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MODE OF ACTION OF 2-AMINO-6-CHLORO-1-DEAZAPURINE

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Abstract—2-Amino-6-chloro-1-deazapurine is of interest as a purine analog with demonstrated *in vivo* activity against mouse leukemia L1210. That the active form of this agent is a nucleotide and that the nucleotide is formed by the action of hypoxanthine (guanine) phosphoribosyltransferase were shown by the facts that (a) L1210 cells deficient in hypoxanthine phosphoribosyltransferase were insensitive to the analog; (b) hypoxanthine, but not adenine, prevented the formation of the analog nucleotide by enzyme preparations containing activities of both hypoxanthine and adenine phosphoribosyltransferases; and (c) the cytotoxicity of the analog was prevented by hypoxanthine. The ribonucleoside of this analog was not toxic to cell cultures and hence is not phosphorylated or cleaved to the base. In intact HEp-2 cells and L1210 cells, the analog was metabolized to the nucleoside 5′-phosphate which accumulated to concentrations as high as 1000 nmoles/10^9 cells; no di- or triphosphates were detected. In HEp-2 cells, the analog reduced the pools of purine nucleotides with some accumulation of IMP. The toxicity of minimal inhibitory concentrations of the analog to HEp-2 cells could be prevented or reversed by 4(5)-amino-5(4)-imidazolecarboxamide (AIC); the toxicity of higher concentrations could be prevented or reversed by a combination of adenine and guanosine but not by AIC. The analog inhibited the incorporation of formate into purine nucleotides and into macromolecules at concentrations that had no effect on utilization of hypoxanthine; at higher concentrations the incorporation of hypoxanthine was inhibited. Low concentrations also inhibited the utilization of uridine and thymidine. The incorporation of hypoxanthine and AIC into guanine nucleotides, but not adenine nucleotides, was inhibited. These results indicate two sites of inhibition of the biosynthesis of purine nucleotides, the more sensitive one being on an early step of the pathway and the less sensitive one on the IMP–GMP conversion. That the blockade of *de novo* synthesis probably was at the site of feedback inhibition was indicated by the fact that the analog inhibited the accumulation of formylglycinamide ribonucleotide in azaserine-treated cells but did not inhibit the synthesis of 5′-phosphoribosyl 1-pyrophosphate. Comparative studies were performed with the related analog, 2-amino-6-chloropurine, which has been reported to produce a similar dual blockade of the purine pathway. This purine was less toxic than its 1-deaza analog; it produced a modest decrease in adenine nucleotides but increased pools of guanine nucleotides. IMP dehydrogenase from L1210 cells was not inhibited by the nucleotides of 2-amino-6-chloropurine or 2-amino-6-chloro-1-deazapurine at 1 mM comcentrations. The nucleotide of 2-amino-6-chloropurine was dechlorinated by AMP deaminase; the apparent K_m was about the same as that of AMP and the V_{max} was 21% that of AMP. The nucleotide of 2-amino-6-chloro-1-deazapurine was not a substrate. Conversion of the nucleotide of 2-amino-6-chloropurine to GMP by the action of AMP deaminase explains both the lower toxicity of 2-amino-6-chloropurine as compared to its 1-deaza analog and its capacity to increase pools of guanine nucleotides. Thus, of the two compounds the 1-deaza analog may be the more interesting as an antitumor agent because of the greater metabolic stability of its nucleotide.

2-Amino-6-chloro-1-deazapurine (ACDP†) was synthesized many years ago by Markees and Kidder [1] and again later by Schelling and Salemink [2], but its antitumor activity was not examined until it was resynthesized in our laboratory [3]. We have found ACDP to be about as effective as 6-mercaptopurine in prolonging the life spans of mice that bear leukemia L1210 [4]. The corresponding purine, 2-amino-6-chloropurine (ACP), also has activity against leukemia L1210‡ and against adenocarcinoma 755 [5], and its mechanism of action has been investigated by Sartorelli *et al.* [6]. It was therefore of interest to determine if the modes of action of ACDP and ACP were the same and if the substitution of a carbon atom for the nitrogen atom in position-1 of the purine ring resulted in any significantly altered metabolic properties. Preliminary reports of some of these data have been presented [4, 7].

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† Abbreviations: ACDP, 2-amino-6-chloro-1-deazapurine; ACP, 2-amino-6-chloropurine; H(G)PRTase, hypoxanthine (guanine) phosphoribosyltransferase; APRTase, adenine phosphoribosyltransferase; AIC, 4(5)-amino-5(4)-imidazolecarboxamide; TCA, trichloroacetic acid; and PRPP, 5′-phosphoribosyl 1-pyrophosphate.

‡ Unpublished results, Division of Cancer Treatment, National Cancer Institute.

MATERIALS AND METHODS

Compounds. ACDP and ACDP-ribonucleoside were synthesized in our laboratories [3, 8] and were

provided by Drs. C. Temple and R. D. Elliott. ACP and ACP-ribonucleoside were obtained from the Sigma Chemical Co. (St. Louis, MO), which was also the source of *p*-nitrophenylphosphate, alkaline phosphatase (17 units/mg protein), 5′-nucleotidase (29 units/mg protein) and AMP deaminase from rabbit muscle. [8-^{14}C]IMP (40 mCi/mmole), [8-^{14}C]adenine (5.1 mCi/mmole), sodium [^{14}C]formate (6.5 mCi/mmole), and [*methyl*-^{3}H]thymidine (6.7 Ci/mmole) were obtained from the New England Nuclear Corp. (Boston, MA). Moravek Biochemicals, Inc. (Brea, CA) was the source of [8-^{14}C]hypoxanthine (56 mCi/mmole), [5-^{3}H]uridine (10 Ci/mmole), [2-^{14}C]thymidine (56 mCi/mmole), and [2-^{14}C]uridine (59 mCi/mmole). [4,5-^{3}H]L-Leucine (6 Ci/mmole) was purchased from Schwarz-Mann, Spring Valley, NY. AIC was tritiated by New England Nuclear, and the product (sp. act. 40.8 mCi/mmole) was purified in our laboratory by Dr. R. F. Struck. Azaserine was a gift from Warner-Lambert/Parke-Davis Laboratories, Detroit, MI.

Synthesis of ribonucleotides of ACDP and ACP. These nucleotides were prepared by the use of the nucleoside phosphotransferase (EC 2.7.1.77) isolated from carrots as described by Brunngraber [9]. The enzyme was incubated with ACP-ribonucleoside (165 μmoles) or ACDP-ribonucleoside (149 μmoles) and *p*-nitrophenylphosphate (2,152 μmoles) in 0.1 M ammonium acetate buffer, pH 5.0, for 21 hr. The reaction mixture was added to a suspension of 10 g each of Norit A and celite in 100 ml of water which had been prepared by the following sequential treatments: (a) suspension in 100 ml of H_2O and adjustment of the pH to 2.0 with HCl; (b) stirring overnight at room temperature; (c) filtration and washing with H_2O until the pH of the filtrate was 3; (d) suspension in H_2O to give a total volume of 100 ml. The resulting mixture (enzymic reaction plus Norite–celite suspension) was stirred at room temperature for 1 hr. The Norit and celite were then removed by filtration; the filtrate was assayed by high pressure liquid chromatography (HPLC) on an ion exchange column to ascertain that all of the nucleotide had been absorbed on the charcoal. The Norit–celite was washed with H_2O until the filtrate was free of chloride ion, after which the phosphates were eluted with 2% ammoniacal ethanol. The eluate, containing the desired nucleotide and *p*-nitrophenylphosphate, was lyophilized, and the residue was dissolved in 20 ml of 2.5 M ammonium acetate, pH 8.5. Separation of the nucleotide from *p*-nitrophenylphosphate was accomplished by chromatography on a column (1 cm × 12 cm) of Affi-Gel 601 (Bio-Rad Laboratories, Richmond, CA), which consists of a boronate derivative attached to polyacrylamide. The solution was applied to the column 2 ml at a time. After each addition, the column was washed with 90 ml of 2.5 M ammonium acetate (which eluted essentially all of the *p*-nitrophenylphosphate) and the nucleotide was then eluted with 0.1 N formic acid. The formic acid eluate was lyophilized, and the residue was dissolved in H_2O and treated with Norit–celite as described above. The Norite–celite was extracted with ammoniacal ethanol which was evaporated to dryness to yield the nucleotide in approximately 50% yield.

Cytotoxicity determinations and reversal studies. Stock cultures of HEp-2 cells were maintained in SRI-14 [10] medium and stock cultures of L1210 cells in Fischer's [11] medium. For HEp-2 cells, cytotoxicities were determined by the effects of the agent on colony formation when 100 cells were incubated in 8 oz prescription bottles for 7–10 days in 10 ml of medium containing the agent at various concentrations; these procedures have been described in detail [12]. For L1210 cells, cytotoxicities were also determined by effects on colony formation, but the cloning was in soft agar. The capacities of various compounds to prevent or reverse the toxicities to HEp-2 cells were also determined similarly by colony counts; additional details are given in Fig. 1.

Effects on incorporation of precursors into DNA, RNA, protein, and soluble nucleotides. The effects of ACDP on the incorporation of various radioisotope-labeled precursors into macromolecules were determined by incubating HEp-2 cells for periods of 1–6 hr with the labeled compound in the presence or absence of ACDP and measuring the specific activities of the total TCA-insoluble material and the alkali-stable acid-insoluble material. The activity of the TCA-insoluble material is a measure of protein synthesis when [^{3}H]leucine is the precursor; a measure of synthesis of RNA + DNA when labeled hypoxanthine, formate or uridine is the precursor; and a measure of DNA synthesis when labeled thymidine is the precursor. Radioactivity in the alkali-stable fraction is a measure of synthesis of DNA from [5-^{3}H]uridine or [^{3}H]thymidine. Radioactivity in RNA was determined as the difference between the activities in the TCA-insoluble fraction and the alkali-stable fraction. These procedures, which are modifications of those of Hershko *et al.* [13], have been described in detail [14].

Effects on the incorporation of precursors into soluble nucleotides were determined by analysis of nucleotide pools by high pressure liquid chromatography as described below.

Measurement of PRPP pools. Pools of PRPP in HEp-2 cells were determined by the method of Bagnara *et al.* [15], which involves measurement of the amount of [^{14}C] AMP formed when a cell extract is incubated with adenine phosphoribosyltransferase (APRTase) (EC 2.4.2.7), excess [^{14}C]adenine, and $MgCl_2$.

Determination of effects on synthesis of purine nucleotides de novo. The effects of ACDP on synthesis of purine nucleotides *de novo* were determined by measurement of inhibition of the synthesis of [^{14}C]formylglycinamide ribonucleotide in cells grown in the presence of azaserine and [^{14}C]formate. The method used is a previously described modification [16] of the procedure of LePage and Jones [17]. Additional details are given in Table 5.

ACDP as a substrate for hypoxanthine (guanine) phosphoribosyltransferase [H(G)PRTase] EC 2.4.2.8). To identify the phosphoribosyltransferase responsible for converting ACDP to the nucleotide, studies were performed with an enzyme preparation from HEp-2 cells that had activities of both H(G)PRTase and APRTase. The enzyme preparation was a 100,000 *g* supernatant fraction that had

been dialyzed overnight against 0.05 M Tris. The incubation mixture contained, in a final volume of 0.20 ml, 0.8 μmole PRPP, 0.4 μmole Mg^{2+}, 0.2 μmole ACDP, and hypoxanthine at various concentrations ranging between 0.01 μmole and 10 μmoles. After incubation for 30 min the reaction was stopped by immersion of the reaction vessel in a boiling water bath. Portions of the solution were subjected to HPLC on a Partisil-10 SAX ion exchange column. ACDP-nucleotide (retention time about 9 min) was separated from IMP (retention time about 8 min). Quantitation of the ACDP nucleotide peak was made by integration of the peak area (see HPLC below).

Studies with IMP dehydrogenase (EC 1.2.1.14). IMP dehydrogenase was prepared from L1210 cells essentially as described by Nelson *et al.* [18]. The enzyme preparation was further purified by chromatography on GMP-agarose. The enzyme solution was applied to a GMP-agarose column (1.5 cm × 11 cm) that had been equilibrated with 10 mM Tris–HCl, pH 7.8, containing 1 mM EDTA, 0.5 mM reduced glutathione, and 25 mM KCl. The column was washed with 10 mM Tris–HCl buffer and then eluted with 10 mM Tris–HCl buffer containing 10 mM GMP. Fractions containing IMP dehydrogenase activity were pooled and dialyzed overnight against 150 vol. of 10 mM Tris–HCl buffer with one change of buffer. Enzyme activity was assayed by the spectrometric method of Jackson *et al.* [19].

AMP deaminase (EC 3.5.4.6). AMP deaminase was assayed spectrophotometrically by measurement of change in optical density at 265 nm when enzyme and substrate (AMP or ACP-ribonucleotide) were incubated in 0.01 M citrate buffer, pH 7.0. When ACP-ribonucleotide was the substrate, HPLC analysis was also performed to substantiate that the product was GMP.

High pressure liquid chromatography. Analyses by high pressure liquid chromatography were performed at ambient temperature with a Waters Associates (Milford, MA) model 202 apparatus. For determination 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 3.7; the flow rate was 1 ml/min. For analysis of nucleosides a reverse phase μBondapak C_{18} column (Waters) was used; elution was achieved at a flow rate of 1 ml/min with one of two solvents: (a) H_2O–acetic acid–dioxane (100:1.5:2.0, by vol.); (b) H_2O–acetonitrile (97:3, v/v). Integration of peak areas was accomplished with a Hewlett–Packard model 3380-A digital electronic integrator. These procedures are described in detail elsewhere [20].

RESULTS

Cytotoxicities (Table 1). ACDP was more toxic than ACP to both HEp-2 and L1210 cells. L1210/MP cells were insensitive to both ACDP and ACP. ACDP-ribonucleoside was not toxic to any of the three cell lines.

Prevention or reversal of cytotoxicity of ACDP (Fig. 1). The toxicity produced by 0.5 μM ACDP could be almost completely prevented or reversed by AIC. As the concentration of ACDP was raised, AIC became less effective and was completely ineffective when the concentration of ACDP was 1.5 μM. The toxicity of an even higher concentration of ACDP (3 μM) was prevented completely by hypoxanthine or by a combination of adenine and guanosine and was partially prevented by adenine alone. In Fig. 1, the results shown for AIC are for a single experiment that is typical of the several that were performed. The results shown for reversal of the effects of higher concentrations of ACDP by purines are for a single experiment, typical of some, but not all, of the experiments that were performed. Hypoxanthine reproducibly prevented completely cytotoxicity in all of the six experiments. The prevention of toxicity by adenine or by adenine + guanosine occurred in most, but not all, experiments. In two of the six experiments adenine alone gave no reversal, and in two experiments the combination of adenine and guanosine gave only partial reversal.

Effects of hypoxanthine and adenine on conversion of ACDP to the nucleotide in cell-free systems (Fig.

Table 1. Cytotoxicities of 2-amino-6-chloropurine (ACP) and 2-amino-6-chloro-1-deazapurine (ACDP)*

Cell line	EC_{50} (μM) ACDP	ACDP ribonucleoside	ACP
L1210	0.3 (4)	> 65 (2)	8.7 (2)
L1210/MP	60.0 (4)	> 65 (2)	>65.0 (2)
HEp-2	0.3 (4)	>120 (2)	10.0 (2)

* Approximately 100 cells were incubated in medium containing inhibitor at various concentrations, and the number of colonies formed was determined 7–10 days later. See text for details. The EC_{50} is that concentration inhibiting colony formation by 50%. The number in parentheses indicates the number of experiments.

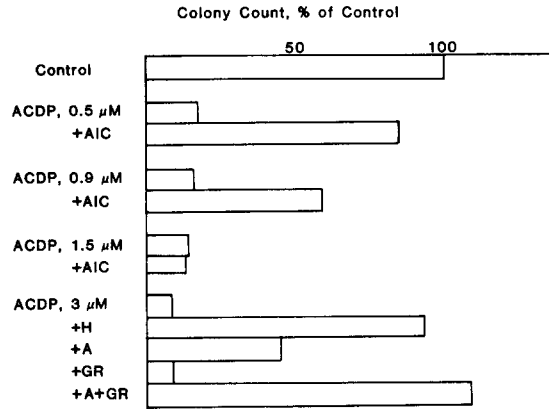

Fig. 1. Effectiveness of AIC and purines in preventing or reversing inhibition of HEp-2 cells by ACDP. Cytotoxicities were determined by the colony count method described in the text. ACDP and the candidate reversal agents were added simultaneously to 10 ml of medium containing 100 cells, and the numbers of colonies formed were counted 7 days later. Abbreviations: H, hypoxanthine; A, adenine; and GR, guanosine. The concentrations (μM) of the reversal agents were: AIC, 317; A, 37; H, 147; and GR, 70.

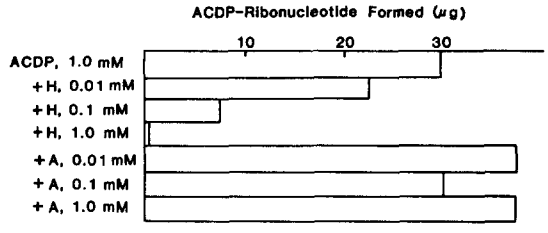

Fig. 2. Effects of adenine (A) and hypoxanthine (H) on the conversion of ACDP to ACDP-ribonucleotide by cell-free extracts from HEp-2 cells. The incubation mixture contained the enzyme, PRPP, $MgCl_2$ (see text for concentrations), and ACDP, A, or H at the concentrations indicated in the chart. The reaction mixture was analyzed for ACDP-nucleotide by HPLC (see text).

2). Cell-free extracts incubated with PRPP, $MgCl_2$, and ACDP formed ACDP-nucleotide, and this conversion was prevented by hypoxanthine but not by adenine. Hypoxanthine at a concentration of 0.1 mM inhibited the formation of ACDP ribonucleotide by 75%, whereas adenine at 1.0 mM gave no inhibition. Not shown are similar experiments in which the effects of ACDP were determined on the conversion of [8-^{14}C]hypoxanthine to [8-^{14}C]IMP. ACDP at concentrations up to 20-fold that of hypoxanthine was without effect on this conversion.

Metabolism of ACDP and effects on nucleotide pools. ACDP has an absorption maximum at 312 nm; this fact makes it possible, in the same HPLC experiment, to determine the effects of ACDP on the pools of natural nucleotides by monitoring the effluent at 254 nm and to measure the amounts of ACDP-nucleotide formed by monitoring the effluent at a wavelength greater than 300 nm. ACDP also has significant u.v. absorption at 254 nm; therefore, when ACDP-nucleotide is present at high concentrations, it is also visible when the effluent is monitored at 254 nm. The results of a typical experiment with HEp-2 cells are shown in Fig. 3. In Fig. 3C it is apparent that the only metabolite of ACDP detected at 340 nm was a compound with a retention time of 8.0 min. Stop-scan determination of the u.v. absorption spectrum of this peak yielded a spectrum indistinguishable from that of ACDP-ribonucleoside (result not shown). When the cell extract was treated with alkaline phosphatase and then subjected to HPLC analysis on a reverse phase column, monitoring of the effluent showed a single peak with a retention time of 25.3 min (Fig. 3D); the same result was obtained when 5′-nucleotidase was substituted for alkaline phosphatase. The retention time and the u.v. absorption spectrum were identical with those of a synthetic sample of ACDP-ribonucleoside (result not shown). When synthetic ACDP-ribonucleoside was added to the phosphatase-treated cell extract, reverse phase chromatography, monitored at 340 nm, revealed only a single peak with a retention time of 25.3 min (result not shown). Some reduction in pools of ATP and GTP, with no appreciable effect on those of pyrimidine nucleotides, is also apparent in Figs. 3A and 3B.

Similar and more extensive studies were performed with L1210 cells; a higher concentration of ACDP was used and its effects were examined at periods from 1 to 24 hr. Under these conditions, the accumulation of ACDP-ribonucleotide was such that it could be detected by scanning at 254 nm (Fig. 4). In addition, another peak appeared with a retention time of about 1 min less than that of ACDP-ribonucleotide. This is the retention time of IMP, which was present in the control at low concentrations (5–35 nmoles/10^9 cells). Stop-scan determination of the u.v. spectrum of the suspected IMP peak in ACDP-treated cells yielded a spectrum identical with that of IMP. When IMP was added to the cell extract and HPLC analysis was repeated, the suspected IMP peak was reinforced (result not shown).

The metabolism of ACDP in L1210 cells and its effects on pools of natural nucleotides are summarized in Table 2. The maximum concentration of ACDP-ribonucleotide was reached within 2 hr; at this time it was about 1000 nmoles/10^9 cells, which was about 20% that of TP in control cells. After 2 hr, the concentration of ACDP-ribonucleotide steadily decreased, but at 24 hr it was still about 25% of its maximum concentration. Decrease in the purine nucleotide pools with no effect on pyrimidine nucleotides occurred by 1 hr. By 4 hr, the pools of both purine and pyrimidine nucleotides were strongly depressed. Accumulation of IMP occurred rapidly, but by 4 hr this pool had fallen to control levels. It is to be noted that, because of the very low concentrations of IMP in control cells, the 5-fold increased pools of IMP shown in Table 2 represent pools that are still small and that, because of the difficulty of determining such small pools, the increase in IMP should be regarded as a qualitative rather than a quantitative observation.

Effects of ACP on nucleotide pools. A limited number of experiments, similar to those of Table 2 with ACDP, were performed with ACP (Table 3). Exposure of HEp-2 cells to this compound for 2 hr produced little effect on nucleotide pools. At 4 hr guanine nucleotides were increased. After 6 hr pools of adenine and pyrimidine nucleotides were decreased, whereas pools of guanine nucleotides remained elevated. To determine whether the elevated GTP pool was due to GTP and not to the triphosphate of ACP-ribonucleoside which (if formed) might co-elute with GTP, the GTP fraction was collected and treated with phosphatase, after which the mixture was subjected to reverse-phase chromatography [elution with the aqueous acetonitrile solvent (see Materials and Methods)]. A single peak with the retention time (9.0 min) of guanosine was obtained. The ribonucleoside of ACP is well separated (retention time 44.4 min) from guanosine under these conditions. No attempt was made in those experiments to identify nucleotides of ACP that might have been formed; very small new peaks were apparent in the monophosphate and triphosphate area.

Effects of ACDP on synthesis of macromolecules (Fig. 5). At a concentration of 15 μM, ACDP inhibited the incorporation of formate into RNA + DNA but was without effect on the incorporation of hypoxanthine. This concentration of ACDP also inhibited almost completely the incorporation of thymidine and the incorporation of

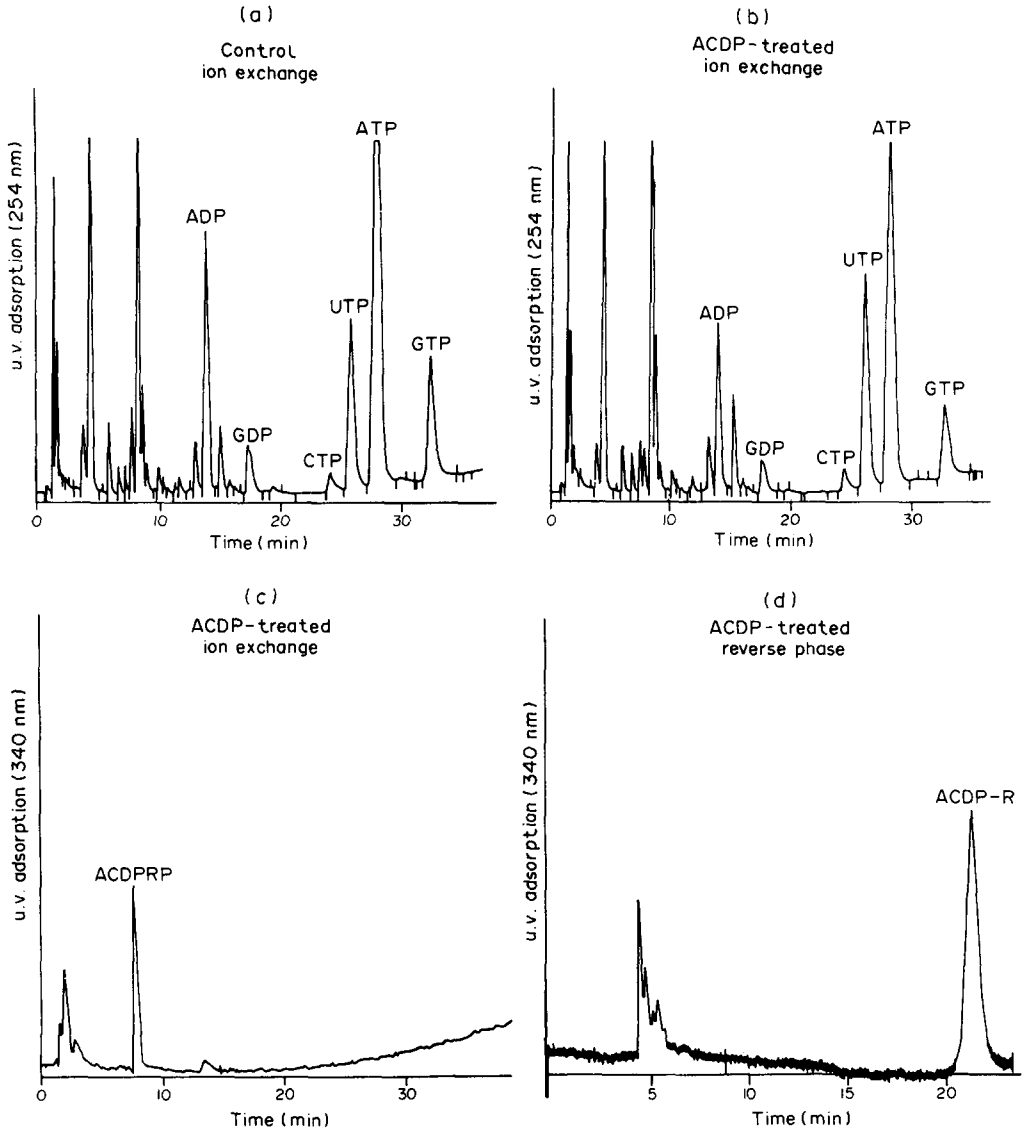

Fig. 3. HPLC analysis of the nucleotides of HEp-2 cells treated with ACDP. To cells in suspension culture, ACDP was added to a concentration of 5.8 μM, and the cells were harvested 4 hr thereafter. Cold perchloric extracts of the cells were subjected to chromatography on an ion exchange column or were treated with phosphatase and analyzed by reverse phase chromatography, as described in the text. (A) Control cells (no ACDP), ion exchange chromatography monitored at 254 nm; (B) ACDP-treated cells, ion exchange chromatography monitored at 254 nm; (C) ACDP-treated cells, ion exchange chromatography monitored at 340 nm; and (D) reverse phase chromatography (solvent, H_2O–acetic acid–dioxane) (see text) of phosphatase-treated extract of ACDP-treated cells, monitored at 340 nm.

uridine into both DNA and RNA. Higher concentrations of ACDP inhibited the incorporation of hypoxanthine and leucine. The results shown for formate and hypoxanthine are for incorporation of precursors into the acid-insoluble fraction; the inhibitions were about the same when the incorporations into RNA and DNA were determined (results not shown).

Effects of ACDP on incorporation of precursors into soluble nucleotides. ACDP (5.8 μM) almost completely inhibited the incorporation of [^{14}C]formate into purine nucleotides (Fig. 6, A and B), but was without marked effect on the incorporation of [^{14}C]hypoxanthine (Fig. 6, C and D). At a higher concentration (15 μM) ACDP did inhibit incorporation of [^{14}C]hypoxanthine, as well as that of [^{3}H]AIC, into guanine nucleotides, and slightly stimulated incorporation into adenine nucleotides, as is shown in the data of Table 4. Similar experiments (not shown) demonstrated that ACDP did not inhibit the conversion of labeled thymidine and uridine to mono-, di-, and triphosphates.

Inhibition of synthesis of formylglycinamide ribonucleotide. ACDP inhibited the synthesis of [^{14}C]formylglycinamide ribonucleotide in cells that had been treated with [^{14}C]formate and azaserine.

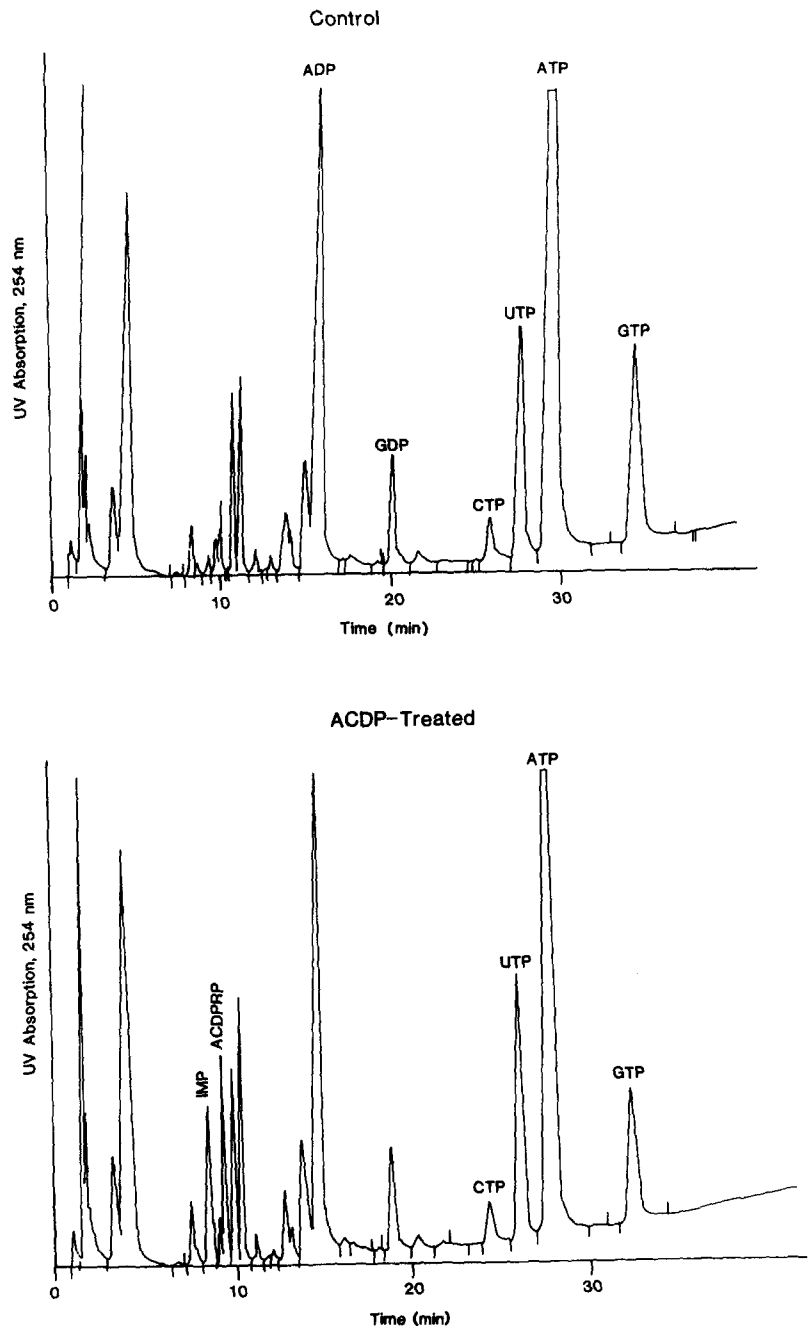

Fig. 4. HPLC analysis of L1210 cells treated with ACDP. To cells in suspension culture, ACDP was added to a final concentration of 29 μM, and the cells were harvested 1 hr thereafter. Cold perchloric acid extracts were prepared and analyzed on an ion exchange column, with monitoring at 254 nm, as described in the text. New peaks appearing in the treated cells were identified as IMP and ACDP-ribonucleotide (ACDPRP); see text for details.

A concentration of 6 μM gave about 80% inhibition, and concentrations of 15 μM and higher gave essentially complete inhibition (Table 5).

Effects on IMP dehydrogenase. The ribonucleotides of ACDP and ACP were assayed as inhibitors of IMP dehydrogenase partially purified from L1210 cells. Using the lowest concentration of IMP (1.8×10^{-5} M) that would give significant changes in u.v. absorption, we were unable to obtain inhibition with ACDP-ribonucleotide or ACP-ribonucleotide at concentrations up to 1 mM. To confirm that the enzyme was functioning properly, we analyzed product formation by HPLC. These analyses confirmed that XMP was being formed and that neither nucleotide inhibited its formation. These negative results are not shown.

Table 2. Metabolism and metabolic effects of ACDP in L1210 cells*

Time (hr)	ACDP-nucleotide (nmoles/10^9 cells)	Nucleotides (% of control) IMP	ADP + ATP	GDP + GTP	Pyrimidine nucleotides
1	619	516	58	72	114
2	1111	538	46	58	86
4	822	106	14	20	31
8	687	<100	25		49
12	477	<100	19	33	33
24	249	<100	26	9	64

* To L1210 cells, ACDP was added to a concentration of 29 μM, and the cells were harvested at periods of 1–24 hr thereafter. Cold perchloric acid extracts were prepared and subjected to HPLC analysis on an anion exchange column. The effluent was monitored at 254 nm to detect the natural nucleotides and at 340 nm to detect ACDP-ribonucleotide. Pools of IMP in the 8, 12 and 24 hr experiments were very low and hence unreliable, and are therefore indicated as <100% that of controls.

ACP-ribonucleotide as a substrate for AMP deaminase. ACP-ribonucleotide was assayed as a substrate for the AMP deaminase from rabbit muscle. The results, subjected to Lineweaver–Burk analysis, gave an apparent K_m of 1.4×10^{-3} M. AMP was also analyzed as a substrate; the K_m was 1.0×10^{-3} M, about the same as reported by others for this enzyme [21]. The V_{max} for ACP-ribonucleotide was 21% that of AMP. These plots are not shown. The reaction of ACP-ribonucleotide with the deaminase produced a change in u.v. absorption of the reaction mixture from that characteristic of ACP(λ_{max}, 312 and 249 nm) to that characteristic of GMP (λ_{max}, 252 nm). To confirm that the product was GMP, the reaction mixture was subjected to HPLC on an anion exchange column. A single peak was obtained with a retention time the same as that of GMP (8.6 min); determination of the spectrum of this peak in a stop-scan spectrophotometer showed a spectrum indistinguishable from that of GMP. A sample of the reaction mixture was treated with alkaline phosphatase and then subjected to HPLC on a reverse phase column (H_2O-acetonitrile solvent). A single peak was obtained with the retention time (9.0 min), and the u.v. absorption spectrum, of guanosine (results not shown). When ACDP-ribonucleotide was evaluated as a substrate for the deaminase, no change in u.v. absorption was observed, even after incubation periods of several hours (results not shown).

Table 3. Effects of ACP on nucleotide pools of HEp-2 cells*

Time (hr)	Nucleotides (% of control) Adenine	Guanine	Pyrimidine
2	90	100	90
4	98	170	89
6	78	150	90
9	72	186	57
12	64	194	58

* ACP was added to cultures of logarithmically growing cells at a concentration of 117 μM, and the cells were harvested at various times thereafter. Nucleotides were analyzed by HPLC (see text and Fig. 3).

DISCUSSION

The results indicate that ACDP was converted to the nucleoside 5′-phosphate, which blocked the biosynthesis of purine nucleotides at two points: an early step of the pathway and the IMP–GMP conversion. That the compound was converted to the nucleotide by the action of H(G)PRTase and that nucleotide formation was required for inhibition were indicated by (a) the observed capacity of hypoxanthine, but not adenine, to prevent the conversion of ACDP to its nucleotide by cell-free preparations that contained activities of both H(G)PRTase and

Table 4. Effects of ACDP on the conversion of hypoxanthine and AIC to nucleotides of adenine and guanine*

	% of total radioactivity Adenine nucleotides	Guanine nucleotides
[^{14}C]Hypoxanthine		
Control	73	23
ACDP (15 μM)	78	16
[^{3}H]AIC		
Control	61	21
ACDP (15 μM)	64	17

* To HEp-2 cells in suspension culture (500 ml) ACDP was added to a concentration of 15 μM, followed 0.5 hr thereafter by [8-^{14}C]hypoxanthine (10 μCi) or [^{3}H]AIC (80 μCi). The cells were harvested 1 hr after addition of the tracer; perchloric acid extracts were prepared and subjected to HPLC analysis on an ion exchange column as described in the text. Fractions (1 ml each) were collected and analyzed for ^{14}C or ^{3}H in a liquid scintillation spectrometer. The isotope content for the adenine nucleotides is that appearing in ADP + ATP; that shown for the guanine nucleotides is that appearing in GDP + GTP.

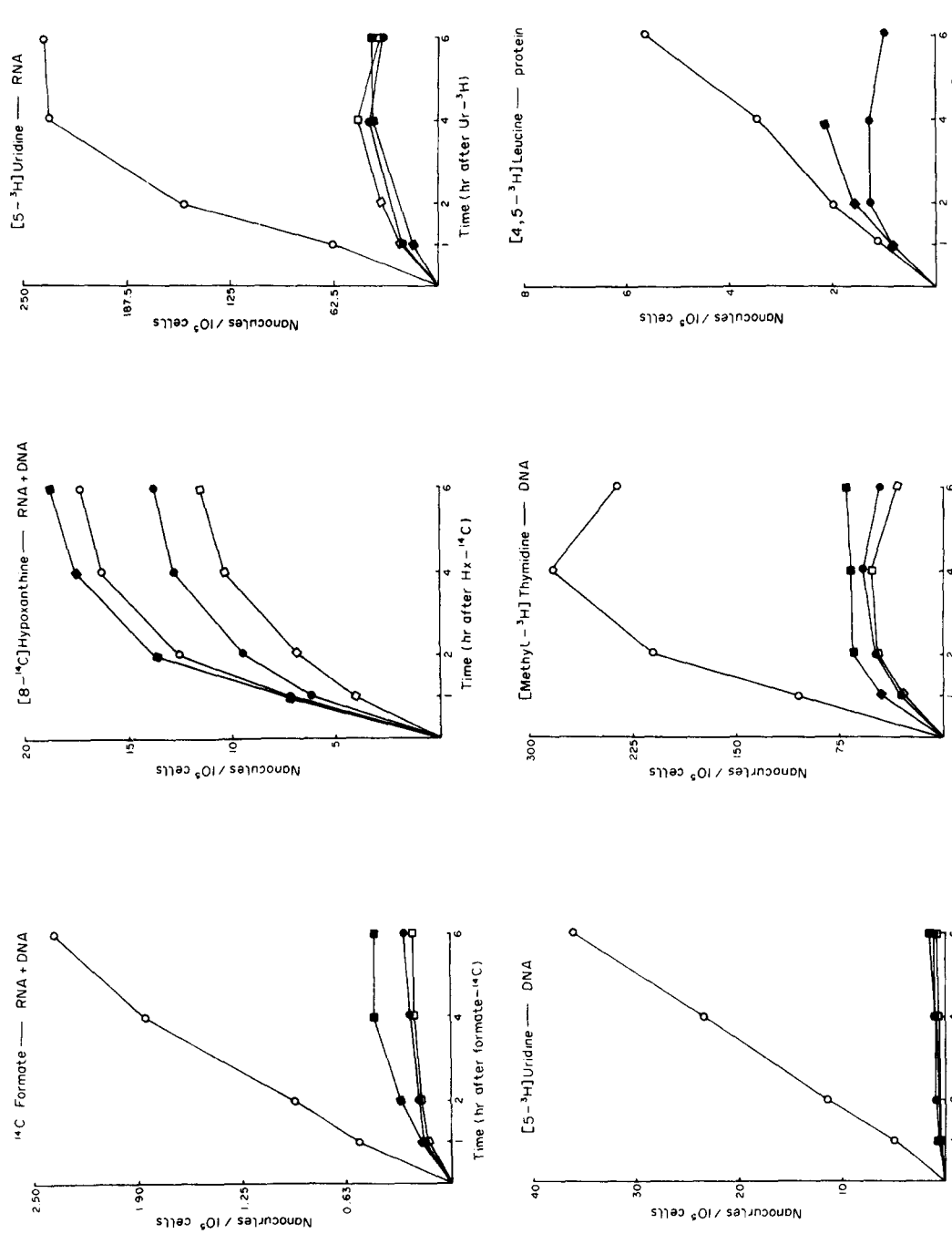

Fig. 5. Effects of ACDP on the incorporation of labeled precursors into macromolecules of L1210 cells. To cells in suspension culture, ACDP was added 0.5 hr before the labeled precursor ([8-^{14}C]hypoxanthine, 2 μCi/ml; [^{14}C]formate, 4 μCi/ml; [*methyl*-^{3}H]thymidine, 2 μCi/ml; [5-^{3}H]uridine, 2μCi/ml; and [4,5-^{3}H]leucine, 2 μCi/ml), and the cells were harvested at periods of 1–6 hr after addition of the labeled compound. See text for methods of isolation and assay. Key: (○) control; (■) ACDP, 15 μM; (●) ACDP, 30 μM; and (□) ACDP, 60 μM.

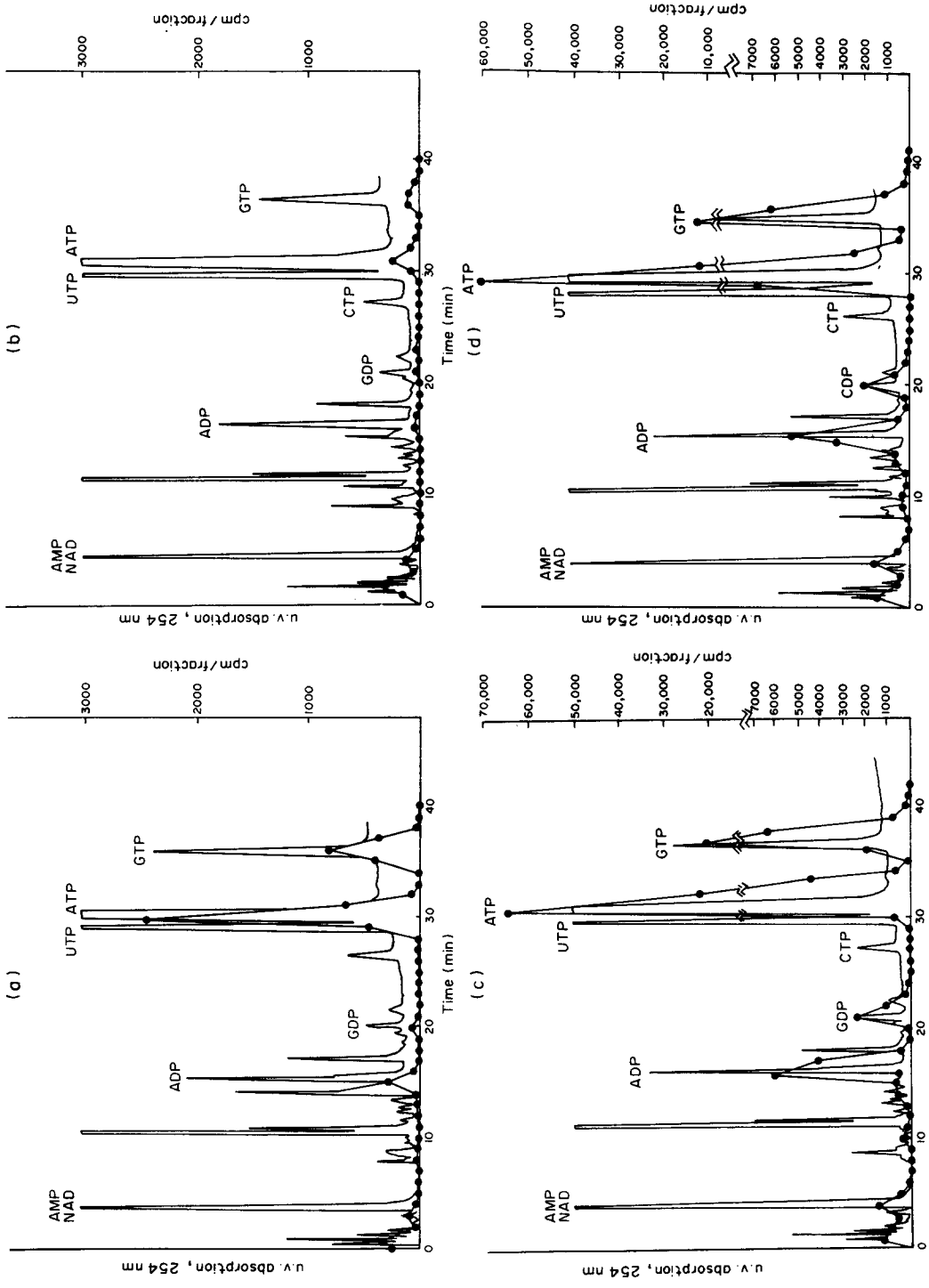

Fig. 6. Effects of ACDP on the incorporation of [^{14}C]formate and [8-^{14}C]hypoxanthine into nucleotides of HEp-2 cells. ACDP was added to cells in suspension culture (500 ml) to a concentration of 5.8 μM, and the labeled precursor [formate (25 μCi) or hypoxanthine (10 μCi)] was added 0.5 hr thereafter. Four hours after addition of the labeled precursor, the cells were harvested. Perchloric acid extracts were prepared and subjected to HPLC on a Partisil-10 SAX column as described in the text. Fractions of 1 ml were collected for radioactivity determinations. (A) Control, [^{14}C]formate; (B) ACDP-treated, [^{14}C]formate; (C) control, [8-^{14}C]hypoxanthine; (D) ACDP-treated [8-^{14}C]hypoxanthine. Key: (smooth lines) u.v. absorption; and (●—●) observed counts per min per 1 ml fraction.

Table 5. Inhibition by ACDP of the synthesis of formylglycinamide ribonucleotide in azaserine-treated HEp-2 cells*

Concn of ACDP (μM)	[^{14}C]Formylglycinamide derivatives (% of control)
0	100
6	17
15	6
29	4

* To suspension cultures of HEp-2 cells ($\sim 4 \times 10^7$ cells in 100 ml of medium) azaserine was added to a concentration of 58 μM, followed 30 min later by ACDP at the indicated concentrations. Thirty minutes after addition of ACDP, sodium [^{14}C]formate (25 μCi) was added. The control culture was treated in the same way except that no ACDP was added. Two hours after addition of formate, the cells were harvested and formylglycinamide ribonucleotides and ribonucleoside were isolated and quantitated by paper chromatography and autoradiography. The formylglycinamide derivatives include the ribonucleotide, the ribonucleoside, and ribonucleoside polyphosphates. Results are for duplicate assays of a single experiment.

APRTase; (b) the lack of toxicity of ACDP to L1210/MP cells that were deficient in H(G)PRTase; and (c) the capacity of hypoxanthine to prevent or reverse the cytotoxicity of ACDP. ACDP was a relatively poor substrate for H(G)PRTase, as shown by the facts that low concentrations of hypoxanthine prevented formation of ACDP-ribonucleotide and high concentrations of ACDP were without effect on the conversion of hypoxanthine to IMP. The reversal or prevention of cytotoxicity by AIC or by combinations of adenine and guanosine indicates that blockade of synthesis of purine nucleotides was responsible for the cytotoxicity of ACDP. Furthermore, the fact that AIC reversed or prevented the toxicity of low concentrations, but not high concentrations, of ACDP is evidence that the early *de novo* site was the more sensitive. The sensitivity of this early site is shown by the potency of ACDP as an inhibitor of the synthesis of formylglycinamide ribonucleotide (Table 5). The fact that PRPP synthesis was not inhibited is consistent with inhibition of the *de novo* pathway being due to feedback inhibition of PRPP amidotransferase, as has been shown for many purine analogs [22]. The marked depression of pools of both adenine and guanine nucleotides in HEp-2 cells (Fig. 3) and L1210 cells (Fig. 4) is consistent with the blockade of *de novo* synthesis being the primary blockade established by ACDP.

Evidence for the second blockade, namely of the conversion of IMP to GMP, was the failure of AIC to prevent or reverse the cytotoxicity of higher concentrations of ACDP (Fig. 1) and the inhibition by ACDP of the incorporation of AIC or hypoxanthine into guanine, but not adenine, nucleotides (Table 4). The observed accumulation of IMP in ACDP-treated cells is consistent with such a blockade but does not prove it, because some, or all, of the IMP might have arisen from degradation of ATP, which was expected to occur in cells whose growth had been inhibited, whatever the mechanism of inhibition might be. At best, only a small accumulation of IMP would be expected to result from inhibition of the IMP–GMP conversion because of the greater potency of the blockade of *de novo* synthesis. Only a small and transient accumulation of IMP was, in fact, observed (Table 2).

Since the nucleotide of the corresponding purine, ACP, has been shown to inhibit IMP dehydrogenase of sarcoma 180 cells [6], it was presumed that the block of the IMP–GMP conversion by ACDP was also due to inhibition of this enzyme. We were unable to inhibit IMP dehydrogenase from L1210 cells with 1 mM concentrations of the nucleotides of ACDP or ACP. This finding with ACP-ribonucleotide is inconsistent with the results of Sartorelli *et al.* [6], who found that the 50% inhibitory concentration of ACP-ribonucleotide for the dehydrogenase from sarcoma 180 cells was 3.7×10^{-4} M. We can conclude only that IMP dehydrogenase from L1210 cells is less sensitive to ACP-ribonucleotide than the enzyme from sarcoma 180 cells. However, with respect to the effects of ACP in intact cells, it is to be noted that the increased pools of guanine nucleotides, resulting from the dechlorination ACP-nucleotide, would produce feedback inhibition of IMP dehydrogenase, and they thus could be responsible for the observed specific inhibition of incorporation of AIC and hypoxanthine into guanine nucleotides [6]. The failure of the nucleotide of ACDP to inhibit IMP dehydrogenase is apparently in conflict with our observations in intact cells. This apparent inconsistency perhaps can be rationalized by the facts that (a) the cellular pools of IMP are very small whereas the nucleotide of ACDP accumulates to a considerable concentration (Table 2), and (b) in the *in vitro* enzyme assay, IMP must be present at a concentration that gives sufficient product for the course of the reaction to be followed. As a result, the ratio of ACDP-nucleotide to IMP may be much higher in intact cells than that which can be used in the *in vitro* enzyme assay.

ACDP at a low concentration inhibited the incorporation of formate, uridine, and thymidine, but not of hypoxanthine, into macromolecules. The fact that ACDP did not inhibit conversion of uridine and thymidine to their triphosphates indicates that the inhibition of incorporation of these precursors into the polynucleotides resulted from interference with polymerization. The failure to inhibit utilization of hypoxanthine at a concentration that inhibited utilization of the other precursors may appear anomalous. This apparent inconsistency can be rationalized if the effects on RNA and DNA synthesis (as measured by incorporation of uridine and thymidine) resulted from depletion of the purine nucleoside triphosphates produced by the *de novo* blockade, and if the amounts of [^{14}C]hypoxanthine used were sufficient, over the short time period of a tracer experiment, to restore purine nucleotide pools to levels that permit normal rates of synthesis of RNA and DNA.

ACDP-ribonucleoside 5′-monophosphate apparently was the inhibitory form of ACDP, since no other metabolites were detected. The presence of small amounts of di- and triphosphates was not excluded, but no evidence for their presence was detected even in cells in which the concentration of

the monophosphate reached 1000 nmoles/10^9 cells, a concentration about 20% that of ATP in control cells. The fact that ACDP-ribonucleoside, which has also been prepared by Cline *et al.* [23], was without cytotoxicity indicates that it was neither phosphorylated nor cleaved to the free base. Thus, ACDP-ribonucleoside was not a substrate for purine nucleoside phosphorylase as is to be expected from the fact that 1-deazaguanosine is not a substrate for this enzyme [24].

A comparison of the data on ACDP in this study and that on ACP from this study and that of Sartorelli *et al.* [6] shows that ACDP is the more toxic compound, but that the two agents apparently have the same mechanism of action. It would be expected that the chief difference in metabolic properties of the two compounds would result from the greater stability of the chlorine atom in ACDP. This difference in stability was apparent in the fact that ACP-ribonucleotide was dechlorinated by AMP deaminase whereas ACDP-ribonucleotide was not. The fact that ACP-ribonucleotide was a substrate for AMP-deaminase is not surprising in view of earlier reports [25, 26] that 6-chloropurine ribonucleotide was a substrate. Presumably dechlorination of ACP-ribonucleotide by AMP deaminase was responsible for the increased pools of guanine nucleotides in the ACP-treated cells. The conversion of ACP-ribonucleotide to guanine nucleotides probably also explains the lesser toxicity of ACP relative to ACDP, because the guanine nucleotides would partially reverse the *de novo* blockade and completely reverse the blockade of IMP dehydrogenase. ACDP and ACP have not been compared in the same experiment with respect to antitumor activity, but a comparison of our results with ACDP [4], and the results from the Division of Cancer Treatment with ACP*, indicates that the compounds have comparable effects in increasing the life spans of mice that bear leukemia L1210. ACDP might, however, be the preferred compound because of the greater stability of its active form.

The lethal event in cells treated with ACDP is not defined by the results. The synthesis of DNA and RNA was inhibited by low concentrations of ACDP and the synthesis of protein by moderate concentrations (Fig. 5). Decreased synthesis of both DNA and RNA would be expected from the marked reduction of purine nucleotide pools produced by ACDP, and inhibition of DNA synthesis would be expected to be the probable lethal action. An observation pertinent to the mode of action of agents that inhibit either *de novo* purine synthesis or the IMP–GMP conversion is the recent one of Cohen *et al.* [27] that GTP plays a specific and as yet undefined role in DNA synthesis. It may be then that the mechanism of cell kill by ACDP is lowered availability of GTP for this role in DNA synthesis, rather than decreased availability of dATP and dGTP for DNA polymerase.

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