J. Robins. The nucleoside analogue 5-NH_2-dCyd was synthesized by Dr. David Goldman as described in Ref. 24. All remaining chemicals were of the highest purity available and obtained from the Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Cellular techniques and enzyme assays. Mouse leukemia L1210 cells were grown in the peritoneum of 6- to 8-week-old DBA/2HA mice (West Seneca Laboratories, West Seneca, NY). Cells were harvested 5–7 days after inoculation of 10^5 cells/mouse. Five milliliters of a hypotonic sodium phosphate buffer (0.002 M, pH = 7.6, containing 0.01 M NaCl) was added to disrupt red blood cells, and 10 ml of Eagle's liquid BME medium minus folate (Gibco, Grand Island, NY) was then added. Cells were separated by centrifugation (121 *g* for 5 min), washed with ice-cold fresh medium and resuspended in Eagle's medium to a final concentration of 2.5×10^7 cells/ml.

For the measurement of tritium release, 200-μl aliquots of the above cell suspension were placed in tubes and incubated at 37° using a shaking water bath. The reaction was started by the addition of [5-^{3}H]dUrd or [5-^{3}H]dCyd (1 μM, in a total volume of 250 μl) after a 40-min preincubation period in the presence or absence of drugs and allowed to proceed for an additional 15 min, unless stated otherwise. The reaction was terminated by transferring 100-μl aliquots into 0.5-ml centrifuge tubes containing 100 μl of a 15% activated charcoal suspension in 4% aqueous perchloric acid. The tubes were vigorously stirred by vortexing and then centrifuged for 2 min in a Beckman Microfuge. The radioactivity of a 100-μl aliquot of supernatant fraction from each tube was counted in a Packard Tri-Carb (model 2450 or 3255) liquid scintillation spectrometer using a toluene-based scintillation mixture. The release of tritium was expressed as a percentage of the total amount of radioactivity added. Blank tubes containing only Eagle's medium were included in the experiments and generally gave background counts of less than 1% of the total radioactivity (50,000–200,000 cpm) added. All values obtained were corrected for background counts. I_{50} values determined from dose-response curves represent the concentrations of inhibitors required for 50% inhibition of the release of tritium. All experiments were repeated at least twice on separate days; experimental data are reported as average values of the results of two to five independent determinations.

For the determination of the cellular uptake of [2-^{14}C]dUrd, 200-μl aliquots of cell suspension were placed on glass fiber filters (Reeve Angel) and washed repeatedly (5×) with 600 μl of ice-cold buffer (0.08 M potassium phosphate containing 0.9% NaCl, pH 7.45). The filters were dried and processed for the measurement of radioactivity (see below).

Incorporation [2-^{14}C]dUrd into DNA was assessed by measuring the radioactivity in TCA precipitable material. After incubation at 37°, 100-μl aliquots of cell suspension were placed on glass fiber filters impregnated with ice-cold 10% TCA containing 0.1 M sodium pyrophosphate. The filters were washed twice with 5 ml of the above TCA solution and allowed to dry in 20-ml glass scintillation vials. A solution (200 μl) of 1 mg/ml DNAse I (bovine pancreas, Sigma) in 50 mM Tris buffer, pH 7.6, containing 3 mM $MgCl_2$ was then added to each filter, and the vials were incubated at 37° overnight followed by measurement of radioactivity.

For the simultaneous measurement of nucleoside uptake or incorporation and tritium release, a mixture of [2-^{14}C]dUrd (10 mCi/mmole) and [5-^{3}H]dUrd (75 mCi/mmole) was used at a final concentration of 1–5 μM. Incubation with L1210 cells with radiolabeled dUrd was followed by taking duplicate samples at a number of time points and processing cell suspensions separately for the determination of tritium release and for the measurement of radioactivity associated with whole cells or TCA precipitates. Channel settings were optimized for carbon-14 and tritium dual-label counting, using a refrigerated Packard Tri-Carb liquid scintillation spectrometer (model 2450 or 3255).

Nucleoside kinase assays in L1210 cell free extracts were performed using a modification [25] of the method of Ives *et al.* [26].

RESULTS

Figures 2 and 3 show the time dependence of the release of tritium when suspensions of L1210 cells harvested 5, 6, or 7 days post-inoculation were incubated with [5-^{3}H]dUrd or [5-^{3}H]dCyd. Using [5-^{3}H]dUrd, there was a brief lag period (*ca.* 5 min) followed by a linear rate of release of tritium which continued for at least 30 min. The initial rate was highest for 5-day and lowest for 7-day harvest of cells. The release of tritium from [5-^{3}H]dCyd was initially faster than from [5-^{3}H]dUrd, but levelled off earlier and at a lower value.

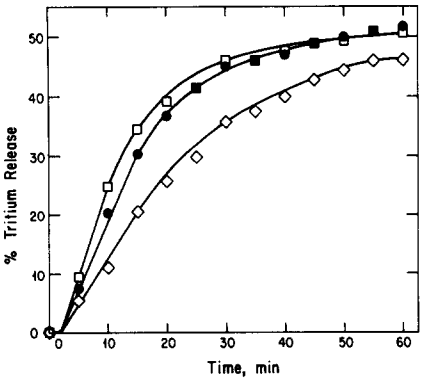

Fig. 3. Time dependence of the release of tritium from [5-^{3}H]dCyd by L1210 cells. The experimental conditions were identical to those described in the legend to Fig. 2.

The plots in Fig. 4 show that the release of tritium was proportional to the cell number using both radiolabeled precursors. Using 5×10^6 cells per tube (standard assay conditions), there was about a 4-fold greater release of tritium from [5-^{3}H]dCyd than from [5-^{3}H]dUrd. Likewise, using crude extracts of L1210 cells, we found that the phosphorylation of [5-^{3}H]-dCyd was greater (10-fold) than the conversion of [5-^{3}H]dUrd to [5-^{3}H]dUMP (data not shown).

The dependence of the release of tritium on the extracellular concentration of radiolabeled precursor is illustrated in Fig. 5. The linearity of the double-reciprocal plots indicates that the kinetics of the overall process involving radiolabeled nucleoside transport, metabolic formation of [5-^{3}H]dUMP and subsequent release of tritium during dTMP synthesis, conforms to the Michaelis–Menten equation. An apparent K_m of 2.7 μM was obtained for both [5-^{3}H]-dCyd and [5-^{3}H]dUrd.

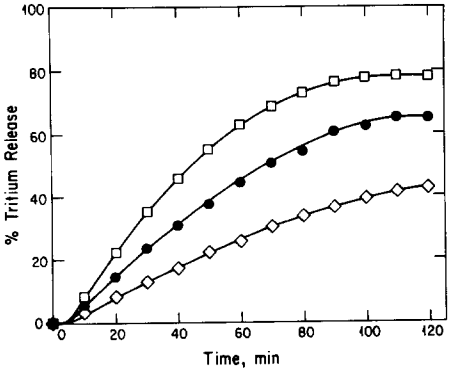

Fig. 2. Time dependence of the release of tritium from [5-^{3}H]dUrd by L1210 cells. Cells, harvested 5 (□), 6 (●), or 7 (◇) days after inoculation into mice, were incubated with the radiolabeled nucleosides at 37° for the indicated periods of time. The amount of tritium released was measured as described in Materials and Methods.

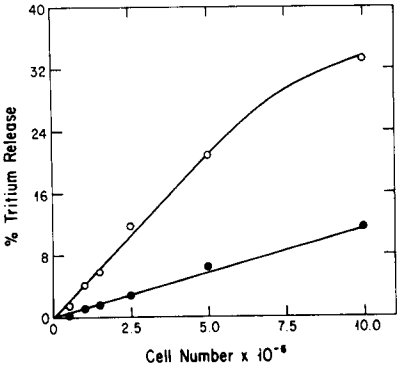

Fig. 4. Dependence of the release of tritium from [5-^{3}H]dUrd and [5-^{3}H]dCyd on the cell number. L1210 cells harvested 6 days after inoculation were incubated at 37° for 15 min in the presence of [5-^{3}H]dUrd (●) or [5-^{3}H]dCyd (○), and the amounts of tritium released were determined as described in Materials and Methods. The number of cells per assay tube was varied as indicated.

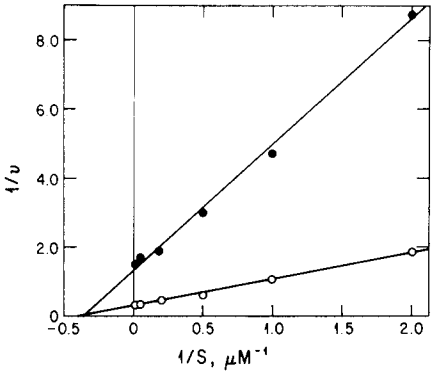

Fig. 5. Dependence of the release of tritium on the concentration of [5-^{3}H]dUrd and [5-^{3}H]dCyd. L1210 cells were incubated at 37° for 15 min with various concentrations of [5-^{3}H]dUrd (●) or [5-^{3}H]dCyd (○); v = pmoles tritium released/min/10^6 cells (see Materials and Methods).

The relationship between nucleoside uptake and subsequent release of tritium was investigated by incubating L1210 cells simultaneously with [2-^{14}C]-dUrd and [5-^{3}H]dUrd. During a 30-min incubation period, the ratio of cell-associated carbon-14 to tritated water was 1.05 ± 0.06 (pmoles/pmole). A correspondence between the release of tritium from [5-^{3}H]dUrd and incorporation of [2-^{14}C]dUrd into acid-precipitable material was established in a similar manner. During a 60-min incubation period the ratio of carbon-14 incorporated to tritium released was 1.11 ± 0.03 (pmoles/pmole).

Figure 6 shows the dose–response curves for the effects of 5-FdUrd on the release of tritium from [5-^{3}H]dUrd. At 1 μM 5-FdUrd, there was a virtually complete inhibition of the release of tritium which demonstrates the specificity of the assay system for measuring the dTMP synthase reaction. A 40-min preincubation with 5-FdUrd followed by the addition of [5-^{3}H]dUrd or [5-^{3}H]dCyd yielded I_{50} values of 5.7×10^{-9} M and 7.6×10^{-9} M respectively (Table 1), demonstrating the sensitivity of the intact cell assay. When 5-FdUrd was added simultaneously with [5-^{3}H]dUrd, there was a small reduction in the inhibitory potency of the analogue (Fig. 6).

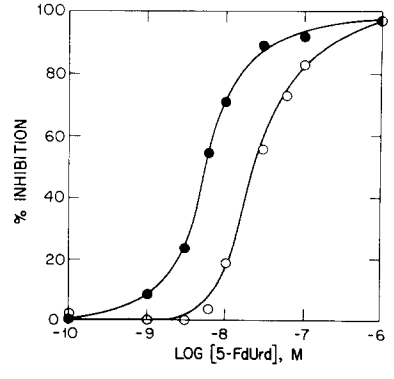

Fig. 6. Dose–response curves for the inhibition by 5-FdUrd of the release of tritium. [5-^{3}H]dUrd was added to the L1210 cell suspension simultaneously with (○) or 40 min after (●) the addition of 5-FdUrd. The percent inhibition of tritium release was determined as described in Materials and Methods.

Table 1. Inhibition of tritium release from [5-^{3}H]dUrd and [5-^{3}H]dCyd in intact L1210 leukemia cells*

Compound	I_{50} (μM) [5-^{3}H]dUrd	[5-^{3}H]dCyd
5-FdUrd	0.0057	0.0076
Methotrexate (MTX)	0.34	0.19
5-Ethynyl-dUrd	0.25	1.0
5-CH_3-dUrd (dThd)	0.45	75.0
5-NH_2-dCyd	>1000	5.0

* Cells were preincubated for 40 min at 37° in the presence of various concentrations of inhibitors before the addition of radiolabeled nucleosides; I_{50} values were determined from dose–response curves (see Fig. 6 and Materials and Methods).

The observed inhibitory activities of some representative nucleoside analogues and of MTX on the release of tritium from [5-^{3}H]dUrd and [5-^{3}H]dCyd are summarized in Table 1. MTX was equally effective as an inhibitor of the release of tritium whether [5-^{3}H]dUrd or [5-^{3}H]dCyd was utilized as the substrate precursor. In contrast, 5-ethynyl-dUrd and 5-CH_3-dUrd (dThd) showed lower I_{50} values with [5-^{3}H]dUrd than with [5-^{3}H]dCyd, whereas the dCyd analog 5-NH_2-dCyd was a more potent inhibitor of the release of tritium from [5-^{3}H]dCyd.

DISCUSSION

The assay system described in this report was developed to measure dTMP synthase activity and its inhibition by antimetabolites in intact mammalian cells. The results in Fig. 6 demonstrate the specificity of the assay using intact L1210 cells by showing that 5-FdUrd, a potent inhibitor of dTMP synthase in its 5′-monophosphate form, was able to completely inhibit the release of tritium from [5-^{3}H]dUrd and [5-^{3}H]-dCyd. Specificity is further indicated by the nearly complete inhibition of the release of tritium by 10 μM MTX [22]. Other antifolates, e.g. aminopterin and triazinate, are also potent inhibitors of the release of tritium in L1210 cells [22]. The marked effect on tritium release, due to indirect inhibition of dTMP formation by these dihydrofolate reductase inhibitors, demonstrates the versatility of the intact cell assay.

Based on the I_{50} values obtained for the inhibition of the release of tritium by MTX and 5-FdUrd, 3.4×10^{-7} M and 5.7×10^{-9} M, respectively (Table 1), these drugs are less potent than their reported growth inhibitory effects in culture, which yield I_{50} values of 5.8×10^{-9} M for MTX [27] and 0.5×10^{-9} M for 5-FdUrd [28]. These potency differences may be accounted for by the fact that growth inhibition is determined over a much longer period of time using lower cell concentrations. Furthermore, growth inhibition by MTX and 5-FdUrd may be due to other effects besides dTMP synthesis inhibition, e.g. the inhibition of purine biosynthesis by MTX [3] and the incorporation of fluoropyrimidines into RNA and/or DNA [29–34].

Inhibition of dTMP synthase by 5-FdUrd and MTX is known to cause significant expansion of the dUMP pools [35–37]. When [5-^{3}H]dUrd or [5-^{3}H]

dCyd is added under these conditions to monitor dTMP synthase activity, isotopic dilution of [5-^{3}H]dUMP may enhance the inhibition of the release of tritium and, therefore, the apparent potency of the drug tested. However, in the absence of dTMP synthase inhibitors and over a range of 1–5 μM extracellular radiolabeled dUrd, a close correspondence between nucleoside uptake and the release of tritium was observed, indicating that the cellular assay accurately reflects the dTMP synthase activity.

It is of particular interest that the release of tritium from [5-^{3}H]dCyd proceeded about four times faster than from [5-^{3}H]dUrd (Fig. 4). Similarly, Balzarini and De Clercq [38,*] found a 2-fold greater release of tritium from [5-^{3}H]dCyd than from [5-^{3}H]dUrd, using different assay conditions. These results suggest a more rapid processing of [5-^{3}H]dCyd by L1210 cells to form intracellular [5-^{3}H]dUMP. This suggestion is consistent with previous findings that there are elevated levels of dCyd kinase [39] and dCMP deaminase [35, 40, 41] in a number of leukemia cell lines. In addition, the pathway involving dCMP deaminase was shown to be the major contributor to the dUMP pool in rat hepatoma cells [41]. Our finding of a 10-fold greater phosphorylation of [5-^{3}H]-dCyd versus [5-^{3}H]dUrd in cell-free extracts suggests that enhanced intracellular nucleotide formation is responsible, at least in part, for the preferential utilization of dCyd L1210 cells, as measured by the tritium release assay.

The observation that the release of tritium from [5-^{3}H]dCyd reached a maximum at *ca.* 50% after 1 hr of incubation (Fig. 3) indicates that about half of dCyd was converted to metabolites other than dUMP. The phosphorylation of dCMP to dCTP may compete with its deamination to dUMP which would account for the observed plateau of the amount of tritium released from [5-^{3}H]dCyd (Fig. 3). In fact, when L1210 cells are incubated with [^{14}C]dCyd, one-half of intracellular radioactivity is associated with cytosine nucleosides and nucleotides and the other half with uracil and thymine nucleosides and nucleotides [42].

For the evaluation of the inhibitory effects of nucleoside analogues, it was considered important to measure the release of tritium from both [5-^{3}H]-dUrd and [5-^{3}H]dCyd in order to distinguish the direct effects on dTMP synthase activity from possible interference with the transport of the radiolabeled precursor and/or its metabolic processing to [5-^{3}H]dUMP. When 5-CH_3-dUrd (dThd) and 5-ethynyl-dUrd were tested, the lower I_{50} values observed for the release of tritium from [5-^{3}H]dUrd compared to [5-^{3}H]dCyd (Table 1) indicate that these nucleosides interfere with the phosphorylation of [5-^{3}H]dUrd by dThd kinase. On the basis of their similar effect on the release of tritium from [5-^{3}H]dUrd alone, both 5-CH_3-dUrd (dThd) and 5-ethynyl-dUrd appear to be dTMP synthase inhibitors of comparable potency (Table 1). However, only 5-ethynyl-dUrd in its phosphorylated form is known to be a sufficiently potent inhibitor of dTMP synthase [23, 43, 44] to account for the observed effect. The much larger I_{50} value of dThd corresponding to the release of tritium from [5-^{3}H]dCyd (Table 1) reveals that the inhibitory effects on the release of tritium from [5-^{3}H]dUrd is primarily due to a competition between dThd and [5-^{3}H]dUrd for phosphorylation by dThd kinase. The absence of any effect of 5-NH_2-dCyd on the release of tritium from [5-^{3}H]dUrd (Table 1) argues against inhibition of dTMP synthase activity. Therefore, the inhibitory effect of this analogue on the release of tritium from [5-^{3}H]dCyd can only be due to interference with the transport of [5-^{3}H]dCyd and/or its conversion to [5-^{3}H]dUMP.

The effects of the potent inhibitor 5-FdUrd on the release of tritium from both [5-^{3}H]dUrd and [5-^{3}H]-dCyd are comparable (Table 1), indicating direct inhibition of dTMP synthase. It is unlikely that 5-FdUrd interferes with the uptake and/or metabolic processing of [5-^{3}H]dUrd at the concentration required for 50% inhibition (5.7×10^{-9} M), since this level is two orders of magnitude lower than the extracellular concentration of added [5-^{3}H]dUrd (1 μM). If there were interference by 5-FdUrd with the formation of [5-^{3}H]dUMP, then simultaneous addition of the inhibitor and the radiolabeled precursor would result in more inhibition of the release of tritium. In fact, the opposite was observed (Fig. 6).

In the presence of MTX, similar I_{50} values were obtained for the inhibition of the release of tritium from [5-^{3}H]dUrd and [5-^{3}H]dCyd (Table 1). These results could be anticipated, since MTX has no effect on the formation of [5-^{3}H]dUMP from either radiolabeled precursor, but rather interferes with the production of the other substrate of dTMP synthase, the cofactor 5,10-methylenetetrahydrofolate, via inhibition of dihydrofolate reductase.

The initial description of the development of the intact cell assay system [18–23] has been followed by a number of reports in the literature which utilize this method [38, 45–49]. The tritium release assay has been employed to study the possible cell cycle regulatory effects on dTMP synthase activity in synchronous cultures of L1210 cells [45], to examine the relative importance of 5-flourouracil incorporation into RNA versus the inhibition of dTMP synthase in intact Novikoff hepatoma cells [46], to study the short-term effects of various antifolates in L1210 cells [21, 22, 47], to establish the role of polyglutamate derivatives of MTX as effective dTMP biosynthesis inhibitors in H-35 hepatoma cells in culture [48], and to study 5-FdUMP effects on dTMP formation and subsequent DNA synthesis [49]. In addition, Balzarini and De Clercq [38] have recently examined the effects of a number of dThd analogues on dTMP synthase activity in L1210 cells. These investigators found a better correlation between grown inhibitory effects and the release of tritium when [5-^{3}H]dCyd was used instead of [5-^{3}H]dUrd. These results could be explained by interference with [5-^{3}H]dUrd phosphorylation by these dThd analogues and support the conclusions reached in the present study.

It is clear that the intact cell dTMP synthase assay is a useful tool for examining the short-term effects of antimetabolites on both the salvage and *de novo* biosynthesis of dTMP *in vitro*. Further studies should

* J. Balzarini and E. De Clercq, *FEBS Abstr.* 5–13, FR-240 (1982).

explore the potential application of this assay system for monitoring *in vivo* the therapeutic effectiveness of drugs which inhibit the dTMP biosynthetic pathway.

Acknowledgements—This work was supported in part by research grants CA-13,604 from the National Cancer Institute, NIH, and CH-192 from the American Cancer Society. We are grateful to Drs. M. Bobek and M. J. Robins for providing us with samples of 5-ethynyl-dUrd.

REFERENCES

1. D. Bowen, J. C. White and I. D. Goldman, *Cancer Res.* **38**, 219 (1978).
2. W. D. Sedwick, M. J. Fyfe, O. E. Brown, T. A. Fraser, M. Kutler and J. Laszlo, *Molec. Pharmac.* **16**, 607 (1979).
3. W. M. Hryniuk, *Cancer Res.* **35**, 1085 (1975).
4. T. J. Bardos, J. Aradi, Y. K. Ho and T. I. Kalman, *Ann. N.Y. Acad. Sci.* **255**, 522 (1975).
5. J. B. Block, R. T. Chlebowski, M. F. Dietrich and W. H. Isacoff, *Cancer Treat. Rep.* **68**, 631 (1984).
6. V. Herbert, G. Tisman, H. Edlis, L. T. Go and L. Brenner, *Clin. Res.* **19**, 738 (1971).
7. V. Herbert, G. Tisman, L. T. Go and L. Brenner, *Br. J. Haemat.* **24**, 713 (1973).
8. J. Zittoun, J. Marquet and R. Zittoun, *Blood* **51**, 119 (1978).
9. T. T. Pelliniemi and W. S. Beck, *J. clin. Invest.* **65**, 449 (1980).
10. D. J. Perez, P. Slowiaczek and M. H. N. Tattersall, *Cancer Res.* **44**, 457 (1984).
11. D. Roberts, *Biochemistry* **5**, 3546 (1966).
12. M. I. Smith Lomax and G. R. Greenberg, *J. biol. Chem.* **242**, 109 (1967).
13. M. I. Smith and G. R. Greenberg, *Fedn. Proc.* **23**, 271 (1964).
14. R. B. Dunlap, N. G. L. Harding and F. M. Huennekens, *Biochemistry* **10**, 88 (1971).
15. M. Kawai and B. L. Hillcoat, *Analyt. Biochem.* **58**, 404 (1974).
16. R. D. Armstrong and R. B. Diasio, *J. biochem. biophys. Meth.* **6**, 141 (1982).
17. P. K. Tomich, C.-S. Chiu, M. G. Wovcha and G. R. Greenberg, *J. biol. Chem.* **249**, 7613 (1974).
18. J. C. Yalowich, *Diss. Abstr. Int.* **41**, 3729-B (1981).
19. T. I. Kalman and J. C. Yalowich, *174th Am. chem. Soc. Meeting Abstr.* BIOL 127 (1977).
20. T. I. Kalman and J. C. Yalowich, *Proc. Am. Ass. Cancer Res.* **19**, 153 (1978).
21. T. I. Kalman and J. C. Yalowich, *Fedn. Proc.* **37**, 500 (1978).
22. T. I. Kalman and J. C. Yalowich, in *Chemistry and Biology of Pteridines* (Eds. R. L. Kisliuk and G. M. Brown), p. 671. Elsevier/North-Holland, New York (1979).
23. T. I. Kalman and J. C. Yalowich, in *Drug Action and Design: Mechanism-Based Enzyme Inhibitors* (Ed. T. I. Kalman), p. 75. Elsevier/North-Holland, New York (1979).
24. D. Goldman and T. I. Kalman, *Nucleosides Nucleotides* **2**, 175 (1983).
25. G. Bendek and A. J. Patel, *Analyt. Biochem.* **79**, 631 (1977).
26. D. H. Ives, J. P. Durham and V. S. Tucker, *Analyt. Biochem.* **28**, 192 (1969).
27. M. H. N. Tattersall, R. C. Jackson, S. T. M. Jackson and K. R. Harrap, *Eur. J. Cancer* **10**, 819 (1974).
28. W. Washtien, A. Matsuda, Y. Wataya and D. V. Santi, *Biochem. Pharmac.* **27**, 2663 (1978).
29. S. J. Caradonna, and Y-C. Cheng, *Molec. Pharmac.* **18**, 513 (1980).
30. D. W. Kufe, P. P. Major, E. M. Egan and E. Loh, *J. biol. Chem.* **256**, 8885 (1981).
31. H. A. Ingraham, B. Y. Tseng and M. Goulian, *Molec. Pharmac.* **21**, 211 (1982).
32. R. I. Glazer and A. L. Peale, *Molec. Pharmac.* **16**, 270 (1979).
33. R. I. Glazer and K. D. Hartman, *Molec. Pharmac.* **17**, 245 (1980).
34. R. I. Glazer and M. Legraverend, *Molec. Pharmac.* **17**, 279 (1980).
35. C. E. Myers, R. C. Young, D. G. Johns and B. A. Chabner, *Cancer Res.* **34**, 2682 (1974).
36. M. H. N. Tattersall and K. R. Harrap, *Cancer Res.* **33**, 3086 (1973).
37. A. Fridland, *Cancer Res.* **34**, 1883 (1974).
38. J. Balzarini and E. De Clercq, *Biochim. biophys. Acta* **785**, 36 (1984).
39. J. P. Durham and D. H. Ives, *Molec. Pharmac.* **5**, 358 (1969).
40. G. F. Maley and F. Maley, *J. biol. Chem.* **234**, 2975 (1959).
41. R. C. Jackson, *J. biol. Chem.* **253**, 7440 (1978).
42. A. W. Schrecker and M. J. Urshel, *Cancer Res.* **28**, 793 (1968).
43. M. Bobek and A. Bloch, in *Chemistry and Biology of Nucleosides and Nucleotides* (Eds. R. E. Harmon, R. K. Robins and L. B. Townsend), p. 135. Academic Press, New York (1978).
44. E. De Clercq, J. Balzarini, P. F. Torrence, M. P. Mertes, C. L. Schmidt, D. Shugar, P. J. Barr, A. S. Jones, G. Verhelst and R. T. Walker, *Molec. Pharmac.* **19**, 321 (1981).
45. W. Rode, K. J. Scanlon, B. A. Moroson and J. R. Bertino, *J. biol. Chem.* **255**, 1305 (1980).
46. J. G. Cory, J. C. Breland and G. L. Carter, *Cancer Res.* **39**, 4905 (1979).
47. R. L. Kisliuk, Y. Gaumont, T. I. Kalman, N. T. Nanavathi and M. G. Nair, *Proc. Am Ass. Cancer Res.* **25**, 309 (1984).
48. J. Galivan, *Molec. Pharmac.* **17**, 105 (1980).
49. D. J. Fernandes, S. K. Crawford and M. G. Playa, *Proc. Am. Ass. Cancer Res.* **25**, 347 (1984).