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ANALOGS OF 2′-DEOXYADENOSINE:

FACILE ENZYMATIC PREPARATION AND GROWTH INHIBITORY EFFECTS ON HUMAN CELL LINES

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Abstract—Nucleoside deoxyribosyltransferase has been extensively purified from extracts of *Lactobacillus leichmannii*. The enzyme was initially co-purified with ribonucleotide reductase and then separated from the latter by affinity chromatography on dGTP-Sepharose. A new procedure is described for the synthesis of 6-(*p*-aminobenzylamino)purine. The latter was diazotized and reacted with Sepharose substituted with *m*-phenylenediamine to produce an affinity material for chromatography of the transferase. Some properties of the purified transferase are described, including remarkable stability to heat and to guanidine hydrochloride. With thymidine as donor, the transferase had a very broad specificity for the purine acceptor, the only modifications not tolerated being the loss of the tautomeric N in the imidazole ring and, possibly, substitution at the 8-position. The enzyme was used for the convenient synthesis of twelve analogs of 2′-deoxyadenosine on the 100–400 mg scale with an average yield of 64 per cent. The method was readily applicable to larger scale preparations and the enzyme was recoverable from the final reaction mixtures in good yield. Of the nucleosides synthesized, the 2-chloro and 2-bromo analogs of 2′-deoxyadenosine were especially good inhibitors of the growth of KB and CCRF-CEM cells in culture.

Analogs of 2′-deoxyadenosine are of considerable interest because of the potent effects of the parent substance on cells [1–6]. Although millimolar concentrations of deoxyadenosine are required in the medium to inhibit cell growth and intracellular DNA synthesis, the requirement for these high levels is largely due to rapid destruction of deoxyadenosine by adenosine deaminase and the subsequent action of purine nucleoside phosphorylase. When deoxyadenosine cytotoxity is determined in the presence of adenosine deaminase inhibitors such as deoxycoformycin [7–9] or erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) [8, 10], however, cell growth is inhibited by deoxyadenosine at concentrations in the range 1–100 μM. It therefore seems possible that analogs of 2′-deoxyadenosine, which are poor substrates for the deaminase, might be powerful inhibitors of cell replication even in the absence of a deaminase inhibitor. This could provide a better approach to the control of cell growth, since the effect of deoxycoformycin on the susceptibility of L cells to 2′-deoxyadenosine is irreversible [8], suggesting that serious toxicity problems might be encountered with the use of deoxycoformycin *in vivo*.

We previously reported an enzymatic route to the 5′-triphosphates of 2′-deoxytubercidin, 2′-deoxytoyocamycin and 2′-deoxyformycin [11]. A new, facile enzymatic route is presented here for the preparation of analogs of 2′-deoxyadenosine, most of which were modified in the six-membered ring of the base. This method requires only the adenine analog and thymidine as starting materials and utilizes the nucleoside deoxyribosyltransferase (EC 2.4.2.6) from *Lactobacillus leichmannii*, to catalyze the reaction:

adenine analog + thymidine
⇌ 2′-deoxyadenosine analog + thymine

The substrate specificity of the enzyme and several of its properties have been investigated, and it has been used to prepare several deoxyadenosine analogs on a scale of 100–400 mg. These analogs have been tested for growth inhibitory effects on human cells in culture.

MATERIALS AND METHODS

Purines were synthesized by the procedures given in the references in brackets: 2,8-dichloroadenine [12], 7-deazaadenine [13], 6-allylaminopurine [14], 2-amino-6-methylthiopurine [15], 2-azaadenine [16], 8-aza-2,6-diaminopurine [17], 8-aza-2,6-dimethylthiopurine [18], 8-aza-6-methylthiopurine [19], 2-azidoadenine [20], 6-benzylaminopurine [21], 2-chloroadenine [22], 1-deazaadenine [23], 2-dimethylaminoadenine [24], 6-dimethylamino-2-methylthiopurine [25], 6-dimethylaminopurine [26], 2-fluoroadenine [27], 2-fluoro-6-methylpurine [27], 2-hydroxy-6-methylaminopurine [28], isoguanine sulfate [29], 2-mercaptoadenine [29], 2-mercapto-6-methylaminopurine [28], 6-methoxypurine [30], 2-methyladenine [31], 6-methylaminopurine [32], 6-methylpurine [33], and 2-methylthioadenine [31].

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2-Bromoadenine was prepared by amminolysis of 2,6-dibromopurine [34] according to the method used for amminolysis of 2,6-dichloroadenine [22]. The following bases were purchased: 2-aminoadenine from Vega; 4-aminopyrazolo-[3,4-*d*] pyrimidine and 8-azaadenine from the Aldrich Chemical Co. (Milwaukee, WI); and purine from HetChem.

Affinity column for purification of nucleoside deoxyribosyltransferase. Holguin and Cardinaud [35] have reported the preparation of several types of affinity materials for chromatography of nucleoside deoxyribosyltransferase from *Lactobacillus helveticus*. We were unable to obtain 6-(*p*-aminobenzylamino)purine by the procedure they describe and used the following procedure instead. 6-Chloropurine (32 mmoles), *p*-nitrobenzylamine (65 mmoles) and Na_2CO_3 (32 mmoles) were suspended in 50 ml of dimethylformamide and stirred at 50°. All dissolved except a part of the Na_2CO_3. Heating was continued and samples were removed at 2-hr intervals to examine the progress of the reaction by t.l.c. on silica plates developed with chloroform–methanol (24:1). Since the reaction was still incomplete after 15 hr, the temperature was raised to 100° for 3 hr and the mixture was then cooled to room temperature. After removal of the solvent under vacuum on a rotary evaporator, the residue was redissolved in 40 ml of dimethylformamide, and 120 ml of water was added to precipitate the product. After standing overnight at room temperature, the precipitate was collected by filtration, washed thoroughly with water, and dried under vacuum (15 μm Hg) at room temperature over P_2O_5. The crude product was extracted twice with boiling 95% ethanol and three times with acetone at room temperature and was finally crystallized from dimethylformamide. Yield: 1.53 g (5.66 mmoles, 17%) of crude product from which 1.12 g of crystallized material was obtained. Elemental analysis was as follows: Found: C, 53.15%; H, 3.92%; N, 30.93%. Calculated for $C_6H_{10}O_2N_6$: C, 53.27%; H, 3.72%; N, 31.08%. The melting point was 288–289°. A value of 220–226° has been reported previously [36], presumably for an impure material. Proton n.m.r. results confirmed the identity of the product. ^1H n.m.r. at 360 MHz in d_6-DMSO (chemical shifts in ppm relative to internal TMS): 4.87(—NH—C$\underline{H}_2$); (7.63, 7.65, 8.21, 8.24 (CH_2—$C_6\underline{H}_4$—NO_2)); 8.19 (H-8); 8.21 (H-2); 8.41 (—N$\underline{H}$—CH_2—); 13.02 (N9-H). The material had an R_F of 0.23 in the t.l.c. system described above. Ultraviolet absorbance maxima in aqueous solution were 279 nm at pH 1.0, 270 nm at pH 7.0, and 277 nm at pH 12.5.

6-(*p*-Aminobenzylamino)purine was prepared by reduction of the nitro derivative as follows. 6-(*p*-Nitrobenzylamino)purine (0.22 mmole) in 20 ml of glacial acetic acid was hydrogenated in the presence of 40 mg of 10% palladium on charcoal at one atmosphere hydrogen pressure and room temperature. The reaction was complete in 8 hr as indicated by assays for the aromatic amine [37] and t.l.c. in the system described above. The catalyst was removed by filtration through Celite, and the filtrate was evaporated to dryness at reduced pressure on a rotary evaporator. Without further purification the product was reacted with Sepharose to which *m*-phenylenediamine had been coupled.

m-Phenylenediamine was coupled to Sepharose 4B-200, which had been activated by cyanogen bromide according to the method of Porath *et al.* [38]. Immediately after 500 g of Sepharose had been activated and washed, *m*-diphenylamine (25 mmoles) dissolved in water was added to the stirred suspension at 5° and the mixture was stirred overnight at 5°. The product was washed with 5 liters of 0.5 M NaCl and then with water until the absorbance of washings at 270 nm was less than 0.05.

The 6-(*p*-aminobenzylamino)purine was diazotized for coupling with the substituted Sepharose by the method of Cuatrecasas [39] as follows. 6-(*p*-Aminobenzylamino)purine diacetate (577 mg, 1.47 mmoles) in 21 ml of 1.5 N HCl at 0° was mixed with sodium nitrite (9.8 mmoles), in 7 ml of water at 0°, stirred for 1 min and kept a further 8 min at 0°. The mixture was then added to a suspension of 60 g of the *m*-phenylenediamine-substituted Sepharose in 60 ml of 0.2 M Na_2CO_3 at 8°. The pH was immediately adjusted to 9.4. After 3 hr the substituted Sepharose, which had now changed from colorless to brown, was packed into a column and washed with 8 M guanidine hydrochloride in 0.1 M sodium phosphate buffer, pH 6.0, until the effluent was colorless, and then with water until the absorbance of the effluent at 260 nm was below 0.05.

Preparation and purification of nucleoside deoxyribosyltransferase. The purification of ribonucleotide reductase from *Lactobacillus leichmannii* has been described previously [40, 41]. The nucleoside deoxyribosyltransferase co-purified with the ribonucleotide reductase up to the last step which involves affinity chromatography on Sepharose to which P^1-(6-aminohex-1-yl)-dGTP has been coupled via cyanogen bromide activation. The nucleoside deoxyribosyltransferase passed through this column without retention and was subsequently purified on the affinity material described in the previous section which will be referred to as adenine-Sepharose.

This final affinity chromatography was performed by a modification of the method of Holguin and Cardinaud [35]. Enzyme from the dGTP-Sepharose column was dialyzed against 0.1 M sodium phosphate buffer, pH 6.0, and applied to an adenine-Sepharose column (2 × 35 cm) that had been equilibrated previously with the same buffer. The column was eluted first with 500 ml of the same buffer, and then with 300 ml each of 1 M, 2 M and 3 M guanidine hydrochloride in 0.1 M sodium phosphate buffer, pH 6.0. Fractions of 20 ml were collected and those containing guanidine hydrochloride were dialyzed overnight against 0.1 M sodium phosphate buffer, pH 6.0. Fractions were subsequently assayed for enzyme activity and their protein concentrations determined. The data for a complete purification are shown in Table 1.

Assay of nucleoside deoxyribosyltransferase. The standard reaction mixture contained 0.25 mM adenine, 2 mM thymidine, 25 mM sodium phosphate buffer, pH 6.0, and enzyme. The mixture was incubated at 37° for 10 min and terminated by addition of 2 ml of diphenylamine reagent [41]. After color development at 37° for 4 hr, absorbance at 595 nm

was compared with that of a standard containing 0.25 μmole deoxyadenosine, enzyme and all other components of the reaction mixture except adenine. Absorbance values were corrected by subtracting the absorbance found in controls containing enzyme and all components except adenine. A unit of activity is defined as the enzyme required to synthesize 1 μmole of deoxyadenosine per min under the standard assay conditions.

Enzymatic synthesis of analogs of 2'-deoxyadenosine. A suspension of the purine (0.46 to 1.6 mmoles) was stirred at 37° in 25 mM citrate buffer, pH 6.0, containing thymidine (1.0 to 1.64 moles/mole of purine) and nucleoside deoxyribosyltransferase (10–30 units, usually purified through Step 6, i.e effluent from the dGTP-Sepharose column: in the case of 2-bromoadenine purified through Step 7). The total volume was 20–40 ml. Incubation and stirring at 37° were continued for 2–4 days. Formation of the deoxyadenosine analog was monitored either by t.l.c. on cellulose (with development by 5% aqueous disodium phosphate saturated with isoamyl alcohol) or by the colorimetric assay involving the diphenylamine reagent. When the reaction had reached equilibrium, the reaction mixture was diluted to 40–60 ml with water and applied to a Dowex 50 × 8 column (NH_4^+, 400 mesh, 4 × 91 cm) that had been equilibrated with 0.1 M ammonium borate buffer, pH 7.3. The column was initially washed with the same buffer, which successively eluted thymidine and thymine. When elution of thymine was complete, as determined by monitoring absorbance of the eluent at 260 or 280 nm, the eluting fluid was changed to 0.05 M ammonia, and elution of the deoxyadenosine analog was monitored in the same way (Fig. 1).

Fractions (20 ml) containing the deoxyadenosine analog were pooled, concentrated to a small volume under reduced pressure and applied to a Dowex 1 × 2 column (OH^-, 400 mesh, 6 × 26 cm) to remove borate. The nucleoside was eluted from the column with water, the ultraviolet-absorbing fractions were pooled and concentrated under reduced pressure, and the concentrate was freeze-dried.

In some cases it was necessary to remove contaminating bases and thymidine by further chromatography on a Biogel P-2 column (2.8 × 200 cm) that had been equilibrated previously with 0.05 M ammonia. Elution was performed with the same concentration of ammonia. The nucleoside product was examined for ultraviolet-absorbing contaminants by t.l.c. on cellulose in 5% disodium phosphate saturated with isoamyl alcohol. In some cases the nucleoside was crystallized from a suitable solvent (Table 3). Elemental analysis was performed by Galbraith Laboratories, Knoxville, TN, on a sample of the crystallized material after drying for 2 hr at 117° under reduced pressure (50 μm Hg) over P_2O_5.

Growth inhibition of cultured human cells by deoxyadenosine analogs. Human lymphoblastic cells (CCRF-CEM, [42]) were grown in RPMI 1640 medium supplemented with 10% of heat-inactivated fetal calf serum and kanamycin (200 μg/ml). Cells at an initial density of 1×10^6 per ml were grown in a volume of 4.5 ml in Falcon flasks (No. 3013, 25 cm^2 growth area, canted neck). Nucleoside solutions were sterilized by millipore filtration, and the concentration of the filtrate was determined spectrophotometrically before aseptic addition of a suitable volume to each flask. Cell counts were determined in duplicate by withdrawing 0.1 ml-samples of the cell suspension from the Falcon flask and mixing each of them with 5 vol of a 0.08% solution of erythrosin B; the numbers of dye-excluding cells were then counted in a hemocytometer. Samples were taken for counting at 24, 48, 72 and 96 hr.

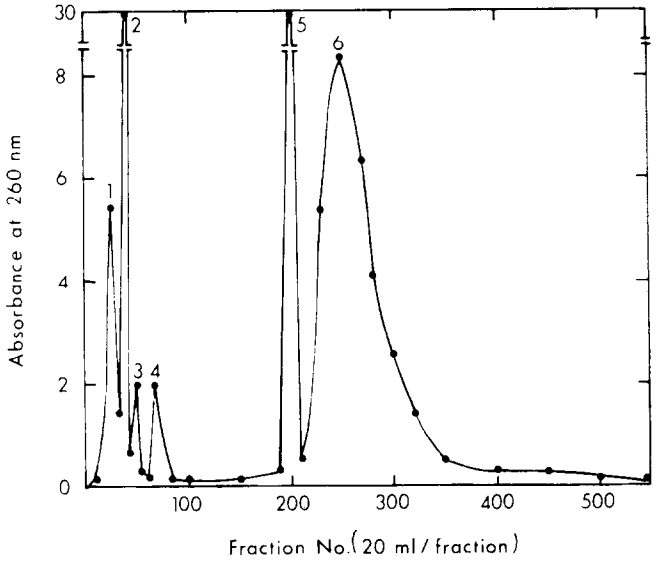

Fig. 1. Purification of enzymatic products from thymidine and 2-bromoadenine by ion-exchange chromatography. The reaction mixture (40 ml) was applied to a Dowex 50 × 8 column (400 mesh, NH_4^+ form), and elution was performed at a flow rate of approximately 3.5 ml/min. Major components of the peaks were as follows: (1) enzyme and thymidine, (2) thymidine, (3) thymidine and thymine, (4) thymine, (5) 2-bromoadenine, and (6) 2-bromo-2'-deoxyadenosine. For other details see Materials and Methods.

Table 1. Purification of nucleoside deoxyribosyltransferase*

Purification step	Volume (ml)	Total activity (units)	Protein conc. (mg/ml)	Specific activity (units/mg)	Yield (%)	Purification factor
1. Crude extract	3620	1370	10.0	0.038	100	
2. Protamine sulfate precipitation	3800	1440	8.3	0.045	105	1.2
3. Ammonium sulfate precipitation	450	735	43.3	0.038	53	
4. Sephadex G-100 filtration	1040	700	3.30	0.20	51	5.4
5. Second ammonium sulfate precipitation	73	599	25.5	0.32	43	8.5
6. dGTP-Sepharose chromatography	200	577	7.8	0.37	42	9.8
7. Adenine-Sepharose affinity chromatography	465	385	0.085	9.8	29	260

* Extract from 320 g of bacterial paste.

Duplicate cultures were grown at each nucleoside concentrate and for each time period. Results reported are for the 48-hr period. In the case of the more potent nucleosides, the EC_{50} values increased on successive days but were still in the same ratio.

Cytotoxicity for KB cells was determined according to the protocols of Geran *et al.* [43].

RESULTS

Purification of nucleoside deoxyribosyltransferase. As shown in Table 1, the procedure for purification of ribonucleotide reductase (Steps 1–6) caused a 10-fold purification of nucleoside deoxyribosyltransferase, with about 40 per cent recovery of activity in the fraction not retarded on the dGTP-Sepharose column (Step 6). Ribonucleotide reductase was eluted from the d-GTP-Sepharose column subsequently. Further purification of the nucleoside deoxyribosyltransferase by affinity chromatography on the adenine-Sepharose (Step 7) produced an overall purification of about 260-fold, with about 28 per cent yield. The elution profile obtained with the affinity column is shown in Fig. 2. Virtually all the activity was eluted by 2 M guanidine hydrochloride, whereas earlier washes brought off most of the protein. This in in contrast to the results of Holguin and Cardinaud [35] who found that this activity from *L. helveticus* required only 1 M guanidine hydrochloride for elution from an affinity column in which the ligand was 6-(*p*-aminophenyl-*n*-hexylamino)purine.

Enzyme at either Stage 6 or Stage 7 caused no metabolic transformations of reactants or products other than deoxyribosyl transfer, and enzyme from

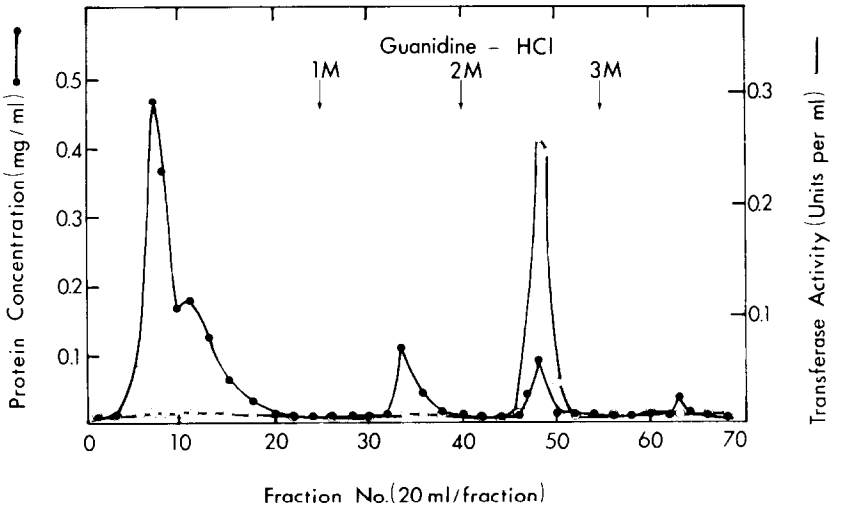

Fig. 2. Affinity chromatography of nucleoside deoxyribosyltransferase. An enzyme solution (15 ml, 4.5 mg protein/ml), eluted from the dGTP-Sepharose column, was applied to the column. Elution was performed at a flow rate of 1.5 ml/min. Other details are given in Materials and Methods.

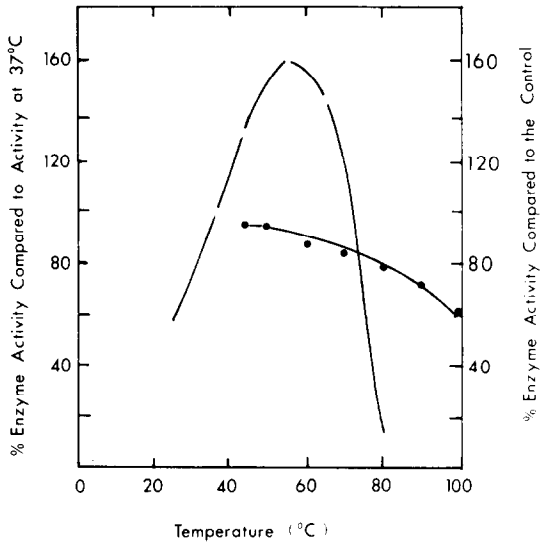

Fig. 3. Effect of temperature on the stability of nucleoside deoxyribosyltransferase. Effect of temperature on enzyme activity during the assay (○). The standard reaction mixture was incubated at the temperature indicated for 10 min before color development. Thermal inactivation of the enzyme prior to enzyme assay (●). Enzyme was heated at the temperature indicated for 10 min, cooled, and stored at 4° overnight before subsequent assay of activity at 37° under standard conditions.

Stage 6 was used in most preparations of deoxyadenosine analogs. For the investigation of the characteristics of the nucleoside deoxyribosyltransferase, enzyme from Stage 7 was used, but enzyme at Stage 6 had rather similar properties.

Properties of the purified nucleoside deoxyribosyltransferase. Several properties of the transferase were investigated so that conditions for the purification of the enzyme and its utilization as a preparative tool could be optimized.

The enzyme exhibited a pH optimum at pH 5.9 with a symmetrical decline in activity as the pH was raised or lowered from this value. At pH 4.5 and 7.5 the activity was approximately 60 per cent that at pH 5.9. Reaction rates at various thymidine concentrations under otherwise standard conditions gave a linear double-reciprocal plot and indicated an apparent K_m of 0.48 mM. When the adenine concentration was varied under otherwise standard assay conditions, the highest velocity was reached at 0.5 mM, with significant inhibition as the concentration was increased to 2 mM. These results are in good agreement with those reported previously by Beck and Levin [44] for the nucleoside deoxyribosyltransferase from *L. leichmannii*. The transferase from *L. helveticus* also exhibits substrate inhibition by adenine [45, 46], although hypoxanthine apparently does not exhibit substrate inhibition of this enzyme [47].

Since previous experience had indicated that the transferase might be rather stable to heat [48, 49], we further investigated this aspect of the *L. leichmannii* enzyme. The results (Fig. 3) indicated that the *L. leichmannii* enzyme is even more stable than the *L. helveticus* enzyme [49], losing only about 35 per cent of its activity after exposure to 100° for 10 min. When the assay of enzymatic activity was conducted at various temperatures, however, a rather different picture emerged. The highest activity was found at 55° (with a 10-min incubation), and at 80° only slight activity remained.

Since guanidine hydrochloride was used to elute the enzyme from the adenine-Sepharose affinity column it was also of interest to determine the stability in the presence of this reagent. When enzyme was exposed to various concentrations of guanidine hydrochloride for 4 hr at room temperature and subsequently dialyzed against buffer at 4° overnight, it was found that there was little loss in activity. Even after exposure to 7 M guanidine hydrochloride there was only a 12 per cent loss of activity. Indeed, even

Table 2. Specificity for the purine acceptor of nucleoside deoxyribosyltransferase*

Inactive	Active (continued)
7-Deazaadenine	3-Deazaadenine
2,8-Dichloroadenine	2,6-Diaminopurine
	2-Dimethylaminoadenine
Active	6-Dimethylamino-2-methylthiopurine
6-Allyaminopurine	6-Dimethylaminopurine
2-Amino-6-methylthiopurine	2-Fluoroadenine
4-Aminopyrazolo[3,4-*d*]pyrimidine	2-Fluoro-6-methylpurine
2-Azaadenine	2-Hydroxy-6-methylaminopurine
8-Azaadenine	Isoguanine
8-Aza-2,6-diaminopurine	2-Mercaptoadenine
8-Aza-2,6-dimethylthiopurine	2-Mercapto-6-methylaminopurine
8-Aza-6-methylthiopurine	6-Methoxypurine
2-Azidoadenine	2-Methyladenine
6-Benzylaminopurine	6-Methylaminopurine
2-Bromoadenine	6-Methylpurine
2-Chloroadenine	2-Methylthioadenine
1-Deazaadenine	Purine

* Thymidine (40 mM), purine (25 mM), sodium citrate buffer, pH 6.0 (25 mM), and enzyme (2.5 units), in a volume of 0.25 ml, were incubated for 6 hr at 37°. Reaction was detected by t.l.c. on cellulose in the phosphate-isoamyl alcohol system.

Table 3. Deoxyadenosine analogs prepared enzymatically

Purine base	Elemental analysis found C (%)	H (%)	N (%)	X* (%)	For	Elemental analysis calc. C (%)	H (%)	N (%)	X* (%)	Amt. syn. (mg)	Yield (%)	R_f†
8-Azaadenine	41.03	4.77	30.24		$C_9H_{12}N_6O_3$‡	40.00	5.22	31.10		335	75	0.64
8-Aza-7-deazaadenine	44.19	5.50	24.99		$C_{10}H_{13}N_5O_3$‡	44.61	5.62	26.01		114	63	0.63
8-Aza-2,6-diaminopurine	38.76	4.96	34.59		$C_9H_{13}N_7O_3$‡	37.89	5.30	34.37		176	66	0.42
2,6-Diaminopurine	42.62	5.39	29.38		$C_{10}H_{14}N_6O_3$‡	42.25	5.67	29.57		370	94	0.35
6-Methylaminopurine	47.03	5.61	24.35		$C_{11}H_{15}N_5O_3$‡	46.64	6.05	24.22		182	49	0.66
6-Benzylaminopurine§	59.62	5.60	20.34		$C_{17}H_{19}N_5O_3$	59.81	5.61	20.51		323	49	0.61
6-Dimethylaminopurine	49.22	6.07	23.54		$C_{12}H_{17}N_5O_3$‡	48.48	6.44	23.56		316	86	0.67
Isoguanine	42.07	4.89	21.87		$C_{10}H_{13}N_5O_4$‖	43.89	6.32	21.32		310	67	0.65
2-Methylthioadenine	43.91	5.08	24.17	11.11	$C_{11}H_{15}N_5O_3S$	44.43	5.08	23.56	10.78	403	82	0.36
2-Fluoroadenine	42.12	4.66	24.46	6.61	$C_{10}H_{12}N_5O_3F$	41.80	4.90	24.37	6.61	134	51	0.55
2-Chloroadenine§	41.85	4.31	24.32	12.49	$C_{10}H_{12}N_5O_3Cl$	42.04	4.23	24.53	12.41	118	40	0.48
2-Bromoadenine¶	36.60	3.79	21.00	24.05	$C_{10}H_{12}N_5O_3Br$	36.38	3.66	21.21	24.21	156	47	0.48

* S, F, Cl or Br as appropriate.
† Thin layer chromatography; cellulose, 5% Na_2HPO_4–isoamylalcohol.
‡ Plus H_2O.
§ Recrystallized from water.
‖ Plus CH_3COOH.
¶ Recrystallized from acetone–cyclohexane.

this loss may have been due to incomplete removal of guanidine hydrochloride before assay, since quite low concentrations of guanidine hydrochloride in the assay caused marked loss of activity. Thus, the presence of 0.1 and 0.2 M guanidine hydrochloride decreased activity by 41 and 53 per cent respectively.

Specificity of the enzyme for purine acceptor. The specificity of the *L. leichmannii* transferase for the purine acceptor was studied by Beck and Levin [44], and Holguin *et al.* [50] made a detailed study of this aspect of the enzyme from *L. helveticus*. These studies showed that large modifications could be made in the six-membered ring without loss of substrate activity, although they frequently caused large changes in the equilibrium constant. Changes in the five-membered ring were tolerated less, and a tautomeric proton was found to be necessary on the imidazole ring. We have further investigated the acceptor specificity of the *L. leichmannii* enzyme; the results are shown in Table 2. An adenine analog that was substituted at the 8-position appeared to be inactive. This is consistent with a result obtained with the *L. helveticus* enzyme. Although 7-deazaadenine did act as a substrate, 8-azaadenine and its derivatives were active, and positional interchange of N-7 was tolerated because 4-aminopyrazolo[3,4-*d*]pyrimidine was active. Also in parallel with the results with the *L. helveticus* enzyme, many substitutions were tolerated in the pyrimidine ring at the 2- and 6-positions, even by bulky groups, and N could be replaced by C at the 1- or 3-positions (and probably both together) or by adding N instead of C at the 2-position.

Enzymatic preparation of analogs of 2′-deoxyadenosine. The scale of the reaction can be readily increased for the production of analogs. To prevent the reaction volume from becoming very large, sparingly soluble adenine analogs were suspended in the reaction mixture which was stirred throughout the reaction period. The equilibrium constant for the reaction varies considerably with the purine acceptor [50], so that the theoretical yield varies from preparation to preparation. Less favorable equilibria were overcome to some extent by using a greater excess of thymidine. In some cases the deoxynucleoside product was also sparingly soluble and crystallized as the reaction proceeded. In such cases, the material that was insoluble at the end of the reaction period was recovered by centrifugation, dissolved in ammonia, and purified by the same procedures used for the dissolved products in the reaction mixture, as described in Materials and Methods.

It may be seen from Table 3 that yields varied from 40 to 94 per cent with a mean of 64 per cent. Elemental analyses of the final materials gave satisfactory data, although in many cases they indicated the presence of a molecule of solvent. Purity was also checked by t.l.c. which indicated the absence of ultraviolet-absorbing impurities.

Growth inhibitory effects of analogs of 2′-deoxyadenosine. The effects of the various analogs on the growth of the two human cell lines are shown in Table 4. In the CCRF-CEM system, 2′-deoxyadenosine itself was found to have an EC_{50} of about 300 μM. It can be seen that nucleosides of isoguanine and 2-methylthioadenine caused little more inhibi-

Table 4. Growth inhibitory effect of deoxyadenosine analogs on CCRF-CEM and KB cells in culture

Purine base	EC_{50} (μM)* CCRF-CEM	KB
8-Azaadenine	43	> 10
8-Aza-7-deazaadenine	0.52	>100
8-Aza-2,6-diaminopurine	>>100	> 10
2,6-Diaminopurine	13	> 10
6-Methylaminopurine	87	> 10
6-Benzylaminopurine	27	> 10
6-Dimethylaminopurine	~ 5	>1.7
Isoguanine	>100	> 10
2-Methylthioadenine	>200	
2-Fluoroadenine	0.23	1.7
2-Chloroadenine	0.015	0.087
2-Bromoadenine	0.017	

* Concentration of nucleoside required to decrease the number of dye-excluding cells (compared with uninhibited control) by 50 per cent at 48 and 72 hr.

tion of growth than deoxyadenosine. Introduction of nitrogen at the 8-position or introduction of a 2-amino group into the adenine structure increased the growth inhibitory effects of the nucleoside, but introduction of both modifications simultaneously, instead of further increasing inhibitory potency, produced a nucleoside no more active than deoxyadenosine itself. Alkyl substitution on the exocyclic nitrogen at C-6 caused modest increases in activity, but the most effective change in the base moiety was introduction of a halogen at the 2-position, the 2-chloro and 2-bromo analogs of 2′-deoxyadenosine being the most effective. In general, nucleosides most inhibitory for the growth of CCRF-CEM were also strongly inhibitory for the growth of KB cells, but an interesting exception was the 8-aza-7-deaza analog (deoxyribonucleoside of 4-amino-pyrazolo[3,4-*d*-]pyrimidine), which was fairly inhibitory for the growth of CCRF-CEM but not for KB cells. Possible differences in transport or metabolism of the nucleoside by the lymphoblastic (CCRF-CEM) and epidermoid carcinoma (KB) cells may account for this.

In the case of 2′-deoxyadenosine, growth inhibitory effects were greatly increased in the presence of the deaminase inhibitor EHNA, the EC_{50} for deoxyadenosine becoming 25 μM for CCRF-CEM cells in the presence of 1 μM EHNA. When the growth inhibitory effects of the 2-fluoro and 2-chloro analogs of deoxyadenosine were determined in the presence of EHNA, however, there was no significant change in the EC_{50}.

DISCUSSION

Suitability of the transferase as a preparative tool. The nucleoside deoxyribosyltransferase of *L. leichmannii* has proven to be very similar to the extensively studied enzyme from *L. helveticus*. This is particularly true in regard to the specificity for the purine acceptor in transfers from thymidine to purine. Holguin and Cardinaud [35] reported that a second nucleoside deoxyribosyltransferase which specifically catalyzes deoxyribosyl transfer between purine bases is present in *L. helveticus*. In affinity chromatography of the *L. leichmannii* enzyme on adenine-Sepharose, purine–purine transferase activity eluted coincidently with purine–pyrimidine activity, so that we were unable to confirm the existence of two distinct transferases.

One feature of the transferase from *L. leichmannii* which is relevant for its use as a tool in the preparation of 2′-deoxyribonucleosides is its unusual stability. Even 10 min at 100° caused only a 35 per cent loss in activity, a much greater stability than reported for the transferase from *L. helveticus* by Roush and Betz [49]. Similarly, there was little loss of activity on exposure to 7 M guanidine hydrochloride, provided the latter was removed by dialysis before determination of enzyme activity. The small loss in enzyme activity that was observed after exposure to guanidine hydrochloride was due possibly to incomplete removal of this substance by dialysis, since the assay was very sensitive to the presence of quite low concentrations of guanidine hydrochloride. The presence of the latter at 0.1 M caused a 41 per cent loss in apparent activity. The stability of the enzyme to unfolding by heat and guanidine hydrochloride, despite loss of activity at high temperature and in quite low concentrations of guanidine hydrochloride, is most readily explained by assuming that refolding to the native configuration is both kinetically and thermodynamically favorable. In addition, the high sensitivity of the enzymatic activity to guanidine hydrochloride may also be related to decomposition of a deoxyribosyl derivative of the enzyme. Danzin and Cardinaud [46] have shown that the *L. helveticus* transferase has a ping-pong reaction mechanism that implies formation of such a deoxyribosyl derivative, and the inhibition of the enzyme by Tris [35, 49] could be explained by a nucleophilic attack on this intermediate. Guanidine hydrochloride could conceivably act in the same way, although no direct evidence for this has been obtained.

The stability of the enzyme suits it admirably for the preparation of 2′-deoxyribonucleosides. During the reaction period, there was only slight loss of activity (about 3 per cent in 24 hr) and, in the case of ion-exchange chromatography on Dowex 50, the transferase was eluted early (Fig. 1) and could be prepared for repeated use by dialysis and ultrafiltration. It is possible that partially denatured enzyme could be reactivated by treatment with guanidine hydrochloride, followed by dialysis, but this has not been attempted.

Enzyme pure enough for nucleoside preparation is obtained without chromatography on the adenine-Sepharose affinity column, and the preceding chromatography on dGTP-Sepharose (which was designed for the concomitant purification of ribonucleotide reductase) is almost certainly unnecessary also. We have not yet investigated either means of increasing the intracellular level of the enzyme or simplification of the preparation. Improvements are, probably, possible in both directions.

Advantages of enzymatic synthesis of deoxyribonucleosides. Most of the compounds in Table 3 have been synthesized by conventional methods and, in particular, the 2-fluoro [51] and 2-chloro [52] derivatives have been reported. The present method, however, offers advantages of brevity and simplicity once

the appropriate base is available. The enzymatic method offers the specific advantage that the α anomer is not formed and the difficult task of separating α and β anomers is avoided. Since these anomers usually differ markedly in biological activity [53, 54], this is an important consideration. For radioactive syntheses it will usually be more convenient to introduce label into the base, which is then converted to the deoxyribonucleoside by the present method, than to perform a more lengthy synthesis of the deoxyribonucleotide by organic chemical methods. Another important consideration is the excellent yields obtained in the enzymatic method (mean 64 per cent) which compare very favorably with the low overall yields obtained in the organic chemical synthetic methods.

Growth inhibitory activity of the deoxyadenosine analogs. The marked inhibition of the growth of human cell lines caused by the 2-halogen analogs is consistent with results reported by others [51, 52] and is presumably explained in part by the resistance of such analogs to adenosine deaminase [54, 55]. Other explanations of the differences in the inhibitory effects of the various nucleosides are of course possible. These would include differences in rates of transport into cells, or differential susceptibility to degradation by phosphorolysis or hydrolysis, followed by conversion of the base to ribonucleotides by phosphoribosyltransferase action. Evidence to date, however, suggests that these are less likely explanations. Although potent growth inhibition by the 2-halogen analogs has now been reported for *Escherichia coli* (K_{12}), *Streptococcus faecium*, leukemia L1210 [52], HEp-2 [51], CCRF-CEM and KB cells, they do not seem to have been examined for toxicity and possible antitumor activity *in vivo*. In some cases, nucleosides that are highly cytotoxic *in vitro* prove to be too toxic to the host to be able to prolong survival of tumor-bearing animals. An example is 2'-deoxythioguanosine [53], which is a less effective antitumor agent than the much less toxic α anomer of this substance. The relative toxicity to host and neoplasm, however, varies from agent to agent, and it will be interesting to examine this ratio in the potent compounds synthesized in this project.

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REFERENCES

1. H. Klenow, *Biochim. biophys. Acta* **61**, 885 (1962).
2. W. H. Prusoff, *Biochem. Pharmac.* **2**, 221 (1959).
3. J. K. Lowe and G. B. Grindey, *Molec. Pharmac.* **12**, 177 (1976).
4. K. G. Lark, *Biochim. biophys. Acta* **45**, 121 (1960).
5. J. H. Kim, S. H. Kim and M. L. Eidinoff, *Biochem. Pharmac.* **14**, 1821 (1965).
6. F. Wanka, *Cell Res.* **85**, 409 (1974).
7. J. K. Lowe, B. Gowans and L. Brox, *Cancer Res.* **37**, 3013 (1977).
8. L. Lapi and S. S. Cohen, *Biochem. Pharmac.* **26**, 71 (1977).
9. W. E. G. Müller, R. K. Zahn, J. Arendes, A. Maidhof and H. Umezawa, *Hoppe-Seyler's Z. physiol. Chem.* **359**, 1287 (1978).
10. D. A. Carson, J. Kaye and J. E. Seggmiller, *J. Immun.* **121**, 1726 (1978).
11. S. A. Brinkley, A. Lewis, W. J. Critz, L. L. Witt, L. B. Townsend and R. L. Blakley, *Biochemistry* **17**, 2350 (1978).
12. E. Fischer, *Chem. Ber.* **30**, 2226 (1897).
13. J. Davoll, *J. chem. Soc.* 131 (1960).
14. F. S. Okumura, N. Enishi, H. Itoh, M. Masumura and S. Kuraishi, *Bull. chem. Soc. Japan* **32**, 886 (1959).
15. J. A. Montgomery and L. B. Holum, *J. Am. chem. Soc.* **79**, 2185 (1957).
16. D. W. Woolley and E. Shaw, *J. biol. Chem.* **189**, 401 (1951).
17. J. Davoll, *J. chem. Soc.* 1593 (1958).
18. J. A. Montgomery, A. T. Shortnacy, G. Arnett and W. M. Shannon, *J. med. Chem.* **20**, 401 (1977).
19. R. Weiss, R. K. Robins and C. W. Noell, *J. org. Chem.* **25**, 465 (1960).
20. C. Temple, Jr., C. L. Kussner and J. A. Montgomery, *J. org. Chem.* **31** 2210 (1966).
21. T. P. Johnson, G. S. McCaleb and J. A. Montgomery, *J. med. Chem.* **6**, 669 (1963).
22. G. B. Brown and V. S. Weliky, *J. org. Chem.* **23**, 125 (1958).
23. J. A. Montgomery and K. Hewson, *J. med. Chem.* **8**, 708 (1965).
24. J. A. Montgomery and L. B. Holum, *J. Am. chem. Soc.* **80**, 404 (1958).
25. J. A. Montgomery and L. B. Holum, *J. Am. chem. Soc.* **79**, 2185 (1957).
26. B. R. Baker, J. P. Joseph and R. E. Schaub, *J. org. Chem.* **19**, 631 (1954).
27. J. A. Montgomery and K. Hewson, *J. Am. chem. Soc.* **82**, 463 (1960).
28. J. A. Montgomery, T. P. Johnston and G. S. McCaleb, *J. Am. chem. Soc.* **81**, 3963 (1959).
29. A. Bendick, J. F. Tinker and G. B. Brown, *J. Am. chem. Soc.* **70**, 3109 (1948).
30. G. Huber, *Chem. Ber.* **90**, 698 (1957).
31. R. K. Robins, K. J. Dille, C. H. Willits and B. E. Christensen, *J. Am. chem. Soc.* **75**, 263 (1953).
32. A. Albert and D. J. Brown, *J. chem. Soc.* 2060 (1954).
33. S. Gabriel and J. Colman, *Chem. Ber.* **34**, 1234 (1901).
34. G. B. Elion and G. H. Hitchings, *J. Am. chem. Soc.* **78**, 3508 (1956).
35. J. Holguin and R. Cardinaud, *Eur. J. Biochem.* **54**, 505 (1975).
36. F. S. Okumura, Y. Kotani, T. Arica, M. Masumura and S. Kuraishi, *Bull. chem. Soc. Japan* **32**, 883 (1959).
37. R. L. Blakley, *Biochem. J.* **65**, 331 (1957).
38. J. Porath, K. Aspberg, H. Drevin and R. Axen, *J. Chromat.* **86**, 53 (1973).
39. P. Cuatrecasas, *J. biol. Chem.* **245**, 3059 (1970).
40. D. Singh, Y. Tamao and R. L. Blakley, *Adv. Enzyme Regulat.* **15**, 81 (1977).
41. R. L. Blakley, *Meth. Enzym.* **51**, 246 (1978).
42. G. E. Foley, H. Lazarus, S. Farber, B. G. Uzman, B. A. Boone and R. E. McCarthy, *Cancer, N.Y.* **18**, 522 (1965).
43. R. I. Geran, N. H. Greenberg, M. M. Macdonald, A. M. Schumacher and B. J. Abbott, *Cancer Chemother. Rep.* (Part 3), **3**, 17 (1972).
44. W. S. Beck and M. Levin, *J. biol. Chem.* **238**, 702 (1963).
45. C. Danzin and R. Cardinaud, *Eur. J. Biochem.* **48**, 255 (1974).
46. C. Danzin and R. Cardinaud, *Eur. J. Biochem.* **62**, 365 (1976).
47. W. Uerkvitz, *Eur. J. Biochem.* **23**, 387 (1971).
48. R. L. Blakley, *J. biol. Chem.* **240**, 2173 (1965).

49. R. H. Roush and R. F. Betz, *J. biol. Chem.* **233**, 261 (1958).
50. J. Holguin, R. Cardinaud and C. A. Salemink, *Eur. J. Biochem.* **54**, 515 (1975).
51. J. A. Montgomery and K. Hewson, *J. med. Chem.* **12**, 498 (1969).
52. L. F. Christensen, A. D. Broom, M. J. Robins and A. Bloch, *J. med. Chem.* **15**, 735 (1972).
53. G. A. LePage, I. G. Tunga and B. Bowman, *Cancer Res.* **24**, 835 (1964).
54. H. P. Baer, G. I. Drummond and E. L. Duncan, *Molec. Pharmac.* **2**, 67 (1966).
55. J. G. Cory and R. J. Suhadolnik, *Biochemistry* **4**, 1729 (1965).