Metabolic Studies with Carbocyclic Analogs of Purine Nucleosides

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SUMMARY

In carbocyclic analogs of nucleosides a methylene group replaces the ring oxygen atom of the ribofuranosyl moiety. When evaluated for cytotoxicity against H. Ep. #2 cells in culture, the carbocyclic analog of adenosine (C-Ado) was highly cytotoxic (ED_{50} 0.7 μM), whereas the carbocyclic analogs of inosine, 6-mercaptopurine ribonucleoside, and 6-methylthiopurine ribonucleoside were not cytotoxic at concentrations up to 150 μM. Sublines of H. Ep. #2 cells that lacked adenosine kinase were resistant to C-Ado, but the degree of resistance was less than that to other cytotoxic adenosine analogs. In H. Ep. #2 cells in culture C-Ado inhibited an early step of *de novo* purine biosynthesis, as determined by its ability to reduce the amount of formylglycinamide derivatives accumulating in azaserine-treated cells.

In crude cell-free supernatants from H. Ep. #2 cells, C-Ado interfered with the phosphorylation of 6-methylthiopurine ribonucleoside; but none of the four carbocyclic analogs interfered with the phosphorylation or deamination of adenosine or the cleavage of inosine to hypoxanthine. In crude supernatants supplemented with ATP and Mg^{2+}, C-Ado was phosphorylated and, to some extent, deaminated. In intact cells ^{3}H-C-Ado was converted to mono-, di-, and triphosphates and, to a small extent, deaminated; no compound migrating like NAD was detected, and little radioactivity was found in polynucleotides. C-Ado was a substrate for a partially purified adenosine kinase from H. Ep. #2 cells ($K_m = 7.8 \times 10^{-5}$ M) and for calf intestinal adenosine deaminase ($K_m = 3.3 \times 10^{-3}$ M).

INTRODUCTION

Carbocyclic analogs of purine nucleosides are compounds with the structure shown below, in which a methylene group replaces the O-atom of the ribofuranosyl ring of a nucleoside. Many analogs of purine nucleosides have potent biological activity (1–3); the carbocyclic compounds are, therefore, of interest as a new type differing from others in that the labile glycosidic linkage has been replaced by a stable C-N bond. Having this bond, the carbocyclic analogs should not be subject to biochemical conversions proceeding via removal of
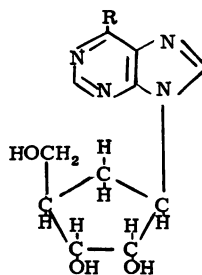
I.   R = NH$_2$
II. R = OH
III. R = SH
IV. R = SCH$_3$

the sugar; however, since they still closely resemble the parent nucleosides, they might be expected to be substrates for enzymes acting at positions other than the glycosidic bond. We report here studies with carbocyclic analogs of two natural nucleosides, adenosine (I) and inosine (II), and of two

cytotoxic nucleosides, 6-MP-ribonucleoside (III) and 6-MeMP-ribonucleoside (IV).[2] A preliminary report of some of these observations has appeared (4).

MATERIAL AND METHODS

Compounds. Dr. Y. F. Shealy and Mr. J. D. Clayton provided samples of 9-[β-DL-2α,3α-dihydroxy-4-β-(hydroxymethyl)-cyclopentyl]adenine (C-Ado[2]), synthesized as described elsewhere (5), and also samples of the carbocyclic analogs of inosine, 6-MP-ribonucleoside, and 6-MeMP-ribonucleoside, prepared by similar procedures.[3] Tritiation of a sample of C-Ado was performed by New England Nuclear Corporation by catalytic (Pt on charcoal) exchange between C-Ado and ^3H_2O. The crude product was purified and freed of labile tritium by repeated equilibration with H_2O and by crystallization; recrystallization to constant specific activity yielded a final product with an activity of 51.8 mC/μmole. The position of labeling with ^3H was not determined; however, tritiation of nucleosides by this procedure is known to give labeling in the purine ring after labile ^3H has been removed. ^{35}S-6-MeMP-ribonucleoside was prepared as described earlier (6). The following compounds or preparations were obtained from the indicated sources: sodium formate-^{14}C, New England Nuclear Corporation, Boston, Massachusetts; adenosine-8-^{14}C and inosine-8-^{14}C, Schwarz BioResearch, Inc., Orangeburg, New York; ATP-γ-^{32}P, Nuclear-Chicago, Des Plaines, Illinois; calf intestinal adenosine deaminase, Sigma Chemical Co., St. Louis, Missouri; crude snake venom (*Crotalus atrox*), Ross Allen's Reptile Institute, Silver Springs, Florida; azaserine, Dr. John Dice, Parke, Davis, and Company, Detroit, Michigan, through the Cancer Chemotherapy National Service Center.

Cell cultures. The cell cultures used in these studies were the H. Ep. #2 line

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originally isolated by Moore *et al.* (7) and two resistant sublines, one designated H. Ep. #2/MeMPR and one designated H. Ep. #2/FA/FAR. The H. Ep. #2/MeMPR line, which was selected for resistance to 6-MeMP-ribonucleoside, is without adenosine kinase activity. The H. Ep. #2/FA/FAR line was selected for resistance first to 2-fluoroadenine and then to 2-fluoroadenosine, and is without activities of AMP pyrophosphorylase and adenosine kinase. The parent cell line is designated H. Ep. #2/S to distinguish it from the resistant cell lines. Earlier papers have described the isolation and characterization of the resistant cell lines (8) and the method used for growing cells in either static or suspension cultures (9). Cytotoxicities of candidate compounds were determined by cloning procedures: 100 cells were placed in 4-ounce prescription bottles containing SRI-14 medium (9) and varying amounts of inhibitor, and the number of macroscopic colonies that formed were counted 7–10 days later (8).

Autoradiography of ^3H-labeled compounds. For autoradiography of chromatograms containing ^3H-C-Ado and its metabolites, the technique described in a bulletin from Schwarz BioResearch (10) was used. In this procedure the chromatogram and X-ray film (Eastman Kodak, single emulsion, blue sensitive) were placed in contact in a bath consisting of organic scintillators in toluene, a solution used conventionally for assays in our Packard Tri-Carb spectrometers. After storage in the dark for the desired period of time, the film was removed from the bath and developed as usual. This method makes possible, within several days, the detection of ^3H-labeled compounds that would be detected only after several weeks if the film were exposed to the chromatogram in the dry state.

Enzyme assays. Assay of C-Ado as a substrate for adenosine deaminase was determined by the measurement of the rate of conversion of ^3H-C-Ado to the corresponding analog of inosine. For assay of its activity as a substrate for adenosine kinase, a 175-fold purified enzyme from H. Ep. #2 cells was used (11), and the reaction was

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[2] Abbreviations: 6-MP, 6-mercaptopurine; 6-MeMP, 6-methylthiopurine; C-Ado, carbocyclic analog of adenosine.

[3] Y. F. Shealy and J. D. Clayton, paper in preparation.

measured by the conversion of unlabeled C-Ado to the phosphate derivative upon incubation with the enzyme in the presence of ATP-γ-^{32}P and Mg^{2+}. Further details of these assays are given in Figs. 2 and 3. Radioactive substrates were used for assays of the effects of carbocyclic analogs on nucleoside-metabolizing enzymes; the methods are described in Table 6.

Metabolism of C-Ado in intact cells. ^{3}H-C-Ado was added to a suspension culture of H. Ep. #2 cells (0.5 to 1 × 10^7 cells/ml) to a final concentration of 1 μg/ml (0.2 μC/ml). At 4 or 24 hr thereafter the cells were harvested and washed twice with 0.9% NaCl, after which the cells were suspended in 10 ml of water and poured into 40 ml of boiling ethanol. The water-soluble portion of the ethanolic extract was subjected to chromatography on paper, either two-dimensionally, using the phenol-H_2O and butanol-propionic acid solvents described in Table 2, or one-dimensionally in butanol-propionic acid. Radioactive compounds were located by autoradiography, and the areas of paper containing radioactivity were cut out and assayed in a Packard

Tri-Carb liquid scintillation counter. From the residue remaining after the ethanol extraction, sodium nucleates were isolated by extraction with hot 10% NaCl and assayed for radioactivity. The nucleates were then treated with 1.0 N KOH at 37°, the mixture was neutralized with $HClO_4$, and $KClO_4$ was removed by centrifugation. The supernatant was acidified with HCl to pH 1 and centrifuged to remove DNA. The resulting supernatant containing RNA nucleotides was subjected to paper chromatography, and areas containing ^{3}H were located by autoradiography; these methods are described in detail elsewhere (12).

Inhibition of purine synthesis de novo. For measurement of the effectiveness of C-Ado in inhibiting *de novo* purine synthesis, cells were grown in the presence of azaserine and formate-^{14}C, and the amount of ^{14}C-labeled formylglycinamide ribonucleotide accumulating in the presence and absence of C-Ado was determined. The methods used were previously described modifications (12–14) of methods described by LePage and Jones (15), and Henderson (16); further details are given in Table 2.

TABLE 1
Inhibition of cell cultures by analogs of purine nucleosides

The inhibitory concentration given is that inhibiting colony formation by 50% or more; for conditions of assay and characteristics of the cell lines, see text. Each value is derived from several independent experiments. The values given for 6-mercaptopurine ribonucleoside and 6-methylthiopurine ribonucleoside are taken from earlier experiments (3).

Compound	Inhibitory concentration (μM)		
	H. Ep. #2/S	H. Ep. #2/ FA/FAR	H. Ep. #2/ MeMPR
6-Mercaptopurine ribonucleoside	0.6	0.5–1.0	0.5–1.0
6-Methylthiopurine ribonucleoside	1.0	>300	>300
Carbocyclic analog of:			
Adenosine	0.7	20[a]	20[a]
Inosine	>150	>150	>150
6-Mercaptopurine ribonucleoside	>150	>150	>150
6-Methylthiopurine ribonucleoside	>150	>150	>150

[a] In some experiments there was some degree of inhibition of the two resistant cell lines by C-Ado at lower concentrations, but the 50% inhibitory concentrations were poorly reproducible. The given value is a concentration that consistently inhibited by more than 50%.

Cytotoxicities of Carbocyclic Analogs (Table 1)

Of the four carbocyclic analogs assayed, only C-Ado was cytotoxic to H. Ep. #2 cells, and its cytotoxic concentration was about the same as that of the ribonucleosides of 6-MP and 6-MeMP. The carbocyclic analogs of inosine, 6-MP-ribonucleoside, and 6-MeMP-ribonucleoside were not inhibitory at concentrations 200-fold the cytotoxic concentration of C-Ado. Two sublines of H. Ep. #2 cells lacking adenosine kinase and resistant to adenosine analogs (8) were not inhibited by concentrations of C-Ado up to 30-fold greater than that inhibiting the parent line. Concentrations of 20 μM or greater did, however, inhibit both resistant lines. In contrast, these cell lines were shown earlier to

TABLE 2
Inhibition by C-Ado and adenosine of the azaserine-induced accumulation of formylglycinamide derivatives in H. Ep. #2/S Cells

To suspension cultures of H. Ep. #2/S cells (4×10⁷ in 100 ml of medium) azaserine was added to a concentration of 58 μM, followed 30 min later by adenosine or C-Ado. Thirty minutes after addition of adenosine or C-Ado, 25 μC of sodium formate-¹⁴C (sp. act. 151 mC/mmole) was added. Cells were harvested 2 hr after addition of formate, washed twice with 0.9% NaCl, and extracted with boiling 80% ethanol. The extract was lyophilized to dryness and taken up in H_2O. The H_2O-soluble portion was subjected to two-dimensional paper chromatography first in aqueous phenol (72% v/v), then in butanol: propionic acid:H_2O [equal volumes of 93.8% aqueous 1-butanol (v/v) and 44% aqueous propionic acid (v/v)]. The radioactive areas were located by autoradiography, cut out, and assayed in a Packard liquid scintillation spectrometer.

Inhibitor	Cpm in FGAR + FGA-ribonucleoside[a]
Control	65,400
+Adenosine (3.7 μM)	30,100
+Adenosine (7.4 μM)	1,300
+C-Ado (7.4 μM)	39,900
+C-Ado (15 μM)	20,300
+C-Ado (37 μM)	700

[a] Abbreviations: FGA, formylglycinamide; FGAR, formylglycinamide ribonucleotide.

have very high (>300-fold) resistance to many other adenosine analogs (8).

Inhibition of Purine Synthesis de novo (Table 2)

C-Ado was an effective inhibitor of the azaserine-induced accumulation of formylglycinamide ribonucleotide, but comparable inhibition required greater amounts of C-Ado than of adenosine. A comparison with previously published results (14) indicates that the amount of C-Ado that was required for inhibition was also larger than those required for inhibition by 2-fluoroadenosine, 7-deazaadenosine, and 8-azaadenosine.

Metabolism of C-Ado

In intact cells C-Ado was converted almost exclusively to nucleotides; the polyphosphates accounted for 75% of the total soluble intracellular activity (Fig. 1, Table 3). There was little unmetabolized

TABLE 3
Metabolism of ³H-C-Ado in H. Ep. #2/S cells

Suspension cultures of H. Ep. #2/S cells were grown for 24 hours in medium containing ³H-C-Ado at a concentration of 3.6 μM. See text and Table 2 for methods of isolation and assay of radioactive metabolites and Table 4 for identification of metabolites. The di- and triphosphates are grouped together because they were incompletely separated and because some triphosphate was degraded to diphosphate during chromatography.

Metabolite	Cpm/10⁶ cells	Percentage of total cpm
Di- and triphosphates of C-Ado	1412	75
C-Ado-monophosphate	320	17
C-Ado	65	3.6
Carbocyclic analog of inosine	49	2.5
Unidentified materials	39	2.0

C-Ado in the cells, and the degree of deamination was small (2.5%). No degradation product other than the deamination product was found, and there was no detectable activity in the area of the chromatogram to which NAD migrates. In crude cell-free preparations supple-

TABLE 4
Chromatographic and electrophoretic migration of C-Ado, metabolites of C-Ado, and related compounds

Compound or mixture	R_f values in solvent[a]				Electrophoretic migrations in buffer[b] (cm)	
	A	B	C	D	E	F
AMP	0.18	0.44	0.26		+3.5	+15.5
ATP	0.05	0.24	0.40		+11.0	+12.5
C-Ado phosphates	0.05, 0.19	0.26, 0.45	0.20, 0.34		+2.5; +9.1	+12.4
Adenosine	0.60	0.76	0.16	0.52	—	—
C-Ado	0.56	0.82	0.12	0.50	−6.0	+6.3
C-Ado phosphates after treatment with snake venom	0.56	0.76	0.11	0.45	—	—
Carbocyclic analog of inosine	0.38	0.72	0.38	0.73	—	—
Deamination product of C-Ado[c]	0.38	0.72	0.34	0.68	—	—

[a] Chromatographic separations were made on Whatman No. 1 or Schleicher-Schuell white paper. The compositions of the solvents were: (A) butanol-propionic acid (see Table 2); (B) 2, 2, 3, 3-tetrafluoro-1-propanol, H_2O, 90% formic acid [25:15:0.5 (v:v:v)] (39); (C) 0.1 M sodium phosphate, pH 6.8, solid $(NH_4)_2$ SO_4, n-propanol [100:60:2 (v:w:v)] (40); (D) 16.4 g ammonium bicarbonate in 100 ml H_2O.

[b] Electrophorectic separations were carried out on Whatman No. 1 paper under the following conditions and in buffers of the following composition: (E) 0.05 M formate; pH 3.6; 2000 V for 60 min, (F) 0.05 M borate; pH 9.2; 1500 V for 60 min.

[c] Product formed upon incubation of C-Ado with cell-free supernatants from H. Ep. #2 cells.

mented with ATP and Mg^{2+}, the metabolism of C-Ado was similar to that in intact cells, except that relatively more deamination occurred. Data on the identification of the metabolites are presented in Table 4. The phosphates were identified by their migration like the corresponding adenosine phosphates upon two-dimensional chromatography (Fig. 1), upon chromatography in three one-dimensional solvents, upon electrophoresis in two buffers, and also by their conversion to C-Ado upon incubation with crude snake venom (Table 4). Additional evidence for the formation of a monophosphate is that when C-Ado was incubated with ATP-γ-^{32}P, Mg^{2+}, and adenosine kinase (Table 5), a ^{32}P-labeled compound was formed which migrated to the position expected for AMP. In the identifications summarized in Table 4 no attempt was made to differentiate between the di- and triphosphates of C-Ado other than by their migration in the two-dimensional solvents, because experience with ATP has shown that there is always some conversion to ADP during chromatography.

When the ^{3}H-labeled phosphates were

treated with snake venom and the resulting mixture was chromatographed on paper, ^{3}H-C-Ado was the only radioactive product detectable. The butanol–propionic acid solvent system used separates C-Ado from its deamination product, and also from guanosine and xanthosine. The carbocyclic analogs of guanosine and xanthosine were not available for study; but since C-Ado and its deamination product migrate much like adenosine and inosine, it would be expected that the carbocyclic analogs of guanosine and xanthosine would also migrate like the natural nucleosides. The absence of radioactivity from these areas of the chromatograms therefore indicates that only phosphates of C-Ado were present on the initial two-dimensional chromatogram.

The crude polynucleotide fraction (DNA and RNA) isolated from H. Ep. #2 cells grown for 24 hr in the presence of ^{3}H-C-Ado had a specific activity of 0.008 μC/mg. If one assumes that all the ^{3}H represents ^{3}H-C-Ado incorporated into the polynucleotides, then the maximum incorporation would represent a replacement of one out of each 2000 adenosine moieties. The amount
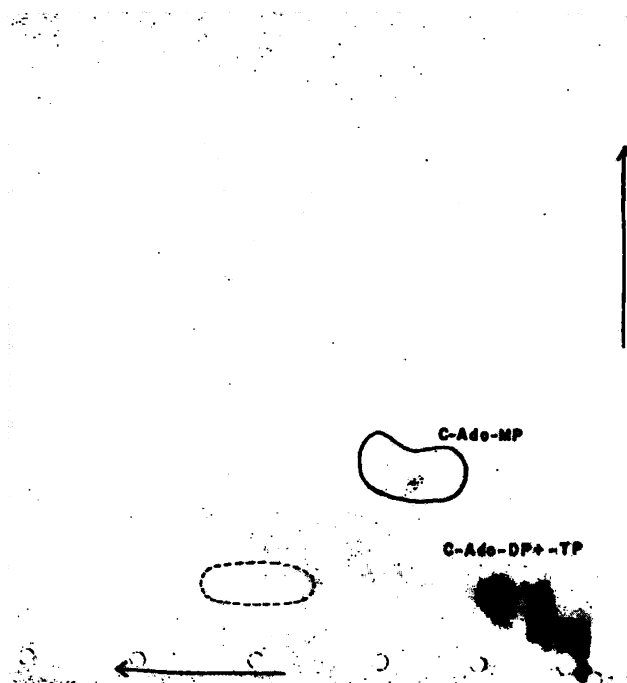
Fig. 1. *Metabolism of* ^3H-*C-Ado by H. Ep. #2/S cells*

The figure is a reproduction of the autoradiogram prepared from the chromatogram from which the data of Table 3 were obtained. The area to which NAD migrates is shown by the dotted line to emphasize the absence of detectable radioactivity in this area. Abbreviations: MP = monophosphate; DP = diphosphate; TP = triphosphate.

of ^{3}H associated with polynucleotides was so small that ^{3}H-labeled C-Ado-monophosphate, if in fact present, could not be identified in the alkaline hydrolyzate of the polynucleotides.

C-Ado as a Substrate for Adenosine Kinase and Adenosine Deaminase

C-Ado was a substrate for a partially purified adenosine kinase prepared from H. Ep. #2 cells and free of adenosine deaminase and AMP kinase activities (11) (Table 5). The K_m was 7.8 × 10^{-5} M (Fig. 2) as compared to K_m values of 1.8 × 10^{-6} M for adenosine and 5.0 × 10^{-5} M for 6-MeMP-ribonucleoside that have been obtained with this enzyme system (11). The rate of phosphorylation of C-Ado was less than those of adenosine and 6-methylthiopurine ribonucleoside, of the same order as that of 2-aminoadenosine, and greater than those of 2'-deoxyadenosine, 3'-deoxyadenosine, and arabinosyladenine. The phos-

phorylation of the carbocyclic analog of 6-MeMP-ribonucleoside was at the borderline of detection by the assay used; at best the phosphorylation of this analog relative to 6-MeMP-ribonucleoside was much poorer than was the phosphorylation of C-Ado relative to adenosine.

C-Ado was also a substrate for calf intestinal adenosine deaminase (Fig. 3). The K_m was approximately 100-fold greater than that for adenosine.

Effects of Carbocyclic Analogs on the Metabolism of Nucleosides

Table 6 presents data on the effects of each of the four carbocyclic analogs on the metabolism of adenosine, 6-MeMP-ribonucleoside, and inosine by crude, cell-free supernatants from H. Ep. #2 cells. When present at a concentration 10-fold that of the substrate, C-Ado decreased the rate of phosphorylation of 6-MeMP-ribonucleoside, but was without effect on the phos-

phorylation of adenosine; these findings are in accord with the fact that the K_m value for C-Ado is about the same as that for 6-MeMP-ribonucleoside but much larger than that for adenosine. None of the analogs inhibited the deamination of adenosine or the cleavage of inosine to hypoxanthine.

TABLE 5
Phosphorylation of nucleosides and analogs by adenosine kinase

The enzyme used was purified 175-fold from H. Ep. #2/S cells. Nucleotide formation was determined by assay of the ^{32}P-monophosphate formed when the nucleoside or analog was incubated with ATP-γ-^{32}P, Mg^{2+}, and enzyme (6.4 μg) for 30 minutes. The procedures for the purification of the enzyme and for the assay are described in an earlier paper (11), from which were also taken the values in the table for the substrates other than the carbocyclic analogs.

Substrate	Nucleotide formed (nanomoles/min/mg protein)
Adenosine	148
C-Ado	62
6-Methylthiopurine ribonucleoside	487
Carbocyclic analog of 6-methylthiopurine ribonucleoside	10[a]
2-Aminoadenosine	54
9-β-D-Arabinofuranosyladenine	<10
2'-Deoxyadenosine	<10
3'-Deoxyadenosine	18

[a] This value is just above the limit of detection, and therefore it is questionable if this analog is phosphorylated.

DISCUSSION

In the carbocyclic analogs of nucleosides the C-N linkage between the cyclopentane and purine rings is a stable bond that should not be subject to the action of nucleoside phosphorylases or hydrolases or otherwise be broken easily. The metabolic stability of this bond is indicated by the observations that (a) C-Ado was not degraded below the nucleoside level, and (b) the carbocyclic analog of 6-MP-ribonucleoside was not cytotoxic, as it would have been had cleavage to 6-MP occurred. Al-
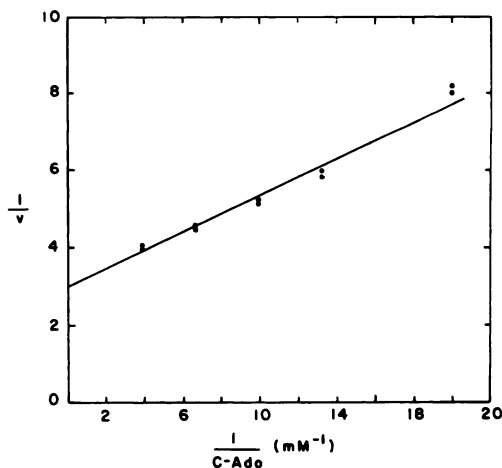

FIG. 2. *Lineweaver-Burk plot for determination of the K_m value for the phosphorylation of C-Ado by adenosine kinase from H. Ep. #2/S cells*

The enzyme was purified 220-fold. The reaction was stopped after 30 min by boiling, and the radioactive products were separated by paper chromatography in butanol-propionic acid. Experimental details are given in Table 5. Values for the ordinate are the reciprocals of the reaction rate in nanomoles per minute. The calculated K_m value was 7.8×10^{-5} M. The K_m value for the phosphorylation of adenosine, determined by the same procedure, was 1.8×10^{-4} M.

though the substitution of the carbocyclic ring for the ribofuranosyl ring protects the resulting analog from reactions proceeding via cleavage of the glycosidic bond, it still permits activity as a substrate for adenosine kinase and adenosine deaminase. Furthermore, the formation of the triphosphate of C-Ado in intact cells indicates that C-Ado-monophosphate is a substrate for a nucleotide kinase.

Results with other nucleoside analogs indicate that only those that can be converted intracellularly to nucleotides are markedly cytotoxic (8). If nucleotide formation is requisite for cytotoxicity, then it is not surprising that the carbocyclic analogs of 6-MP-ribonucleoside and inosine are not toxic, since inosine kinase is weak or absent in mammalian cells (for pertinent literature see references 8 and 11). However, the inactivity of the carbocyclic analog of 6-MeMP-ribonucleoside as a growth inhibitor and its essential inactivity as a
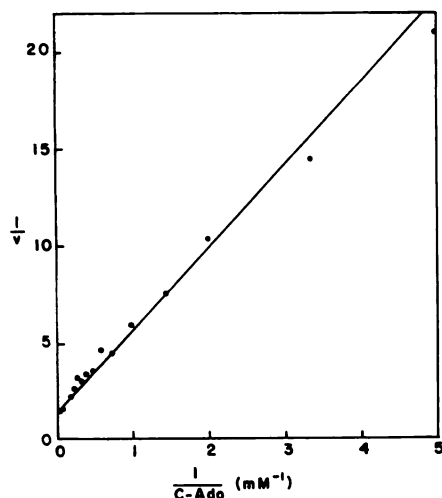
FIG. 3. *Lineweaver-Burk plot for determination of the K_m value for the deamination of C-Ado*

The incubation mixtures contained ^{3}H-C-Ado at the specified concentrations, Sigma calf intestinal adenosine deaminase (2 μg), and 5 μmoles of potassium phosphate buffer, pH 7.5, in a final volume of 0.2 ml. Incubation was for 5 min at 25°. The reaction was stopped by immersion into a boiling water bath for 1 min. The radioactive products were isolated by paper chromatography in butanol–propionic acid; radioassays were performed on a Packard model 7201 radiochromatogram scanner. Values for the ordinate are the reciprocals of the reaction rate in nanomoles per 5 min. The calculated K_m value was 3.3×10^{-3} M. The K_m for deamination of adenosine, determined with the same preparation and by the same procedures, was 3.6×10^{-3} M.

substrate for adenosine kinase are unexpected in view of the cytotoxicity and ready phosphorylation of 6-MeMP-ribonucleoside (8, 11, 17). The fact that the cell lines lacking adenosine kinase showed a considerable degree of resistance to C-Ado is evidence that a phosphate derivative is responsible, at least in part, for the observed cytotoxicity. But the fact that the degree of resistance was only 30-fold, as contrasted to resistance of >300-fold to other adenosine analogs (8), suggests that C-Ado as such may be toxic. Toxicity resulting from the direct action of C-Ado itself could, of course, not be detected in cells containing adenosine kinase and therefore capable of forming the more toxic

phosphate. In this regard, it is notable that a number of 9-alkylpurines, which cannot be converted to nucleotides, are toxic to mammalian cells (18), as is also 9-β-D-arabinofuranosyl-6-MP, which is neither phosphorylated nor cleaved (19). It is possible that the cytotoxic action of C-Ado in cells lacking adenosine kinase is similar to the action of these compounds.

The metabolism of C-Ado in H. Ep. #2 cells represents a pattern remarkable in its simplicity: the phosphates were almost the exclusive products. Although the triphosphate was formed in large amount, there was no detectable conversion to an NAD analog (in which C-Ado would replace adenosine). In contrast, 2-aminoadenosine in H. Ep. #2 cells (20) and 7-deazaadenosine in *Streptococcus faecalis* (21) are each anabolized to the triphosphate level, and each forms an NAD analog. It is notable that C-Ado-monophosphate apparently does not undergo the nucleotide interconversions characteristic of AMP, since the corresponding analogs of IMP, XMP, and GMP were not detected on chromatograms.

The metabolic block responsible for the cytotoxicity of C-Ado is a matter yet to be investigated fully. C-Ado was a moderately good inhibitor of purine synthesis *de novo*, presumably by a feedback-type inhibition that has been observed with many analogs of purines and nucleosides that can be converted intracellularly to nucleotides (for references, see reference 13). Although C-Ado produces this type of inhibition, the importance of this metabolic effect to growth inhibition cannot be evaluated in the absence of data on other possible sites of action. Incorporation into polynucleotides would appear to be an unlikely mechanism of action, because of the very small amount of ^{3}H from ^{3}H-C-Ado associated with polynucleotides.

Since C-Ado is a new type of purine nucleoside analog, the data of the present paper contribute to the already reported correlations of structure of nucleosides and their activities as substrates or inhibitors for adenosine kinase (11, 22) and adenosine deaminase (23–28). The data indicate that the O-atom of the ribonfuran-

TABLE 6

Effects of carbocyclic analogs of nucleosides on the metabolism of adenosine inosine and, 6-methylthiopurine ribonucleoside in cell-free supernatants from H. Ep. #2 cells.

For all determinations, the enzyme fraction was a crude 26,000 g supernatant from H. Ep. #2 cells. Each incubation mixture contained substrate (10^{-4} M), crude enzyme (5 mg protein), the carbocyclic compound at the indicated concentration, and potassium phosphate buffer (50 μmoles), pH 7.0, in a final volume of 1.0 ml. For assays of inosine phosphorylase and adenosine deaminase, there was no supplementation of the mixture; for assay of adenosine kinase, the mixture contained ATP (2.5 μmoles) and $MgCl_2$ (0.25 μmole). Incubation was for 3 min and the reactions were stopped by immersion of the incubation mixture in boiling water. Portions of the reaction mixtures were subjected to paper chromatography in butanol-propionic acid; and the radioactive areas were located and assayed for radioactivity (see Table 2 for details). The results given are for a single experiment and were confirmed in another experiment in which substrate concentrations and incubation times were different.

Enzyme activity measured	Substrate	Concentration of inhibitor (M)	Reaction rate: percent of control in presence of carbocyclic analog of			
			Adenosine	Inosine	6-MP-ribonucleoside	6-MeMP-ribonucleoside
Adenosine kinase	³⁵S-6-MeMP-ribonucleoside	10^{-4}	85	80	87	90
		10^{-3}	42	96	93	107
Adenosine kinase	Adenosine-8-¹⁴C	10^{-4}	100	98	107	118
		10^{-3}	82	112	126	159
Adenosine deaminase	Adenosine-8-¹⁴C	10^{-4}	100	100	99	99
		10^{-3}	101	99	98	96
Inosine phosphorylase	Inosine-8-¹⁴C	10^{-4}	100	100	100	100
		10^{-3}	100	101	99	101

osyl ring is important, but not essential, for substrate activity for these enzymes and also that a glycosidic linkage to the purine ring is not required. 4'-Thioadenosine, in which an S-atom replaces this O-atom, is also a substrate for adenosine kinase (22) and adenosine deaminase (23).

By definition, a carbocyclic analog of a nucleoside differs from the nucleoside only in the replacement of the ring O-atom of the ribofuranosyl group by a methylene group. Other than C-Ado, the only reported carbocyclic nucleoside analog is the analog of thymidine, which was synthesized by Murdock and Angier (29) and found to be without biological activity. However, a number of purine derivatives have been synthesized which have cyclopentyl, cyclohexyl, or substituted cyclopentyl or cyclohexyl groups at the 9-position and hence conceivably may function as nucleoside analogs (30–35). Many compounds of this type have biological activity. 9-Cyclopentyl and 9-cyclohexyl derivatives of adenine, hypoxanthine, and 6-MP inhibit growth of

H. Ep. #2 cells in culture (18); 9-cyclopentyl-6-MP has antitumor activity *in vivo* (36); and 9-cyclopentyladenine and 6-chloro-8-aza-9-cyclopentylpurine markedly inhibit the steroid-induced synthesis of Δ^5-3-ketosteroid isomerase in *Pseudomonas testosteroni* (37). In addition, a number of these compounds inhibit adenosine deaminase *in vitro* (25, 33–35, 38). The modes of action of these 9-cycloaliphatic derivatives are still unknown; however, since a number of 9-aliphatic derivatives also have similar biological activity, the activity of 9-cycloaliphatic derivatives may not be related closely to the structural similarity of the ribofuranosyl and cycloaliphatic rings.

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