Biochemical Pharmacology, Vol. 34, No. 15, pp. 2749–2754, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

METABOLISM AND ACTION OF NEPLANOCIN A IN CHINESE HAMSTER OVARY CELLS

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(*Received* 25 *September* 1984; *accepted* 19 *December* 1984)

Abstract—Neplanocin A is a naturally occurring carbocyclic analog of adenosine which contains a cyclopentene moiety in place of ribose and has demonstrated antitumor and antimicrobial activity. This compound was highly toxic to Chinese hamster ovary (CHO) cells; the approximate minimum inhibitory concentration of neplanocin A for inhibition of clone formation was 0.1 μM. The toxicity of the agent was greatly reduced by prior treatment with adenosine deaminase. [^{3}H]Uridine incorporation into perchloric acid insoluble material in growing cells was inhibited by neplanocin A more dramatically than that of [^{3}H]thymidine or [^{3}H]leucine. Treatment with the drug resulted in a marked depression of ATP pool levels. High pressure liquid chromatographic analysis of cellular nucleotide pools from cells treated with neplanocin A revealed the formation of an apparent drug metabolite (NpcTP) that eluted in the triphosphate region of the chromatographic profile. Treatment of NpcTP with alkaline phosphatase produced a nucleoside with properties similar to neplanocin A. An adenosine-kinase-deficient cell line formed little, if any, NpcTP but demonstrated only slight resistance to the agent. These observations suggest that neplanocin A was efficiently metabolized to the triphosphate level but that this metabolite was responsible for only a fraction of the observed toxicity.

Neplanocin A (Fig. 1) is a carbocyclic analog of adenosine which contains a cyclopentene moiety in place of ribose. It was originally isolated from culture filtrates of *Ampullariella regularis* [1, 2] and has since demonstrated significant activity against L1210 leukemia in mice [1]. Metabolic studies with neplanocin A have shown that it can be converted to an analog of *S*-adenosyl-L-methionine, by mouse L 929 cells [3] and also that it is a potent inhibitor of *S*-adenosylhomocysteine hydrolase and vaccinia virus multiplication in these cells [4]. It has also been shown to be a substrate for adenosine deaminase [5]. The implications of these observations to the cytotoxic activity of neplanocin A are not clear. This paper describes the metabolism of neplanocin A to the triphosphate level and its effects on nucleotide metabolism in CHO cells§.

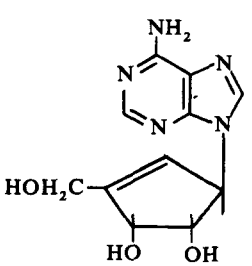

Fig. 1. Structure of neplanocin A.

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§ Abbreviations: CHO cells, Chinese hamster ovary cells; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; NpcTP, neplanocin A metabolite; ara-C, 1-β-D-arabinofuranosylcytosine; AK, adenosine kinase; dCK, deoxycytidine kinase; and ADA, adenosine deaminase.

MATERIALS AND METHODS

Chemicals. Neplanocin A was provided by the Warner–Lambert Corp., Ann Arbor, MI. [*Methyl*-^{3}H]Thymidine (50 Ci/mmole), [5,6-^{3}H]uridine (42 Ci/mmole, [4,5-^{3}H]leucine (61 Ci/mmole), and [^{14}C]formate (56 mCi/mmole) were purchased from ICN Pharmaceuticals, Inc. (Irvine, CA). All other reagents were purchased from appropriate commercial sources.

Cells and medium. CHO cells were routinely carried in monolayer culture using McCoy's 5a growth medium supplemented with 10% fetal bovine serum as previously described [6]. Medium supplemented with dialyzed serum was employed in all experimental procedures. The mutant cell line, Rb^R-1, was derived from the CHO line, is deficient in adenosine kinase, and has been described elsewhere [7]. Line aC^R-7, similarly derived, is deficient in deoxycytidine kinase and has also been described previously [6]. The minimum inhibitory drug concentration was determined by methods described elsewhere [6]. Briefly, cells were allowed to form clones for 7 days in a broad range of drug concentrations. The minimum inhibitory concentration is defined as that concentration of drug which resulted in the formation of clones containing fewer than 50 cells.

Measurement of incorporation of macromolecule precursors into perchloric acid insoluble material. Logarithmically growing cells were trypsinized and dispensed into sterile 20 ml glass vials,

1.5×10^5 cells/vial in 2 ml of medium, and incubated for 16–18 hr in a humidified CO_2 incubator at 37°. The medium in each vial was then removed and replaced with 1 ml of fresh medium containing dialyzed serum and the indicated concentrations of drug. After 3 hr of preincubation, isotopically labeled precursors were added and incubation was continued for 2 more hr. The media were then removed, and the cells were washed once with 5 ml of cold PBS followed by the addition of 5 ml of cold 0.4 N perchloric acid. The vials were allowed to stand in the cold (4°) for 30 min, after which the perchloric acid was removed and the residues were washed once with 5 ml of the same. The radioactivity in the residues was counted after the addition of 5 ml of Aquasol (New England Nuclear Corp., Boston, MA).

Analysis of cellular nucleotide pools. Cells were dispensed into 125-ml tissue culture flasks (2×10^6 cells/flask) in 5 ml of medium and incubated overnight in humidified CO_2 at 37°. The media were then replaced with 5 ml of fresh medium containing dialyzed serum and the desired drug concentrations. After 5 hr of incubation, the media were aspirated and the monolayers were quickly washed with cold PBS followed by extraction of nucleotide pools with cold 0.4 N perchloric acid. Duplicate flasks were used to determine the number of cells present after incubation. In studies relating to the effects of drug on metabolism of isotopically labeled nucleotide precursors, cells were incubated with drug for 3 hr prior to the addition of isotope and then for another 2 hr. The perchloric acid extracts were neutralized with KOH, and the nucleotide pools were fractionated using a Waters Associates (Milford, MA) ALC 204 high pressure liquid chromatograph equipped with two model 6000A pumps, System Controller, and a column of Partisil-10 SAX anion exchange resin (25 × 4.6 mm; Whatman, Inc.). Cell samples (1×10^6 cell equivalents) were injected with the U6K-LC injection system and eluted with a gradient (curve 7) from 60% buffer A to 100% buffer B in 25 min at a flow rate of 2 ml/min. Buffer A was composed of 0.15 M $NH_4H_2PO_4$, pH 2.8 and buffer B contained 0.75 M $NH_4H_2PO_4$, pH 3.5. When radioisotope measurements were required, 1-ml fractions were collected directly into scintillation vials and counted after mixing with 9 ml of Aquasol. The eluted compounds were detected at 254 nm by model 440 detector and quantitated using a Data Module. Nucleotides were identified by comparison with the retention times of known standards. Nucleoside separations were accomplished with a 3.9 mm × 30 cm reversed phase column of μBondapak C_{18} from Waters Associates. Isocratic elution was carried out with 5% methanol at a flow rate of 2 ml/min.

Isolation of nucleotides and hydrolysis by alkaline phosphatase. After HPLC fractionation of nucleotides as described above, those fractions containing ATP and the drug metabolite (NpcTP) (18–20 min) were pooled and the nucleotides were removed from the high salt buffer by absorption onto activated charcoal as described by Shewach and Plunkett [8]. Removal of nucleotides from the charcoal was effected with 50% ethanol containing 2% NH_4OH. The sample was evaporated to dryness under a stream of nitrogen, and the residue was dissolved in a minimum amount of water. ATP from 4.5×10^5 cell equivalents and ATP + NpcTP from 6×10^6 drug-treated cell equivalents were each incubated with 10 μg of *Escherichia coli* alkaline phosphatase in 0.04 M Tris–HCl, pH 8.0, in a final volume of 0.5 ml. The reaction was stopped after 2 hr by placing the tubes in a boiling water bath for 3 min followed by HPLC analysis.

Preparation of deaminated neplanocin A. Neplanocin A was deaminated in a reaction mixture containing 650 μM neplanocin A, 25 μM potassium phosphate, pH 7.5, and adenosine deaminase at a concentration of 2.5 units/ml. After incubation for 2 hr at 37°, the reaction was terminated by placing the mixture in a boiling water bath for 5 min and centrifuged. HPLC analysis of a sample of the reaction mixture on a reversed phase column as described indicated complete conversion of neplanocin A (retention time = 9 min) to its deaminated derivative (retention time = 3.7 min). The solution was sterilized by passage through a 0.2 μm filter and used directly.

RESULTS

Table 1 describes the growth inhibitory properties of neplanocin A toward CHO cells and two nucleoside kinase deficient cell lines derived from them. The inhibitory concentrations of tubercidin and ara-C were included in the table to show that the kinase-deficient lines demonstrate the appropriate resistance properties. Growth of the parent CHO cell line was inhibited by 0.1 μM neplanocin A. The adenosine-kinase-deficient cell line, Rb^R-1, demonstrated the appropriate resistance to tubercidin which is known to be phosphorylated by adenosine kinase, and only slight resistance to neplanocin A. The deoxycytidine kinase deficient cell line, aC^R-7, which

Table 1. Growth inhibition of CHO and variant cell lines*

Cell line	Enzyme deficiency	Approximate minimum inhibitory concentration (μ)			
		Neplanocin A	Deaminated neplanocin A	Tubercidin	Ara-C
CHO		0.1	>2.0	5	0.5
Rb^R-1	AK	0.5	ND	>500	0.5
aC^R-7	dCK	0.1	ND	10	50

* Procedures are as described in the text. Abbreviations: AK, adenosine kinase; dCK, deoxycytidine kinase; and ND, not determined.

is resistant to ara-C, was not resistant to neplanocin A.

Since neplanocin A has been shown to be a substrate for adenosine deaminase, we considered this reaction as a possible means of either detoxifying the drug or producing an active metabolite. To estimate the toxicity of the deaminated compound, we carried out growth inhibition experiments with an adenosine-deaminase-treated preparation. The result is shown in Table 1. Treatment of neplanocin A with adenosine deaminase clearly reduced its toxicity to CHO cells. Thus, this reaction has the potential of detoxifying neplanocin A rather than activating it. The addition of an adenosine deaminase inhibitor to the culture medium with neplanocin A did not, however, alter significantly the observed inhibitory drug concentrations (not shown). This suggests that cellular levels of ADA are not sufficient to detoxify the agent.

The observations described above suggest either that neplanocin A is phosphorylated by an enzyme other than adenosine kinase or that the agent does not require phosphorylation to be active. This was clarified by a series of experiments utilizing HPLC analysis of the nucleotide pools of treated and untreated cells. Analysis of the nucleotide pools of 10^6 CHO cells incubated for 5 hr in the presence and absence of 25 μM neplanocin A revealed the appearance of an additional peak, labeled NpcTP, which eluted at approximately 20 min (Fig. 2). While this peak could reflect the formation of neplanocin A triphosphate, it could as well be the accumulation

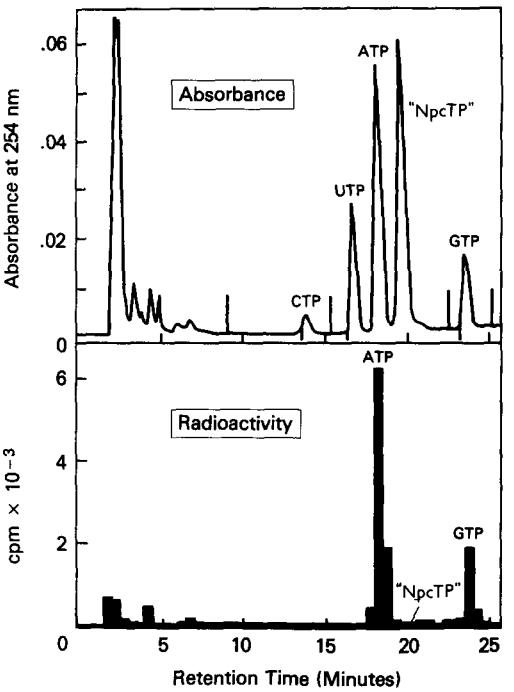

Fig. 3. Metabolism of [^{14}C]formate into purine nucleotides by cells incubated with 25 μM neplanocin A. The upper graph shows the absorbance profile of the HPLC fractionation. The lower graph shows the corresponding profile of radioactivity. The procedure is described in Materials and Methods.

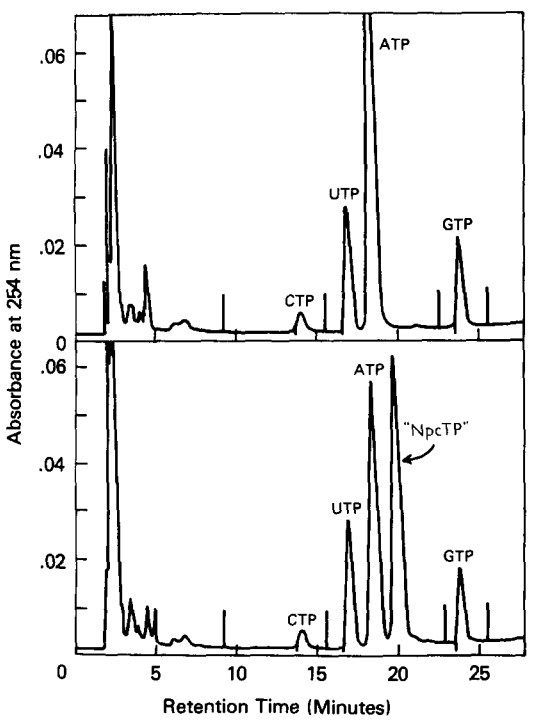

Fig. 2. HPLC analysis of nucleotides from CHO cells incubated with (lower graph) and without (upper graph) 25 μM neplanocin A. Cell samples (1×10^6 cell equivalents) were analyzed for each graph. The procedure is described in Materials and Methods.

of a natural cell constituent such as dATP, which, in fact, elutes with a very similar retention time. To distinguish between these possibilities, cells were exposed to neplanocin A and provided with [^{14}C]-formate to label the purine pools in the cell. The upper graph of Fig. 3 shows the absorbance profile from these cells in which there was a substantial accumulation of the peak in question, termed NpcTP. The lower graph shows the profile of radioactivity in these peaks. ^{14}C appeared in both ATP and GTP but there was no accumulation in the region of the NpcTP peak, suggesting that this peak was probably drug derived. Incubation of cells with deaminated neplanocin A did not give rise to the NpcTP peak (not shown). Further proof of the identity of NpcTP was obtained by treatment with alkaline phosphatase and subsequent identification of the resulting products. Nucleotides eluting in those fractions corresponding to ATP and NpcTP (18–21 min) were collected, removed from the high salt buffer by adsorption onto charcoal, and eluted with an alkaline ethanol solution. Alkaline phosphatase treatment of this fraction from cells incubated without drug gave rise to only adenosine (graph B of Fig. 4), as identified by chromatography on a μBondapak C_{18} reversed phase column. Similar treatment of these nucleotide fractions from cells incubated with neplanocin A gave rise to both adenosine and neplanocin A (graph C of Fig. 4), indicating that the peak appearing at 20 min (Figs. 2 and 3), NpcTP, was indeed a triphosphate derivative of neplanocin A.

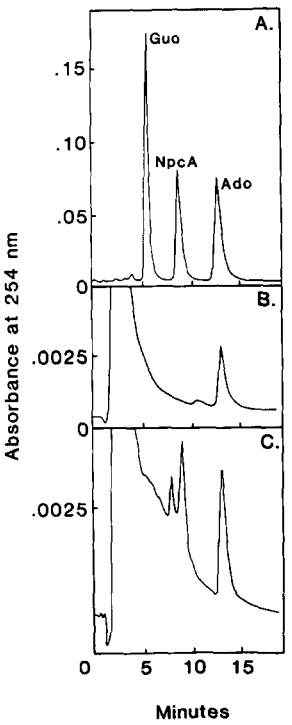

Fig. 4. HPLC analysis of nucleoside products of alkaline-phosphatase-treated nucleotides. ATP and NpcTP were isolated and hydrolyzed, and the products were analyzed by HPLC as described in Materials and Methods. Standards are shown in Graph A, the products of ATP hydrolysis from control cells in graph B, and the products of ATP and NpcTP hydrolysis from cells incubated for 5 hr with neplanocin A in graph C.

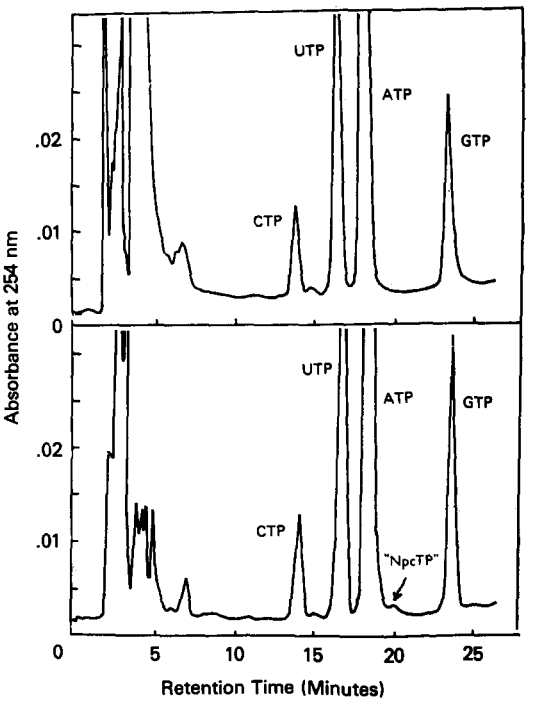

Fig. 5. HPLC analysis of nucleotides from adenosine-kinase-deficient cells (Rb^R-1) incubated with (lower graph) and without (upper graph) 25 μM neplanocin A. The procedure was as described for Fig. 2.

The additional peak appearing at 7.8 min (graph C of Fig. 4) eluted with a retention time very similar to that of thymidine. Since TTP tends to co-elute with ATP from the Partisil column, this may well reflect an accumulation of TTP resulting from inhibition of DNA synthesis in the presence of drug.

The above experiments were carried out at very high drug concentrations, 25 μM, with the purpose of accentuating the effects of drug. At lower concentrations, 0.5 to 1.0 μM, the NpcTP metabolite was still observed; however, the peak was very small, and the depressive effect on ATP levels was, accordingly, much less, but still evident, when observed in the same time frame (5-hr incubation with drug).

To determine if the presence of adenosine kinase is necessary for the metabolism of neplanocin to the triphosphate derivative, the nucleotide pools of the adenosine kinase deficient line, Rb^R-1, were analyzed. The upper graph of Fig. 5 shows the nucleotides from control cells, without drug. The lower graph shows the analysis of an extract of comparable cells incubated in the presence of drug for 5 hr as in the previous experiments with CHO cells. In this case, however, there appeared little, if any, absorbance in the region of NpcTP. We have on occasion observed small NpcTP peaks from Rb^R-1 cells that had been carried for long periods. When

Table 2. Effect of 25 μM neplanocin A on nucleotide pool levels and the incorporation of [^{14}C]formate into ATP and GTP in CHO cells*

Expt. No.	Neplanocin A (μM)	CTP (Abs)	UTP (Abs)	ATP (Abs)	ATP (cpm)	GTP (Abs)	GTP (cpm)
	None	100	100	100	100	100	100
1	25	92.2	111	52.9	47.9	88.1	64.0
2	25	87.7	102	53.7	65.0	75.2	65.4
3	25	90.8	115	49.3		92.7	
4	25	85.6	102	53.5		97.4	

* The procedure is described in Materials and Methods. Values are expressed as percent of control and are the mean of duplicate determinations. Abs refers to determinations based on absorbance.

fresh cells (from frozen stocks) were used, the peak was barely detectable or absent, suggesting the appearance of revertant cells during long-term culture.

Nucleotide pool levels in CHO cells treated with neplanocin A are shown in Table 2. To determine the effects of neplanocin A on *de novo* purine synthesis, the flow of isotope from [^{14}C]formate into ATP and GTP was also measured. The primary effect was a significant depression in ATP concentration with a lesser effect on GTP. *De novo* synthesis, as measured by [^{14}C]formate incorporation, more or less correlates with these observations.

The overall effects of these phenomena on the synthesis of macromolecules in CHO cells are illustrated in Fig. 6. The data in the left panel indicate that RNA synthesis, as measured by [^{3}H]uridine incorporation, was most affected by neplanocin A with the maximum inhibition in the range of 40–50%. If phosphorylation of the drug was essential for this activity, inhibition of RNA synthesis in the CHO line should be more efficient than that in the adenosine-kinase-deficient line, Rb^{R}-1. To determine whether neplanocin A triphosphate was responsible for this inhibition, the effects of the drug on uridine incorporation in the kinase-deficient lines were compared (right panel). Although there was a slight difference in the curves, it was not dramatic, suggesting that inhibition of RNA synthesis by neplanocin A may not require phosphorylation of the drug. This experiment was also carried out with lower drug concentrations (0.01 to 1.0 μM, not shown). At these concentrations, inhibition of [^{3}H]-uridine incorporation was consistently somewhat greater in line Rb^{R}-1 than the parent CHO line.

DISCUSSION

Neplanocin A shares several structural and biological properties with aristeromycin, another carbocyclic analog of adenosine in which a methylene group replaces the oxygen atom of the ribofuranosyl ring. Like neplanocin A, aristeromycin is phosphorylated by adenosine kinase to form the monophosphate derivative which is phosphorylated further to the triphosphate level [9, 10]. It is also metabolized to a carbocyclic GMP analog which is an effective inhibitor of HGPRT activity in H.Ep-2 cells [11], a phenomenon which does not appear to occur with neplanocin A. Both neplanocin A and aristeromycin [10] are active in the absence of significant synthesis of their triphosphate derivatives (in adenosine-kinase-deficient cells) and both have been shown to interfere with methylation reactions [3, 4, 10] which could play an important role in the action and/or toxicity of these compounds. There is, however, little direct evidence in the literature indicating that interference with methylation reactions is actually cytotoxic to cultured cells. Thus, a firm interpretation of these observations is difficult and must await further clarification of the relevance of interference with methylation reactions to cytotoxic mechanisms.

Acknowledgement—This work was supported by PHS Grant CA 35788 awarded by the National Cancer Institute, DHHS.

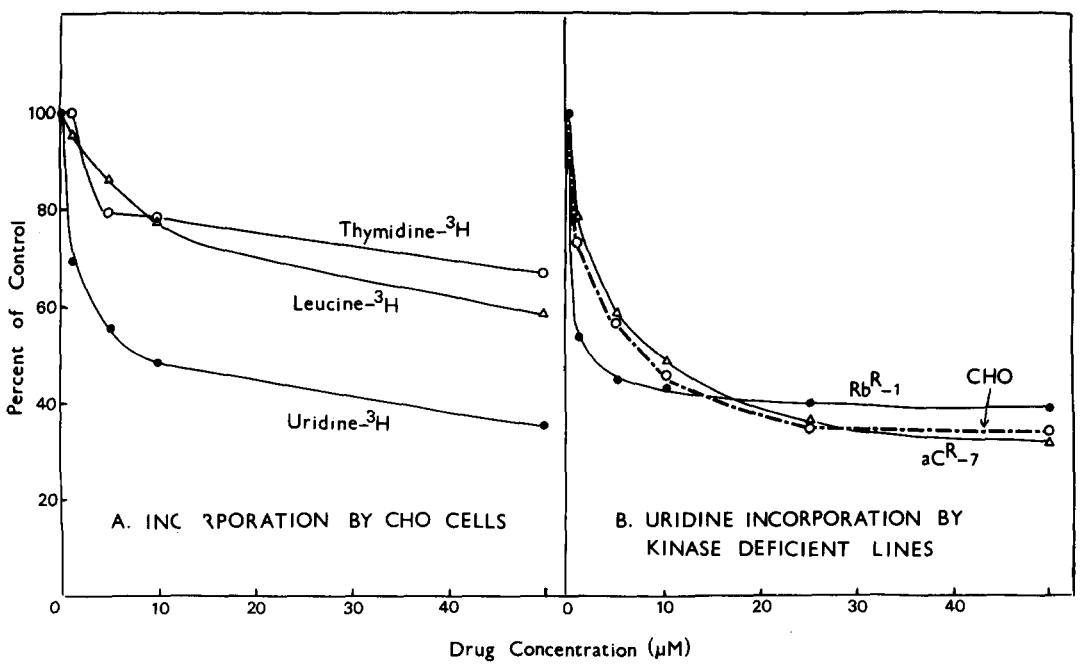

Fig. 6. Effects of neplanocin A on incorporation of radioactive precursors into perchloric acid insoluble material in CHO cells (A) and kinase-deficient mutants (B). The procedure is described in Materials and Methods. Graph A shows incorporation of [*methyl*-^{3}H]thymidine (○), [5-^{3}H]uridine (●), and [4,5-^{3}H]leucine (△) in CHO cells. Graph B compares the incorporation of [5-^{3}H]uridine in the parent CHO (○), adenosine-kinase-deficient Rb^{R}-1 (●), and deoxycytidine-kinase-deficient aC^{R}-7 (△) cells.

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