Biochemical Pharmacology, Vol. 23, pp. 3199–3209. Pergamon Press, 1974. Printed in Great Britain.

INHIBITION OF CELL GROWTH AND OF PURINE BIOSYNTHESIS BY ALLOPURINOL AND 4-AMINOPYRAZOLO(3,4-d)PYRIMIDINE— A COMPARATIVE STUDY*,†

KUMIKO IWAMOTO and DAVID W. MARTIN, JR.‡

Departments of Medicine and Biochemistry and Biophysics, School of Medicine, University of California, San Francisco, Calif. 94143, U.S.A.

(*Received* 10 *November* 1973; *accepted* 1 *March* 1974)

Abstract—We compared the effects of two chemically similar purine analogs, 4-hydroxypyrazolo(3,4-d)pyrimidine (allopurinol) and 4-aminopyrazolo(3,4-d)pyrimidine (4-APP) on the growth and on *de novo* purine biosynthesis of rat hepatoma cells in culture. Both compounds were found to significantly inhibit their growth as well as their *de novo* purine synthesis, the effective concentrations of 4-APP being nearly two orders of magnitude less than those of allopurinol. For both drugs, there was a good correlation between the ability to inhibit *de novo* purine synthesis and to produce growth inhibition, suggesting a causal relationship. Although the two compounds inhibit purine synthesis to a similar extent, the mechanisms by which they affect the early portion of the purine pathway differ: allopurinol apparently acts by end product-mediated inhibition and/or depletes the cell of substrates, whereas 4-APP, in addition, regulates gene expression by causing a repression of an early function in the *de novo* pathway.

PURINE analogs and their derivatives have become important pharmacologic tools for the treatment of neoplasms,[1,2] certain inflammatory diseases[3] and hyperuricemia.[4] The mechanisms by which most of these compounds act are not fully understood.

4-Aminopyrazolo(3,4-d)pyrimidine (4-APP), an analog of adenine, has potent toxic properties in experimental animals[5] and in man[6] that have precluded its clinical use. It is known to inhibit biosynthesis of purines,[7–9] but the site of its interference is unknown. It is not clear whether this effect accounts for its ability to inhibit growth of tumors and cultured cells.[6,10,11] In contrast, 4-hydroxypyrazolo(3,4-d)pyrimidine (4-HPP, allopurinol), an analog of hypoxanthine and chemically similar to 4-APP, has relatively little toxic effect,[12–15] but it also effectively inhibits synthesis of purines.[16,17] Its additional potent capacity to inhibit xanthine oxidase, an enzyme which catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid, has led to its clinical use in the treatment of hyperuricemia.[12,18]

* A brief preliminary report of this work was presented at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, San Francisco, Calif., August 1973 (*Pharmacologist*, **15**, 235, 1973).

† This investigation was supported in part by U.S. Public Health Service Genetics Center Grant GM-19527. Kumiko Iwamoto was supported as a research trainee by U.S. Public Health Service Grant 5T1-GM-00063-15, 16.

‡ Reprint requests should be addressed to: David W. Martin, Jr., M.D., Department of Medicine, School of Medicine, University of California, San Francisco, Calif. 94143, U.S.A.

The liver is thought to be a major site of *de novo* purine synthesis in intact animals,[19,20] and studies *in vivo* and clinical trials have shown the liver to be the site of toxic effects of 4-APP.[5] Accordingly, we have chosen to study the mechanisms of action of these drugs in a model of the mammalian hepatocyte, the HTC cell.[21] HTC cells are an established line of rat hepatoma cells in continuous culture in which the regulation of *de novo* purine synthesis has been previously studied.[22] Because their growth is inhibited by the presence of purines in the culture medium,[22] HTC cells provide a suitable experimental system for a comparative investigation of the effects of the two analogs at the molecular level.

We measured the inhibitory effects of allopurinol and 4-APP on cell growth and on the *de novo* pathway of purine synthesis in HTC cells. Our observations led to an examination of the different mechanisms by which these two drugs influence the early steps of purine anabolism.

MATERIALS AND METHODS

Reagents. Uniformly labeled [^{14}C]glycine (100 mCi/m-mole), [^{3}H]hypoxanthine (0·77 mCi/m-mole), and Omnifluor scintillant were purchased from New England Nuclear Corp.; cellulose 300 u.v. thin-layer chromatographic sheets from Brinkmann Instrument Co.; and 4-aminopyrazolo(3,4-d)pyrimidine (4-APP) and cycloheximide from Sigma Chemical Co. 4-Hydroxypyrazolo(3,4-d)pyrimidine (4-HPP, allopurinol) was a gift of Dr. Gertrude Elion of Burroughs Wellcome Research Laboratories, Research Triangle Park, N.C., and was also obtained from Sigma Chemical Co. Azaserine was a gift of the Drug Development Branch, National Cancer Institute. All other materials were commercially available products of reagent grade. The liquid scintillation fluid consisted of 4 g Omnifluor in 300 ml Triton and 700 ml toluene. Samples were counted in a liquid scintillation counter.

The purity of 4-HPP and 4-APP was confirmed by ascending thin-layer chromatography on 20 × 20 cm cellulose 300 sheets using a solvent system of butanol–water (86:14, v/v). Both drugs were greater than 99·5% pure.

Conditions of cell culture. Rat hepatoma (HTC) cells were grown as suspension cultures in Swims 77 medium containing 2 mM L-glutamine and 10% calf serum as previously described.[21-23] Stock (wild-type) cultures, maintained at 37° in logarithmic growth by daily dilution with fresh medium, doubled approximately every 30 hr. Cultures were periodically monitored for the presence of *Mycoplasma*,[24] fungi and bacteria.

A clone of HTC cells, deficient in hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) activity, was selected for resistance to 6-thioguanine after chemical mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. This clone was maintained under nonselective conditions as described above for the wild-type cells. Less than 0·1 per cent of normal HGPRTase activity could be detected.*

Growth experiments were initiated by diluting cells with fresh medium to a density of 1–2 × 10^5 cells/ml in a total volume of 100 ml; each of these aliquots was maintained in spinner culture in a 500-ml Wheaton bottle at 37°. During the experimental period, duplicate determinations of cell densities were made daily in a hemacytometer. Cell viability was determined by trypan blue exclusion. We used the

* N. T. Owen and D. W. Martin, Jr., unpublished observations.

change in relative cell number at the end of 72 hr of exposure to drug as an index of growth.

Concentrated aqueous solutions of 4-HPP and 4-APP were sterilized by filtration and added to cultures at appropriate concentrations. Control cultures received equivalent volumes of water. When necessary, each medium with or without added drugs was adjusted to an initial pH of 7·4–7·8 by the addition of hydrochloric acid or potassium hydroxide.

When drugs were removed from cultures after 18 hr of exposure, procedures previously described for washing and resuspension of cells were employed.[22]

Assay for rate of formation of α-N-formyl glycinamide ribonucleotide. The rate of incorporation of [^{14}C]glycine into α-*N*-formyl glycinamide ribonucleotide (FGAR) in cells incubated in the presence of 10 μg azaserine and 0·5 μCi [^{14}C]glycine/ml of medium was determined by the method previously described.[22]

Assay of xanthine oxidase activity. Xanthine oxidase activities were determined in crude extracts of HTC cells and of male Sprague–Dawley rat liver. The extracts were gel-filtered at 4° on a column of Sephadex G-25 fine that had been equilibrated with 0·05 M potassium phosphate buffer, pH 7·5. The reaction was carried out at 37° in 6 × 50 mm tubes containing in 25 μl: 8 nmoles hypoxanthine, 0·8 μCi [^{3}H]hypoxanthine, 1·25 μmoles potassium phosphate buffer, pH 7·5, and 140–360 μg protein. The reaction was stopped by the addition of 25 μl of cold 1 N perchloric acid; after centrifugation, 2 μl of the supernatant was applied to a plastic-backed thin-layer sheet of 300 u.v. cellulose, 20 × 20 cm, and chromatographed with known hypoxanthine, xanthine and urate standards vs *n*-butanol–methanol–water–concentrated ammonium hydroxide (60:20:20:1). The radioactivity present as hypoxanthine was determined for each reaction by liquid scintillation counting, and the rates of disappearance of hypoxanthine were calculated. This assay is linear with time for 30 min and proportional to quantity of extract added up to 400 μg. The method of Lowry *et al.*[25] was used to estimate protein concentrations.

RESULTS

Effects of 4-HPP (allopurinol) and 4-APP on cell growth. The addition of 4-HPP to the culture medium at a concentration of 0·25 mM or greater inhibited the growth of HTC cells (Fig. 1, left panel). A dose-related response was observed: the doubling time of cells lengthened as the concentration of drug was increased. This was reflected by the number of viable cells remaining after 72 hr of exposure to the drug. At a concentration of approximately 0·6 mM, there was no net change in cell number (i.e. relative cell increase value of 0). Cytotoxic effects were apparent at 2·0 mM; at this concentration, the number of cells decreased to less than the initial inoculum. The dose–response curve illustrating the growth inhibitory effects of 4-APP was similar to that of 4-HPP (Fig. 1, right panel), although much lower concentrations of 4-APP affected cell growth.

The biological action of a purine analog is frequently dependent upon its conversion to its corresponding ribonucleotide. Because evidence suggests that this may also be true for 4-HPP and because the conversion of this drug to its ribonucleotide appears to require HGPRTase activity,[26] we examined the effects of 4-HPP and 4-APP on the growth of HGPRT-deficient cells. 4-HPP did not significantly affect

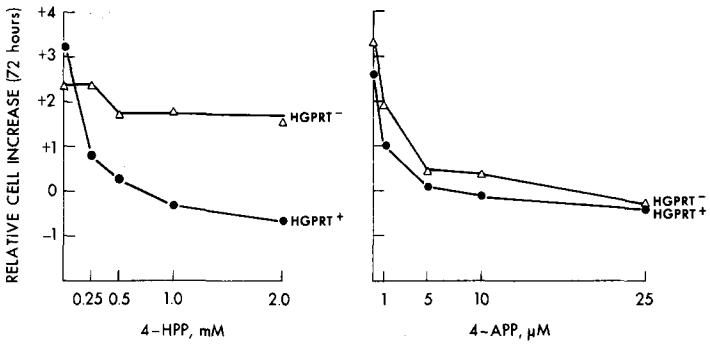
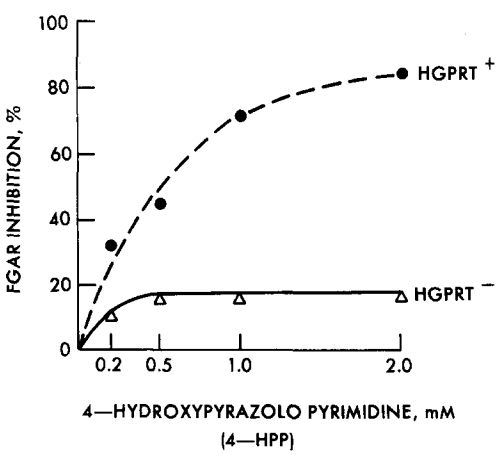


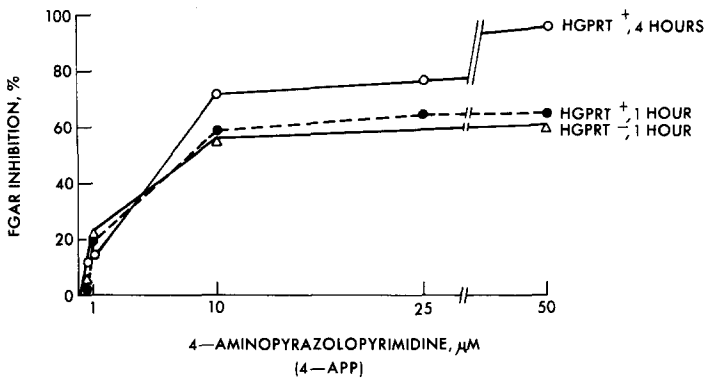
FIG. 1. Inhibition of growth of HTC cells by increasing concentrations of drug. Experimental procedure is outlined in Materials and Methods. Relative cell increase $[(N_{72} - N_0)/N_0]$ is the ratio of the increase in cell number at the end of the 72-hr drug exposure period (N_{72}) to the cell number in the initial inoculum (N_0). A negative value represents loss of cells; zero represents no change in cell number. Two cell populations were studied: wild-type HTC cells (HGPRT†, ●) and a mutant strain deficient in HGPRTase activity (HGPRT⁻, △). Each curve represents the results of one experiment and is typical of the six experiments performed with each of the two cell populations. Left panel, exposure to 4-HPP; right panel, exposure to 4-APP.

the growth of these cells (Fig. 1, left panel), whereas 4-APP again was an effective inhibitor (Fig. 1, right panel). Thus, the activity of HGPRTase appears to be necessary for the inhibition of cell growth by 4-HPP.

Effects of 4-HPP and 4-APP on de novo *purine biosynthesis.* The rate of incorporation of glycine into FGAR in the presence of azaserine is a measure of the rate of the early steps of purine biosynthesis *de novo*, and can be used to study end product-mediated regulation of these steps. Both 4-HPP and 4-APP inhibit the rate of FGAR formation in wild-type cells (Fig. 2). Again, as in the growth experiments, the absence of HGPRTase activity results in a significant resistance to inhibition by 4-HPP, whereas 4-APP remains an effective inhibitor of *de novo* purine synthesis. In wild-type cells, the maximal inhibitory effect of 4-HPP occurred promptly (within 60 min). Although 4-APP produced rapid inhibition of FGAR formation in wild-type



(A)



(B)

FIG. 2. Inhibition of FGAR accumulation measured after 1 and 4 hr of exposure to drug in wild-type cells (HGPRT$^+$) and in cells lacking HGPRTase activity (HGPRT$^-$). Suspension cultures of 6–7 × 10^5 cells/ml of complete medium in a total volume of 50 ml were exposed to various concentrations of drug; 10-ml aliquots were removed after 0 and 3 hr of drug exposure and preincubated for 30 min at 37° with azaserine (6·4 × 10^{-6} M). [^{14}C]glycine (0·5 μCi/ml) was added and its incorporation into [^{14}C]FGAR was measured after 30 min of incubation as described in Materials and Methods. Values represent the times at the ends of the labeling periods. (A) Inhibition after exposure to 4-HPP; inhibition was identical after both 1 and 4 hr of exposure. (B) Inhibition after exposure to 4-APP; inhibition in HGPRT$^-$ cells was similar after both 1 and 4 hr of exposure to drug, the variation being no greater than 4 per cent for any concentration.

cells, its maximal effect on FGAR production was observed only after at least 4 hr of exposure to the drug. The concentration of each drug that prevents any net change in cell number in the growth experiments is approximately equal to that which exerts half-maximal inhibitory effect on *de novo* purine synthesis (Table 1). Although HTC cells were sensitive to concentrations of 4-APP approximately two orders of magnitude less than concentrations of 4-HPP, the data are consistent with the premise that these two analogs inhibit HTC cell growth as a consequence of their inhibition of *de novo* purine biosynthesis.

Mechanisms by which 4-HPP and 4-APP inhibit de novo *purine synthesis.* Our findings indicate that the two related pyrazolopyrimidines had similar effects on cell growth and purine biosynthesis, although these effects were manifested at markedly different concentrations. Since purine biosynthesis is known to be sensitive to regula-

TABLE 1. EFFECT OF 4-HPP AND 4-APP ON RATES OF CELL GROWTH AND ON *de novo* PURINE SYNTHESIS*

	4-HPP Concn (mM)		4-APP Concn (μM)	
	Cell growth	Purine synthesis	Cell growth	Purine synthesis
No effect	0·10	0·10	0·5	0·5
Minimal detectable effect	0·25	0·20	1·0	1·0
Half-maximal effect	0·60	0·40	1–5	5·0
Maximal effect	2·0	2·0	10–25	10–25

* The dose-related inhibition of growth of HTC cells are those values depicted in Fig. 1. Concentrations eliciting half-maximal effects correspond to the approximate midpoints on the dose–response curves. Inhibition of *de novo* purine synthesis as measured by decreased incorporation of [^{14}C]glycine into FGAR is presented in graphic form in Fig. 2. The estimated concentrations for half-maximal effect were determined from the midpoints of these curves.

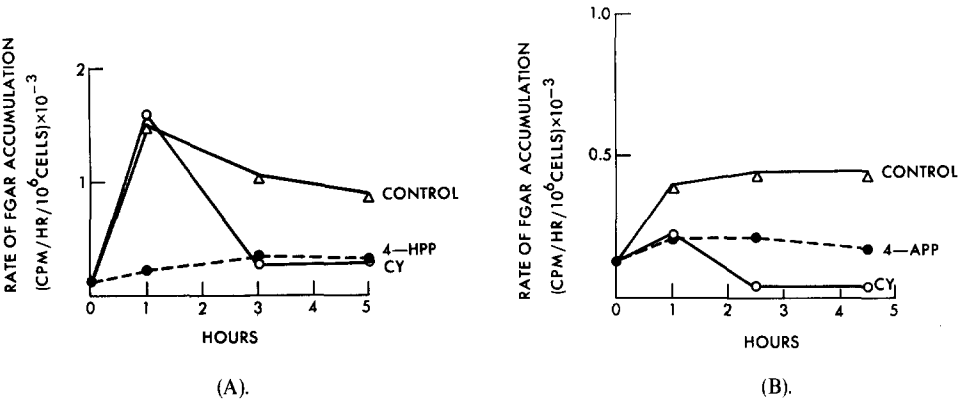


FIG. 3. Rate of FGAR accumulation in HGPRT$^+$ cells after 18 hr of exposure to drug. Suspension cultures of 4–5 × 10^5 cells/ml were incubated at 37° for 18 hr after the addition of drug to the medium. Treatment of cells after removal of drug is described in Materials and Methods. Rates of [^{14}C]FGAR accumulation were determined as outlined in the legend to Fig. 2 and in Ref. 22. (A) Incubation with 2·0 mM 4-HPP. Control (△), cells resuspended in fresh medium without 4-HPP; 4-HPP (●), 2 mM 4-HPP added to fresh medium; Cy (○), 2 × 10^{-4} M cycloheximide added to fresh medium in the absence of 4-HPP. (B) Incubation in the presence of 20 μM 4-APP. Control (△), cells in fresh medium without drug; 4-APP (●), 20 μM 4-APP added to fresh medium; Cy (○), 2 × 10^{-4} M cycloheximide added to fresh medium in the absence of 4-APP. At 24 hr, control cells regained an uninhibited capacity to form purines: 0·79 × 10^{-3} cpm/hr/10^6 cells (not plotted on graph).

tion by feedback inhibition[27] and by end product-mediated repression,[22,28] we compared the mechanisms by which 4-HPP and 4-APP inhibit the early portion of the purine pathway.

After 18 hr in culture medium containing 2·0 mM 4-HPP, HTC cells were washed free of inhibitor and suspended in fresh medium containing no drug, 2·0 mM 4-HPP, or 2 × 10^{-4} M cycloheximide, a drug that inhibits protein synthesis in HTC cells. In cells free of drug (control), there was immediate resumption of *de novo* purine synthesis as measured by production of FGAR (Fig. 3(A)). Similarly purine synthesis was rapidly resumed in cells incubated in medium containing cycloheximide; thus, concomitant protein synthesis was not necessary for the resumption of purine synthesis in cells exposed to 4-HPP. In the absence of drugs, the rate of accumulation of FGAR at 1 hr was greater than the rate at which the cells eventually stabilized their purine synthesis. The difference between these two rates of FGAR synthesis might be due to the response of the cells to fresh medium and to depleted intracellular pools of purine nucleotides; the decline in rate would then result from the rapid reaccumulation of the nucleotides. Obviously, such an overshoot could not occur in cells in which any of the enzyme(s) leading to the synthesis of FGAR had been depleted. This is further indirect evidence that 4-HPP does not repress the synthesis of a rate-limiting enzyme in the first third of the *de novo* purine pathway.

In contrast, cells exposed to 20 μM 4-APP under similar conditions, and then suspended in medium free of 4-APP, required concomitant protein synthesis to resume their rapid rate of purine production (Fig. 3(B)). Furthermore, in the absence of cycloheximide, the resuspended cells required up to 24 hr to regain their

uninhibited capacity to form purines (see legend to Fig. 3). Thus, 4-APP appears to repress the formation of a protein molecule(s) required during an early step in the *de novo* purine pathway. The identity and function of the macromolecule(s) are unclear.

DISCUSSION

Our studies demonstrate the ability of allopurinol to inhibit growth of HTC cells, the first reported evidence of its toxicity in any experimental cell line. Because high concentrations of the drug were required to affect cell growth, the physiologic or pharmacologic significance of the toxicity is not apparent. Its relative lack of cytotoxicity has previously been well established by studies in cultured cells,[13,17] experimental animals[14,29] and in man.[12,15] As is the case with many purine analogs and their derivatives, allopurinol appears to require the presence of HGPRTase activity in order to inhibit cell growth and *de novo* purine biosynthesis. The slight decrease in the growth rate of these cells at higher concentrations of allopurinol may be due to its ability to inhibit pyrimidine biosynthesis, an effect which is not dependent on the presence of HGPRTase activity and is unrelated to its ability to inhibit xanthine oxidase.[30]

The site of action and mechanism by which allopurinol or its metabolites could bring about death of HTC cells is a question of interest. Based on the many investigations regarding mechanisms of action of other purine analogs,[31] two likely possibilities can be proposed. If the nucleotide were incorporated into nucleic acids, synthesis of faulty DNA with or without eventual mutagenicity might occur. We have not yet determined whether allopurinol or its ribonucleotide is incorporated into the nucleic acids of HTC cells. Kelley and Wyngaarden[17] could not demonstrate the incorporation of allopurinol into nucleic acids of cultured human fibroblasts. Neither could Elion *et al.*[29] detect allopurinol incorporation into nucleic acids of mouse liver *in vivo*; however, allopurinol was not a cytotoxic agent in either experimental system. An alternative possibility suggests that allopurinol ribonucleotide inhibits purine and, perhaps, pyrimidine synthesis, leading to an inhibition of over-all nucleotide synthesis and growth. And, indeed, it has been demonstrated in cell-free extracts that allopurinol ribonucleotide can inhibit by allosteric mechanisms the first enzyme unique to *de novo* purine synthesis. glutamine phosphoribosylpyrophosphate amidotransferase (PRPP amidotransferase),[26] and orotidylic decarboxylase,[30] an enzyme in the pathway of *de novo* pyrimidine synthesis. The correlation observed in our experiments between concentrations necessary for inhibition of HTC cell growth and for inhibition of *de novo* purine synthesis suggests that purine starvation is a contributory factor (Fig. 4).

The inhibitory effect of allopurinol on purine synthesis has become well recognized in patients taking the drug[32,33] and in cultured human fibroblasts exposed to allopurinol.[17] Our data confirm these findings in HTC cells. It has not been determined which mechanism(s) is responsible for the inhibitory effect of allopurinol on purine synthesis. Three hypotheses have been postulated: (a) allopurinol ribonucleotide-mediated inhibition of PRPP amidotransferase,[26] (b) depletion of intracellular phosphoribosylpyrophosphate (PRPP), a rate-limiting substrate for purine biosynthesis,[34] and (c) an increased conversion of hypoxanthine to purine ribonucleotides,

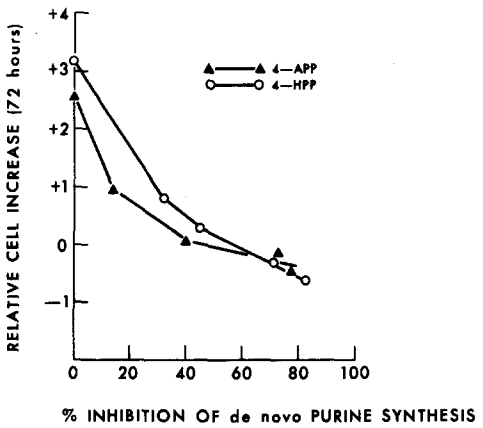


FIG. 4. Correlation between inhibition of growth and inhibition of *de novo* purine synthesis of wild-type HTC cells. Data from Figs. 1 and 2 (4-hr values) are replotted to show the relationship between inhibition of HTC cell growth, indicated by relative cell increase, and inhibition of [^{14}C]FGAR formation in the presence of increasing concentrations of 4-HPP or 4-APP. 4-HPP (○); concentrations (mM) corresponding to points on the graph from left to right are: 0, 0·25, 0·50, 1·0 and 2·0. 4-APP (▲); concentrations (μM) are: 0, 1·0, 5·0, 10 and 25 from left to right.

which in turn inhibits purine synthesis,[17,35] as a result of inhibition of the enzyme, xanthine oxidase, which catabolizes hypoxanthine. Since HTC cells lack xanthine oxidase, and contain no apparent inhibitor of the xanthine oxidase from rat liver (Table 2), our data are inconsistent with the last proposal. From the present study, we cannot determine whether the inhibitory effects of allopurinol are the result of depletion of intracellular PRPP during conversion of the unmodified base to its

TABLE 2. CATALYTIC ACTIVITY OF XANTHINE OXIDASE IN EXTRACTS OF HTC CELLS AND RAT LIVER*

Source of enzyme preparation	Addition	Specific catalytic activity†
Rat liver	None	13·2
	4-HPP‡	<0·32
	Methylene blue§	9·0
	Methylene blue and 4-HPP	<0·32
HTC cells	None	<0·32
	4-HPP	<0·32
	Methylene blue	<0·32
	Methylene blue and 4-HPP	<0·32
HTC cells and rat liver	None	7·1

* Catalytic activity of xanthine oxidase was assayed in extracts of HTC cells and rat liver as described in Methods. The lower limit of the assay was 0·32 μmole hypoxanthine metabolized/hr/mg of protein. The assay containing extracts from both HTC cells and rat liver was performed at equal protein concentrations of each and one-half the concentration of each used in the other assays.

† Micromoles of hypoxanthine oxidized/mg of protein/hr.

‡ Allopurinol, 40 μM final concentration.

§ Final concentration, 0·001%.

ribonucleotide or a result of an allosteric inhibitory effect of its ribonucleotide. However, our experiments (Fig. 3, A) suggest strongly that, unlike adenine, allopurinol does not repress the synthesis of a rate-limiting enzyme(s) of the first third of the *de novo* purine pathway.

4-APP, an adenine analog, is also capable of inhibiting *de novo* purine biosynthesis. In contrast to studies in H. Ep. no. 2 cells,[9] a short exposure (60 min) of HTC cells to 4-APP inhibits their purine synthesis, as indicated by a decreased rate of accumulation of FGAR. Our evidence suggests that this analog, like adenine itself, directly or indirectly represses the formation of an unidentified macromolecule(s) which functions early in the *de novo* purine pathway. However, the marked inhibition of synthesis of FGAR after a short exposure to 4-APP suggests that this acute decline in purine synthesis may be due to feedback inhibition or to depletion of PRPP and not to repression. This is supported further by previous observations in HTC cells that the most rapidly turning over rate-limiting protein in the purine pathway possesses a half-time of approximately 2 hr.[22]

It is not clear whether the active form of 4-APP is 4-APP itself, a 4-APP ribonucleotide, or whether it is a deaminated metabolite such as allopurinol (4-HPP). Even if deamination of 4-APP occurs in this cell line, the latter possibility is unlikely since the cells are less sensitive to allopurinol than to 4-APP. Clearly, HGPRTase activity is not necessary for the conversion of 4-APP to its active moeity. This is consistent with earlier work *in vitro* utilizing specific mutants of H. Ep. no. 2 cells and demonstrating that adenine phosphoribosyltransferase activity is necessary for the formation of the 4-APP ribonucleotide.[36] Studies in Ehrlich ascites cells[8] suggested that 4-APP or its ribonucleotide may affect a metabolic function that occurs after the formation of IMP. Thus, it is possible that the repression is mediated by the accumulation of a normal ribonucleotide proximal to such a drug-mediated block.

Our data establish the sensitivity of HTC cells to the growth-inhibitory properties of 4-APP. The concentration which causes half-maximal inhibition of cell growth is similar to that demonstrated in other tissue–culture systems.[37] Inhibition of *de novo* purine biosynthesis in HTC cells by 4-APP was effected by a concentration of the drug that inhibits growth of cells. In H. Ep. no. 2 cells,[32] a cell line in which the effects of 4-APP have been studied rather extensively, such a correlation for the effects of the ribonucleoside[38] of 4-APP, but not 4-APP itself,[9] exists. The correlation noted between growth-inhibitory concentrations and concentrations resulting in the inhibition of purine synthesis strengthens the view that these may be causal relationships (Fig. 4). The possibility that 4-APP may directly disturb or participate in synthesis of nucleic acids was not pursued at this time. Incorporation of 4-APP, 4-APP ribonucleotide, or both into nucleic acids of normal and neoplastic mouse tissues has been reported,[39] but no correlation has been demonstrated with the cytotoxic effects of the drug. In *Escherichia coli*, no incorporation of 4-APP into nucleic acids was detected.[40]

The greater potency of 4-APP in comparison to 4-HPP has been substantiated by the parameters used in this study. Investigations of 4-APP-resistant cells in bacterial systems[41,42] and H. Ep. no. 2 cells[37,43] have not clarified the basis for its marked toxicity. Despite the difference in potency, the relationship frequently described between inhibition of purine synthesis *de novo* by other purine analogs and their growth-inhibitory activities[31] also applies to both 4-APP and allopurinol in our sys-

tem (Fig. 4). Even though our data demonstrate that these relationships in the two drugs are identical, the molecular mechanisms by which these two compounds inhibit *de novo* purine synthesis differ. Increased knowledge of the intracellular kinetics of ribonucleotide formation, enzyme specificities, and pool sizes of metabolites may further our understanding of the effects and potencies of these drugs.

Acknowledgements—The authors express their appreciation to Ms. N. T. Owen for her technical assistance during the initial phases of this study.

REFERENCES

1. P. CALABRESI and R. E. PARKS, JR., *The Pharmacological Basis of Therapeutics: A Textbook of Pharmacology, Toxicology, and Therapeutics for Physicians and Medical Students* (Eds. L. S. GOODMAN and A. GILMAN), 4th Edn, pp. 1371–5. MacMillan, New York (1970).
2. M. DAWSON, *Cellular Pharmacology: The Effects of Drugs on Living Vertebrate Cells In Vitro*, pp. 48–54. Charles C. Thomas, Springfield (1972).
3. R. L. MILLER and K. L. MELMON, in *Clinical Pharmacology: Basic Principles in Therapeutics* (Eds. K. L. MELMON and H. F. MORRELLI), p. 399. MacMillan, New York (1972).
4. R. W. RUNDLES, J. B. WYNGAARDEN, G. H. HITCHINGS and G. B. ELION, *A. Rev. Pharmac.* **9,** 345 (1969).
5. J. SCHOLLER, F. S. PHILIPS and S. S. STERNBERG, *Proc. Soc. exp. Biol. Med.* **93,** 398 (1956).
6. R. K. SHAW, R. N. SHULMAN, J. D. DAVIDSON, D. P. RALL and E. FREI, III, *Cancer, N.Y.* **13,** 482 (1960).
7. J. S. GOTS and E. G. GOLLUB, *Proc. Soc. exp. Biol. Med.* **101,** 641 (1959).
8. B. A. BOOTH and A. C. SARTORELLI, *J. biol. Chem.* **236,** 203 (1961).
9. L. L. BENNETT, JR. and D. SMITHERS, *Biochem. Pharmac.* **13,** 1331 (1964).
10. T. C. HSU, R. K. ROBINS and C. C. CHENG, *Science, Wash.* **123,** 848 (1956).
11. R. W. BROCKMAN and E. P. ANDERSON, *A. Rev. Biochem.* **32,** 463 (1963).
12. R. W. RUNDLES, *Cancer Res.* **23,** 1220 (1963).
13. G. H. HITCHINGS, *Cancer Res.* **23,** 1225 (1963).
14. J. A. ALEXANDER, G. P. WHEELER, D. D. HILL and H. P. MORRIS, *Biochem. Pharmac.* **15,** 881 (1966).
15. R. A. CHALMERS, H. KRÖMER, J. T. SCOTT and R. W. E. WATTS, *Clin. Sci.* **35,** 353 (1968).
16. L. L. BENNETT, JR., D. SMITHERS, C. TEAGUE, H. T. BAKER and P. STUTTS, *Biochem. Pharmac.* **11,** 81 (1962).
17. W. N. KELLEY and J. B. WYNGAARDEN, *J. clin. Invest.* **49,** 602 (1970).
18. J. B. WYNGAARDEN and W. N. KELLEY, in *The Metabolic Basis of Inherited Disease* (Eds. J. B. STANBURY, J. B. WYNGAARDEN and D. S. FREDRICKSON), 3rd Edn. p. 889. McGraw-Hill. New York (1972).
19. A. W. MURRAY, *A. Rev. Biochem.* **40,** 811 (1971).
20. L. G. LAJTHA and J. R. VANE, *Nature, Lond.* **182,** 191 (1958).
21. E. B. THOMPSON, G. M. TOMKINS and J. F. CURRAN, *Proc. natn. Acad. Sci. U.S.A.* **56,** 296 (1966).
22. D. W. MARTIN, JR. and N. T. OWEN, *J. biol. Chem.* **247,** 5477 (1972).
23. D. W. MARTIN, JR., G. M. TOMKINS and D. GRANNER, *Proc. natn. Acad. Sci. U.S.A.* **62,** 248 (1969).
24. L. HAYFLICK, *Tex. Rep. Biol. Med.* **23** (suppl. 1), 285 (1965).
25. O. H. LOWRY, N. J. ROSEBROUGH, A. C. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 256 (1951).
26. R. J. MCCOLLISTER, W. R. GILBERT, JR., D. M. ASHTON and J. B. WYNGAARDEN, *J. biol. Chem.* **239,** 1560 (1964).
27. J. F. HENDERSON, *Regulation of Purine Biosynthesis* (ACS Monograph 170), pp. 163–95. American Chemical Society, Washington, D.C. (1972).
28. E. MCFALL and B. MAGASANIK, *J. biol. Chem.* **235,** 2103 (1960).
29. G. B. ELION, A. KOVENSKY, G. H. HITCHINGS, E. METZ and R. W. RUNDLES, *Biochem. Pharmac.* **15,** 863 (1966).
30. W. N. KELLEY, T. D. BEARDMORE, I. H. FOX and J. C. MEADE, *Biochem. Pharmac.* **20,** 1471 (1971).
31. J. A. MONTGOMERY, *Prog. med. Chem.* **7,** 69 (1970).
32. R. W. RUNDLES, J. B. WYNGAARDEN, G. H. HITCHINGS, G. B. ELION and H. R. SILBERMAN, *Trans. Ass. Am. Phycns* **76,** 126 (1963).
33. B. T. EMMERSON, *Ann. rheum. Dis.* **25,** 622 (1966).
34. I. H. FOX, J. B. WYNGAARDEN and W. N. KELLEY, *New Engl. J. Med.* **283,** 1177 (1970).
35. R. S. POMALES, S. BIEBER, R. FRIEDMAN and G. H. HITCHINGS, *Biochim. biophys. Acta* **72,** 119 (1963).
36. R. W. BROCKMAN, G. G. KELLEY, P. STUTTS and V. COPELAND, *Nature, Lond.* **191,** 469 (1961).
37. L. L. BENNETT, JR., M. H. VAIL, P. W. ALLAN and S. C. SHADDIX, *Biochem. Pharmac.* **22,** 1221 (1973).
38. L. L. BENNETT, JR. and J. A. MONTGOMERY, in *Methods in Cancer Research* (Ed. H. BUSCH), Vol. III, p. 588. Academic Press, New York (1967).

39. J. F. Henderson and I. G. Junga. *Cancer Res.* **21**, 118 (1961).
40. F. F. Zimmerman and H. G. Mandel. *Expl Cell Res.* **33**, 138 (1964).
41. W. R. Pickering and R. A. Woods. *Biochim. biophys. Acta* **264**, 45 (1972).
42. B. J. Bedair and R. Fuerst. *Antimicrob. Agents Chemother.* 260 (1962).
43. L. L. Bennett, Jr., P. W. Allan, D. Smithers and M. H. Vail, *Biochem. Pharmac.* **18**, 725 (1969).