Effects of Allopurinol and Oxipurinol on Purine Synthesis in Cultured Human Cells

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ABSTRACT In the present study we have examined the effects of allopurinol and oxipurinol on the *de novo* synthesis of purines in cultured human fibroblasts. Allopurinol inhibits *de novo* purine synthesis in the absence of xanthine oxidase. Inhibition at lower concentrations of the drug requires the presence of hypoxanthine-guanine phosphoribosyltransferase as it does in vivo. Although this suggests that the inhibitory effect of allopurinol at least at the lower concentrations tested is a consequence of its conversion to the ribonucleotide form in human cells, the nucleotide derivative could not be demonstrated. Several possible indirect consequences of such a conversion were also sought. There was no evidence that allopurinol was further utilized in the synthesis of nucleic acids in these cultured human cells and no effect of either allopurinol or oxipurinol on the long-term survival of human cells in vitro could be demonstrated.

At higher concentrations, both allopurinol and oxipurinol inhibit the early steps of *de novo* purine synthesis in the absence of either xanthine oxidase or hypoxanthine-guanine phosphoribosyltransferase. This indicates that at higher drug concentrations, inhibition is occurring by some mechanism other than those previously postulated.

INTRODUCTION

Allopurinol and oxipurinol are effective inhibitors of xanthine oxidase in vitro and in vivo (1–5). Since xanthine oxidase catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid (Fig. 1), the use of these compounds in man leads to a substantial and consistent reduction in uric acid synthesis (6–10). In addition to this inhibitory effect on the final steps of purine catabolism, allopurinol also appears to inhibit the *de novo* synthesis of purines in many patients. This was initially suggested by the finding that the decrease in uric acid excretion produced by allopurinol was not stoichiometrically repaced by the increase in urinary hypoxanthine and xanthine which occurred (11). Further evidence for this inhibitory effect of allopurinol on *de novo* purine synthesis in vivo subsequently came from studies which demonstrated that allopurinol reduces the incorporation of glycine-1-^{14}C, a precursor of purine synthesis *de novo,* into uric acid (12).

In the present study we have examined the effect of allopurinol and its major metabolic product, oxipurinol, on the early steps of *de novo* purine synthesis in cultured human fibroblasts and have attempted to evaluate the possible mechanisms by which inhibition might occur. In addition, since the long-term safety of allopurinol in man has yet to be established with certainty, an effort has been made to test in these human cells in vitro certain potential toxic effects which might be anticipated in vivo.

METHODS

Sodium formate-^{14}C (58.7 mCi/mmole) and hypoxanthine-8-^{14}C (4.14 mCi/mmole) were obtained from New England Nuclear; xanthine-8-^{14}C (17.6 mCi/mmole) from Calbiochem; and allopurinol-6-^{14}C (0.07 mCi/mmole) was a gift from Dr. Gertrude Elion of Burroughs Wellcome Research Laboratories, Tuckahoe, N. Y. Azaserine was a gift from American Cyanamid Co., Lederle Laboratories Div., Pearl River, N. Y. Allopurinol (4-hydroxypyrazolo (3,4-d) pyrimidine) and oxipurinol (4,6 dihydroxypyrazolo (3,4-d) pyrimidine) were provided by Burroughs Wellcome & Co.

Punch biopsies, 3 mm in diameter, were removed from the forearm skin of subjects of either sex with no detectable abnormality in purine synthesis. The specimens were minced and three small fragments were placed on a 60 mm Falcon plastic sterile disposable Petri dish (Falcon Plastics, Los Angeles, Calif.) and immobilized with a cover slip which was anchored to the Petri dish with sterile stopcock grease. The tissue was flooded with Eagle's medium containing Earle's balanced salt solution (GIBCO F-15), 14.3% fetal

Received for publication 25 July 1969 and in revised form 11 November 1969.

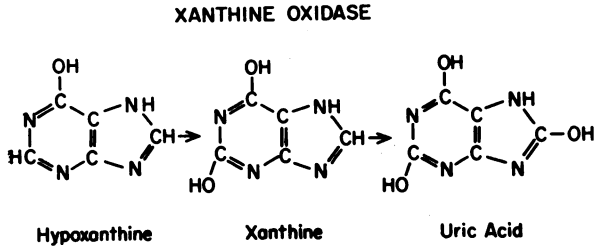

FIGURE 1 Xanthine oxidase reaction.

calf serum, penicillin (5000 U/ml), streptomycin (50 μg/ml), and incubated at 37°C in a 5% CO_2–95% air environment until outgrowth was apparent. The cells were then subcultured and serially propagated in 100-mm Falcon plastic sterile disposable Petri dishes until sufficient quantities were available for study. The method described for cultured fibroblasts is essentially that developed by Dr. John Littlefield and we are indebted to him for his assistance.[1] The 100-mm Petri dishes, containing confluent monolayers of cells, yield between 8 and 12 mg of cells wet weight which corresponds to 0.16–0.24 mg of protein.

Cells from patients genetically deficient in hypoxanthine-guanine phosphoribosyltransferase (PRT^-) (J.R., M.W., D.F., and S.M.) were obtained frozen in ampules at −70°C from Dr. J. E. Seegmiller of the National Institutes of Health. These cells were rapidly thawed at 37°C and subcultured in a manner similar to that described above.

Fibroblasts which had grown to confluency were harvested for biochemical studies by brief incubation in 0.25% trypsin in a buffered isotonic salt solution at room temperature to release them from attachment to the plastic surface. The cells were then collected by centrifugation at 600 *g* and washed with phosphate-buffered 0.154 M sodium chloride (PBS), pH 7.4. Cell suspensions were prepared by determining the total wet weight and adding sufficient volume of PBS to obtain a concentration of 80 mg/ml.

The early reactions of purine biosynthesis *de novo* were assessed by measuring the formation of radioactive *N*-formylglycinamide ribonucleotide (FGAR) in the presence of 0.3 mM azaserine, 5.5 mM glucose, 4 mM glycine, 20 mM L-glutamine, and 1.52 mM sodium formate-^{14}C and 8 mg wet weight of cells essentially as described by Rosenbloom, Henderson, Caldwell, Kelley, and Seegmiller (13), except for the modifications described below. The major radioactive, ethanol-soluble product in this reaction mixture cochromatographed with authentic FGAR-^{14}C (provided by Dr. Stanley Appel) by ascending chromatography in four different solvent systems (butanol: acetic acid: H_2O, 70:20:10, R_f 0.04; 95% ethanol: 6N ammonium acetate: H_2O, 70:5:25, R_f 0.18; *N*-propanol: concentrated ammonium hydroxide: H_2O, 60:30:10, R_f 0.18; isobutyric acid: 2 N ammonium hydroxide, 66:34, R_f 0.13) and by high voltage electrophoresis in two different buffer systems (0.05 M sodium citrate, pH 2.9; 0.05 M sodium borate, 0.001 M EDTA, pH 8.8).

FGAR was extracted in 80% ethanol and routinely separated from other radioactive compounds by high voltage electrophoresis in either 0.05 M borate buffer, pH 8.8, containing 0.001 M ethylenediaminetetraacetate or 0.05 M citrate buffer at pH 2.9. Inosinic acid is not separated from FGAR by electrophoresis in the borate buffer system as it is in the citrate buffer system. However, no inosinic acid-^{14}C accumulates under these experimental conditions since that which is formed is rapidly catabolized to inosine by the very high 5′-nucleotidase activity present in the cells. The results obtained therefore are similar using either buffer system. The FGAR-^{14}C present in the ethanolic extract was located by radioautography of the electrophoretogram and compared with the migration of authentic FGAR-^{14}C. In subsequent experiments, since inosinic acid migrated with authentic FGAR in the borate buffer system, the electrophoretograms were spotted with sufficient carrier inosinic acid to be identified by ultraviolet absorbance after electrophoresis and this area which also contained the FGAR-^{14}C was cut out, placed in a scintillation vial containing 10 ml of phosphor, and counted in a Tricarb liquid scintillation counter at 60% efficiency. The recovery of authentic FGAR-^{14}C using this system ranged from 92 to 98%. Duplicate samples agreed within 15%. Two radioactive derivatives of FGAR which probably represent the ribonucleoside and a polyphosphate were noted as previously reported. These compounds were present at a relatively low concentration and were always proportional to the amount of FGAR formed under the conditions used and therefore they were not counted as part of the FGAR counts formed.

Fibroblast extracts used for the assay of xanthine oxidase activity were prepared from a suspension containing 80 mg/ml wet weight in 0.1 M Na pyrophosphate buffer, pH 8.0, by rapidly freezing and thawing twice in dry ice and methyl Cellosolve. Xanthine oxidase was assayed spectrophotometrically by following the increase in optical density at 292 mμ which occurred at room temperature over a 1 hr period in a reaction mixture containing 20 μmoles Na pyrophosphate, 200 μl of cell extract, and 10 mμmoles hypoxanthine in a final volume of 3.0 ml. 50 μl of cell extracts prepared in a similar fashion were also incubated with 10 mμmoles of either allopurinol-6-^{14}C (0.07 mCi/mmole), hypoxanthine-8-^{14}C (4.1 mCi/mmole), or xanthine-8-^{14}C (17.6 mCi/mmole) in a final volume of 60 μl for 3 hr at 37°C. 20 μl of this reaction mixture was then spotted on 3MM paper and on Whatman DE81 (diethylaminoethyl cellulose) paper with hypoxanthine, xanthine, and uric acid as carriers or with allopurinol and oxipurinol as carriers depending on the isotopic precursor present. The reaction products spotted on 3MM paper were separated from each other by electrophoresis at 4000 v and 250 ma for 1 hr in 0.05 M borate buffer. Those on DEAE paper were separated by ascending chromatography in 0.2 M ammonium formate.

The incorporation of radioactive purine bases into nucleic acids was studied during cell growth in monolayer since no nucleic acid synthesis could be detected in cells kept in a sterile suspension at 37°C for up to 48 hr. Cell strains derived from normal individuals and patients deficient in hypoxanthine-guanine phosphoribosyltransferase were subcultured onto 100-mm sterile Petri dishes. Hypoxanthine-8-^{14}C and xanthine-8-^{14}C were diluted with appropriate quantities of the purine-^{12}C bases to obtain a specific activity of 0.07 mCi/mmole in order that they would have the same specific activity as the allopurinol-6-^{14}C which was available. 1 μmole of each radioactive base was added to each of four Petri dishes containing 10.0 ml media and fibroblasts in exponential growth and the cells were incubated at 37°C in 95% air–5% CO_2 atmosphere. In studies designed to test the effect of allopurinol on the incorporation of hypoxanthine into nucleic acids, duplicate monolayer cultures were set up to contain allopurinol-^{12}C at a final concentration of either 1×10^{-5} or 1×10^{-4} mole/liter and hypoxanthine-8-^{14}C (4.1 mCi/mmole) at a final concentration of 1×10^{-6}

[1] Littlefield, J. W. Personal communication.

TABLE I

Absence of Xanthine Oxidase Activity in Fibroblast Extracts Using Hypoxanthine-8-^{14}C as Substrate

Fibroblast extract		Hypoxanthine	Xanthine and uric acid
		cpm	*cpm*
E. T.	Boiled extract	38,940	36
	Exp. 1	48,806	46
	Exp. 2	45,611	43
S. M.	Boiled extract	36,461	34
	Exp. 1	43,859	36
	Exp. 2	41,052	41

mole/liter. After growth for 3–5 days, the medium was removed, the cells were washed thoroughly with PBS, and harvested in the usual manner. The cell pellet was resuspended in PBS, an aliquot removed for protein determination, and an equal volume of cold 10% trichloroacetic acid added to the suspension. After at least 10 min at 4°C, the samples were filtered on 0.45 Millipore filters, washed thoroughly with cold 5% trichloroacetic acid and water, dried and immersed in a scintillation vial containing 6.6 ml phosphor and 3.3 ml of Triton X-100, and counted.

Protein concentration was estimated by the method of Lowry, Rosebrough, Farr and Randall (14).

RESULTS

No xanthine oxidase activity could be demonstrated in fibroblast extracts by a spectrophotometric assay. As indicated in Table I, the enzyme was also found to be absent when a more sensitive radioisotopic assay was used. There was no detectable conversion of hypoxanthine, the natural substrate for xanthine oxidase, to either xanthine or uric acid even though it would have been possible to detect xanthine oxidase activity as low as 5.6×10^{-6} mole/mg protein per hr, under these conditions. This would indicate that if xanthine oxidase activity were present, it would be less than 0.2% of that noted in normal human jejunal mucosa (15). Similar negative results were obtained with an extract of cells (S. M.) lacking an enzyme, hypoxanthine-guanine phosphoribosyltransferase, which normally would tend to compete with xanthine oxidase for their common substrate, hypoxanthine. Finally, we have been unable to detect the formation of radioactive uric acid with xanthine-8-^{14}C as substrate or the formation of labeled oxipurinol with allopurinol-6-^{14}C as substrate in these cell extracts, providing additional evidence that xanthine oxidase is not present.

The effect of allopurinol on the incorporation of hypoxanthine-8-^{14}C into cold trichloroacetic acid–precipitable nucleic acids was examined. If xanthine oxidase were present in these cells at any time during their growth in monolayer cultures, the presence of a potent inhibitor such as allopurinol should inhibit the conversion of hypoxanthine to xanthine and thus allow more substrate to be available for conversion to inosinic acid and hence nucleic acids. As illustrated in Table II, the presence of relatively high concentrations of allopurinol (up to 1×10^{-3} mole/liter) during log growth of fibroblasts in monolayer did not alter the incorporation of hypoxanthine into nucleic acids indicating that in addition to the absence of xanthine oxidase in fibroblast extracts, this enzyme also is apparently not functional during any stage of the cell cycle.

TABLE II

Effect of Allopurinol on the Incorporation of Hypoxanthine-8-^{14}C into Cold Acid-Insoluble Material in Cultured Human Fibroblasts

Cell strain	Cold acid-insoluble material	
	RLV (3 days)*	JWI (5 days)*
	cpm/mg protein	
Control	116,770	143,490
Allopurinol 1×10^{-4} M	117,120	135,400
1×10^{-3} M	116,230	144,950

* Duration of growth in hypoxanthine-8-^{14}C and allopurinol.

The synthesis of FGAR-^{14}C in the presence of azaserine, a measure of the rate of the first three steps of purine biosynthesis *de novo,* is indicated for seven different cell strains in Table III. The values in this table represent the control data for all of the inhibitory studies reported later. Three of the seven cell strains, under these experimental conditions, had no detectable hypoxanthine-guanine phosphoribosyltransferase activity (PRT^-), whereas the other four cell strains exhibited normal activity of this enzyme (PRT^+). As previously

TABLE III

Synthesis of FGAR-^{14}C in Seven Different Cell Strains in the Presence of Azaserine

Cells	Sex	Age	FGAR-^{14}C
			cpm/μg protein per hr
Normal hypoxanthine-guanine phosphoribosyltransferase			
E. H. A.	Male	45	6.7
C. L. L.	Male	57	7.1
J. W. B.	Male	45	7.9
H. H.	Female	16	7.6
Deficient hypoxanthine-guanine phosphoribosyltransferase			
D. F.	Male	15	41.1
J. R.	Male	17	55.1
S. M.	Male	15	161.7

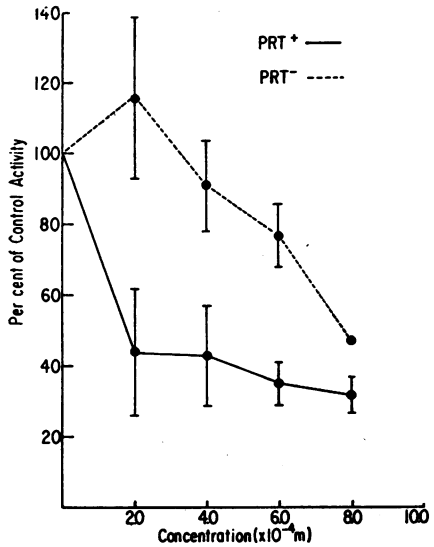

FIGURE 2 The effect of hypoxanthine on the synthesis of FGAR-^{14}C in four cell strains of human fibroblasts with normal hypoxanthine-guanine phosphoribosyltransferase activity (PRT$^+$) and in three cell strains genetically deficient in hypoxanthine-guanine phosphoribosyltransferase activity (PRT$^-$).

noted, PRT$^-$ cells accumulate FGAR much more extensively than do PRT$^+$ cells (13).

Figs. 2 and 3 illustrate the inhibitory effect of hypoxanthine and allopurinol respectively on the early steps of *de novo* purine synthesis in each of the four PRT$^+$ cell strains and the three PRT$^-$ cell strains. Although the results obtained were quantitatively rather variable in different cell strains, they were qualitatively similar and in any given cell strain studied under similar conditions, the inhibition observed was quite reproducible.

In all PRT$^+$ cell strains, both hypoxanthine and allopurinol inhibited the synthesis of FGAR at concentrations as low as 2×10^{-4} mole/liter. Although hypoxanthine was consistently a slightly better inhibitor of FGAR synthesis than was allopurinol, these studies demonstrate that allopurinol or one of its metabolic products is an effective inhibitor of the early steps of *de novo* purine synthesis in these cells despite the absence of xanthine oxidase.

Concentrations of hypoxanthine and allopurinol of 2×10^{-4} mole/liter which consistently inhibit FGAR accumulation in normal cells actually lead to a modest increase in FGAR accumulation in these PRT deficient cells. At concentrations of 6×10^{-4} mole/liter and greater, however, inhibition is observed. It appears therefore that the inhibition of *de novo* purine synthesis produced in normal cells at low concentrations of either hypoxanthine or allopurinol is dependent on the presence of hypoxanthine-guanine phosphoribosyltransferase. At the higher concentrations used, inhibition occurs in the absence of hypoxanthine-guanine phosphoribosyltransferase.

The effect of oxipurinol on FGAR synthesis in the four PRT$^+$ cell strains and in one PRT$^-$ cell strain is illustrated in Fig. 4. A consistent inhibitory effect of this compound in PRT$^+$ cell strains could only be demonstrated at a concentration of 6×10^{-4} mole/liter and greater. At the higher concentrations tested the inhibition observed was quite similar to that produced by allopurinol. In the PRT$^-$ cell strain studied the effects were essentially the same as those produced with allopurinol.

Table IV compares the incorporation of hypoxanthine-8-^{14}C into nucleic acids with that of radioactive xanthine and allopurinol of the same specific activity and at the same concentration. In normal fibroblasts grown in monolayer, xanthine was incorporated into nucleic acids at a very low level not exceeding 0.1% of that observed with hypoxanthine. No incorporation of allopurinol into cold trichloroacetic acid–precipitable counts could be demonstrated. Under the conditions of this experiment it is probable that incorporation approaching 0.02% of that observed with hypoxanthine could have been detected.

The purine bases up to a concentration of 1×10^{-3} mole/liter had no discernible effect on protein synthesis or cell survival.

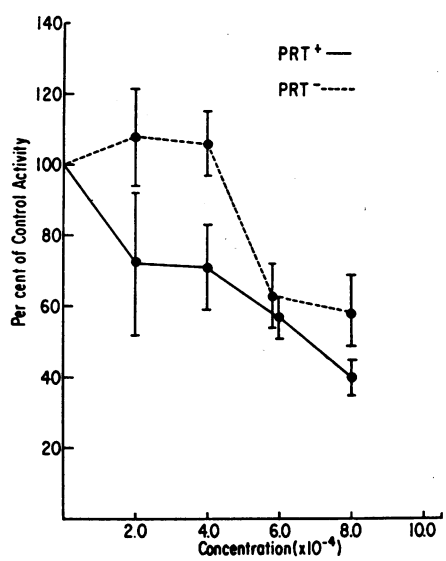

FIGURE 3 The effect of allopurinol on the synthesis of FGAR-^{14}C in four cell strains of human fibroblasts with normal hypoxanthine-guanine phosphoribosyltransferase activity (PRT$^+$) and in three cell strains genetically deficient in hypoxanthine-guanine phosphoribosyltransferase activity (PRT$^-$).

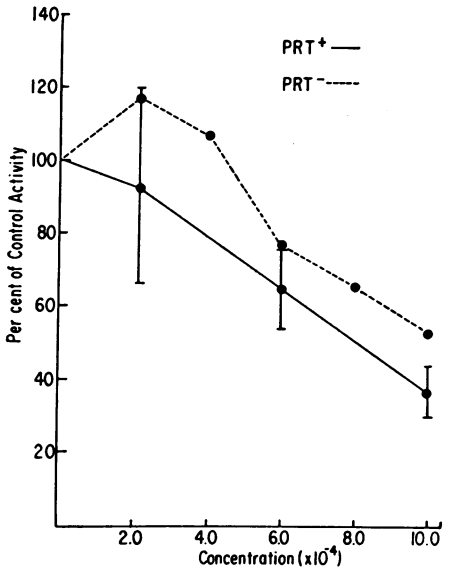

FIGURE 4 The effect of oxipurinol on the synthesis of FGAR-^{14}C in four cell strains of human fibroblasts with normal hypoxanthine-guanine phosphoribosyltransferase activity (PRT$^+$) and in one cell strain genetically deficient in hypoxanthine-guanine phosphoribosyltransferase activity (PRT$^-$).

DISCUSSION

In addition to its well recognized inhibitory effect on xanthine oxidase, allopurinol also inhibits the *de novo* synthesis of purines. Based on in vivo studies in man as well as in vitro studies in pigeon liver, several mechanisms have been proposed to account for this inhibitory effect of allopurinol on purine synthesis. Allopurinol, as its ribonucleotide, may act directly on the rate-limiting step of purine biosynthesis *de novo,* PRPP [2] amidotransferase, to produce feedback inhibition by an allosteric mechanism (mechanism 1). The enzyme hypoxanthine-guanine phosphoribosyltransferase (PRT), which is capable of catalyzing the conversion of allopurinol to its ribonucleotide (16), is present in man (17). The latter compound, allopurinol ribonucleotide, is an effective inhibitor of PRPP amidotransferase obtained from pigeon liver whereas the free base, allopurinol, is not (16).

An alternative hypothesis suggests that allopurinol, by inhibiting xanthine oxidase and therefore the conversion of hypoxanthine to xanthine, produces a higher concentration of hypoxanthine that could in turn increase the formation of inosinic acid, a conversion again requiring the enzyme PRT (mechanism 2). This compound and the adenine and guanine nucleotides formed from it are allosteric inhibitors of purine synthesis *de novo* at the level of the rate-limiting step, PRPP amidotransferase (18). Support for this hypothesis is derived from studies in mice which demonstrate that allopurinol produces a substantial increase in the incorporation of labeled hypoxanthine into soluble nucleotides and into the normal purine components, adenine and guanine, of nucleic acids (19, 20) and by the observation in man that allopurinol enhances the reutilization of hypoxanthine derived from exogenous inosine (8). Clearly, one postulated mechanism depends on the presence of xanthine oxidase whereas the other does not.

The increased conversion of either allopurinol or hypoxanthine to its respective ribonucleotide could also deplete intracellular PRPP, an essential substrate for PRPP amidotransferase. This reduction in substrate concentration could also account for the observed decrease in purine synthesis *de novo* (mechanism 3). These three potential mechanisms are summarized in Fig. 5.

Human fibroblasts in culture synthesize purines both *de novo* and by the reutilization of free purine bases (13). In addition, the molecular regulation of these purine pathways appears so far to be quite similar to that previously elucidated in lower organisms. Unlike lower organisms or human liver and gastrointestinal mucosa, however, these cultured human fibroblasts lack the enzyme xanthine oxidase. Such cells, therefore, provide an opportunity for determining the relative importance of the inhibitory effects of allopurinol which are not dependent on the presence of xanthine oxidase.

The data presented here indicate that allopurinol inhibits the early steps of *de novo* purine synthesis in cultured human fibroblasts when assessed by following the incorporation isotopic formate into formylgly-

TABLE IV

Incorporation of Radioactive Allopurinol, Hypoxanthine, and Xanthine into Cold Trichloroacetic Acid–Precipitable Nucleic Acids

Radioactive base	Specific activity	Acid-insoluble material
	mCi/mmole	*cpm/mg protein*
Hypoxanthine-8-^{14}C	0.07	65,397
		78,471
		50,507
		56,168
Xanthine-8-^{14}C	0.07	67
		25
		26
		39
Allopurinol-6-^{14}C	0.07	<10
		<10
		<10
		<10

[2] Phosphoribosylpyrophosphate.

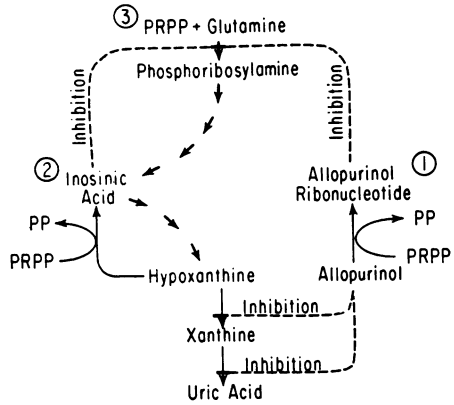

FIGURE 5 Possible mechanisms to account for the inhibitory effect of allopurinol on the *de novo* synthesis of purines. These include (1) conversion of allopurinol to its ribonucleotide; (2) increased conversion of hypoxanthine to inosinic acid; and (3) depletion of intracellular PRPP.

cinamide ribonucleotide (FGAR). Since xanthine oxidase is not present in these cells, it would seem quite unlikely that the inhibitory effect observed is due to an increased conversion of hypoxanthine to inosinic acid. The alternative possibility which must be considered, therefore, is that allopurinol is converted to its ribonucleotide and that it is this compound or the depletion of PRPP resulting from this reaction that leads to the decrease in *de novo* purine synthesis. The finding that cell strains genetically deficient in PRT and thus unable to convert allopurinol to its ribonucleotide exhibit resistance to the feedback effect of relatively low concentrations of allopurinol is consistent with this hypothesis. Resistance to this effect of allopurinol has also been demonstrated in vivo in several of the patients who lack hypoxanthine-guanine phosphoribosyltransferase from whom these cells were cultured (21).

Higher concentrations of allopurinol as well as hypoxanthine produced a decrease in FGAR accumulation in the PRT-deficient cells. This finding might be the result of low concentrations of PRT activity in these cells as recently reported by Fujimoto and Seegmiller (22). The similarity of the inhibitory effects observed with hypoxanthine and allopurinol seem inconsistent with this hypothesis. In addition, we have been unable to demonstrate more than trace amounts of PRT activity in these cells. It is also possible that at higher concentrations of these compounds, the synthesis or regulation of some component other than the PRPP amidotransferase is affected which is crucial to the synthesis of FGAR. Such an interpretation would imply that there are at least two different mechanisms by which allopurinol may inhibit *de novo* purine synthesis in normal fibroblasts in the absence of xanthine oxidase.

Oxipurinol, the major product of allopurinol oxidation in vivo, is not a substrate for hypoxanthine-guanine phosphoribosyltransferase (17). An inhibitory effect of this compound on *de novo* purine synthesis would therefore also have to be attributed to some mechanism other than those summarized in Fig. 5. Although the effects of lower concentrations of oxipurinol were quite variable, relatively high concentrations of this agent inhibited FGAR synthesis in a manner similar to that observed with comparable concentrations of allopurinol. Oxipurinol also appears to reduce the *de novo* synthesis of purines when administered to both normal and gouty subjects in vivo although it is clearly not possible to exclude the role of xanthine oxidase inhibition in these studies (23).

The concentration of allopurinol and oxipurinol which effected significant inhibition in these cells in culture is approximately equal to the concentration of oxipurinol which occurs in extracellular fluid of patients being treated with allopurinol (5). The relative intracellular concentration of these drugs in vivo and in vitro, however, is unknown.

These studies indicate that allopurinol and its primary metabolic product, oxipurinol, can inhibit the *de novo* synthesis of purines in cultured human fibroblasts in the absence of xanthine oxidase and suggest that although allopurinol ribonucleotide may play a prominent inhibitory role at lower concentrations, the inhibitory effect observed with oxipurinol and at higher concentrations of allopurinol in normal cells and cells lacking PRT must be due to some mechanism other than those previously suggested. In the last several years it has become apparent that allopurinol as the free base actually inhibits several enzymes in addition to xanthine oxidase. This list now includes tryptophane pyrrolase (24) and pyrimidine deoxyribosyltransferase (25). It is doubtful that inhibition of either of these enzymes could lead to the changes observed here but such data do emphasize the potential existence of other enzymes in which an alteration in activity might theoretically lead at least in part to the changes in FGAR synthesis observed in the present study. Enzymes which catalyze the destruction or utilization of the natural purine nucleotides as well as those responsible for the synthesis of PRPP and glutamine would seem to be particularly germane as sites of potential inhibition. Preliminary data in this laboratory indicate that free bases, allopurinol and oxipurinol, are not inhibitors of 5′-nucleotidase, the major enzyme responsible for the catabolism of guanylic and inosinic acids.

We have not been able to detect the ribonucleoside or the ribonucleotide derivatives of allopurinol either during routine incubation conditions or under optimal assay conditions in which an excess of PRPP and optimal

concentration of magnesium were maintained. It must be emphasized however, that the sensitivity for detection of either of these compounds under our in vitro conditions is currently very poor and relatively large quantities of either derivative could have escaped detection. The ribonucleoside derivative of allopurinol is found in the urine of most patients being treated with allopurinol, but it is not clearly established whether this is formed directly from the drug by the action of a purine nucleoside phosphorylase or results from the breakdown of the nucleotide derivative by an enzyme with 5′-nucleotidase activity (26, 27).

If nucleotide derivatives of allopurinol are formed, they would have the further capability of being incorporated into nucleic acids, as occurs with some of the other purine analogs, and possibly leading to DNA instability, chromosomal breaks, reading errors, or being otherwise mutagenic. Elion, Kovensky, Hitchings, Metz, and Rundles were unable to detect incorporation of radioactive allopurinol or oxipurinol into liver nucleic acids 24 hr after their administration to mice in vivo (26). We have also not been able to demonstrate the incorporation of allopurinol into nucleic acids in human cells cultured in vitro. Under the experimental conditions reported here, it should have been possible to detect the incorporation of allopurinol into nucleic acids if it occurred as often as 0.02% of that observed with hypoxanthine. In addition, there was no apparent difference in growth rate or survival of these cultured human cells grown in 1 mM allopurinol, 1 mM oxipurinol, or 1 mM hypoxanthine through 57 generations. These studies of course in no way guarantee the ultimate safety of allopurinol *in vivo* though they are reassuring. Unfortunately, it has not been possible to adequately assess somatic mutation rate in these cells so that this important aspect of the long-term toxicity of allopurinol must await further evaluation.

It should be emphasized that the inhibitory effect of both allopurinol and oxipurinol on *de novo* purine synthesis *in vivo* could still be in part at least an indirect result of the inhibition of xanthine oxidase. The present study was designed to test and has demonstrated other potential mechanisms of inhibition in an in vitro system where the role of xanthine oxidase inhibition can be disregarded. The administration of allopurinol to a patient with xanthinuria, a condition characterized by a genetically determined absence of xanthine oxidase, produced no decrease in total purine excretion (28). However, it is possible that in this in vivo situation the PRT enzyme was already saturated by the large concentrations of hypoxanthine present. If this were the case, an additional substrate for the enzyme such as allopurinol probably could not be converted to its ribonucleotide form to a significant degree and therefore no inhibition would be observed.

ACKNOWLEDGMENTS

We wish to acknowledge the excellent technical assistance provided by Mrs. Jean Meade and Mrs. Margaret Evans.

This investigation was supported by U. S. Public Health Service Grant No. AM12413-02 and United Medical Research Foundation Grant 177 to Duke University.

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