

a typical experiment the spin-labeled colchicine analogue ($1-5 \times 10^{-5}$ M) in 0.1 M MES buffer containing 0.5 mM GTP was incubated at either 25 or 37 °C, for 1.5 h with an equal concentration of purified calf brain tubulin. The sample was then transferred to a quartz aqueous flat cell and the ESR spectrum recorded using the following instrumental parameters: microwave power, 20 mW; modulation, 2 g; scan time, 4 min; scan range, 100 G. In some experiments the sample was transferred immediately to the flat cell and the spectrum recorded as a function of time at either 25 or 37 °C. In other experiments the spin label (5×10^{-5} M) and tubulin (5×10^{-5} M) were incubated at 37 °C for 1.5 h and then passed over a short column (1 × 25 cm) of Sephadex G-25 and the column eluted at 4 °C with 0.1 M MES buffer. The fractions were then placed in the flat cell and their ESR spectra recorded.

Microtubule Binding Assay. The binding of the spin labels to rat brain microtubular protein was measured as previously described.⁴ Briefly, the method consisted of incubating a 200- μ L aliquot of rat brain supernatant containing about 1.0 mg of protein, with 800 μ L of a solution containing 10 mM sodium phosphate

buffer (pH 7.0), 5 mM MgCl₂, 0.1 mM GTP, 240 mM sucrose, and 2.5 μ M [³H]colchicine (0.2 Ci/mmol; New England Nuclear). The colchicine analogues were added at the beginning of the incubation to a final concentration of 25 μ M. After a 2-h incubation at 37 °C, the reaction was stopped by the addition of 1 mL of an ice-cooled solution containing 100 μ M colchicine. The [³H]colchicine complex was isolated by adsorption onto DE 81 Whatman Chromedia filter paper. After washing the filters were transferred to counting vials, 10 mL of Aquasol (New England Nuclear) was added, and the sample was counted in a Mark III Searle scintillation counter. The results for the colchicine analogues were expressed in terms of the percent inhibition of [³H]colchicine binding. Under our assay conditions, the addition of 25 μ M unlabeled colchicine to the incubation decreased [³H]colchicine binding by about 90%.

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Bis(*m*-nitrophenyl) and Bis(*p*-nitrophenyl) Esters and the Phosphorodiamidate of Thymidine 5'-Phosphate as Potential Sources of Intracellular Thymidine 5'-Phosphate in Mouse Cells in Culture

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Thymidine 5'-phosphate (TMP) derivatives with masked phosphate groups were synthesized in tritiated form from [methyl-³H]thymidine. They were of interest as models for 5' nucleotide derivatives that might be able to permeate mammalian cells and then liberate intracellular antimetabolite 5' nucleotides by loss of the masking groups. Mouse L fibroblasts were grown in vitro in the presence of 1 mM 5'-amino-5'-deoxythymidine, which was found to suppress >99% of cellular thymidine kinase activity while inhibiting the rate of cell division by only 30%. The TMP derivatives were less effective than thymidine in labeling the deoxyribonucleic acid (DNA) of the L cells. The labeling was inhibited 95-99% by 5'-amino-5'-deoxythymidine, indicating that it represented incorporation into DNA of [³H]thymidine formed from degradation of the test compounds. No evidence was obtained that the compounds acted as sources of intracellular TMP by cell permeation followed by loss of phosphate blocking groups. Similar studies yielded no evidence that the bis(*m*-nitrophenyl) ester of TMP produced intracellular TMP by that route in the LM(TK⁻) strain of L cells that are genetically deficient in thymidine kinase.

The development of prodrugs that can act as extracellular sources of intracellular 5' mononucleotides is of chemotherapeutic interest due to extensive evidence that the growth-inhibitory action of almost all antineoplastic purine and pyrimidine base and nucleoside analogues requires their anabolism, intracellularly, to the corresponding 5' mononucleotides and that the intracellular level of these nucleotides in drug-resistant neoplasms is frequently low.¹ The nucleotides are poorly membrane permeable,²⁻⁴ are dephosphorylated by plasma enzymes,⁵⁻⁷ and have been chemotherapeutically ineffective against resistant neo-

plasms. The ability of various types of potential nucleotide prodrugs to inhibit resistant neoplasms has been studied, but as yet only partial successes have been reported.⁸ In the present study we synthesized thymidine 5'-phosphate (TMP, 3) derivatives that possessed masked phosphate groups and were radioactively labeled in the thymidine (TdR) moiety and utilized them as models of candidate prodrugs of antimetabolite 5' nucleotides. They were evaluated by bioassay of incorporation of the radioactive TdR into deoxyribonucleic acid (DNA) in cultured cells in which TdR phosphorylation was blocked genetically or by an enzyme inhibitor. Under these conditions, labeling of DNA would be expected only as a result of the liberation of intracellular TMP by loss of the masking groups. TMP diesters (1a,b) with lipophilic and electron-withdrawing esterifying groups were studied in view of their potential to hydrolyze, intracellularly, to monoesters (2a,b) that might furnish intracellular 3 by phosphodiesterase action. The diamidate 1c of TMP was also studied because of recent evidence that the diamidate of 5-fluoro-2'-deoxyuridine 5'-phosphate (FdUMP) may be capable of gener-

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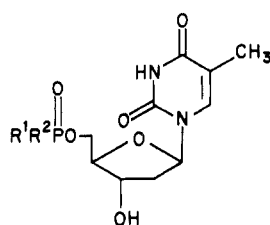
Table I. Rates of Hydrolytic Conversions of **1a-c** to **2a-c**, Respectively

compd	hydrolysis conditions		$t_{1/2}$, ^a h
	temp, °C	medium	
1a	28	phosphate, ^b pH 7.6 + 10% <i>p</i> -dioxane	2.0
	27	phosphate, pH 7.6 + 10% <i>p</i> -dioxane	0.58
1a	27	phosphate, pH 7.6	0.83
1a	37	culture medium ^c	2.1
1b	23	phosphate, pH 7.6	4.75
1b	37	culture medium	8.5
1c	37	phosphate, pH 7.6	>72 ^d
1c	37	phosphate, pH 6.0	>72 ^e

^a Time for hydrolysis of half the starting material. ^b Aqueous 50 mM potassium phosphate. ^c See Experimental Section. The pH was 7.65. ^d No hydrolysis after 72 h. ^e 10% hydrolysis after 72 h.

ating FdUMP in mammalian cells.^{9,10}

Syntheses. TdR was treated in sulfolane at 22 °C for 16 h with 1 equiv of tetrakis(*p*-nitrophenyl) pyrophosphate¹¹ generated in situ from bis(*p*-nitrophenyl) hydrogen phosphate and dicyclohexylcarbodiimide. Silica gel TLC revealed a major product, two minor products, and 10% of TdR. The major product (**1a**) was isolated by silica gel chromatography and obtained in crystalline form in 36% yield. It was concluded to be substituted on O(5') from paper chromatographic and electrophoretic evidence that hydrolysis at pH 7.6 yielded *p*-nitrophenol and thymidine 5'-(*p*-nitrophenyl phosphate) (**2a**). The minor products possessed R_f values that indicated they might be the 3'-O-substituted positional isomer of **1a** and the corresponding 3',5'-di-O-substituted derivative of TdR. Mild basic treatment of the more polar byproduct produced *p*-nitrophenol and a compound indistinguishable from thymidine 3'-(*p*-nitrophenyl phosphate).



- 1a**, $R^1 = R^2 = p\text{-NO}_2\text{C}_6\text{H}_4\text{O}$
b, $R^1 = R^2 = m\text{-NO}_2\text{C}_6\text{H}_4\text{O}$
c, $R^1 = R^2 = \text{NH}_2$
2a, $R^1 = \text{OH}$; $R^2 = p\text{-NO}_2\text{C}_6\text{H}_4\text{O}$
b, $R^1 = \text{OH}$; $R^2 = m\text{-NO}_2\text{C}_6\text{H}_4\text{O}$
c, $R^1 = \text{OH}$; $R^2 = \text{NH}_2$
3, $R^1 = R^2 = \text{OH}$

The TMP diester **1b** was prepared, similarly to **1a**, by reaction of TdR with in situ generated tetrakis(*m*-nitrophenyl) pyrophosphate. [*Methyl*-³H]-labeled **1a** and **1b** were obtained from [*methyl*-³H]TdR in satisfactory yields by adaptations of the above procedures. Treatment of [*methyl*-³H]TdR with POCl₃ in triethyl phosphate and amination of the resulting 5'-phosphorodichloridate gave thymidine 5'-phosphorodiamidate (**1c**)¹² in [*methyl*-³H]-labeled form. Acidic hydrolysis of **1c** to **3** and treatment of this with 5'-nucleotidase gave no thymidine 3'-phosphate, showing that [*methyl*-³H]-**1c** was free of thy-

Table II. Incorporation of ³H from [*methyl*-³H]-Labeled **1b** into the DNA Fraction of Mouse LM(TK⁻) Cells Growing in Culture

[<i>methyl</i> - ³ H]- labeled compd ^a	concn in medium, μM	period of incubation with cells, h	rate of incorporation of ³ H into DNA, cpm (10 ⁶ cells) ⁻¹ h ⁻¹
2'-deoxycytidine ^b	0.017	2	21960
thymidine	0.9	19	53.0
1b	0.9	19	18.1
2b	0.9	19	37.6
1b	0.9	23 ^c	48.5
1b	10	19	21.6

^a The medium contained, initially, 1 μCi/mL of each compound. ^b [1',2',5-³H]-2'-deoxycytidine. ^c Cells were exposed for 4 h at 37 °C to ³H-labeled **1b** in serum-free medium and for an additional 19 h after addition of fetal bovine serum to a 10% level.

midine 3'-phosphorodiamidate.

Table I lists hydrolytic properties of **1a-c**. In a buffer of pH 7.6 **1a** hydrolyzed 2-3 times faster than ethyl bis-(*p*-nitrophenyl) phosphate¹³ and 6-fold faster than **1b**. At pH 7.6 **1c** was stable, but at pH 6.0 acid-catalyzed formation of **2c** was detectable. In tissue culture medium, hydrolysis of **1a** gave **2a** as a minor product except during the initial 0.5 h, the major products being **3** and TdR. These were not formed in the absence of serum and presumably arose by sequential phosphodiesterase attack on **2a** and phosphatase attack on **3**.

Studies with Cells in Culture. Initial studies employed the LM(TK⁻) strain of mouse fibroblasts that lacks the cytoplasmic form of TdR kinase (TK), one of several enzymes required for the incorporation of Tdr into DNA. Cytoplasmic TK is the predominant form in mouse L cells; trace amounts of a mitochondrial form are also present in both L cells and LM(TK⁻) cells.^{14,15} When LM(TK⁻) cells were exposed for 19 h to 0.9 μM of [³H]-**1b** the rate of incorporation of ³H into the DNA-containing fraction was one-third that from [³H]TdR and 0.08% that from [³H]-deoxycytidine (Table II). [LM(TK⁻) cells express levels of deoxycytidine kinase similar to those in L cells, so that exogenous deoxycytidine is an efficient precursor of DNA cytosine.] Some or all of the ³H in the DNA could thus have arisen via intra- and/or extracellular degradation of [³H]-**1b** to [³H]TdR and subsequent formation of [³H]-**3** mediated by mitochondrial TK. [³H]-**2b** labeled the DNA more effectively than **1b**, possibly because it served as a more direct source of extracellular TdR. The ineffectiveness of **1b** was not ascribable to adsorptive losses of **1b** onto the polystyrene dishes used in cell culture or onto serum constituents in the culture medium, effects that were found to be small under the conditions of the study (see Table II). In addition, **1b** showed no evidence of cell toxicity at the level employed in the DNA labeling experiments. When the **1b** level was increased 10-fold by addition of unlabeled **1b**, the rate of ³H incorporation remained constant, indicating a proportionality between rate of incorporation and **1b** level and arguing against complexation of **1b** or **2b** to cellular receptors as a cause of the weak precursor activity of **1b**.

5'-Amino-5'-deoxythymidine (5'-NH₂-TdR) selectively inhibits rat cytoplasmic TK with respect to rat mitochondrial TK.¹⁶ At a level of 1 mM in the culture me-

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Table III. [*methyl*-³H]-Labeled Thymidine 5'-Phosphate Derivatives **1a-c** as Precursors of [*methyl*-³H]Thymine in the Deoxyribonucleic Acid (DNA) Fraction of Mouse L Cells Growing in Culture

[<i>methyl</i> - ³ H]-labeled compd ^a	period of incubation with cells, h	concn of 5'-NH ₂ -TdR present, mM	rate of incorporation of [³ H]thymine into DNA, cpm (10 ⁶ cells) ⁻¹ h ⁻¹	inhibition by 5'-NH ₂ -TdR of rate of ³ H incorporation, %
thymidine	2	0.5	2276	98.5
	2	1.0	1168	99.2
1a	2	1.0	492	98.8
1b	19	1.0	519	94.7
1c	2	1.0	14	99.4
	20	1.0	68	92.3

^a The medium initially contained a level of 0.5 μ M of each compound and 1 μ Ci/mL of radioactivity.

dium, it inhibited the rate of incorporation of exogenous TdR into DNA of mouse L fibroblasts by more than 99% (Table III) but appeared to cause no cytopathic effects other than a 30% inhibition of the rate of increase in cell population during 20 h. In the presence of 1 mM 5'-NH₂-TdR, **1a** and **1b** were 50% as effective as [³H]TdR as exogenous sources of [³H]thymine in L cell DNA, while [³H]-**1c** was only 1.2% as effective as [³H]TdR during a test period of 2 h (Table III). At 20 h the rate with **1c** was seemingly elevated, possibly due to partial degradation of the 5'-NH₂-TdR. In all cases 5'-NH₂-TdR strongly inhibited ³H uptake, indicating that this represents incorporation into DNA of [³H]TdR formed from **1a-c**. The studies thus provide no evidence with either of the two cell strains that **1a-c** act as sources of intracellular 3 by cell permeation and subsequent loss of phosphate masking groups.

Experimental Section

Chemical Synthesis. General Procedures. Sulfolane and triethyl phosphate were dried by distillation from CaH₂. [*methyl*-³H]Thymidine was purchased from New England Nuclear, [1',2',5-³H]deoxycytidine was from Amersham, and 5'-nucleotidase (Grade III, from *Crotalus adamanteus*) and thymidine 5'- and 3'-mono-*p*-nitrophenylphosphates were from Sigma Chemical Co. Ultraviolet spectra were determined with a Cary Model 15 spectrophotometer. Melting points were determined in capillary tubes and are uncorrected. Radioactivity was determined with a Packard Model 2425 liquid scintillation spectrometer. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, and unless otherwise indicated are within $\pm 0.4\%$ of the theoretical values. Electrophoresis was carried out on Whatman No. 1 paper at pH 7.6 (0.05 M triethylammonium bicarbonate). Paper chromatography (descending technique) employed Whatman No. 1 paper (A) 1-butanol-water (86:14), (B) 1-butanol-acetic acid-water (5:2:3), (C) 2-propanol-H₂O (8:2), or (D) 2-propanol-NH₄OH-water (7:1:2). Thin-layer chromatography was carried out on Merck F-254 silica gel plates in (E) chloroform-methanol (3:1) or (F) chloroform-methanol (19:1).

Bis(*m*-nitrophenyl) Hydrogen Phosphate. Tris(*m*-nitrophenyl) phosphate, mp 110–112 °C (lit.¹⁷ mp 109–110 °C), was prepared by reaction of sodium *m*-nitrophenoxide with POCl₃ under conditions used for the synthesis of tris(*p*-nitrophenyl) phosphate.¹¹ Hydrolysis of tris(*m*-nitrophenyl) phosphate with LiOH in aqueous dioxane as described for the corresponding para-substituted compound¹¹ gave bis(*m*-nitrophenyl) phosphate in 78% yield as yellow needles (from water), mp 221–225 °C dec. Addition of pentane to a solution of this product in CHCl₃ gave an apparently isomorphous form of mp 108–110 °C (lit.¹⁷ mp 109–110 °C).

Thymidine 5'-[Bis(*m*-nitrophenyl) phosphate] (1b**).** To a solution of bis(*m*-nitrophenyl) hydrogen phosphate (782 mg, 2.3 mmol) in anhydrous sulfolane (10 mL) was added dicyclohexylcarbodiimide (206 mg, 1 mmol). Dicyclohexylurea began

to separate immediately and after 30 min at 22 °C thymidine (242 mg, 1 mmol, dried at 78 °C in vacuo) was added, and the mixture was stirred at 45 °C for 16 h. Ethyl acetate (25 mL) was added, and the mixture was filtered from dicyclohexylurea. The filtrate was extracted with water until the extracts were neutral. The ethyl acetate solution was concentrated and applied to two 2-mm silica gel plates (20 \times 20 cm). These were developed twice in system F. Thymidine (*R*_f 0.05) amounting to ca. 10% of the UV-absorbing components was present as well as two minor UV-absorbing products of *R*_f 0.21 and 0.34, respectively. The major band (*R*_f 0.12) was eluted with CHCl₃-MeOH (18:2). Addition of light petroleum to the concentrated eluate yielded 190 mg (33%) of **1b** as a pale yellow powder: mp 110–112 °C; UV λ_{\max} (H₂O) 263 nm (ϵ 17 500). Anal. (sample dried at 78 °C) (C₂₂H₂₁N₄O₁₂P₂H₂O \cdot 0.25CHCl₃) C, H, N, P, Cl.

Thymidine 5'-[Bis(*p*-nitrophenyl) phosphate] (1a**).** This was prepared at 22 °C from bis(*p*-nitrophenyl) hydrogen phosphate (Aldrich Chemical Co.) and thymidine (1 mmol) under the conditions used for synthesis of **1b**. **1a** (*R*_f 0.12) was separated by preparative TLC in system F from thymidine (10%) and byproducts of *R*_f 0.22 (16%) and 0.34 (8%) and was obtained as white needles (205 mg, 36% yield) from CHCl₃-MeOH (1:4): mp 138–140 °C; UV λ_{\max} (H₂O) 269 nm (ϵ 22 800). Anal. (dried at 25 °C) (C₂₂H₂₁N₄O₁₂P₂H₂O \cdot 0.6CHCl₃) C, H, N, P, Cl: calcd, 10.05; found, 10.79.

A solution (0.1 mM) of **1a** in aqueous potassium phosphate (10 mM, pH 7.6) was stored at 22 °C overnight and then evaporated to dryness in vacuo (bath at 25 °C). The residue was extracted with EtOH-H₂O (1:1) and the solution was chromatographed on paper in solvent systems A, B, and D, together with *p*-nitrophenol and the potassium salts of **2a** and thymidine 3'-(mono-*p*-nitrophenyl phosphate). The only UV-absorbing components detected in the aqueous ethanolic extract were *p*-nitrophenol and a second spot (*R*_f values 0.13, 0.57, and 0.77, respectively) corresponding to **2a** and differing from thymidine 3'-(mono-*p*-nitrophenyl phosphate) (*R*_f 0.20, 0.64, and 0.83, respectively). Analysis of the aqueous ethanolic extract by paper electrophoresis at pH 3.6 revealed only two components, and these possessed the same mobilities as *p*-nitrophenol and **2a**.

Acidic Hydrolysis of **1c.** A solution of **1c**¹² (6 mg) in 0.1 N HCl (2 mL) was stored at 22 °C for 16 h. Chromatography in solvent E showed that no **1c** remained; chromatography in solvent D and paper electrophoresis at pH 7.6 indicated that the only UV-absorbing component was **3**. The volatiles were removed under reduced pressure, and the residue was dissolved in a solution of 5'-nucleotidase (2 mg) in 2 mL of 50 mM Tris buffer, pH 9.0. The solution was kept at 37 °C for 30 min, after which chromatography in solvent F and paper electrophoresis at pH 7.6 indicated that **3** had been completely converted to thymidine. No thymidine 3'-phosphate was detected in the incubation mixture.

[*methyl*-³H]Thymidine 5'-[Bis(*m*-nitrophenyl) phosphate]. A solution of [*methyl*-³H]thymidine (0.15 mg, 2 Ci/mmol) in MeOH (100 μ L) was transferred portionwise to the bottom of a cone-shaped microvial during which the MeOH was evaporated with a stream of argon. The vial was dried at 78 °C for 2 h under vacuum. To a solution of 39 mg of bis(*m*-nitrophenyl) hydrogen phosphate in dry sulfolane (1.0 mL) was added dicyclohexylcarbodiimide (20 mg). The mixture was stored at 50 °C for 1 h after which a portion (14 μ L) was added to the vial containing the [*methyl*-³H]thymidine and the mixture was stored at 50 °C

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for 60 h. The mixture was then applied to a 5×10 cm analytical silica gel plate. This was developed first with CHCl_3 and then twice with system F. The desired compound (λ_{max} 263 nm in H_2O , 2.0 optical density units at 263 nm) was eluted with EtOH. Unreacted thymidine (3.1 optical density units at 263 nm) was recovered by extraction with EtOH of the appropriate zone of lower R_f .

[methyl- ^3H]Thymidine 5'-Phosphorodiamidate. To [methyl- ^3H]thymidine (0.12 mg, 1 mCi) (dried at 78°C) was added 20 μL of a solution of 25 μL of POCl_3 in 1.5 mL of dry triethyl phosphate. The mixture was stored at 5°C for 16 h and for 4 h at 22°C . To this was added 100 μL of dry dioxane saturated with NH_3 . After 2 h, the precipitate was collected by centrifugation and dissolved in EtOH. The EtOH was evaporated to dryness in vacuo. A solution of the residue in MeOH (50 μL) was applied to two 5×10 cm analytical silica gel plates that were developed in system E. A zone of R_f 0.18 corresponding to 1c was eluted with EtOH to give 0.325 mCi of [methyl- ^3H]-1c.

Rates of Hydrolysis of 1a-c. Hydrolysis rates of 1a,b at 22°C in 50 mM aqueous potassium phosphate buffer, pH 7.6, were determined by spectrophotometric measurement of the formation of *p*- or *m*-nitrophenol at 400 nm in solutions initially 0.05 mM with respect to 1a or 1b. The stability of 1c in 50 mM phosphate buffer, pH 7.6 or 6.0, was studied by paper electrophoresis.

Solutions (0.5 mM) of 1a or 1b in the culture medium were maintained at 37°C in a 5% CO_2 -air mixture. At intervals, 100- μL portions were applied to a 5×10 cm silica gel analytical plate and this was developed in solvent F. Appropriate bands were eluted with MeOH, and the amount of 1a or 1b was determined spectrophotometrically. Plots of log (residual 1a or 1b) against time were linear, indicating pseudo-first-order kinetics. These plots were used to furnish the half-life of 1a or 1b. The values are given in Table I.

[methyl- ^3H]-1a (10 μCi) in 0.1 mL of EtOH was added to the culture medium (2 mL) and the mixture was maintained under the above conditions. At intervals, portions (250 μL) were cooled in ice and then treated with 50% aqueous $\text{CCl}_3\text{CO}_2\text{H}$ (25 μL). The mixture was centrifuged at 4000 rpm at 3°C for 10 min. A portion (10 μL) of each supernate was cochromatographed on paper in solvent C with unlabeled 1a, 2a, 3, and thymidine. The four UV-absorbing areas on the chromatogram were cut out and directly assayed for ^3H .

Studies with Cells in Culture. Mouse L-strain fibroblasts (clone 929) were obtained from the American Type Culture Collection (CCL-1); a 5-bromodeoxyuridine-resistant thymidine-kinase deficient variant of this line, LM(TK⁻), was obtained from Dr. Richard Axel, Columbia University; LM(TK⁻) cells were originally developed by Kit et al.¹⁸ Cells were propagated as

monolayers in plastic flasks in Dulbecco's modified Eagle medium (GIBCO Laboratories, glucose 4.5 g/L), supplemented with 10% fetal bovine serum, at 37°C in 5% CO_2 in air.

For experiments, 5×10^6 cells were seeded in 6-cm tissue culture grade (i.e., surface-sulfonated) polystyrene Petri dishes and incubated overnight before use. Isotopically labeled compounds to be tested were diluted appropriately from freshly prepared stock solutions in 50% EtOH to yield an activity of 1 $\mu\text{Ci}/\text{mL}$ of medium and a final EtOH concentration of 0.5%. The EtOH did not affect the rate of cell division. At zero time, the medium was aspirated and replaced with 2 mL of medium containing the test compound and, when required, 5'- NH_2 -TdR. In experiments involving 5'- NH_2 -TdR, this was added to the medium 0.5 h before zero time to a level of 1 mM, to ensure inhibition of TK prior to addition of the test compound.

Uptake of isotope into acid-soluble cellular macromolecules was determined with use of whole $\text{CCl}_3\text{CO}_2\text{H}$ -fixed cells. Monolayers were washed once with 2 mL of Puck's saline A containing 0.2 g/L of ethylenediamine tetraacetate. The wash was aspirated and replaced with 5 mL of the same mixture containing 0.04% trypsin, and the plates were incubated 10 min at 37°C . The cell suspension was collected on a Millipore filter (0.45- μm pore size), washed once with 5 mL of Dulbecco's phosphate-buffered saline, four times with 5-mL portions of 5% $\text{CCl}_3\text{CO}_2\text{H}$, and once with 5 mL of 70% ethanol. The dried filters were counted in 5 mL of a toluene solution of scintillation phosphors; under these conditions, counting efficiency for ^3H was about 10%. Zero time blank values in the presence and absence of 5'- NH_2 -TdR were obtained by immediately removing the medium containing each radioactive compound. The blanks were less than 200 cpm/ 10^6 cells for 1a-c.

The number of cells per petri dish was determined with a Coulter electronic cell counter, using the above trypsinization procedure on parallel control cultures. Prior to the addition of the isotopically labeled compounds the number of cells per dish was in the range $5.5\text{--}7.5 \times 10^5$.

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Registry No. 1a, 92220-88-7; [methyl- ^3H] 1a, 92220-91-2; 1b, 92220-87-6; [methyl- ^3H] 1b, 92220-90-1; 1c, 56945-94-9; [methyl- ^3H] 1c, 92220-92-3; bis(*m*-nitrophenyl) hydrogen phosphate, 28022-29-9; tris(*m*-nitrophenyl) phosphate, 3862-04-2; tetrakis(*m*-nitrophenyl) pyrophosphate, 92220-89-8; tetrakis(*p*-nitrophenyl) pyrophosphate, 52625-61-3; thymidine, 50-89-5; [methyl- ^3H]thymidine, 67336-82-7.

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