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Differences in the Metabolism and Metabolic Effects of the Carbocyclic Adenosine Analogs, Neplanocin A and Aristeromycin

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SUMMARY

Neplanocin A and aristeromycin are carbocyclic adenosine analogs that differ only in that neplanocin A contains a double bond in the carbocyclic ring, whereas this ring in aristeromycin is saturated. We have compared the metabolism and some of the metabolic effects of neplanocin A and synthetic (±)-aristeromycin (*C*-Ado) in murine leukemia L1210 cells in culture. *C*-Ado, as shown earlier, was not only converted to its own phosphates but also was metabolized to phosphates of carbocyclic guanosine. Both rapidly proliferating and slowly proliferating or resting cells phosphorylated *C*-Ado, but *C*-Ado was not converted to phosphates of carbocyclic guanosine in detectable amounts in cells whose growth had reached a plateau. When the metabolism of neplanocin and *C*-Ado was examined in the same experiment, both analogs were converted to the triphosphate analogs of ATP; no conversion of neplanocin A to the corresponding carbocyclic analogs of guanine nucleotides was detected, whereas *C*-Ado was converted to the carbocyclic analog of GTP in amounts that approximated the GTP pool. This difference in metabolism was associated with a marked difference in effects of the two analogs on the utilization of hypoxanthine and guanine which was inhibited by *C*-Ado but not by neplanocin. The failure of neplanocin A to be converted to analogs of guanine nucleotides apparently is the result of poor capacity of its monophosphate to serve as a substrate for AMP deaminase; the V_{max} for deamination of neplanocin-5′-monophosphate by this enzyme was only 5% of that for *C*-Ado monophosphate. In contrast, neplanocin A was a better substrate than *C*-Ado for adenosine deaminase.

Np-A (Fig. 1) is a recently isolated, naturally occurring carbocyclic analog of adenosine (1, 2), which has proved to be of considerable interest because of its antiviral, antileukemic, and antimalarial activities and its potency as an inhibitor of adenosylhomocysteine hydrolase and of methylations (2–7). Np-A is closely related to another carbocyclic adenosine analog, aristeromycin, which was isolated from natural sources as an optically active isomer (8) shortly after it had been synthesized (9) as the racemate (*C*-Ado, (±)-aristeromycin). The two analogs differ only in that Np-A has a double bond in the carbocyclic ring, whereas this ring in aristeromycin is saturated (Fig. 1). One would expect that these two structurally related agents would have similar modes of action. Both *C*-Ado (10) and Np-A (5, 6, 11) are metabolized to their triphosphates, and *C*-Ado, like Np-A, is a potent inhibitor of adenosylhomocysteine hydrolase (12). *C*-Ado produces multiple metabolic effects (10, 13). Among these is the essentially complete blockade of the utilization of hypoxanthine and guanine (13, 14), which was rationalized by the observations that (a) *C*-Ado, in addition to being converted to phosphates of *C*-Ado, was also converted to phosphates of carbocyclic guanosine, and (b) that the carbocyclic analog of GMP was a good inhibitor of hypoxanthine (guanine) phosphoribosyltransferase (14). In the course of comparing Np-A and *C*-Ado we have found, as reported here, that Np-A was not converted to phosphates of the Np-A analog of guanosine in detectable amounts and that this failure can be ascribed to the fact that Np-A-5′-monophosphate, as compared to *C*-Ado-5′-monophosphate, is a poor substrate for AMP deaminase.

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Materials and Methods

Synthesis of Np-A-5′-monophosphate. A solution of Np-A (10 mg, 38 μmol) in triethyl phosphate (0.5 ml) under a nitrogen atmosphere was chilled in an ice bath. To this solution was added $POCl_3$ (70 mg, 42 μl, 12-fold excess) via syringe over a 1-min period and the mixture was stirred at 0° for 2.25 hr, by which time the reaction was complete as judged by HPLC analysis. The solution was diluted with ice water (5 ml), extracted with CH_2Cl_2 (twice in 5 ml, CH_2Cl_2 extracts discarded), and pumped to a dry, white residue at room temperature. This residue was applied to three cellulose thin layer plates (5 × 20 cm) and eluted with 2-propanol/H_2O/concentrated NH_4OH (65:25:10). Extraction of the product bands (R_f = 0.1) with 1:1 EtOH/H_2O, filtering, and removal of solvents gave a white solid, 5 mg (37% yield assuming ammonium salt had formed), R_f = 0.2 (CH_3CN/1 N NH_4OH, 65:35, silica gel). This solid gave a positive Hanes-Isherwood (15) test for the presence of monophosphates, and was a single peak by HPLC

ABBREVIATIONS: NP-A, neplanocin A; *C*-Ado, the carbocyclic analog of adenosine, (±)-aristeromycin; HPLC, high pressure liquid chromatography.

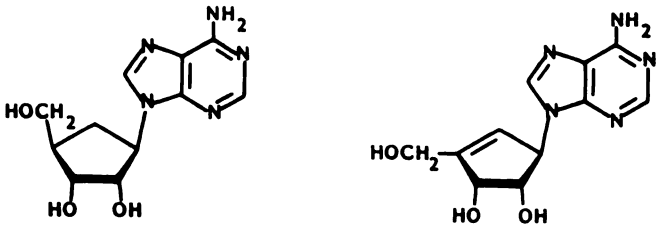

Fig. 1. Structures of aristeromycin and Np-A.

analysis. HPLC (reverse phase): Gradient elution, A (0.01 M $NH_4H_2PO_4$) 90% → 50%, B (MeOH) 10% → 50%; retention time: Np-A, 6.5 min, Np-A-monophosphate, 3.9 min. The principal by-product of this reaction is the 5′-chloro compound, which is easily separated by the above procedure (retention time 13 min). To prove that the phosphate was at the 5′-position, the Np-A-monophosphate was treated with both 5′-nucleotidase and 3′-nucleotidase. Treatment with the 5′-nucleotidase converted the Np-A monophosphate back to Np-A, as judged by HPLC analysis, whereas treatment of the Np-A monophosphate with 3′-nucleotidase caused no change. 5′-AMP and 3′-AMP were assayed concurrently to assure that the nucleotidases had the expected specificities.

Other materials. Np-A was provided by the National Cancer Institute. *C*-Ado, *C*-Ado-5′-monophosphate, and *C*-IMP were synthesized in our laboratories (9, 14). *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). [8-^{14}C]Guanine (57 mCi/mmol) and [8-^{14}C]hypoxanthine (56 mCi/mmol) were obtained from Moravek Biochemicals, Inc. (Brea, CA). Alkaline phosphatase from *Escherichia coli*, adenosine deaminase from bovine intestine, AMP deaminase from rabbit muscle, 5′-nucleotidase from *Crotalus adamanteus*, and 3′-nucleotidase from rye grass were obtained from Sigma Chemical Co. (St. Louis, MO).

Experiments with cell cultures. L1210 cells were grown in Fischer's medium (16). For metabolic studies, cells were grown in suspension; at the initiation of the experiments the cells were growing exponentially at a concentration of approximately 5×10^5 cells/ml. Cells were harvested by centrifugation and washed free of medium with 0.85% NaCl solution, after which the cells were extracted with cold 0.5 N $HClO_4$. To this extract, $KHCO_3$ was added, and $KClO_4$ was removed by centrifugation, after which the supernatant solution was lyophilized. The residue was dissolved in H_2O, and the resulting solution was subjected to HPLC.

High pressure liquid chromatography. HPLC analyses were performed with a Waters Associates (Milford, MA) model 202 apparatus. Analysis of nucleotides was accomplished on a Partisil-10 SAX anion exchange column (Whatman, Inc., Clifton, NJ) with either a linear or a #7 (slightly concave) 50-min gradient, from 5 mM $NH_4H_2PO_4$ (Fisher Scientific, Fairlawn, NJ), pH 2.8, to 750 mM $NH_4H_2PO_4$, pH 3.7, and a flow rate of 2 ml/min. Nucleosides were analyzed on a reverse phase Spherisorb ODS 5-μm column (Phase-Sep, Norwalk, CT); elution was accomplished with 25 mM $NH_4H_2PO_4$ (pH 5.5)/acetonitrile (95:5, v/v) at a flow rate of 1 ml/min.

Deaminase assays. Np-A and *C*-Ado were assayed as substrates for adenosine deaminase (EC 3.5.4.4) from calf intestine by measurement of the decrease in absorbance at 265 nm as the deamination proceeded (17). The reaction was carried out at room temperature in 0.05 M phosphate buffer, pH 7.4 (18). Change in absorbance at 265 nm was also used to assay the 5′-monophosphates of Np-A and *C*-Ado as substrates for AMP deaminase (EC 3.5.4.6) from rabbit muscle (17). The reaction was carried out in 0.01 M citrate buffer, pH 6.5. Since the result of attack of both deaminases on both of the substrates and on the natural substrates (adenosine and AMP) is the replacement of the 6-amino group of the adenine moiety with a hydroxyl group, the change in molecular absorbance is the same for all substrates (8100 M^{-1} cm^{-1}). For the studies with AMP deaminase, HPLC on an anion exchange column was used to confirm the identities of the products. Kinetic constants of substrates for both deaminases were determined by Lineweaver-Burk (19) plots (not shown); for each substrate at least 10 concentrations were used.

Results

Influence of rate of cell proliferation on the metabolism of *C*-Ado. In the course of investigating the metabolism of *C*-Ado over a period of many months, it was noted that, although *C*-Ado was always converted to the triphosphate in amounts approximating or exceeding that of ATP, the extent of conversion to carbocyclic GTP was variable and in occasional experiments was not detected. On the hypothesis that this variability might be caused by differences in the rates of proliferation of the cell cultures during the period of exposure to *C*-Ado, we examined the metabolism of *C*-Ado in cultures at different cell densities. For this purpose, cultures of L1210 cells were initiated at a low cell inoculum, and experiments were performed when cells were in rapid growth, 24 hr later when the growth rate had slackened, and 48 hr later when the cells had ceased to increase in number. The results are shown in Fig. 2. In all three cultures, *C*-Ado-triphosphate was formed in considerable amount, although the slowly proliferating (Fig. 2B) or resting (Fig. 2C) cells formed less than the rapidly proliferating cells did (Fig. 2A). The pools of natural nucleotides were also less in the less rapidly proliferating cells. In contrast, the amount of carbocyclic GTP was sharply reduced in the slowly proliferating cells (Fig. 2B) as compared to rapidly proliferating cells (Fig. 2A) and was barely detectable in resting cells (Fig. 2C). The amounts of carbocyclic triphosphates were calculated by integration of the areas under the peaks using the conversion factors for ATP and GTP. The values thus determined (nmol/10^9 cells) were: carbocyclic ATP: Fig. 2A, 2490; Fig. 2B, 1260; Fig. 2C, 1010; carbocyclic GTP: Fig. 2A, 990; Fig. 2B, 260; Fig. 2C, 20.

Metabolism of Np-A. Fig. 3 shows the results of experiments in which HPLC was used to examine the nucleotide profiles of L1210 cells grown in the presence of either Np-A or *C*-Ado. Np-A-treated cells contained a large new peak in the triphosphate area between ATP and GTP (Fig. 3B). A Perkin-Elmer model L-75 stop-flow scanner was used to determine the UV absorption spectrum of the ascending, middle, and descending portions of this triphosphate peak; all of these scans gave the spectrum of Np-A, which is not distinguishable from that of ATP. The peak immediately preceding the new triphosphate peak has the retention time of ATP of controls and also gave a UV scan typical of ATP. No other new peak was evident in the triphosphate area; the other peaks in this area gave scans typical of CTP (retention time 27 min), UTP (retention time 29 min), and GTP (retention time 37 min). Thus, there was no evidence for a separate peak of the Np-A analog of GTP. However, the possibility remained that this triphosphate was present but co-eluted with GTP, from which it would not be distinguished by UV spectrum. In addition, mono- and diphosphates of the Np-A analogs of guanosine, inosine, or xanthosine might also be present and not resolved from the natural mono- and diphosphates. To investigate this possibility, the entire cell extract was treated for 4 hr with alkaline phosphatase, and the resulting nucleosides were then analyzed by HPLC on a reverse phase column (Fig. 4). The only new peak detected in analyz-

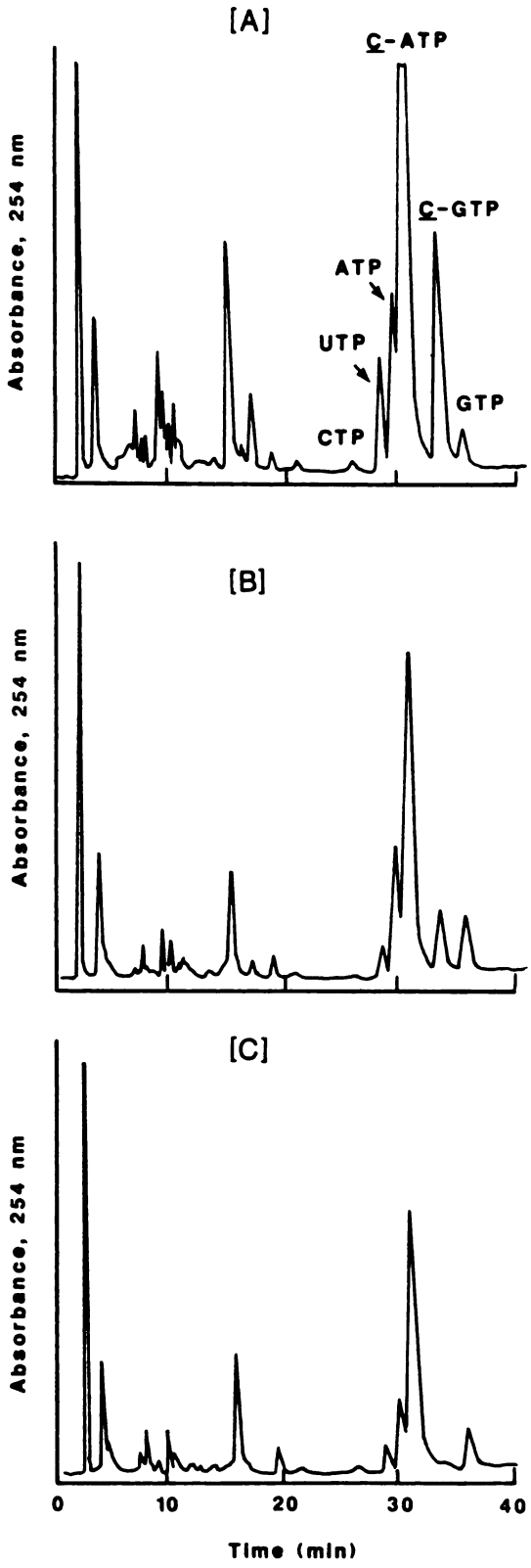

Fig. 2. Metabolism of *C*-Ado by L1210 cell cultures of different growth rates. Six cultures of L1210 cells, all taken from the same batch, were established and allowed to proliferate overnight, at which time the cell density was 6.5 × 10^5 cells/ml. At this time *C*-Ado was added to one culture to a final concentration of 25 μM; after 4 hr this culture and one of the other cultures to which no addition was made (control) were harvested, and extracts were prepared and subjected to HPLC on an anion exchange column (A). After an additional 24 hr, the cell density had reached 10 × 10^5 cells/ml; at this time *C*-Ado (25 μM) was added to the culture and after 4 hr this culture and one untreated culture were harvested and assayed as described above (B). During the next 24 hr the cell number did not change significantly; at this time one of the remaining cultures was treated with *C*-Ado and the other was used as control for analyses as described above (C). The charts show the results with *C*-Ado-treated cells; chromatograms of control cells are not shown. *C-ATP*, 5′-triphosphate of *C-Ado*; *C-GTP*, 5′-triphosphate of the carbocyclic guanosine analog of *C*-Ado.

able amount was one that eluted with the retention time of Np-A (Fig. 4C) and which had the UV absorption of Np-A. When Np-A was added to the phosphatase-treated extract before chromatography, the intensity of the suspected Np-A peak was increased (results not shown). The Np-A-treated cells also contained a very small amount of another material, eluting with a retention time of 9.6 min, which was not present in controls. This peak was too small to permit scanning; hence it is not known whether it is a metabolite of Np-A or a natural metabolite accumulating in Np-A-treated cells. The Np-A analog of guanosine was not available for comparison. Carbocyclic guanosine derived from *C*-Ado also has a retention time of about 9.5 min (Fig. 4A); hence, the data do not exclude the possibility that this peak may be the Np-A analog of guanosine. However, the amount of UV-absorbing material in this peak represents only 1–2% of the total UV absorbing material, whereas the Np-A peak represents about 28%. Even if this peak were the Np-A analog of guanosine, it would represent a conversion that was quite limited in comparison with the conversion of *C*-Ado to carbocyclic guanosine (Fig. 4D).

Experiments with *C*-Ado (Figs. 3C and 4D) were performed in parallel with the Np-A experiments as positive controls. As we have shown earlier (14), *C*-Ado-treated cells contain two new triphosphates: a major peak (retention time 32 min) of *C*-Ado triphosphate and a smaller peak of the triphosphate of carbocyclic guanosine (Fig. 3C). When the extract of *C*-Ado-treated cells was treated with phosphatase and then subjected to reverse phase chromatography, peaks with the retention time of *C*-Ado and carbocyclic guanosine were present (Fig. 4D). It is to be noted that, under the conditions of the reverse phase chromatography (Fig. 4), *C*-Ado and neplanocin have quite different retention times. In these experiments, the pH of the eluting buffer was 5.5. In our earlier experiments with *C*-Ado (14), the pH of the eluting buffer was 3.5, and the retention time of *C*-Ado was only about 8 min as contrasted with a retention time of 19 min at pH 5.5. At pH 3.5, Np-A has a retention time of 7 min, only slightly less than that of *C*-Ado. This difference in relative retention times as the pH is raised presumably reflects a difference in pK_a of the two carbocyclic analogs.

In another experiment, the triphosphate area (peaks with retention times from 26 min to 38 min, Fig. 3) was collected, after which the nucleotides were freed of buffer salts by passage through a charcoal column (Norit A) which retained the nucleotides. The nucleotides were eluted with ammoniacal ethanol; the eluate was evaporated to dryness, and the residue was taken up in 0.01 M Tris buffer (pH 8.0) and treated with alkaline phosphatase. The resulting nucleosides were analyzed by reverse phase HPLC as described above. The only peak appearing on the chromatograms of nucleosides from Np-A-treated cells that was not present on the chromatograms of nucleosides from control cells was one with the retention time

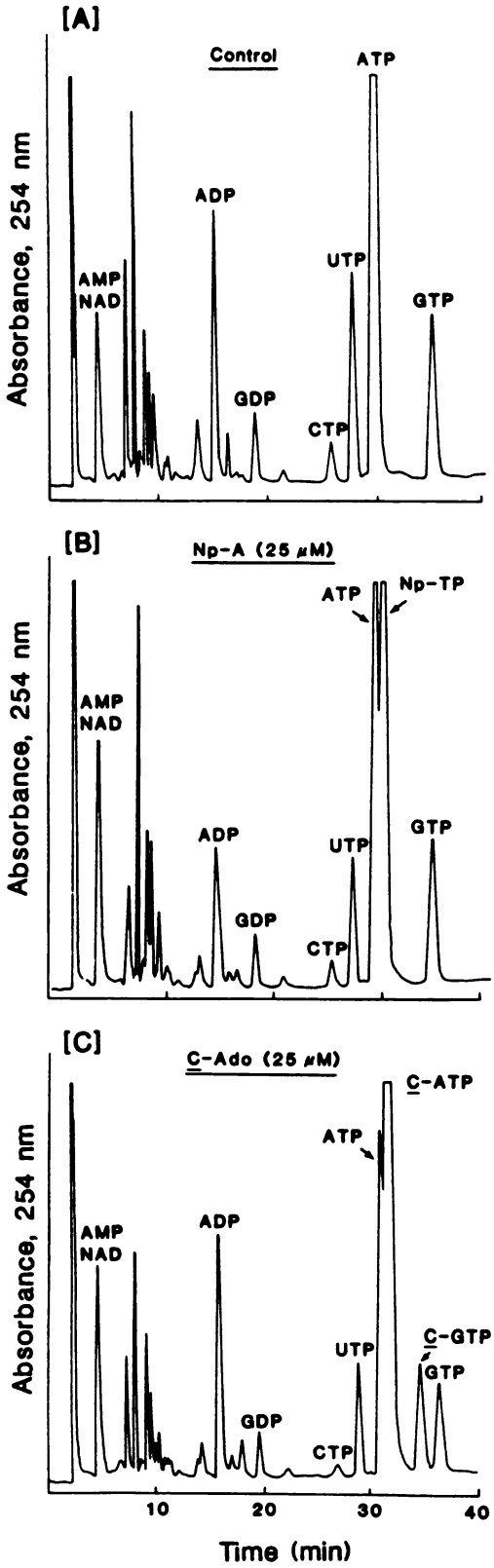

Fig. 3. Metabolism of Np-A and *C*-Ado in L1210 cells. Np-A (B) or *C*-Ado (C) was added to exponentially growing suspension cultures of L1210 cells. Control cells, grown from the same batches of cells as the treated cultures, received no treatment (A). After 4 hr, the cells were harvested and washed free of medium. Perchloric acid extracts of the cells were made and subjected to HPLC on an anion exchange column with a linear gradient as described in the text. *Np-TP*, triphosphate of Np-A; see Fig. 2 legend for other abbreviations.

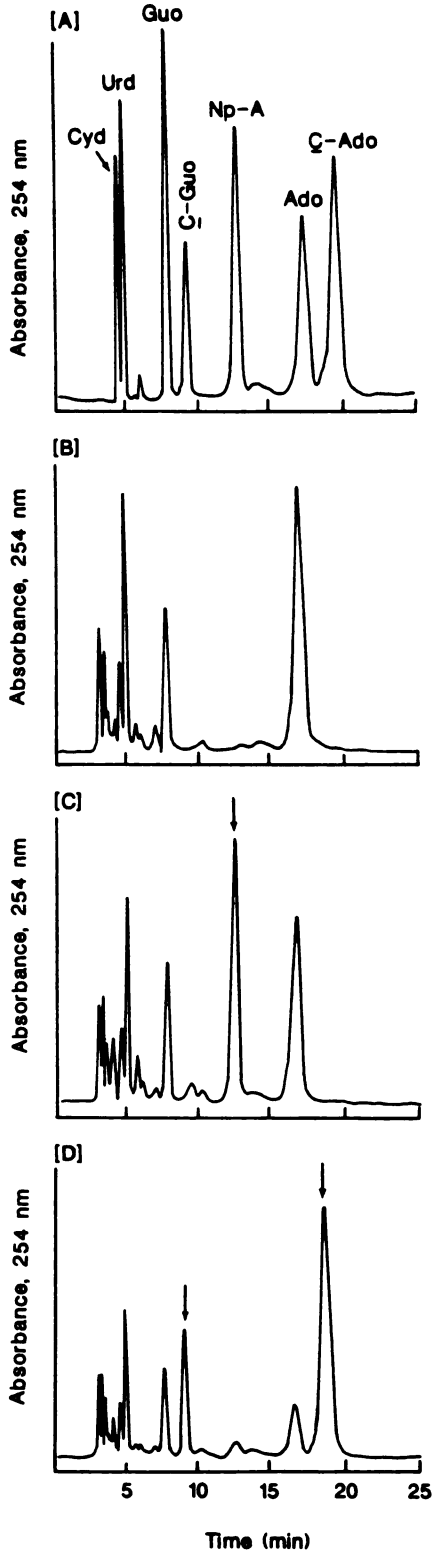

Fig. 4. Reverse phase chromatography of phosphatase-treated extracts from L1210 cells treated with Np-A or *C*-Ado. Extracts from control and D-treated cells (Fig. 3) were incubated 4 hr with alkaline phosphatase after which the resulting mixtures were subjected to reverse phase chromatography (see the text). A, Standards: natural nucleosides, *C*-Ado, Np-A, and carbocyclic guanosine (*C-Guo*, the guanosine analog of *C*-Ado); B, extracts from control cells; C, extracts from cells grown for 4 hr in the presence of Np-A (25 μM); D, extracts from D cells grown for 4 hr in the presence of *C*-Ado (25 μM). *Arrows* mark the peaks that appear in cells treated with Np-A or *C*-Ado but not in control cells.

of Np-A. These chromatograms, which are similar to those of Fig. 4, are not shown.

Effects on utilization of ^{14}C-labeled hypoxanthine and guanine (Table 1). *C*-Ado strongly inhibited the utilization of guanine and hypoxanthine; we have demonstrated this inhibition earlier, but the results in Table 1 are from additional experiments in which *C*-Ado and Np-A were assayed in parallel. Np-A had no marked effect on the utilization of either guanine or hypoxanthine; the utilization of guanine was a little increased and that of hypoxanthine a little decreased. These experiments represent only relatively crude measurements preliminary to the assay of nucleotide pools by HPLC. These results do, however, serve to indicate little or no inhibition by Np-A under conditions under which *C*-Ado produced strong inhibition. These results were confirmed by the HPLC analyses (Fig. 5) which show no marked effects of Np-A on the conversion of these bases to nucleotides. Fig. 5 also shows again the conversion of Np-A to Np-A-triphosphate. It is to be noted that, in determining the radioactivities, 2-ml fractions were taken, and the points at which these happened to fall can change the shape of a peak of radioactivity without changing the total radioactivity associated with a given nucleotide. *C*-Ado, under the same conditions, inhibited almost completely the incorporation of these bases into nucleotides (14).

Activities of Np-A, *C*-Ado and their 5′-monophosphates as substrates for AMP deaminase and adenosine deaminase (Table 2). *C*-Ado-5′-monophosphate, Np-A-5′-monophosphate, and 5′-AMP were compared as substrates for AMP deaminase. The course of the reactions was followed spectrophotometrically and the identities of the products were confirmed by HPLC. The action of AMP deaminase on *C*-Ado-5′-monophosphate produced a new peak with a retention time (anion exchange column) of 7.9 min, the same as that of a known sample (14) of carbocyclic IMP. The action of the deaminase on Np-A-5′-monophosphate produced a new peak with a retention time of 8.0 min and with a UV absorption spectrum the same as that of IMP. The Np-A analog of IMP was not available for comparison, but the disappearance of the peak of Np-A-5′-monophosphate (retention time 3.4 min) and the appearance of a new peak with a longer retention time and with the UV absorption spectrum of IMP are indicative that deamination occurred. The kinetic constants (Table 2) were determined by double reciprocal plots (not shown). 5′-AMP, used as a positive control, had an apparent K_m of about 1000 μM, which is in the range of values reported (20). *C*-Ado-5′-monophosphate did not differ greatly from 5′-AMP in K_m or V_{max}. Np-A-5′-monophosphate had the lowest apparent K_m of the three substrates; its V_{max} was only 4.7% of that of *C*-Ado-5′-monophosphate. Np-A is a known substrate for adenosine deaminase from goat intestine (21); however, we also assayed it as a substrate for the bovine intestinal enzyme in order to have a comparison, in the same laboratory, of the substrate activities of Np-A, *C*-Ado, and their phosphates. The deamination of Np-A proceeded at a rate about 10-fold that of *C*-Ado. *C*-Ado and *C*-Ado-5′-monophosphate are racemates. If only one isomer is a substrate for the deaminase under the conditions of the assay, then the K_m values obtained for the racemates would be twice those for the natural isomers. If this correction is applied, the K_m values for the two carbocyclic nucleosides are about the same for adenosine deaminase and the K_m values for the corresponding 5′-monophosphates are about the same for AMP deaminase. Evidence that only one isomer of a racemic nucleoside or nucleotide may be subject to enzymic attack, or that the two isomers react at quite different rates, is provided by the recent report of Herdewijn *et al.* (22), who were able to resolve *C*-Ado-5′-monophosphate by treating it with 5′-nucleotidase. In preliminary experiments directly pertinent to the present question, we have obtained evidence that adenosine deaminase also attacks selectively one isomer of *C*-Ado, as shown by the facts that the rate of deamination leveled off when about half of the substrate was deaminated and that the product was levorotatory whereas the remaining substrate was dextrorotatory.[1]

TABLE 1

Effects of C-Ado and Np-A on the utilization of hypoxanthine and guanine by L1210 cells

C-Ado or Np-A was added to exponentially growing cultures of L1210 cells 0.5 hr before the addition of [8-^{14}C]hypoxanthine or [8-^{14}C]guanine (25 nCi/ml of medium). One hr after addition of the labeled compound the cells were harvested and washed free of medium; the cell pellet was assayed for radioactivity. The values shown are cpm in 10^6 cells expressed as percentages of those in control cells. The control values (cpm/10^6 cells) were: Experiment 1: guanine, 2,900; hypoxanthine, 25,300; Experiment 2: guanine, 3,700; hypoxanthine, 27,800.

Inhibitor	Utilization: % of control			
	Guanine		Hypoxanthine	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Np-A (25 μM)	126	113	87	83
NP-A (50 μM)	122	131	85	82
C-Ado (25 μM)	22	30	20	33
C-Ado (50 μM)	9	15	6	19

Discussion

The failure of Np-A to be converted to analogs of guanosine phosphates under conditions under which *C*-Ado was converted to the carbocyclic analog of GTP is a striking difference in the metabolism of these related carbocyclic adenosine analogs and one that could not be predicted in advance. In their study of the metabolism of Np-A in Chinese hamster ovary cells, Saunders *et al.* (11) reported no metabolites other than phosphates of Np-A, but Np-A analogs of guanine nucleotides were not specifically sought for and, hence, were not excluded. Since it was found that, under certain metabolic conditions, the conversion of *C*-Ado to carbocyclic GTP was very low or undetectable (Fig. 2), conversion to analogs of guanine nucleotides could be excluded as a pathway for Np-A only if it were shown that the same cell cultures had the capacity to convert *C*-Ado to carbocyclic guanine nucleotide analogs (Fig. 3). We have shown earlier that the conversion of *C*-Ado to the corresponding guanosine nucleotide analogs can be prevented by coformycin at concentrations that inhibit AMP deaminase (14). Therefore, it is likely that the decrease in this conversion as cell density increases (Fig. 2) reflects a decrease in activity of AMP deaminase, which, as a regulatory enzyme (20), would be expected to vary in activity with rate of cell proliferation.

The fact that Np-A is not converted to the Np-A analog of GTP indicates the failure of a nucleotide derived from Np-A to serve as a substrate for some enzyme along the pathway involved in the conversion of AMP to GTP. When the entire cell extract from Np-A-treated cells was treated with phosphatase and then analyzed by reverse phase chromatography (Fig. 4), the only unnatural nucleoside detected was Np-A. The absence

[1] J. A. Secrist III, S. J. Clayton, and J. A. Montgomery, unpublished results.

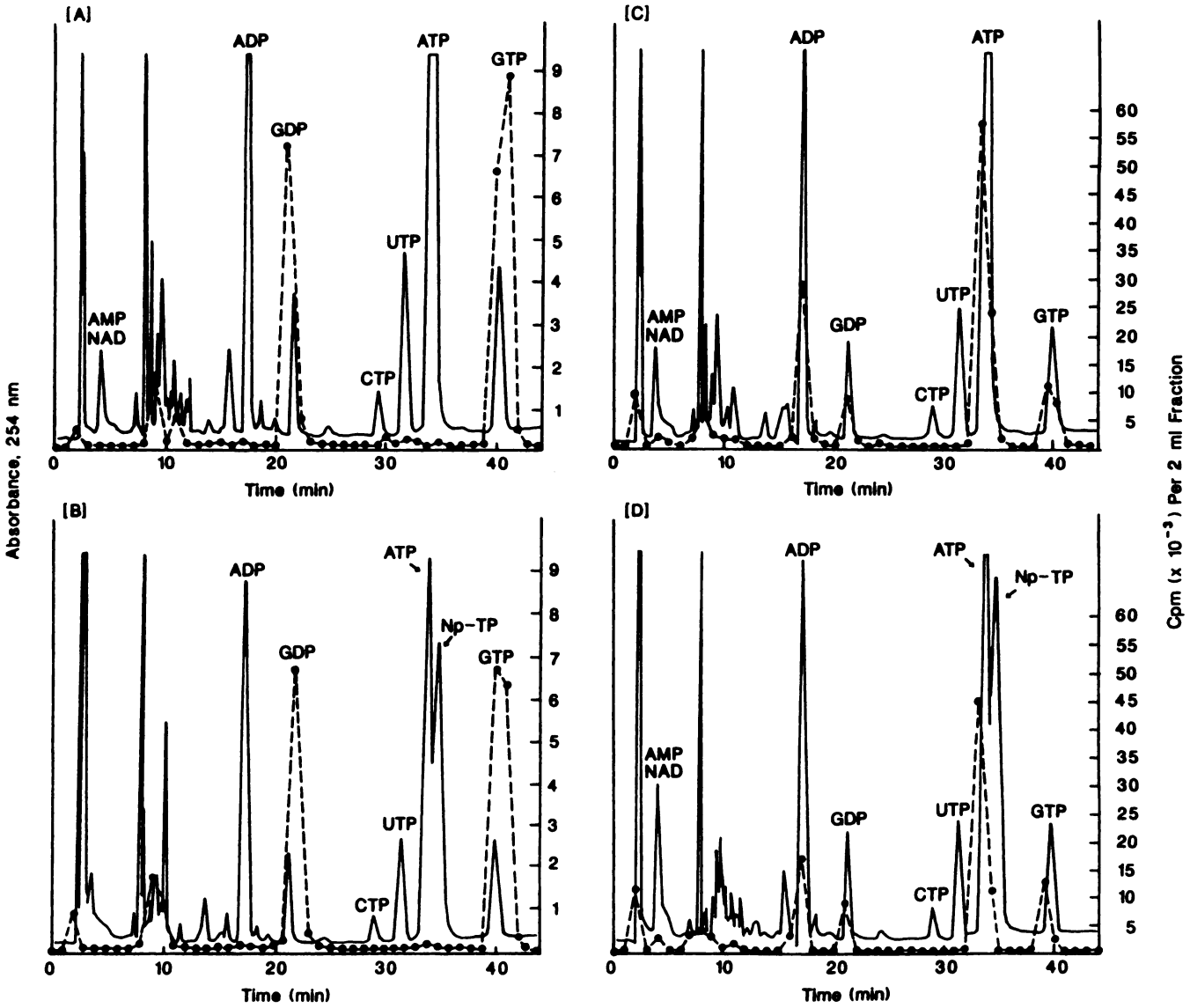

Fig. 5. Conversion of guanine and hypoxanthine to nucleotides in control and Np-A-treated L1210 cells. To cells in suspension culture, Np-A was added to a final concentration of 50 μM followed 0.5 hr thereafter by [8-^{14}C]guanine or [8-^{14}C]hypoxanthine (25 nCi/ml of medium). One hr after addition of the labeled base, the cells were harvested and an extract was prepared and subjected to HPLC on an anion exchange column using a #7 gradient (see the text). 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, Np-A-treated cells; C, [^{14}C]hypoxanthine, control cells; D, [^{14}C]hypoxanthine, Np-A-treated cells. *Solid lines*, UV absorbance; *dashed lines*, radioactivity. *Np-TP*, triphosphate of Np-A.

TABLE 2

Kinetic constants for the deamination of C-Ado, Np-A, and their monophosphates

The nucleosides and nucleotides were assayed as substrates for adenosine deaminase from bovine intestine and AMP deaminase from rabbit muscle by determination of changes in absorbance at 265 nm. Kinetic constants were determined from double reciprocal plots of the resulting data. The reaction mixtures were also assayed by HPLC to verify that deamination had occurred.

Substrate	K_m (μM)	V_{max} (nmol/min/mg)
	Adenosine deaminase	
Adenosine	29	435
C-Ado	1000	9.4
Np-A	500	100
	AMP deaminase	
AMP	1000	3.6
C-Ado-monophosphate	560	1.9
Np-A-monophosphate	146	0.09

of Np-A analogs of inosine, xanthosine, and guanosine indicated that the failure of Np-A-5′-monophosphate to serve as a substrate for AMP deaminase was the most likely reason that Np-A was not converted to guanine nucleotide analogs. This explanation did not appear likely, however, in view of the facts that (a) Np-A has been reported to be a better substrate than *C*-Ado for adenosine deaminase from goat intestine (21), and (b) with the several nucleoside-nucleotide pairs that have been examined, activity of the nucleoside as a substrate for adenosine deaminase is paralleled by activity of the nucleotide as a substrate for AMP deaminase (20). Therefore, it was essential to synthesize Np-A-5′-monophosphate for direct comparison with *C*-Ado-5′-monophosphate. *C*-Ado-5′-monophosphate was deaminated at about 50% of the rate of AMP, but Np-A-5′-monophosphate was deaminated at a rate that was only 2% of the rate of AMP (Table 2). The difference in activities of the

two carbocyclic phosphates as substrates for AMP deaminase appears to be an adequate explanation of why *C*-Ado, but not Np-A, is converted to analogs of guanine nucleotides. This explanation assumes similar behavior of the AMP deaminase of L1210 cells and the rabbit muscle enzyme used in these studies; insofar as they have been examined, AMP deaminases from various sources appear to have similar substrate specificities (20).

From what is known, it is apparent that both Np-A and *C*-Ado produce multiple metabolic effects. The mechanisms of action of these two related carbocyclic analogs would be expected to have points of similarity due to the actions of the unphosphorylated carbocyclic nucleosides and their phosphates, and points of dissimilarity due to the presence of analogs of guanine nucleotides formed from one analog but not from the other. One such difference is noted in the effects on utilization of guanine and hypoxanthine. *C*-Ado strongly inhibited the utilization of both bases, and this inhibition probably is due to the presence of nucleotides of carbocyclic guanosine (14). Np-A did not inhibit utilization of these bases. Thus, these results with hypoxanthine and guanine are consistent with the failure of Np-A to be converted to analogs of guanine nucleotides, which is a consequence of the failure of Np-A-5′-monophosphate to be deaminated. The presence of carbocyclic analogs of guanine nucleotides of *C*-Ado may also have other metabolic consequences that have not yet been noted. Furthermore, it is not unlikely that other, as yet undetected differences in effects of Np-A and *C*-Ado may result from the existence of enzymes other than AMP deaminase for which the phosphates of Np-A and *C*-Ado may differ as substrates or inhibitors.

The results of the deaminase studies are also of interest with respect to substrate specificities of these enzymes. The poor activity of Np-A monophosphate as a substrate for AMP deaminase indicates an interesting structural requirement for activity as a substrate for this enzyme. The presence of the double bond in Np-A requires that C-5′ be essentially in the plane of the cyclopentene ring, as shown in Fig. 1. It is known that at least three moieties of the AMP molecule are involved in its binding to AMP deaminase: the phosphate group, the 3′-hydroxy group, and the adenine ring (23). The rigidity of C-5′ in Np-A-5′-monophosphate obviously changes the location of the phosphate group relative to the rest of the nucleotide and, therefore, prevents the phosphate group from assuming all of the conformations possible in 5′-AMP or *C*-Ado-5′-monophosphate. Presumably, it is this different spatial orientation that is responsible for the poor activity of Np-A-5′-monophosphate as a substrate for AMP deaminase. That the orientation of the phosphate may limit or prevent substrate activity has been shown earlier in studies by Hampton *et al.* (23) with phosphonate analogs of AMP. In one of these analogs the 4′-$CH_2OPO_3H_2$ group of AMP was replaced by —CH═$CHPO_3H_2$ (phosphonate group *trans* to C-4′) and in the other by —$CH_2CH_2PO_3H_2$. The saturated compound was a moderately good substrate for AMP deaminase (V_{max} = 22% of that of AMP), whereas the unsaturated compound was a poor substrate (V_{max} = 0.7% of that of AMP). Np-A-5′-monophosphate appears similar to the unsaturated phosphonate analog in that the presence of a double bond restricts the phosphorus to orientations that permit only poor substrate activity. It is of interest that, in contrast to the results with AMP deaminase, the presence of the double bond in Np-A still permits good substrate activity for adenosine deaminase. Although Np-A was deaminated more slowly than adenosine, the rate was about 10-fold greater than that of *C*-Ado (Table 2). It is known that the presence of the 5′-hydroxy group is critical for substrate activity of a nucleoside for adenosine deaminase (24). The fact that Np-A is an effective substrate would therefore indicate either that the enzyme is not fastidious with respect to the relative spatial orientation of the hydroxymethyl group, or, alternatively, that the conformations to which the hydroxymethyl group is restricted in Np-A include that required for substrate activity. Tsujino *et al.* (21) have compared Np-A and aristeromycin as substrates for adenosine deaminase from goat intestine. Their results were similar to ours in that they found Np-A to be a better substrate than aristeromycin; Np-A was deaminated at a rate 5-fold that of aristeromycin, whereas we found that the ratio was about 10-fold. Our results differ, however, in the rates of deamination of both substrates relative to adenosine. Tsujino *et al.* found the rates for Np-A and aristeromycin to be, respectively, 1.6% and 0.3% of that of adenosine, whereas our values were 22% and 2%. We have no explanation for these differences in relative rates other than that the enzymes were from different sources.

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