Inhibition of the Biosynthesis of Thymidylic Acid by 4-*N*-Hydroxy-2′-Deoxycytidine in L5178Y Leukemic Cells

D. J. Nelson[1] and C. E. Carter

Department of Pharmacology, Western Reserve University School of Medicine, Cleveland, Ohio, and Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut

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SUMMARY

Reproduction of L5178Y leukemic cells in culture was inhibited by 10^{-5} M 4-*N*-hydroxy-2′-deoxycytidine. Thymidine and deoxyuridine specifically protected the cells from growth inhibition. In whole cell incubations *in vitro*, thymidine-^{3}H incorporation into DNA-thymine was slightly stimulated, whereas deoxycytidine-^{3}H, deoxyuridine-^{3}H, and formate-^{14}C incorporation into acid-soluble thymidylate nucleotides and DNA-thymine was markedly inhibited by 4-*N*-hydroxy-2′-deoxycytidine. Thymidylate synthetase activity from partially purified extracts of *E. coli* and cell-free preparations of sonically disrupted L5178Y leukemic cells was competitively inhibited by 4-*N*-hydroxy-2′-deoxycytidylic acid.

Studies in which L5178Y leukemic cells were incubated *in vitro* with 4-*N*-hydroxy-2′-deoxycytidine-^{3}H demonstrated that the analog was phosphorylated to the monophosphate. Although 4-*N*-hydroxy-2′-deoxycytidine did not inhibit phosphorylation of thymidine-^{3}H, deoxyuridine-^{3}H, or deoxycytidine-^{3}H *in vitro*, the phosphorylation of 4-*N*-hydroxy-2′-deoxycytidine-^{3}H in this system was inhibited by thymidine and deoxyuridine. These experiments indicate that 4-*N*-hydroxy-2′-deoxycytidine is a functional analog of deoxyuridine that is phosphorylated to 4-*N*-hydroxy-2′-deoxycytidylic acid, and produces its cellular effects through inhibition of the biosynthesis of thymidylic acid.

INTRODUCTION

Hydroxylamine reacts with a number of pyrimidine derivatives to form hydroxylamino pyrimidines (1–6). Cellular and biochemical effects of hydroxylamino pyrimidines have been restricted to relatively few observations. Hydroxylamino pyrimidine nucleotide residues occur in DNA treated with hydroxylamine (5), and the mutagenic effect of hydroxylamine has been ascribed to the resultant transitions in hydrogen bonding of the altered deoxycytidylic acid residues in the DNA molecule (7). Another biochemical effect produced by this class of compounds was described by Maley and Maley (8), who found that 4-*N*-hydroxy-2′-deoxycytidylic acid (4-NOH-dCMP)[2] inhibited chick embryo deoxycytidylate deaminase.

[1] Part of these data was presented to the faculty of Yale University, New Haven, Connecticut, in partial fulfillment of the requirement for the Ph.D. degree. Present address Department of Pharmacology, Western Reserve University School of Medicine, Cleveland, Ohio.

[2] The following abbreviations have been used: 4-NOH-dCMP, 1-(β-D-2′-deoxyribofuranosyl 5′-phosphate)-4-hydroxylamino-2-(1*H*)-pyrimidinone; 4-NOH-CdR, 1-(β-D-2′-deoxyribofuranosyl)-4-hydroxylamino-2-(1*H*)-pyrimidinone; CdR, 2′-deoxycytidine; UdR, 2′-deoxyuridine; TdR, thymidine; dCMP, 2′-deoxycytidylate; dUMP, 2′-deoxyuridylate; TMP, thymidylate.

In this paper we report the inhibition of thymidylic acid biosynthesis produced by 4-*N*-hydroxy-2′-deoxycytidine (4-NOH-CdR) in mouse leukemia (L5178Y) cells and studies of cell-free extracts of *Escherichia coli* and leukemia cells which demonstrate that 4-NOH-dCMP competitively inhibits thymidylate synthetase.

MATERIALS AND METHODS

Preparation of 4-N-hydroxy-2′-deoxycytidine. A modification of the method described by Brown and Schell (4) was used to synthesize 4-NOH-CdR. Deoxycytidine hydrochloride, 500 mg (Calbiochem, Los Angeles 54, California), was dissolved in 10 ml of 5 M hydroxylamine hydrochloride which had been adjusted to pH 6.0 with sodium hydroxide. After 72 hr at room temperature, the reaction was acidified with 3.4 ml of 12.4 N hydrochloric acid and allowed to stand for 48 hours at room temperature. Sodium chloride and hydroxylamine hydrochloride were precipitated from the reaction mixture with 10 volumes of ethanol, and the ethanol-soluble fraction was dried *in vacuo* at 40°. This residue was dissolved in 5 ml of water and then neutralized with 0.1 M sodium hydroxide. 4-NOH-CdR was completely freed of deoxycytidine, deoxyuridine, and hydroxylamine by column chromatography on Dowex 1-X8 (chloride form) with water elution, as shown in Fig. 1. The fractions containing the analog were pooled and evaporated to dryness *in vacuo*. The solid material obtained by this procedure was extremely hydroscopic, and attempts to crystallize the compound in several solvents were unsuccessful. The amorphous product was obtained in 40–60% yield and had the following elemental composition: calculated for $C_9H_{13}N_3O_5$: C, 44.50%; H, 5.4%; N, 17.7%; found, C, 44.30%; H, 5.71%; N, 17.0%. The analysis was performed by Midwest Microlabs, Indianapolis, Indiana.

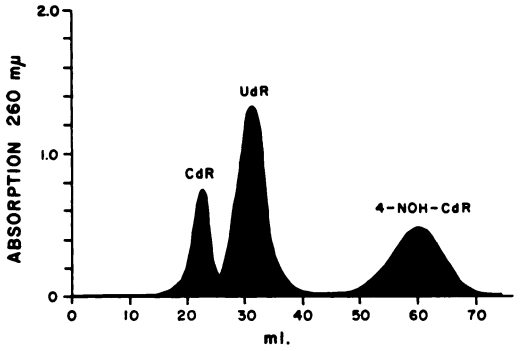

FIG. 1. *Separation of deoxycytidine (CdR), deoxyuridine (UdR), and 4-N-hydroxy-2′-deoxycytidine (4-NOH-CdR) by ion-exchange chromatography*

Column: Dowex 1-chloride, 0.8 × 40 cm, 0.5 μmole of each deoxynucleoside was absorbed on the column, and the eluting agent was distilled water, pH 5.5.

The amorphous 4-NOH-CdR was dissolved in water, and the solution was adjusted to pH 6 and stored at −15°C. Under these conditions the analog was stable for a period of 1 week; after this time traces of deoxyuridine were detected in the preparation.

The conditions employed for the characterization of the compound by paper chromatography are described in Table 1.

TABLE 1
Conditions for characterization by paper chromatography

Compound	R_F			
	A[a]	B	C	D
Deoxycytidine	0.24	0.59	0.73	0.59
Deoxyuridine	0.42	0.75	0.58	0.81
4-*N*-Hydroxy-2′-deoxycytidine	0.37	0.72	0.58	0.63

[a] Solvent A, *n*-butanol:water (85:15); B, pyridine:*n*-butanol:water (1:1:1); C, isobutyric acid:ammonium hydroxide (28%):water (66:1:33); D, isopropanol:2 N hydrochloric acid (65:35).

The pH dependence of the ultraviolet absorption spectrum of 4-NOH-CdR was similar to that of the ribosyl analog, 4-*N*-hydroxy-cytidine, described by Fox (1), and the pK_a of 4-NOH-CdR was 2.53, based on a calculation from spectrophotometric titration data by the method of Albert (9). 4-NOH-CdR was characterized further by reduction with sodium dithionite to form deoxycytidine in 27% yield. It was

also found that periodate oxidation of 4-NOH-CdR quantitatively yielded deoxyuridine and that 1 mole of periodate was consumed per mole of 4-NOH-CdR as measured by the method of Dixon and Lipkin (10).

The method described by Maley and Maley (8) was employed to synthesize 4-NOH-dCMP. The product was purified by elution from a Dowex 1-X8 (formate) ion-exchange column with a linear gradient of 0 to 4 N formic acid. 4-NOH-dCMP was eluted with approximately 2 N formic acid and was separated completely from 2′-deoxycytidylate (dCMP), which was eluted with 0.1 N formic acid, and 2′-deoxyuridylate (dUMP) which eluted with 4 N formic acid. The lithium salt of 4-NOH-dCMP was prepared by the method of Tener (11).

Tritium-labeled 4-NOH-CdR and 4-NOH-dCMP were prepared from deoxycytidine-^{3}H and deoxycytidylic acid-^{3}H obtained from Schwarz BioResearch, Inc.; deoxyuridine-^{3}H was obtained from Calbiochem, Los Angeles, California; thymidine-^{3}H was obtained from Schwarz BioResearch, Inc.; and sodium formate-^{14}C was obtained from Nuclear-Chicago Corporation. *dl*-L-Tetrahydrofolic acid was supplied by Sigma Chemical Company.

The medium and culture techniques used in cell growth experiments have been described by Fischer (12). Cell concentration was measured with a Coulter particle counter, model A. A suspension of logarithmically growing L5178Y leukemic cells (obtained from Dr. G. A. Fischer) was diluted aseptically to approximately 5000 cells/ml. Compounds to be tested were added to duplicate sterile Kimax screw-cap test tubes, 16 mm × 125 mm, in a total volume of 0.2 ml, then 5.0 ml of the cell suspension was added to each tube. The sealed tubes were incubated horizontally at 37° for 60–90 hr. The cells maintained logarithmic growth throughout this period and the population doubled every 12–14 hr.

L5178Y leukemic cells used in biochemical experiments were obtained from $B_6D_2F_1$ male mice (Cumberland View Farms, Cumberland, Tennessee), 7 days after intraperitoneal implantation of 0.1 ml of a 1:10 cell suspension in 0.15 M NaCl. The L5178Y leukemic cells were washed once with ice-cold 0.15 M NaCl. One volume of cold distilled water was added to the packed cells, and the cell suspension was stirred for 30 sec to disrupt osmotically the erythrocytes. Isotonicity was restored by addition of 10 volumes of cold Fischer's medium, and after 5 minutes, the tumor cells were centrifuged and the procedure for erythrocyte lysis was repeated twice. The tumor cells were washed twice with cold Fischer's medium, and the packed cells were suspended in 50 volumes (1:50) or 100 volumes (1:100) of medium. In some experiments the medium contained 10% horse serum.

From L5178Y cells incubated with isotopically labeled precursors, an acid-soluble fraction was obtained by extraction of the cell pellet with 7% perchloric acid. This fraction was neutralized with 5 M potassium hydroxide and then subjected to ion-exchange column chromatography on Dowex 1-X8 by the method of Hurlbert *et al.* (13) for the separation of nucleotides. Chromatography on Dowex 1-X8 (chloride), as shown in Fig. 1, was employed for the separation of nucleosides. Nonradioactive carrier compounds were characterized by paper chromatography in the solvent systems described in Table 1. DNA was isolated by extraction of the neutralized acid-insoluble pellet with 10% NaCl, and base analysis carried out by the methods of Dannberg *et al.* (14).

The procedure described by Wahba and Friedkin (15) was used to purify and measure the activity of thymidylate synthetase from *E. coli* B. Thymidylate synthetase was measured spectrophotometrically in extracts of L5178Y cells using the conditions described by Reyes and Heidelberger (16). Cell-free extracts of L5178Y cells were prepared by sonic disruption of 1 volume of cells in 7 volumes of 0.1 M tris buffer, pH 8.0, at 4°. Three periods of 30 sec exposure to maximum output of an MSE (Instrumentational Associates, New York) instrument were employed. The extract was then centrifuged for 60 min at 30,000 *g*, and the

supernatant fraction was employed for assay of thymidylate synthetase and for deoxyribonucleoside kinase as described in the legend of Table 5. The sonicated preparation of L5178Y cells was the source of deoxyribonucleoside kinase.

Radioactivity was measured with a Nuclear-Chicago liquid scintillation spectrometer, and protein was measured by the Folin procedure as modified by Lowry *et al.* (17).

RESULTS

Inhibition of the Growth of L5178Y Leukemic Cells in Culture by 4-N-Hydroxy-2'-deoxycytidine

Growth of L5178Y leukemic cells in Fischer's medium was inhibited 50% by 12 μM 4-NOH-CdR (Fig. 2). Although the rate of growth was depressed 95% by 50 μM 4-NOH-CdR, the multiplication of the inhibited cells was logarithmic throughout the test period. Microscopic examination showed that the inhibited cells did not differ in size from the normal cells.

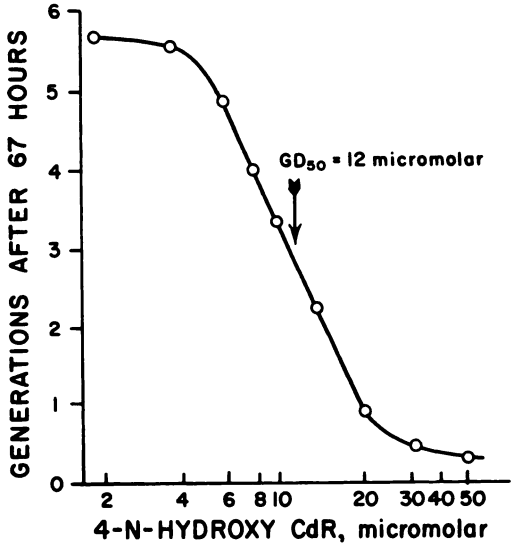

FIG. 2. *Inhibition of growth of L5178Y leukemia in culture by 4-NOH-CdR*

See Methods for the details of the procedure for growing L5178Y leukemia cells in culture.

The growth-inhibitory effect of 4-NOH-CdR upon L5178Y leukemic cells in culture could be prevented when deoxyuridine, deoxycytidine, and thymidine were added to the growth medium. Deoxycytidine at 500 μM concentration only partially prevented the inhibition of 50 μM 4-NOH-CdR, whereas complete protection of the cells was achieved with either thymidine or deoxyuridine at 10 μM concentration. When compared on a molar basis, thymidine was four times more effective than deoxyuridine (Fig. 3) in preventing growth inhibition by 4-NOH-CdR. Several purine and pyrimidine derivatives at a concentration of 10 μM in the medium of growth experiments with L5178Y leukemic cells did not protect against inhibition by 4-NOH-CdR at 50 μM concentration, as shown in Table 2. The growth rate of in-

TABLE 2

The effect of pyrimidines and purines on the inhibition of growth of L5178Y cells by 4-N-hydroxy-2'-deoxycytidine

Initial cell concentration was 3640 cells/ml; the results are the average of duplicate determinations.

Additions	Concentration (μmoles/liter)	Generations after 95 hr
None	—	5.8
4-NOH-CdR	50	0.6
4-NOH-CdR + guanine	10	0.8
4-NOH-CdR + guanosine	10	0.9
4-NOH-CdR + deoxyguanosine	10	0.7
4-NOH-CdR + adenine	10	0.8
4-NOH-CdR + adenosine	10	0.4
4-NOH-CdR + deoxyadenosine	10	0.6
4-NOH-CdR + hypoxanthine	10	0.5
4-NOH-CdR + inosine	10	0.6
4-NOH-CdR + cytidine	10	0.5
4-NOH-CdR + deoxycytidine	10	1.1
4-NOH-CdR + uridine	10	0.8
4-NOH-CdR + pseudouridine	10	1.2
4-NOH-CdR + 5-methyl deoxycytidine	10	2.1
4-NOH-CdR + deoxyuridine	10	5.9
4-NOH-CdR + thymidine	10	5.9

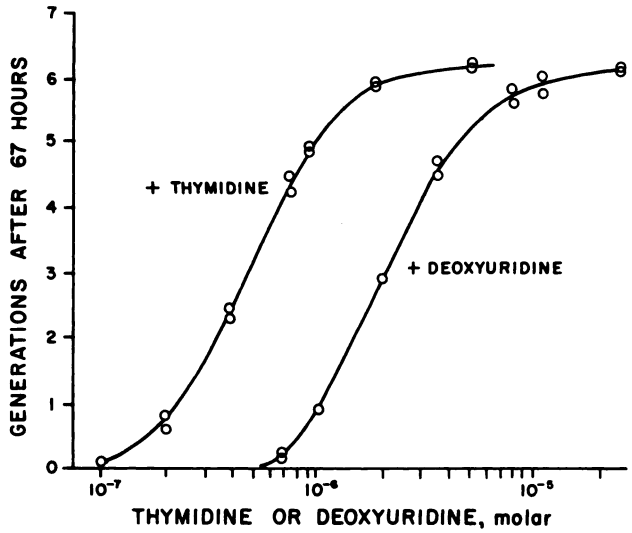

FIG. 3. *Prevention by deoxyuridine and thymidine of the inhibition by 4-NOH-CdR of the growth of L5178Y cells*

Conditions: 4-NOH-CdR, 50 μM, was present in every tube; either thymidine or deoxyuridine was added at the indicated concentration and the tubes were incubated for 67 hr, then the generations of growth were determined.

hibited cells was restored by about 30% when 5-methyl deoxycytidine was present in the medium, an effect which may be due to the presence of a small amount of thymidine. The purines and pyrimidines were not themselves inhibitory at the concentrations used, and hydroxylamine at a concentration of 100 μM did not inhibit cell growth.

Effect of 4-N-Hydroxy-2'-deoxycytidine on Pyrimidine Deoxynucleotide Metabolism

When L5178Y leukemic cells were incubated for periods of 30 min with tritium-labeled deoxycytidine, deoxyuridine, and sodium formate-^{14}C, striking alteration in the incorporation of these substances into acid-soluble deoxynucleotides and DNA were produced by the addition of 4-NOH-CdR to the medium. The effect of 4-NOH-CdR upon deoxycytidine-^{3}H metabolism is shown in Table 3. In the presence of 4-NOH-CdR at 5×10^{-4} M, the radioactivity of acid-soluble deoxycytidylic nucleotides was increased 85%, but incorporation of deoxycytidine-^{3}H into DNA-cytosine was decreased 19%. A greater effect of the analog was the inhibition of incorporation of deoxycytidine-^{3}H into DNA-thymine, where the radioactivity was decreased 96.5% by 5×10^{-4} M 4-NOH-CdR. In addition, labeling of dUMP by deoxycytidine-^{3}H was increased in the presence of 4-NOH-CdR at 5×10^{-4} M. The results of a similar experiment with deoxyuridine-^{3}H in the medium are shown in Table 3. The incorporation of radioactivity into the acid-soluble thymidylate nucleotides was inhibited 91% by the presence of 5×10^{-4} M 4-NOH-CdR in the medium, and under these conditions the formation of DNA-thymine from deoxyuridine-^{3}H was inhibited 93%. It is also apparent from the data of Table 3, that 4-NOH-CdR did not inhibit the incorporation of thymidine-^{3}H into DNA-thymine.

Maley and Maley (8) reported that 4-NOH-dCMP inhibited deoxycytidylic acid deaminase in chick embryo extracts. From the data of Table 3, a similar inhibition of deamination by the analog leading to an increase of radioactivity derived from deoxycytidine-^{3}H in deoxycytidylate nucleotides might be inferred. However, when a direct measurement of the conversion of dCMP to dUMP in L5178Y cell-free extracts was made, the addition of a high

TABLE 3

Incorporation of pyrimidine deoxynucleosides into L5178Y leukemia cells and effect of 4-NOH-CdR

A suspension of L5178Y leukemia cells in Fischer's medium (2×10^6 cells/50 ml) was preincubated for 10 minutes at 37° with or without 4-NOH-CdR at 5×10^{-4} M, then the tritium-labeled pyrimidines were added and the incubation was continued for 30 min. The cells were collected by centrifugation and extracted with 7% perchloric acid. After heating the acid-soluble extract for 15 min at 90°, which hydrolyzed di- and triphosphates to the respective mononucleotides, the radioactive deoxymononucleotides were purified by ion-exchange chromatography on Dowex 1-X8 (formate) and finally by paper chromatography. The counts per minute shown were corrected for percentage of recovery of 1 μmole each of unlabeled carrier compounds added to the initial extracts. These are the results of a single experiment for each of the labeled precursors.

Precursor	Product	Control (cpm)	+4-NOH-CdR 5×10^{-4} M (cpm)	Difference (%)
Deoxycytidine-^{3}H 16 μC; 2.4 C/mmole	Total acid-soluble	221,000	324,000	+68
	Acid-soluble deoxycytidylic nucleotides	167,000	310,000	+85
	Acid-soluble deoxyuridylic acid	100	950	+950
	Thymidylate nucleotides	38,000	2,300	−94
	Total DNA	380,000	110,000	−71
	DNA-cytosine	125,000	101,000	−19
	DNA-thymine	255,000	9,000	−96.5
Deoxyuridine-^{3}H 7 μC; 1.3 C/mmole	Deoxyuridylic acid	100	2,600	+2600
	Acid-soluble thymidylate nucleotides	95,000	8,500	−91
	DNA-thymine	14,400	1,040	−93
Thymidine-^{3}H 16 μC; 4.3 C/mmole	DNA-thymine	2,700,000	3,100,000	+10

concentration of 4-NOH-dCMP produced only a small inhibition of deoxycytidylic acid deaminase.

As shown in Table 4, 4-NOH-CdR was an inhibitor of the incorporation of formate-^{14}C into the 5-methyl group of DNA-thymine in L5178Y leukemic cells incubated *in vitro*. Previous work by Delamore and Prusoff (18), Kit (19), and others demonstrated that deoxyuridine and deoxycytidine stimulated the incorporation of ^{14}C-formate into DNA. Therefore, if the decrease in the amount of radioactivity incorporated into DNA-thymine from precursor deoxycytidine-^{3}H and deoxyuridine-^{3}H were due to dilution of the intracellular pool of these compounds by the breakdown products of 4-NOH-CdR formate-^{14}C incorporation into DNA-thymine would not be inhibited by the presence of the analog and probably would be increased. As shown in Table 4, the incorporation of formate-^{14}C into DNA-thymine was strongly inhibited by 4-NOH-CdR, and this effect was only partly reversed by the addition of deoxycytidine.

Inhibition of Thymidylate Synthetase by 4-N-Hydroxy-2'-deoxycytidylic acid

Thymidylate synthetase was partially purified from *E. coli* and assayed by the methods of Wahba and Friedkin (15). The spectrophotometric measurement of thymidylate synthetase activity, which is dependent on the initial linear increase of 340 mμ absorbancy due to the conversion of $N^{5,10}$-methylene tetrahydrofolate to dihydrofolate, showed that 4-NOH-dCMP was an inhibitor of the reaction. A graphic representation of the data by the double-reciprocal method of Lineweaver and Burk (20), Fig. 4, demonstrated that 4-NOH-dCMP inhibited competitively with respect to dUMP. The value of the Michaelis constant of this preparation of thymidylate synthetase was calculated by the slope-intercept method and found to be K_m (dUMP) $= 4.4 \times 10^{-6}$ M, in agreement with

TABLE 4

Incorporation of formate-^{14}C into the methyl group of DNA-thymine by L5178Y cells in vitro

The incubation mixture, 4.0 ml, contained packed cells, 0.2 ml, suspended in Fischer's medium plus 10% horse serum, 3.6 ml, and other additions were in a volume of 0.2 ml. After 5 min of preincubation at 37°, sodium formate-^{14}C (1 μmole, 10 μC) was added and incubation was continued for 40 min. These are the results of a single experiment.

Additions to the medium	DNA-thymine (cpm/ μmole)	Inhibition (%)
Control	37,000	—
+ 4-NOH-CdR 5×10^{-5} M	11,200	60
+ 4-NOH-CdR 5×10^{-4} M	1,505	96
+ Deoxycytidine 10^{-3} M	45,050	+20
+ 4-NOH-CdR 5×10^{-5} M and deoxycytidine 10^{-3} M	19,400	48
+ 4-NOH-CdR 5×10^{-4} M and deoxycytidine 10^{-3} M	4,060	89

the value reported by Friedkin (15). The inhibition constant, K_i (4-NOH-dCMP) $= 1.2 \times 10^{-6}$ M, was calculated from the Lineweaver-Burk plot in Fig. 4. The ratio of K_m:K_i indicated that 4-NOH-dCMP was bound to the enzyme system with a slightly greater affinity than the natural substrate, dUMP. Incubation of the enzyme with the analog for 5 min prior to the addition of other components did not alter the kinetics of the reaction, and there was no change in 340 mμ absorbancy when 4-NOH-dCMP was tested as a substrate in the reaction mixture. Other compounds closely related to 4-NOH-dCMP, namely 4-NOH-CdR and 4-NOH-CMP, were not inhibitors, and hydroxylamine at 10^{-3} M inhibited only 20%.

Thymidylate synthetase activity in cell-free extracts of mouse leukemia cells was measured by the spectrophotometric method of Wahba and Friedkin under the conditions described by Reyes and Heidelberger (16). A double-reciprocal plot of the kinetic data, shown in Fig. 5, demonstrated that 4-NOH-dCMP inhibited the enzyme competitively with respect to dUMP. The apparent Michaelis constant, K_m (dUMP),

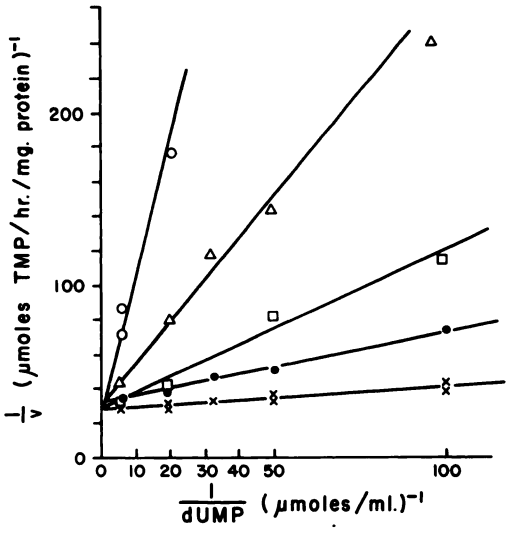

FIG. 4. *Lineweaver-Burk plot of inhibition of thymidylate synthetase of Escherichia coli by 4-NOH-dCMP*

-×- control; -●- 2.7 μM 4-NOH-dCMP; -□- 8.3 μM 4-NOH-dCMP; -△- 27.5 μM 4-NOH-dCMP; and -○- 83.5 μM 4-NOH-dCMP. $1/v = (\mu$moles TMP/hr/mg of protein$)^{-1}$. See Methods for enzyme preparation and assay procedure.

was 5.9×10^{-6} M and the inhibition constant, K_i (4-NOH-dCMP), was 6×10^{-7} M. Incubation of the enzyme with 4-NOH-dCMP produced no change in the 340 mμ absorbancy, which indicated that the analog was not a substrate for thymidylate synthetase. When dUMP was added to the incubation after the enzyme has been exposed to 4-NOH-dCMP for 10 min, there was no change in the kinetics of inhibition, a finding which showed that the analog was reversibly associated with the enzyme. As in the case of *E. coli* thymidylate synthetase, 4-NOH-CdR and 4-NOH-CMP were not inhibitors of the leukemic cell enzyme.

Effect of 4-N-Hydroxy-2'-deoxycytidine on Deoxynucleoside Kinase Activity

Extracts which were prepared from sonically disrupted L5178Y cells converted deoxycytidine-^{3}H, deoxyuridine-^{3}H, and thymidine-^{3}H to the corresponding 5'-nucleotides in the presence of $MgCl_2$ and

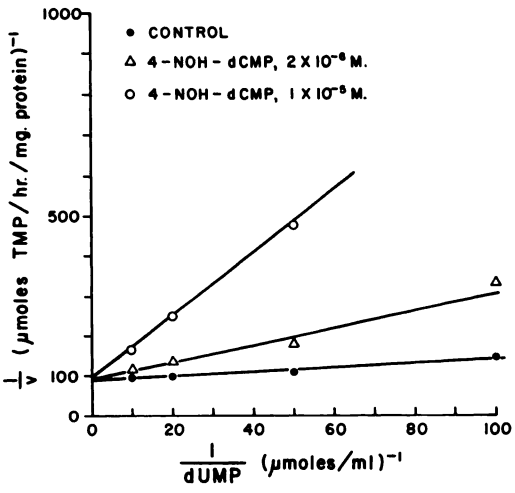

FIG. 5. *Inhibition of mouse leukemia thymidylate synthetase by 4-NOH-dCMP*

The reaction mixture contained 200 mμmoles *dl*-L-tetrahydrofolic acid; 10, 20, 50, 100 mμmoles of dUMP; 3.3 μmoles of formaldehyde; 50 μmoles of mercaptoethanol; 1.7 μmoles of sodium ascorbate; phosphate buffer, 0.1 M, pH 6.8; enzyme extract, 1.86 mg protein; in a total volume of 1.0 ml. All the components were incubated for 10 min at 25°, then dUMP and 4-NOH-dCMP were added. The velocity of the reaction was calculated from the initial linear increase in absorbance at 340 mμ.

ATP. The addition of 10^{-5} M 4-NOH-CdR to the incubation medium did not inhibit phosphorylation of the deoxynucleosides, as shown in Table 5. The phosphorylation of 4-NOH-CdR-^{3}H to the mononucleotide also occurred in this system, but at a much slower rate than that observed for the naturally occurring deoxyribonucleoside substrates. Furthermore, it was found that the conversion of 4-NOH-CdR to 4-NOH-dCMP was inhibited strongly by the addition to the incubation medium of thymidine and deoxyuridine. Under similar conditions, deoxycytidine did not inhibit the phosphorylation of 4-NOH-CdR. These observations are consistent with reports that deoxycytidine kinase is distinct from thymidine-deoxyuridine kinase (21), and support the suggestion that the 4-*N*-hydroxy cytosine derivatives used here are functional analogs of the corresponding uracil compounds.

Metabolism of 4-NOH-CdR by L5178Y Leukemia Cells

Tritium-labeled 4-NOH-CdR was incubated with L5178Y leukemia cells in Fischer's medium for 1 hr, and the distribution of radioactivity in the chromatographically separated pyrimidine deoxyribonucleosides and nucleotides was measured. The results of this analysis are summarized in Table 6. Two concentrations of 4-NOH-CdR-^{3}H were used: 1×10^{-6} M, a noninhibitory level, and 5×10^{-5} M, which in previous experiments inhibited deoxycytidine-^{3}H incorporation into DNA. At both concentrations of the analog which were employed, only 1–2% of the radioactivity added to the incubation medium was recovered from the acid-soluble fraction of washed cells. The acid-soluble fraction which was prepared from the incubated cells was analyzed by chromatography on Dowex 1-formate. The deoxynucleoside fraction under both conditions contained about 75% of the acid-soluble radioactivity, of which over 90% was shown by paper chromatography and Dowex 1-X8-(Chloride) chromatography to be 4-NOH-CdR-^{3}H.

Analysis of the deoxyribonucleotide fraction resulted in recovery of the tritium-labeled analog monophosphate, 4-NOH-dCMP. In cells exposed to 5×10^{-5} M 4-NOH-CdR-^{3}H for 1 hr, the intracellular concentration of 4-NOH-dCMP, based on specific activity measurement, was found to be 1.7×10^{-6} M, an amount sufficient to inhibit thymidylate synthetase in the cell-free enzymic studies.

A significant proportion of the radioactivity in the acid-soluble nucleotide fraction was present in cytosine and thymine deoxyribonucleotides, a finding which is consistent with Maley's observation (8) that 4-NOH-dCMP-^{14}C was converted to dCMP-^{14}C. When an inhibitory concentration of 4-NOH-CdR-^{3}H was employed (5×10^{-5} M), radioactivity in the thymidylic acid fractions was decreased greatly.

A relatively high proportion of the intracellular radioactivity derived from 4-NOH-CdR-^{3}H was also found in deoxycy-

TABLE 5

The effect of 4-NOH-CdR on deoxynucleoside kinase activity of cell-free extract of L5178Y leukemia cells and effect of deoxynucleosides on phosphorylation of 4-NOH-CdR

Series A experiments contained Tris 25 μmoles and sodium phosphate 25 μmoles pH 7.4; ATP, 5 μmoles; $MgCl_2$, 0.5 μmoles; enzyme extract, 0.1 ml, 1.2 mg protein; plus substrates and additions in a total volume of 0.5 ml. In series B the incubation contained Tris, 50 μmoles pH 8.0; $MgCl_2$, 1 μmole; ATP, 2.5 μmoles; enzyme extract, 0.05 ml, 0.35 mg; plus substrate and other additions in a total volume of 0.14 ml. After 30 min incubation at 37°, products of the reactions in series A were separated from substrate by paper chromatography by descending development with isobutyric acid:water:ammonium hydroxide, 28% (66:33:1). The distribution of radioactivity on paper chromatograms was measured with a Vanguard Autoscanner 880, and from the areas the percentage of conversion to nucleotide was calculated. The radioactivity in the products from the series B reaction was measured by elution of the radioactive compounds from the paper chromatogram with 0.1 N HCl and determination of the total radioactivity of substrate and nucleotide. The data are averages of duplicate experiments.

Series	Deoxynucleoside substrate	Unlabeled additions	Conversion to nucleotide (%)	Inhibition (%)
A	CdR-^{3}H 7.6 μM; 1 × 10^6 cpm	—	23	—
		+4-NOH-CdR, 10 μM	20	13
	UdR-^{3}H 1.5 μM; 4 × 10^5 cpm	—	42	—
		+4-NOH-CdR, 10 μM	43	0
	TdR-^{3}H 8.6 μM; 2 × 10^6 cpm	—	46	—
		+4-NOH-CdR, 10 μM	47	0
B	4-NOH-CdR-^{3}H; 70 μM; 61,000 cpm	—	5.05	—
		+UdR, 70 μM	2.44	52
		+UdR, 700 μM	0.23	95
		+TdR, 7 μM	4.20	17
		+TdR, 70 μM	0.28	94
		+CdR, 700 μM	4.75	6

tidylate and thymidylate residues in DNA. The relative amount of radioactivity in cytosine and thymine residues in DNA is consistent with the distribution of radioactivity in the deoxynucleotide fractions described above. When the higher concentration of 4-NOH-CdR-^{3}H was employed, the radioactivity in DNA decreased 50% and all the difference was accounted for by the decrease in radioactivity of thymine. There was no evidence for the occurrence of 4-NOH-dCMP residues in DNA, and no analog di- or triphosphates were found in the acid-soluble fractions.

The distribution of the radioactivity derived from 4-NOH-CdR-^{3}H indicated that small amounts of deoxycytidine and deoxyuridine occurred as transformation products of the labeled analog. As shown in Fig. 1, ion-exchange chromatography satisfactorily separates these deoxynucleosides, and all radioactive 4-NOH-CdR-^{3}H was purified immediately before use by this method. The purity of the preparation was further confirmed by paper chromatography. Nevertheless, zero time analysis of incubation mixtures which contained 4-NOH-CdR-^{3}H always revealed a small amount of radioactive deoxycytidine and deoxyuridine. An enzymic transformation of 4-NOH-CdR or 4-NOH-dCMP to deoxycytidine, or deoxyuridine, could not be demonstrated in cell-free extracts. Nor could an enzymic mechanism be established for the formation of deoxycytidylic and deoxyuridylic acids from 4-NOH-dCMP. It, therefore, appears that the degradation of the analog to cytosine and uracil deoxyribosyl compounds is due to nonenzymic reactions which occur to a small extent

TABLE 6

Metabolic transformation products of 4-N-hydroxy-2'-deoxycytidine-^{3}H isolated from L5178Y leukemia cells

Packed L5178Y leukemia cells, 2 ml, were suspended in Fischer's medium (100 ml) and 4-NOH-CdR-^{3}H was added at a concentration of 1×10^{-6} M (9.6×10^7 cpm/μmole) and 5×10^{-5} M (1.9×10^6 cpm/μmole). After incubation for 30 minutes at 37° the acid-soluble nucleotides were extracted with 7% perchloric acid, appropriate carrier nucleotides were added, and the compounds were separated by chromatography on Dowex 1-formate. Ribosides eluted from the Dowex 1-formate with water were separated by chromatography on Dowex 1-chloride (see Fig. 1) and each compound was identified by its uv spectrum and behavior in paper chromatography. The data are from single experiments.

Concentration of 4-NOH-CdR-^{3}H	1×10^{-6} M	5×10^{-5} M
Acid-soluble extract	134,000 cpm	124,000 cpm
Acid-soluble compounds		
Water eluate	94,000	101,000
CdR	1,010	2,520
UdR	1,100	2,880
4-NOH-CdR	77,790	75,210
dCMP region[a]	11,000	12,100
4-NOH-dCMP	8,720	4,350
dCDP	1,085	445
dCTP	5,100	4,050
TDP	1,150	290
TTP	8,000	800
Acid insoluble fraction		
Sodium nucleate, DNA	31,400	16,200
DNA-cytosine	8,500	7,800
DNA-thymine	22,900	8,400

[a] Paper chromatography showed that the radioactivity in this fraction was not all associated with dCMP and migrated with at least two other unidentified components.

when the analog is exposed to medium and cells.

DISCUSSION

The ability of 4-NOH-CdR to interfere with thymidylate biosynthesis is supported by three lines of evidence: (a) the inhibition of L5178Y leukemia cell growth in culture by the analog and specific protection afforded by thymidine and deoxyuridine; (b) the decrease of incorporation of deoxycytidine-^{3}H, deoxyuridine-^{3}H, and formate-^{14}C into DNA-thymine produced by 4-NOH-CdR in L5178Y cells *in vitro* in short periods of incubation; and (c) the demonstration that the nucleotide, 4-NOH-dCMP, was a competitive inhibitor of thymidylate synthetase in cell-free preparation from *E. coli* and L5178Y cells. The nucleotide, 4-NOH-dCMP, was found in the acid-soluble fraction of L5178Y cells, which were incubated in a medium that contained the analog deoxyribonucleoside. In cell-free extracts of L5178Y cells, the ATP dependent conversion of 4-NOH-CdR to 4-NOH-dCMP was also demonstrated.

A principal mechanism of the inhibitory action of 4-NOH-CdR, therefore, appears to be the competitive inhibition of thymidylate synthetase by 4-NOH-dCMP. The values of the kinetic constants (K_m (dUMP): K_i (4-NOH-dCMP) = 4 for the *E. coli* and 10 for the leukemic cell enzyme) show that 4-NOH-dCMP is somewhat more strongly bound to the enzyme than dUMP. As an inhibitor of thymidylate synthetase, 4-NOH-dCMP is much weaker than 5-fluoro-2'-deoxyuridylic acid (K_m: $K_i = 1800$) (22).

Although the ability of deoxyuridine and thymidine to protect against the inhibitory effects of 4-NOH-CdR in cell culture is correlated with the proposed site of action of the analog on thymidylate synthetase, it is also clear from studies with the cell-free deoxynucleoside kinase that deoxyuridine and deoxythymidine effectively compete with 4-NOH-CdR for the phosphorylation system which converts the analog to 4-NOH-dCMP. Since the latter compound is the active form of the analog in the inhibition of thymidylate synthetase, this effect of deoxyuridine and thymidine on the phosphorylation of 4-NOH-CdR must be taken into account in the interpretation of cell culture experiments.

Attempts to inhibit the growth of the L5178Y ascitic mouse leukemia *in vivo* by frequent intraperitoneal injection of 4-NOH-CdR in amounts up to 7.5 mg/kg per day were unsuccessful. It was found that 4-NOH-CdR-^{3}H was rapidly excreted by the animals and that the rate of development of the ascites tumor was not signifi-

cantly altered. From the results presented on the distribution of tritium in the transformation products of 4-NOH-CdR in L5178Y cell suspension, some of the reasons for this failure are clear. In the first place, the penetration of the analog into the cell is very low, and secondly, the phosphorylation of 4-NOH-CdR to the nucleotide proceeds at only about one-tenth the rate observed for deoxyuridine and deoxycytidine. Since the evidence points to the nucleotide as the active inhibitory form of 4-NOH-CdR at the site of methylation of dUMP by thmidylate synthetase, these factors conspire to prevent growth inhibition of the neoplastic cell in the mouse. Furthermore, the small amounts of deoxyuridine and deoxycytidine which arise from 4-NOH-CdR, in themselves and as phosphorylated products, antagonize the inhibitory effects of 4-NOH-CdR.

The demonstration that the hydroxylamino group is capable of functioning as an analog of the 4-oxy group of a deoxyribosyl pyrimidine is of interest, and suggests the development of other hydroxylamine deoxyribosyl pyrimidine compounds.

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REFERENCES

1. J. J. Fox, D. V. Praag, I. Wempen, I. L. Doerr, L. Cheong, J. E. Knoll, M. L. Eidinoff, A. Bendich and G. B. Brown, *J. Am. Chem. Soc.* **81**, 178 (1959).
2. H. Schuster, *J. Mol. Biol.* **3**, 447 (1961).
3. D. W. Verwoerd, H. Kohlhage and W. Zillig, *Nature* **192**, 1038 (1961).
4. D. M. Brown and P. Schell, *J. Mol. Biol.* **3**, 709 (1961).
5. D. M. Brown and P. Schell, *J. Chem. Soc.* **32**, 208 (1965).
6. C. Janion and D. Shugar, *Biochem. Biophys. Res. Commun.* **18**, 617 (1965).
7. E. Freese, E. Bautz and E. Bautz-Freese, *Proc. Natl. Acad. Sci.* **47**, 845 (1961).
8. G. F. Maley and F. Maley, *J. Biol. Chem.* **239**, 1164 (1964).
9. A. Albert and E. P. Sargeant, *in* "Ionization Constants of Acids and Bases," p. 73. Wiley, New York, 1962.
10. J. S. Dixon and D. Lipkin, *Anal. Chem.* **26**, 1092 (1954).
11. G. Tener, *J. Am. Chem. Soc.* **83**, 159 (1961).
12. G. A. Fischer, *Ann. N.Y. Acad. Sci.* **76**, 673 (1958).
13. R. B. Hurlbert, H. Schmitz, A. F. Brumm and V. R. Potter, *J. Biol. Chem.* **209**, 23 (1954).
14. P. B. Dannberg, B. J. Montag and C. Heidelberger, *Cancer Res.* **18**, 329 (1958).
15. A. J. Wahba and M. Friedkin, *J. Biol. Chem.* **237**, 3794 (1962).
16. P. Reyes and C. Heidelberger, *Mol. Pharmacol.* **1**, 14 (1965).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
18. I. W. Delamore and W. H. Prusoff, *Biochim. Biophys. Acta* **80**, 220 (1964).
19. S. Kit, *Cancer Res.* **17**, 56 (1957).
20. H. Lineweaver and D. Burk, *J. Am. Chem. Soc.* **56**, 658 (1934).
21. F. Maley and G. F. Maley, *Biochemistry* **1**, 847 (1962).
22. K. U. Hartmann and C. Heidelberger, *J. Biol. Chem.* **236**, 3006 (1961).