MOLECULAR PHARMACOLOGY, 27:666–675

Inhibition of Utilization of Hypoxanthine and Guanine in Cells Treated with the Carbocyclic Analog of Adenosine

Phosphates of Carbocyclic Nucleoside Analogs as Inhibitors of Hypoxanthine (Guanine) Phosphoribosyltransferase

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Received February 12, 1985; Accepted March 25, 1985

SUMMARY

In cell cultures treated with the carbocyclic analog of adenosine (*C*-Ado, (±)-aristeromycin), the utilization of hypoxanthine and guanine has been observed to be blocked. In an attempt to define the mechanism of this inhibition, we have reexamined the metabolism of *C*-Ado and its effects on the metabolism of guanine and hypoxanthine. In cultures of L1210 cells, *C*-Ado at a concentration of 25 μM inhibited the utilization of hypoxanthine and guanine for nucleotide synthesis by more than 90% but produced little or no inhibition of the utilization of these bases in cultures of L1210/MeMPR cells which lack adenosine kinase and cannot phosphorylate *C*-Ado. In cultures of mammalian cells (L1210, HEp-2, and colon-26 cells), *C*-Ado was converted to the triphosphate (as previously observed) and also to the triphosphate of the carbocyclic analog of guanosine. The presence of coformycin in the medium at a concentration sufficient to inhibit AMP deaminase almost completely prevented the formation of carbocyclic GTP; thus, the deamination of *C*-Ado monophosphate is essential for the formation of phosphates of carbocyclic guanosine. Since hypoxanthine (guanine) phosphoribosyltransferase is known to be subject to end product inhibition, it was considered likely that phosphates of carbocyclic guanosine or carbocyclic inosine, present in *C*-Ado-treated cells, were responsible for inhibition of utilization of hypoxanthine and guanine. The 5′-phosphates of the carbocyclic analogs of inosine and guanosine were synthesized and found to be effective inhibitors of the phosphoribosyltransferase. Carbocyclic GMP was a better inhibitor than carbocyclic IMP and was also superior to GMP and IMP; the concentration of *C*-GMP that produced a 50% inhibition of GMP formation was approximately 1 μM. It is probable that the presence of phosphates of carbocyclic guanosine accounts for the inhibition of utilization of hypoxanthine and guanine in *C*-Ado-treated cells.

INTRODUCTION

The carbocyclic analog of adenosine (Fig. 1) was first synthesized by Shealy and Clayton (1) and later isolated (as an optically active isomer) from natural sources by Japanese workers (2, 3) who gave it the generally accepted name of aristeromycin. This adenosine analog was a substrate for adenosine kinase, and the triphosphate was the principal metabolite in cell cultures (4). *C*-Ado[1] was cytotoxic at low concentrations (4) and produced diverse biochemical effects, as illustrated by the observations that (*a*) *C*-Ado (presumably as its phosphates) was a strong inhibitor of purine synthesis *de novo* (4); (*b*) *C*-Ado-5′-phosphate was an inhibitor of GMP kinase (5); and (*c*) *C*-Ado as such (i.e., without phosphorylation) was a potent inhibitor of adenosylhomocysteine hydrolase (6). In addition, in our earlier studies, it was noted that in cells treated with *C*-Ado the

This work was supported by Grant CH-2Y from the American Cancer Society and by Public Health Service Grant 5 PO1-CA34200 awarded by the National Cancer Institute.

[1] The abbreviations used are: *C*-Ado, the carbocyclic analog of adenosine; *C*-Guo, the carbocyclic analog of guanosine; *C*-Ino, the carbocyclic analog of inosine; *C*-AMP, *C*-ADP and *C*-ATP, 5′-mono-, di-, and triphosphates of *C*-Ado; *C*-GMP, *C*-GDP, *C*-GTP, 5′-mono-, di-, and triphosphates of *C*-Guo; *C*-IMP, 5′-phosphate of *C*-Ino; H(G)PRT, hypoxanthine (guanine) phosphoribosyltransferase; HPLC, high pressure liquid chromatography; PRPP, 5-phosphoribosyl-1-pyrophosphate.

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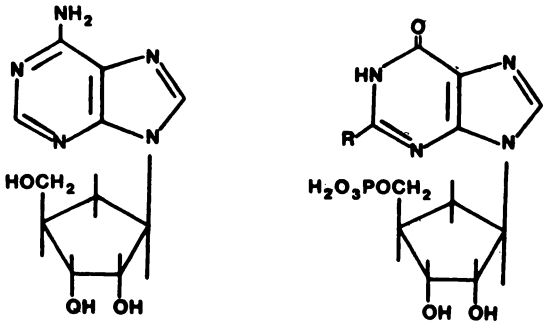

FIG. 1. *Structures of C-Ado, C-IMP, and C-GMP*

utilization of guanine and hypoxanthine was almost completely blocked (5). This action of *C*-Ado has never been rationalized. In an attempt to do so, we have reinvestigated the metabolism of *C*-Ado and its effects on the metabolism of guanine and hypoxanthine. We present here evidence that *C*-Ado, in addition to being metabolized to phosphates of *C*-Ado, is also metabolized to phosphates of *C*-Guo and that *C*-GMP is a good inhibitor of H(G)PRT (EC 2.4.2.8). *C*-GMP therefore may be the metabolite that is responsible for the blockade of metabolism of guanine and hypoxanthine in *C*-Ado-treated cells. A preliminary report of some of these results has been presented (7).

MATERIALS AND METHODS

C-IMP and C-GMP. These nucleotide analogs were synthesized as described below and were characterized by the data summarized with the procedures.

Carbocyclic analog of 2',3'-isopropylideneguanosine. A mixture of 3 ml of dry acetone (dried over $MgSO_4$), 0.64 ml of triethyl orthoformate, 210 mg of 4-toluenesulfonic acid monohydrate, and 81 mg of *C*-Guo (9) was stirred at 25° for 20 min. The mixture became homogeneous. Two additional 100-mg portions of *C*-Guo [total, 281 mg (1 mmol)] were added at intervals, and the resulting mixture was stirred at room temperature for 3 hr. The reaction mixture, containing a white solid, was poured into 9 ml of water containing 0.09 ml of concentrated ammonium hydroxide. Concentration of the solution gave a gelatinous white solid in three crops: total yield, 215 mg. The crude product was recrystallized from water [recovery, 150 mg; m.p., 255–263° dec. (capillary inserted at 240°)]. For analysis, the sample was dried for 2.5 hr at 0.1 mm Hg and at 78° over phosphorous pentoxide: TLC, 1 spot (54 μg applied; 4:1·ethanol/concentrated ammonium hydroxide as developing solvent, detection by UV light).

Analysis. Calculated for $C_{14}H_{19}N_5O_4 \cdot 4H_2O$: C, 42.74; H, 6.92; N, 17.81. Found: C, 42.81; H, 5.36; N, 17.40.

Carbocyclic analog of GMP. A solution of 1.5 ml of trimethyl phosphate, 0.061 ml (0.66 mmol) of phosphorous oxychloride, and 107 mg (0.33 mmol) of the isopropylidene derivative of *C*-Guo was prepared at −10°, stirred for 3 hr at −5°, and poured into 25 ml of water. The resulting solution was allowed to stand at 25° for 1 hr, heated at 78° for 1 hr, and cooled to 25°. The pH of the solution was adjusted to pH 2 with 3 N aqueous ammonia, and this solution was poured onto a column of a cation exchange resin (4 g of Amberlite CG-120, H^+ form). The column was washed with 75 ml of water and then with 50 ml of 3 N aqueous ammonia. The water washings were discarded; the ammonia effluent was concentrated *in vacuo* at 38° to about 2 ml. The concentrated solution was stored at 5°, and a white precipitate was collected by filtration, dried *in vacuo* at room temperature, and identified by TLC and its melting point as *C*-Guo: weight, 20 mg (19% recovery). The filtrate was evaporated (at 38°) to dryness *in vacuo*, the white residue was triturated with ethanol (3 ml), and the mixture was stored at 5°. The white solid was collected by filtration, washed with cold ethanol, and dried *in vacuo* over phosphorus pentoxide at room temperature: weight, 53 mg. This material was dissolved in a mixture of water, ethanol, and aqueous ammonia (0.03 ml of 6 N), the solution was filtered, the filtrate was concentrated to a white solid, and the residue was dried at 0.1 mm Hg (for 15 min at 78° and for 46 hr at room temperature) over phosphorus pentoxide: weight, 46 mg. This material was used for the biochemical studies; HPLC showed that it was a mixture consisting of 84% *C*-GMP and 16% *C*-Guo.

A 30-mg portion of this material was chromatographed on a small column of a cation exchange resin (9 g of Bio-Rad, H^+ form, AG 50W-X4) with 0.1 N aqueous ammonia as the developing and eluting solvent. Thirty 13-ml fractions were collected, the elution being monitored by UV absorbance at 250 nm. Additional *C*-Guo (4 mg; total recovery, 21.5%) was recovered by combining fractions 23–25 and evaporating the solution to dryness *in vacuo*. Fractions 6–17 were combined, the solution was concentrated *in vacuo* at 42° to a solid residue, ethanol was added to and evaporated from the residue, and the white solid was dried at room temperature (0.1 mm Hg) for 20 hr: weight of *C*-GMP, 20 mg; TLC, 1 spot (20 μg applied to a plate of silica gel, 5:3:2 butanol/water/acetic acid, detection by UV light). [The amounts of *C*-Guo and *C*-GMP separated by chromatography are in good agreement with the HPLC analysis (84:16) of the specimen used for the biochemical studies.] Analysis of the isolated *C*-GMP by HPLC on a column of Partisil-10 SAX, as outlined below, gave a value of 99.4%. For further analysis, the sample was dried *in vacuo* at 78° over phosphorus pentoxide for 4 hr: UV_{max} of 255 (ε 11,600) and 279 nm (ε 7,800) at pH 1, 253 (ε 12,700) and 268 nm (shoulder) at pH 7, and 258 (shoulder) and 268 nm (ε 11,000) at pH 13; mass spectra by the fast atom bombardment method: positive mode, *m/z* 362 $(M + 1)^+$; negative mode, *m/z* 360 $(M - 1)^-$. The UV data, with values of ε calculated for the presence of ethanol and water in the amounts indicated by the microanalytical data (below), are in good agreement with the UV data for *C*-Guo (9).

Analysis. Calculated for $C_{11}H_{16}N_5O_7P \cdot 0.3C_2H_5OH \cdot 0.4H_2O$: C, 36.44; H, 4.90; N, 18.32. Found: C, 36.51; H, 4.76; N, 18.15.

Isopropylidene derivative of the carbocyclic analog of inosine. A mixture of 25 ml of dry acetone (dried over $MgSO_4$), 0.085 ml of 2,2-dimethoxypropane, a catalytic amount of perchloric acid (0.5 ml of a solution of 0.11 ml of perchloric acid in 10 ml of acetone), and 60 mg of *C*-Ino was stirred at room temperature overnight. Additional perchloric acid (0.11 ml) was added to the stirring mixture, which became homogeneous. After 2 hr, 0.3 ml of triethylamine was added, and the mixture was concentrated *in vacuo* to about 1 ml of solution. The solution was applied to a thick plate of silica gel (Whatman PLK5F, 1-mm thickness). The chromatogram was developed with chloroform/methanol (3:1), the UV-absorbing band was removed, and the product was extracted from the silica gel with methanol. The methanol was evaporated *in vacuo*, the residue was mixed with ethanol (25 ml), the mixture was filtered to remove a small amount of silica gel, and the filtrate was concentrated *in vacuo* to a colorless syrup. The residue was stirred with ethyl acetate (12 ml), and the white solid that formed was collected by filtration and dried: weight of crude isopropylidene *C*-Ino, 59 mg; TLC, 1 spot (40 μg applied to a plate of silica gel, 3:1 chloroform/methanol as developing solvent, detection by UV light). A specimen of crude material (77 mg) from another run was recrystallized from 1:1 ethanol/ethyl acetate; recovery, 14 mg; m.p., 205–215° dec. (capillary inserted at 170°); TLC, 1 spot; UV_{max} of 250 nm (ε 11,300) at pH 1, 249 nm (ε 12,200) at pH 7, 254 nm (ε 13,200) at pH 13 (values of ε calculated for 1:2 ethanol/isopropylidene *C*-Ino).

Analysis. Calculated for $C_{14}H_{18}N_4O_4 \cdot \frac{1}{2}C_2H_5OH$: C, 54.69; H, 6.43; N, 17.01. Found: C, 54.59; H, 6.20; N, 17.02.

Carbocyclic analog of inosine 5'-phosphate. The isopropylidene derivative of *C*-Ino (109 mg, 0.33 mmol as 2:1 *C*-Ino/ethanol) was added to

a solution, previously cooled to −10°, of 0.12 ml of phosphorous oxychloride and 1.5 ml of trimethyl phosphate. The mixture became homogeneous during 10 min of stirring at 5°. The reaction solution was stirred at −5° for 3.5 hr and then poured into water. The resulting solution was allowed to stand at room temperature for 1 hr, heated at 78° for 1 hr, and cooled to room temperature. The pH of this solution was adjusted to pH 1.5, and the solution was poured onto a column of the formate form of an anion exchange resin (35 ml of Dowex 1-X8, formate). The column was washed successively with water (100 ml), 0.1 N formic acid (400 ml), and 100-ml portions of 0.3, 1, 3, and 5 N formic acid. The effluent obtained by washing the column with the last four portions of formic acid was concentrated to dryness *in vacuo*, the residue was triturated with ethanol, the mixture was kept at −20° for 2 hr, and the resulting white solid was collected by filtration and dried *in vacuo* during 18 hr over phosphorus pentoxide: weight of *C*-IMP, 31 mg; TLC, 1 spot (silica gel plate, 5:3:2 butanol/water/acetic acid as developing solvent, detection by UV light); UV_{max} of 251 nm (ε 11,200) at pH 1, 249 nm (ε 12,400) at pH 7, 254 nm (ε 13,000) at pH 13; mass spectra by the fast atom bombardment method: positive mode + 4-toluenesulfonic acid, *m/z* 347 $(M + 1)^+$; negative mode, *m/z* 345 $(M - 1)^-$. The UV data, with values of ε calculated for a hydrate (analysis, 1:4 *C*-IMP/H_2O), are in good agreement with the UV data for *C*-Ino (8). Analysis by HPLC on a column of Partisil-10 SAX, as outlined below, gave a value of 99.4%.

Analysis. Calculated for $C_{11}H_{15}N_4O_7P \cdot 4H_2O$: C, 31.58; H, 5.54; N, 13.39. Found: C, 31.30; H, 4.60; N, 13.27.

A second portion of *C*-IMP was obtained by concentrating the ethanol filtrate (above) to an oily solid, triturating the residue with ethanol/hexane (1:1), collecting the white solid by filtration: weight after drying *in vacuo* at room temperature, 38 mg. Mass spectral, UV, and TLC data were comparable to those listed above: HPLC analysis, 99%.

Other materials. [8-^{14}C]Hypoxanthine (56 mCi/mmol), [8-^{14}C]guanine (57 mCi/mmol), and [8-^{14}C]adenine (59 mCi/mmol) were obtained from Moravek Biochemicals, Brea, CA. *C*-Ado (1) and the racemic carbocyclic analogs of inosine (*C*-Ino) (8) and guanosine (*C*-Guo) (9) were synthesized as described earlier. Synthetic *C*-Ado is the racemate and is to be distinguished from the naturally occurring aristeromycin which is the 1′*R*, 2′*S*, 3′*R*, 4′*R*-isomer (nucleoside numbering). Coformycin was a gift from Warner-Lambert/Parke-Davis Laboratories, Detroit, MI. Alkaline phosphatase from *Escherichia coli* was obtained from Sigma Chemical Co., St. Louis, MO.

Cell cultures. HEp-2 cells and mouse colon-26 tumor cells were grown in SRI-14 medium (10), and L1210 cells in Fischer's medium (11). A subline of L1210 cells was selected for resistance to 6-(methylmercapto)purine ribonucleoside by methods described earlier for isolation of a similar mutant of HEp-2 cells (12); this subline, designated L1210/MeMPR was essentially devoid of adenosine kinase activity (results not shown). The parent L1210 line is designated L1210/0 to distinguish it from the mutant. For metabolic studies, cells were grown in suspension cultures and used for experiments when the cells were in logarithmic growth at a concentration of about 10^5 cells/ml.

High pressure liquid chromatography. HPLC analyses were performed with a Waters Associates (Milford, MA) model 202 apparatus. For analysis of nucleotides, a Partisil-10 SAX anion exchange column (Whatman, Inc., Clifton, NJ) was used with a linear gradient from 5 mM $NH_4H_2PO_4$, pH 2.8, to 750 mM $NH_4H_2PO_4$, pH 3.8, at a flow rate of 2 ml/min. For analysis of nucleosides by reversed phase chromatography, a Spherisorb ODS (5-μ) column [4.6 × 250 mm (Alltech Associates, Deerfield, IL)] was used; the eluant was 25 mM $NH_4H_2PO_4$ (pH 3.5)/acetonitrile (95:5, v/v), and the flow rate was 1 ml/min. Integration of peak areas was accomplished with a Hewlett-Packard model 3380-A digital electronic integrator.

Phosphoribosyltransferase assays. The enzyme preparation was a 100,000 × *g* supernatant fraction (from HEp-2 cells) that had been dialyzed overnight against 0.05 M (pH 7.5) Tris. The standard reaction mixture contained, in a final volume of 0.2 ml, 8 nmol of $Mg_2PRPP \cdot 2H_2O$, 8 nmol of $MgCl_2$, 2 nmol of [8-^{14}C]hypoxanthine, or 2 nmol of [8-^{14}C]guanine (0.1 μCi). In the inhibition studies, concentrations of inhibitor and substrates were varied as indicated in the tables. After incubation, the reaction was stopped by addition of EDTA in excess of the Mg^{2+} concentration. Base and nucleotide were separated by chromatography on paper with 95% ethanol/1 M ammonium acetate, pH 7.5 (7:3, v/v), as the solvent. The area containing nucleotides (IMP or GMP) was cut out and assayed for ^{14}C in a Packard liquid scintillation spectrometer (Downers Grove, IL).

RESULTS

Metabolism of C-Ado. When HEp-2 cells or L1210 cells were grown in the presence of *C*-Ado, two new peaks appeared in the triphosphate area between ATP and GTP (Fig. 2). Colon-26 cells showed similar nucleotide profiles (results not shown). The new peaks in the triphosphate area, and the ATP and GTP peaks, were scanned with a Perkin-Elmer stop flow scanner; Fig. 3 shows the results with the peaks found in L1210 cells. The new triphosphate eluting just after ATP had an ultraviolet absorption spectrum indistinguishable from that of the ATP peak, and that eluting just before GTP had an absorption spectrum indistinguishable from that of the GTP peak. These spectra are the same as those of known ATP and GTP at this pH. These peaks were therefore tentatively identified as *C*-ATP and *C*-GTP. To obtain further evidence for the presence of derivatives of both *C*-Ado and *C*-Guo, a sample of the entire cell extract was treated overnight with alkaline phosphatase after which the reaction was terminated by immersion of the incubation mixture in a boiling water bath. The mixture was centrifuged, the supernatant solution was lyophilized, and the resulting residue was taken up in water and subjected to reversed phase chromatography (Fig. 4). In addition to the natural nucleosides (Urd, Cyd, Ado, and Guo), the chromatogram showed the presence of two new peaks with retention times of 6.9 and 7.5 min (Fig. 4, B and C). These were the retention times of synthetic samples of *C*-Ado and *C*-Guo as determined in a separate assay (Fig. 4A). These peaks, when scanned, had UV absorption spectra indistinguishable from those of adenosine and guanosine and from those of the two new triphosphate peaks scanned in Fig. 3 (results not shown). To further establish the identities of these peaks as *C*-Ado and *C*-Guo, samples of known *C*-Ado and *C*-Guo were added to the phosphatase-treated fraction prior to reversed phase chromatography; these additions reinforced peaks at 6.9 and 7.5 min and led to no new peaks (Fig. 4D). In another experiment, the triphosphate peaks (peaks with retention times of 30–37 min) were collected as a single fraction. This fraction was then passed through a column of charcoal (Norite) to adsorb the nucleotides and remove buffer salts. The nucleotides were then eluted from the charcoal with ammoniacal ethanol; the extract was concentrated to dryness and taken up in H_2O. The solution was treated overnight with alkaline phosphatase, and the resulting nucleoside mixture was analyzed by reversed phase chromatography as described above. The resulting chromatograms (not shown) were similar to those of Fig. 4C; peaks with retention times of *C*-Ado and *C*-Guo were present. Thus, the new peaks present on the initial chromatograms (Fig.

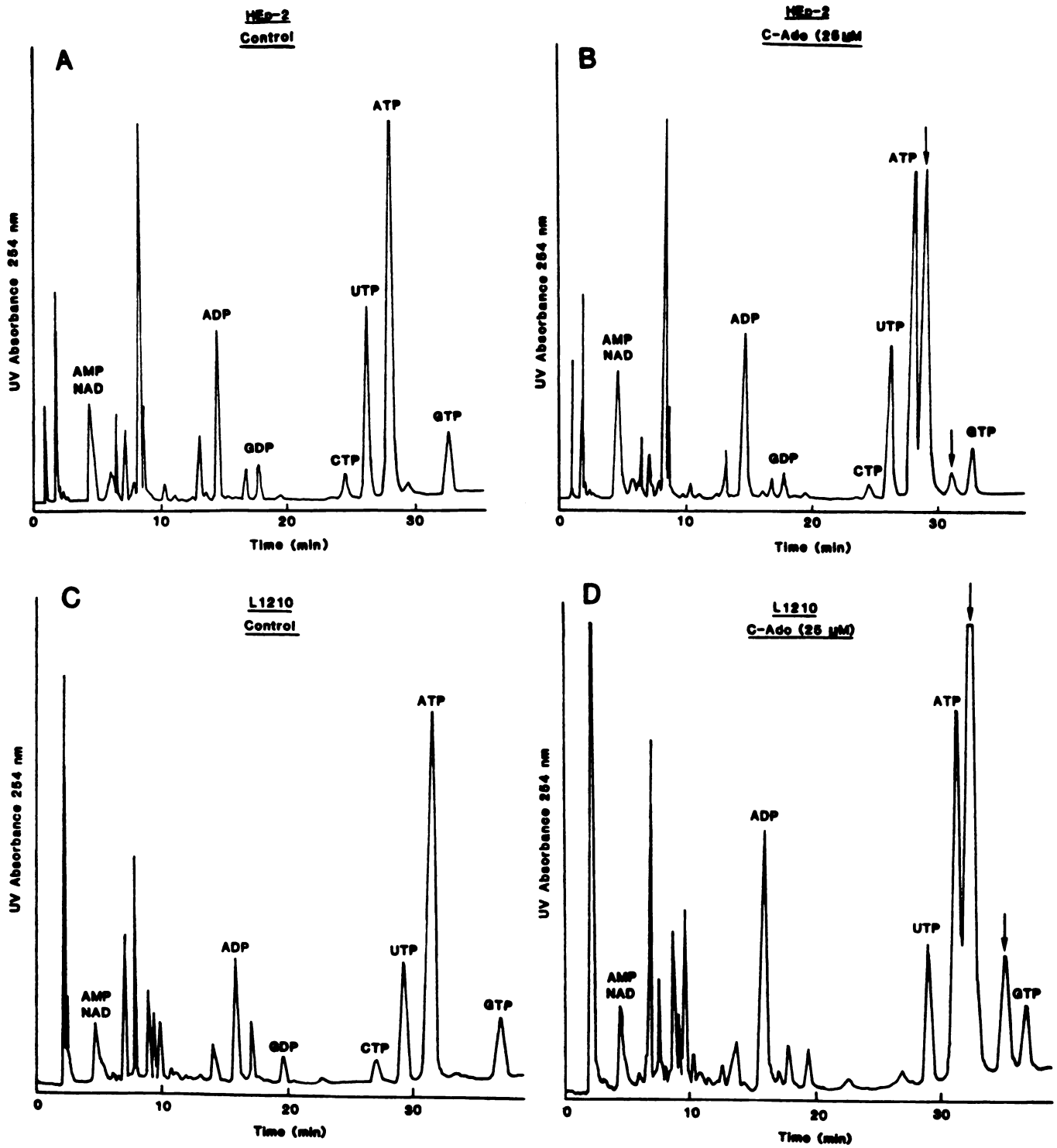

FIG. 2. *Metabolism of C-Ado by L1210 cells and HEp-2 cells*

C-Ado was added to exponentially growing suspension cultures at a concentration of 25 μM. Control cultures were grown concurrently from the same batches of cells as the treated cultures. After 4 hr, the cells were harvested and washed free of medium. Perchloric acid extracts of the cells were prepared and assayed by HPLC as described in the text. New peaks appearing in the *C*-Ado-treated cultures are marked with arrows.

2) apparently are *C*-AdoTP and *C*-GuoTP. New peaks present in HEp-2 cells and colon-26 cells were characterized similarly, but less extensively, and with similar results.

In L1210 cells exposed to *C*-Ado, peaks in the monophosphate area (retention times of approximately 2 and 7 min) and the ADP peak (retention time of approximately 16 min) were increased (Fig. 2, C and D); these increases probably represent respectively *C*-AMP, *C*-IMP, *C*-GMP, and *C*-ADP. Because of the poor resolution in these areas, no attempt was made to identify or quantitate these peaks.

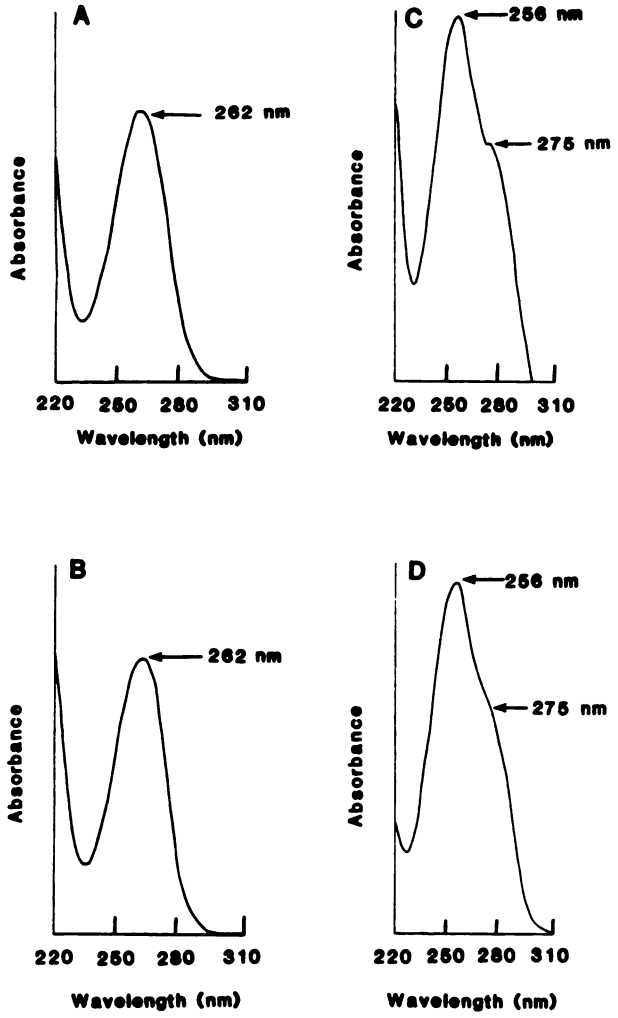

FIG. 3. *Ultraviolet absorption spectra of the peaks appearing in the triphosphate area of chromatograms of C-Ado-treated L1210 cells*

The peaks appearing between 30 and 37 min in the chromatogram of Fig. 2D were subjected to stop flow scanning in a Perkin-Elmer model LC-75 scanner. A, ATP peak (retention time, 30.8 min); B, peak eluting just after ATP (retention time; 31.8 min): C, peak eluting just before GTP peak (retention time, 34.7 min); D, GTP peak (retention time, 36.3 min).

Since *C*-Ino had been shown to be metabolically inert (4), it appeared that the formation of *C*-GTP must proceed via deamination of *C*-AMP and subsequent metabolism of *C*-IMP to *C*-GMP. To obtain direct evidence on this point, experiments were performed with coformycin, which, at appropriate concentrations, has been shown to inhibit AMP deaminase (13). Preliminary experiments were performed to determine the amounts required for strong inhibition of the deamination of AMP under the conditions of our experiments. In these determinations, [^{14}C]adenine was used; it was determined that a concentration of 35 μM coformycin produced a >75% reduction of label in GTP. When this concentration was used in experiments with *C*-Ado, the results shown in Fig. 5 were obtained. Coformycin produced an increase in the AMP-NAD and ADP peaks and caused changes in two peaks (unidentified) eluting just after AMP-NAD; however, there were no significant alterations in the triphosphates

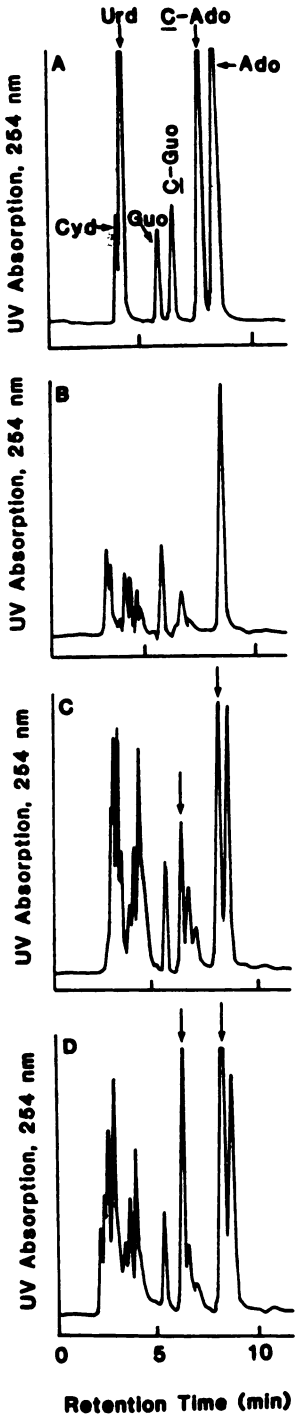

FIG. 4. *Reversed phase chromatography of phosphatase-treated nucleotides from C-Ado-treated L1210 cells*

Extracts of untreated (Fig. 2C) and *C*-Ado-treated (Fig. 2D) L1210 cells were incubated overnight with alkaline phosphatase and the resulting mixtures were subjected to reversed phase chromatography (see text). A, standards: natural nucleosides, *C*-Ado, and *C*-Guo; B, extracts from control cells; C, extracts from cells treated with *C*-Ado (25 μM) for 4 hr; D, extracts from *C*-Ado-treated cells to which *C*-Ado and *C*-Guo were added prior to reversed phase chromatography. Arrows mark new peaks appearing in samples from *C*-Ado-treated cells.

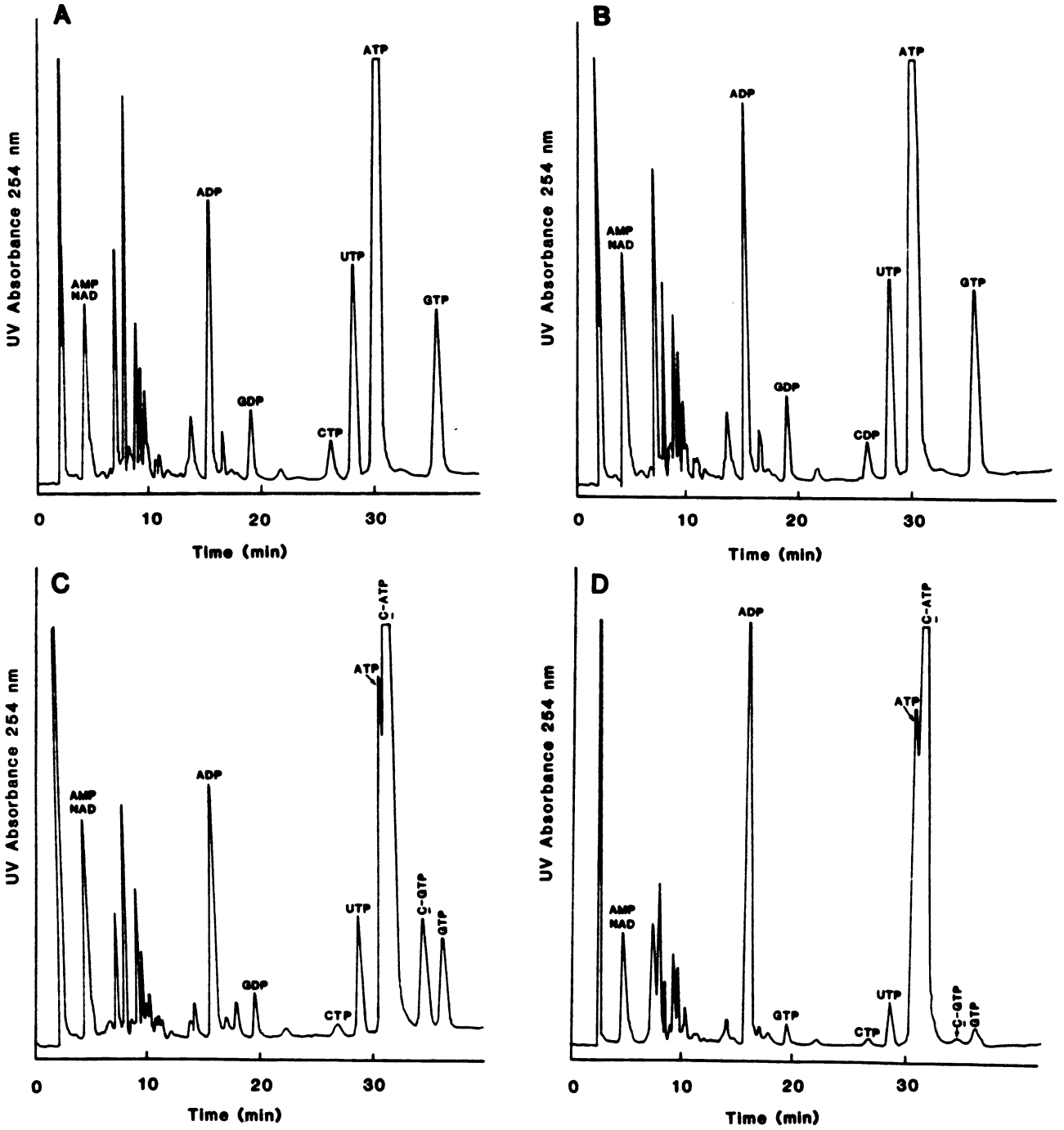

FIG. 5. *Effects of coformycin on the metabolism of C-Ado in L1210 cells*

The experiments were performed as described in Fig. 2 except for the addition of corformycin: A, control cells; B, cells grown in the presence of coformycin (35 μM); C, cells grown in the presence of *C*-Ado (25 μM); D, cells grown in the presence of *C*-Ado (25 μM) and coformycin (35 μM).

(Fig. 5, A and B). *C*-Ado alone produced the expected peaks of *C*-ATP and *C*-GTP (Fig. 5C). When coformycin was present, the pool of *C*-GTP was decreased whereas that of *C*-ATP was little affected (Fig. 5D). It is also to be noted that the combination of *C*-Ado and coformycin decreased the pool of GTP below that in cells treated with *C*-Ado alone; the mechanism of this apparent synergism has not yet been investigated further.

Time relationships in the metabolism of C-Ado and effects on pools of natural nucleotides (Table 1). L1210 cells were grown in the presence of *C*-Ado and the nucleotide pools were determined at times through 24 hr. The concentration of *C*-ATP reached a peak at 2 hr and that of *C*-GTP at 4 hr. After 24 hr, there were still substantial concentrations of both triphosphates (20–25% of peak concentrations). At 2 hr, the concentration of *C*-ATP exceeded that of ATP and remained at a higher level through 24 hr; for example, at 4 hr, the concentrations (nanomoles/10^9 cells) were 1142 for ATP and 1720 for *C*-AdoTP. The concentration of *C*-GTP was greater

TABLE 1

Time study of the metabolism and metabolic effects of C-Ado in L1210 cells

C-Ado was added to exponentially growing L1210 cells to a final concentration in the medium of 10 μM. At the times shown in column 1, the cells were harvested. Extracts were prepared for analysis by HPLC which was performed as described in the text and in Fig. 2. Separate controls were used for each time. Pools of the natural nucleotides represent the sum of the phosphates that were resolved: for adenosine nucleotides, AMP + ADP + ATP; guanine nucleotides, GDP + GTP; uridine nucleotides, UTP; cytidine nucleotides, CTP. The pool sizes (nanomoles/10^9 cells) for 4-hr control cultures (representative of control cultures) were: adenine, 2680; guanine, 670; uracil, 550; cytidine, 290.

Time	Nucleotide pool				*C*-ATP	*C*-GTP
	Adenine	Guanine	Uracil	Cytosine		
hr	*% control*				*nmol/10^9 cells*	
1	72	58	52	50	1274	146
2	68	54	59	33	1878	391
4	43	32	60	27	1720	659
8	38	32	63	20	1190	457
13	54	55	75	42	986	410
24	48	45	66	59	403	175

TABLE 2

Effects of C-Ado on the uptake of purines by L1210 cells

C-Ado was added to exponentially growing suspension cultures of L1210 cells 0.5 hr before the addition of the labeled purine (25 nCi/ml of medium). The cells were harvested and washed free of medium, and the cell pellet was assayed for radioactivity. The values shown are the counts in 10^6 treated cells expressed as percentages of those in control cells. The values for control cells (cpm/10^6 cells) were: for L1210/0, [8-^{14}C]adenine, 23,315; [8-^{14}C]hypoxanthine, 31,500; [8-^{14}C]guanine, 5,115; for L1210/MeMPR, [8-^{14}C]adenine, 32,575; [8-^{14}C]hypoxanthine, 17,857; [8-^{14}C]guanine, 6,442. These are the same experiments for which nucleotide profiles are shown in Figs. 6 and 8.

Concentration of *C*-Ado	Uptake		
	[8-^{14}C]Adenine	[8-^{14}C]Hypoxanthine	[8-^{14}C]Guanine
μM	*% control*		
L1210/0			
10	79	37	34
25	57	7	9
50	59	3	4
L1210/MeMPR			
25	95	122	73

than that of GTP from 2 to 13 hr, but was about the same as that of GTP at 24 hr. The pools of nucleotides of natural bases were depressed within 1 hr and reached a minimum at 4–8 hr, after which they began to recover; however, at 24 hr, they were still well below controls. The greatest effect was on the cytidine nucleotides; this seemingly greater effect may be due to the low concentration of CTP in control cells and the consequently large error in determining it when it is lowered.

Effects of C-Ado on utilization of purines. Table 2 presents data on the effects of *C*-Ado on the incorporation of adenine, guanine, and hypoxanthine into the acid-insoluble fraction of L1210 cells. At a concentration of 10 μM, the utilization of hypoxanthine and guanine was reduced to about 35% of controls, whereas that of adenine was reduced to about 80% of controls. Higher concentrations of *C*-Ado (25 and 50 μM) reduced the incorporation of hypoxanthine and guanine to less than 10% of controls and that of adenine to about 60% of controls. A more detailed examination of effects on uptake of purines is presented in Fig. 6 which shows the effects of *C*-Ado (25 μM) on the conversion of guanine and hypoxanthine to nucleotides. *C*-Ado strongly depressed the incorporation of [^{14}C]guanine into mono-, di-, and triphosphates (Fig. 6, A and B). [^{14}C]Hypoxanthine labeled both adenine and guanine nucleotides, and *C*-Ado depressed incorporation into all of the nucleotides (Fig. 6, C and D). Similar experiments were also performed with L1210/MeMPR cells. Since *C*-Ado was expected not to be phosphorylated in these cells that are deficient in adenosine kinase, these experiments were designed to show whether the observed effects on the utilization of hypoxanthine and guanine might be due to inhibition by *C*-Ado of the transport of these bases. As a preliminary to these experiments, the metabolism of *C*-Ado in L1210/MeMPR cells was determined with the purpose of eliminating the possibility that *C*-Ado might be phosphorylated by a kinase other than adenosine kinase. The HPLC analysis (Fig. 7) showed no peaks between ATP and GTP where *C*-ATP

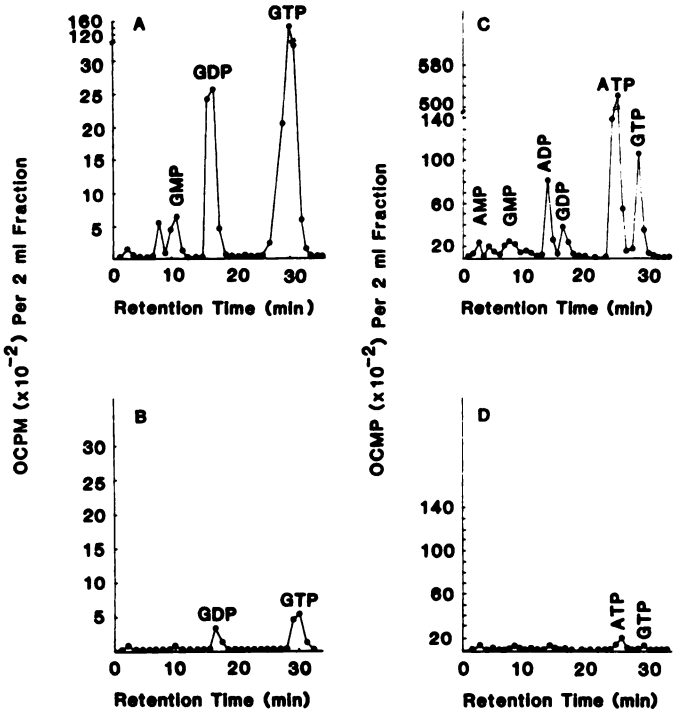

FIG. 6. *Effects of C-Ado on the conversion of guanine and hypoxanthine to nucleotides in L1210 cells*

To cells in suspension culture, *C*-Ado was added at a concentration of 25 μM, followed 0.5 hr thereafter by [8-^{14}C]guanine or [8-^{14}C]hypoxanthine (25 nCi/ml of medium). One hr after addition of the labeled compound, the cells were harvested and an extract was prepared and subjected to HPLC on an anion exchange column as described in the text and in Fig. 2. Fractions of 2 ml were collected and assayed for ^{14}C in a Packard liquid scintillation spectrometer. A, [^{14}C]guanine, control cells; B, [^{14}C]guanine, *C*-Ado-treated cells; C, [^{14}C]hypoxanthine, control cells; D, [^{14}C]hypoxanthine, *C*-Ado-treated cells. *OCPM*, observed counts per minute. The identification of the peaks of radioactivity were assigned on the basis of peaks of ultraviolet absorption (not shown) and the known patterns of the natural nucleotides (Fig. 2).

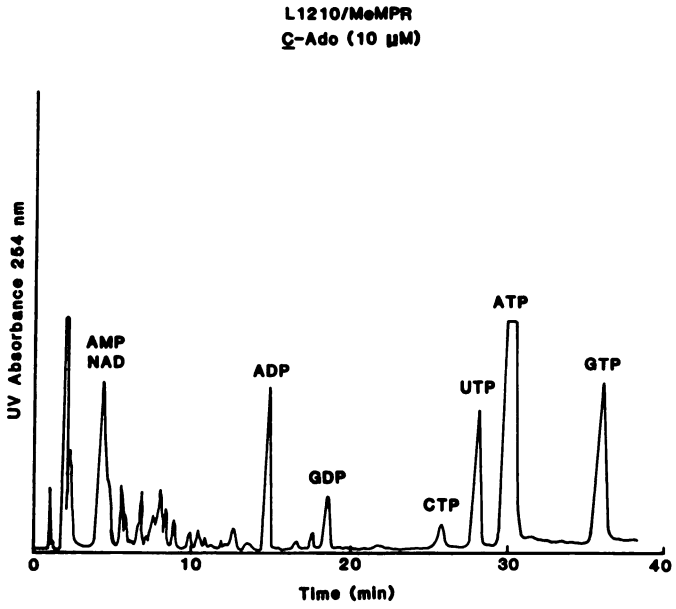

FIG. 7. *Nucleotide profile of L1210/MeMPR cells grown in the presence of C-Ado*

The experimental conditions were the same as those in Fig. 2D, except that L1210/MeMPR cells, rather than L1210/0 cells, were used.

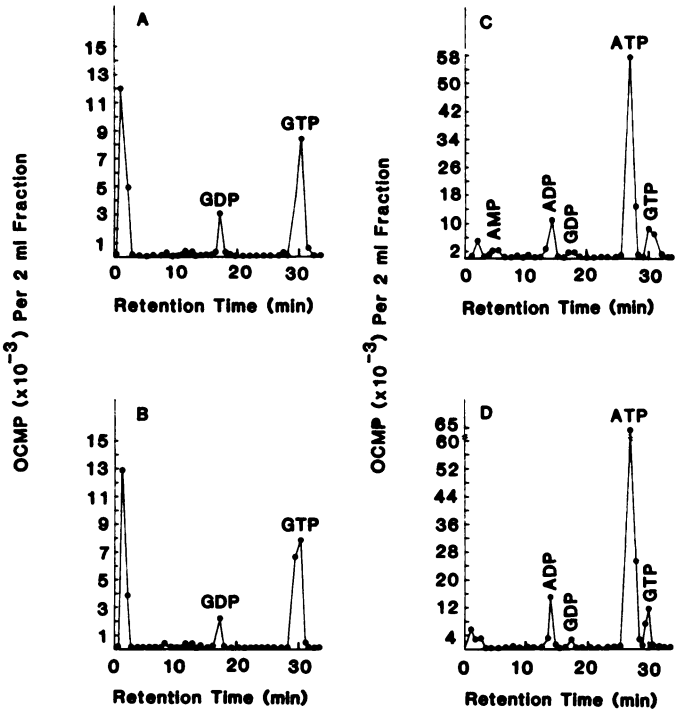

FIG. 8. *Effects of C-Ado on the conversion of guanine and hypoxanthine to nucleotides in L1210/MeMPR cells*

The amounts of labeled guanine and hypoxanthine and the experimental conditions were the same as those in Figs. 6 and 7. A, [^{14}C] guanine, control cells; B, [^{14}C]guanine, *C*-Ado-treated cells; C, [^{14}C] hypoxanthine, control cells; D, [^{14}C]hypoxanthine, *C*-Ado-treated cells.

and *C*-GTP would appear. The nucleotide profile of these *C*-Ado-treated L1210/MeMPR cells was in fact almost identical with that of control L1210/0 cells (Fig. 2C). In L1210/MeMPR cells, *C*-Ado caused some decrease in the overall uptake of guanine (Table 2) and in its conversion to nucleotides (Fig. 8), but these effects were small in comparison with those in L1210/0 cells. No inhibitory effects were observed on the utilization of adenine or hypoxanthine (Table 2, Fig. 8). Thus, these results indicate that the blockade of the utilization of guanine and hypoxanthine observed in L1210/0 cells requires phosphorylation of *C*-Ado and cannot be attributed to interference by *C*-Ado with the entry of these bases into the cell.

Inhibition of H(G)PRT. The synthetic samples of *C*-IMP and *C*-GMP (see above) were assayed by HPLC on a Partisil-10 SAX anion exchange column prior to their study as inhibitors. By this criterion, *C*-IMP had a purity greater than 99%. The initial sample of *C*-GMP contained 16% *C*-Guo. This sample was used as such in some studies with allowance made for the nucleoside content. Subsequently, a specimen of *C*-GMP free of *C*-Guo was obtained by ion exchange chromatography. *C*-IMP and *C*-GMP were compared with IMP and GMP as inhibitors of H(G)PRT from HEp-2 cells (Table 3). Both *C*-IMP and *C*-GMP were effective inhibitors, but *C*-GMP inhibited at concentrations 1 order of magnitude less than the concentration of *C*-IMP required for equivalent in-

TABLE 3

Inhibition of H(G)PRT by IMP, GMP, C-IMP, and C-GMP

Reaction conditions[a]	Concentration for 50% inhibition of rate of formation		
	IMP	*C*-IMP	Ratio *C*-IMP/IMP
		μM	
IMP formation			
Standard	9	14	1.6
E + PRPP + I[b]	10	15	1.5
E + Hyp + I[b]	16	27	1.7
E + I[b]	13	18	1.4
	GMP	*C*-GMP	Ratio *C*-GMP/GMP
GMP formation			
Standard	3	0.87	0.29
E + PRPP + I[b]	5	0.75	0.15
E + Gua + I[b]	5	0.60	0.12
E + I	7	1.2	0.17

[a] The standard reaction mixture consisted of enzyme, [^{14}C]hypoxanthine, or [^{14}C]guanine, and the inhibitors at various concentrations. Incubation was for 20 min at 37° (see text for details of assay).

[b] Preincubation of enzyme with indicated substrates and inhibitor (I) for 30 min prior to completion of reaction mixture and initiation of the enzyme reaction.

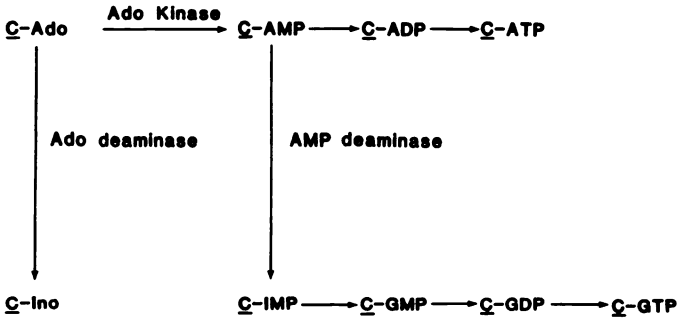

FIG. 9. *Pathways of the metabolism of C-Ado*

hibition. *C*-IMP was less effective than IMP as an inhibitor, whereas *C*-GMP was more effective than GMP. The concentration of *C*-GMP required for 50% inhibition was about 1 μM. Preincubation of the enzyme with substrates or inhibitors did not change significantly the magnitude of inhibition. Under all conditions, the concentration of *C*-GMP required for 50% inhibition of GMP formation was 2–7% the concentration of *C*-IMP required for 50% inhibition of IMP formation.

DISCUSSION

The identification of both *C*-ATP and *C*-GTP as metabolites of *C*-Ado, together with earlier observations that *C*-Ado is a substrate for adenosine kinase (4), indicates that *C*-Ado is metabolized along the same pathways as adenosine (Fig. 9). Although only the triphosphates of the carbocyclic analogs were identified, the monophosphates (carbocyclic analogs of AMP, IMP, XMP, and GMP) and the diphosphates (carbocyclic analogs of ADP and GDP) are obligatory intermediates in their formation. In Fig. 9, the conversion of *C*-AMP to *C*-IMP is shown; although this conversion has not been demonstrated directly, it, rather than the alternate pathway *C*-Ado → *C*-Ino → *C*-IMP, is the probable route of formation of *C*-IMP. Evidence for this conclusion is that (*a*) although *C*-Ado is a substrate for adenosine deaminase, *C*-Ino is itself not phosphorylated in L1210 or HEp-2 cells and is without inhibitory activity (4), and (*b*) coformycin, at a concentration known to inhibit the conversion of AMP to IMP in intact cells, severely reduced the amount of *C*-GTP found in *C*-Ado-treated cells (Fig. 5).

In our earlier work, we noted the profound inhibition by *C*-Ado of the utilization of guanine and hypoxanthine (5) but were unable to rationalize it in terms of the metabolites of *C*-Ado (phosphates of *C*-Ado only) that were identified at that time. The observation that *C*-GTP was also a metabolite of *C*-Ado, when considered together with the fact that IMP and GMP exert strong end product inhibition of H(G)PRT (14), offered a possible explanation: the inhibition of utilization of guanine and hypoxanthine might be due to inhibition of H(G)PRT by *C*-IMP, *C*-GMP, or both. These two nucleotide analogs were prepared and found, in fact, to be good inhibitors of H(G)PRT (Table 3). The guanine analog in particular was an effective inhibitor, and therefore would be the metabolite most likely to account for the observed inhibition in cells treated with *C*-Ado. These facts, of course, do not prove that this is the mechanism by which utilization of hypoxanthine and guanine is restricted or inhibited but they do constitute strong presumptive evidence for this mechanism. The utilization of guanine and hypoxanthine was inhibited severely as compared to inhibition of utilization of adenine (Table 2), and in *C*-Ado-treated cells exogenous guanine and hypoxanthine were excluded from nucleotide pools without the concomitant accumulation of any metabolites of these bases (Fig. 6). These facts could only be explained by an inhibition of transport or by an inhibition of phosphoribosylation. Inhibition of transport by *C*-Ado is excluded by the failure of *C*-Ado to block utilization of these bases in L1210/MeMPR cells, which do not have capacity to phosphorylate *C*-Ado (Table 2, Fig. 8). This inhibition then must be the result of inhibition of the formation of IMP and GMP which occurs by the H(G)PRT-catalyzed reaction. Since *C*-GMP and *C*-IMP were not isolated, the question arises as to whether they are present in *C*-Ado-treated cells in sufficient quantity to produce the observed inhibitions. This question cannot be answered from the available data, except to note that the quantity of the GMP analog required for inhibition of H(G)PRT (approximately 1 μM for 50% inhibition) would be below the limit of detection in our HPLC analyses. Also to be noted is the fact that the natural nucleotides, IMP and GMP, would also be present and contribute to inhibition of H(G)PRT. Thus, in *C*-Ado-treated cells the H(G)PRT activity would be controlled by the combined pools of IMP, GMP, *C*-IMP, and *C*-GMP.

In addition to rationalizing the effects of *C*-Ado on metabolism of hypoxanthine and guanine, the inhibition of H(G)PRT by *C*-GMP is of considerable interest in itself. Many attempts have been made to develop inhibitors of H(G)PRT, but no really potent inhibitors are known (15–21). In fact, no inhibitor has been reported that is as active as is GMP itself. Since *C*-GMP is more active than GMP, it is the best of the known inhibitors of this enzyme. In mammalian cells, H(G)PRT represents a salvage pathway of purine synthesis; hence, inhibition of H(G)PRT would not be a mechanism of cytotoxicity. However, many parasites are dependent on preformed purines for synthesis of purine nucleotides (22) and, hence, inhibitors of this and other salvage enzymes are of potential importance in the therapy of diseases caused by such parasites. *C*-Ado itself is not a good candidate as a therapeutic agent for parasites because it is toxic to mammalian cells. However, *Leishmania* have been shown to phosphorylate allopurinol ribonucleoside, which is not phosphorylated by mammalian cells (23), and this observation suggests that nucleoside analogs, such as *C*-Ino and *C*-Guo, which are inert in mammalian cells (4), might be activated in parasites. Also, although salvage pathways are not essential to mammalian cells, effective and specific inhibitors of adenine phosphoribosyltransferase and H(G)PRT would be of some interest as metabolic tools in the study of mammalian purine metabolism, for example, in studying reutilization of purines.

With respect to elucidation of the mode of action of *C*-Ado, the fact that nucleotides of both *C*-Ado and *C*-Guo are metabolites makes more difficult the designation of one site of action as being responsible for cytotoxicity. The reduction of pools of both adenine and guanine nucleotides in *C*-Ado-treated cells probably is the result of feedback inhibition of synthesis *de novo*, a previously observed effect (4). That sites of inhibition other than the purine pathway are involved is shown by our inability to prevent or reverse the cytotoxicity of *C*-Ado by purines (5), and the marked effects of *C*-Ado on pools of CTP (Table 2). It would be predicted, in fact, that the considerable pools of *C*-ATP and *C*-GTP that are formed could produce effects at multiple metabolic sites.

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