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Oxipurinol-Associated Inhibition of Pyrimidine Synthesis in Human Lymphoblasts

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SUMMARY

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The administration of allopurinol or its major metabolic oxidation product, oxipurinol, to man consistently results in inhibition not only of uric acid synthesis but also of pyrimidine synthesis. Molecular correlates of the latter inhibition produced by oxipurinol have been studied in permanent human lymphoblast lines in tissue culture, which appear to be satisfactory models for the study of this drug effect. Incubation of intact lymphoblasts with oxipurinol resulted within 90 min in significant inhibition of incorporation of [^{14}C]orotic acid but not [^{3}H]uridine into cold acid-precipitable counts. This inhibition of pyrimidine synthesis was correlated with the generation of an orotidylic acid decarboxylase inhibitor which, in cell-free extracts, required 5-phosphoribosyl 1-pyrophosphate and oxipurinol for its formation and could be generated in extracts of cells deficient in hypoxanthine-guanine phosphoribosyltransferase. Stabilization of both orotidylic acid decarboxylase and orotate phosphoribosyltransferase also resulted from incubation of lymphoblasts with oxipurinol. The production of the stabilized activities appeared to coincide temporally, in substrate requirements, and in reversibility with the generation of the inhibitor of orotidylic acid decarboxylase activity, suggesting a common molecular basis for these effects. In addition, oxipurinol treatment resulted in an alteration in the quaternary structure of the bifunctional enzyme complex containing both activities, with an increase in molecular weight from 41,000 to 108,000 as estimated by gel filtration studies. In the higher molecular weight material both increased stability and inhibition of orotidylic acid decarboxylase were demonstrated. These findings are in accord with the suggestion that ribonucleotides of oxipurinol result in inhibition of pyrimidine synthesis through alterations in the activity, stability, and quaternary structure of the enzyme complex. A concentration-dependent increase in the specific activities of orotidylic acid decarboxylase and orotate phosphoribosyltransferase has been demonstrated in both lymphoblasts and fibroblasts incubated with oxi-

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purinol. In lymphoblasts this increase in enzyme activity occurred within 2–3 hr and was not prevented by treatment of the cells with cycloheximide. Stabilization of the enzymes during extraction from the cell or direct activation of the enzyme activities appear to be more likely explanations for the increased enzyme activities than induction of new enzyme synthesis or inhibition of normal enzyme catabolism. No differences were found between normal and hypoxanthine-guanine phosphoribosyltransferase-deficient lymphoblast (or fibroblast) lines in activities of either orotate phosphoribosyltransferase or orotidylic acid decarboxylase.

INTRODUCTION

Allopurinol, an analogue of hypoxanthine, is both a competitive inhibitor and a substrate of the final enzyme involved in uric acid synthesis, xanthine oxidase (EC 1.2.3.2), and is therefore in wide clinical use as a hypouricemic agent (1). The major inhibitory effect of this drug on xanthine oxidase activity is produced by binding of its oxidation product, oxipurinol, to an enzyme-bound molybdenum cofactor of lower valence state than is present in the active enzyme (2). The metabolism of allopurinol results, however, in at least two additional pharmacological effects. In most individuals, particularly those patients who are overproducing uric acid, treatment with this drug diminishes the total amount of purines synthesized and excreted by the body, an effect dependent on activity of the enzyme hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) (3), which catalyzes conversion of allopurinol to allopurinol 1-ribonucleotide. The rate of purine synthesis *de novo* is diminished as a consequence of this reaction, either through depletion of the regulatory substrate 5-phosphoribosyl 1-pyrophosphate[3] (4), through accumulation of inhibitory purine nucleotide derivatives of hypoxanthine and xanthine, or, less likely (5), through inhibition by allopurinol ribonucleotide (6) of the rate-limiting first reaction in purine synthesis *de novo* catalyzed by PP-ribose-P amidotransferase (EC 2.4.-2.14) (7, 8).

An inhibitory effect of metabolites of allopurinol on pyrimidine synthesis has recently been demonstrated *in vivo* in humans (9, 10) and rats (11) as well as in human fibroblasts in tissue culture (12). This effect results from inhibition of the enzyme orotidylic acid decarboxylase (EC 4.1.1.23), which catalyzes conversion of orotidylic acid to uridylic acid, by nucleotide derivatives of oxipurinol, the major metabolite of allopurinol (12–15). Inhibition of pyrimidine synthesis *in vivo* is accompanied in addition by increased activities of orotate phosphoribosyltransferase (EC 2.4.2.10) and orotidylic acid decarboxylase (11, 13, 16), sequential enzymes of pyrimidine synthesis which constitute a bifunctional enzyme complex (13, 17, 18). The molecular basis of the increased enzyme activities is controversial; Fox *et al.* (13) showed evidence for stabilization of these enzymes in erythrocytes from individuals treated with allopurinol, and yeast orotidylic acid decarboxylase is stabilized by oxipurinol ribonucleotides (15). Stabilization in erythrocytes, however, was not confirmed by Beardmore *et al.* (16), who suggested a direct activation of orotate phosphoribosyltransferase by an oxipurinol metabolite. In liver homogenates of rats given allopurinol, Brown and co-workers (11) found increased stability of these enzyme activities to thermal inactivation and an apparent increase in the molecular weight of the enzyme complex. These authors suggested that the observed effects of enzyme stabilization, increased enzyme activity, and inhibition of pyrimidine synthesis were consequences of the demonstrated alteration in quaternary structure of the enzyme complex.

In the present studies we evaluate the effects of oxipurinol on pyrimidine metabolism in diploid human lymphoblasts in tissue culture, using normal cell lines and cell lines severely deficient in hypoxanthine-guanine phosphoribosyltransferase. Our

[3] Abbreviations used are: PP-ribose-P, 5-phosphoribosyl 1-pyrophosphate; TCA, trichloracetic acid.

studies in both intact cells and cell extracts indicate that exposure of human lymphoblasts to oxipurinol result in a number of temporally related changes in pyrimidine synthesis, most likely mediated through an alteration in the quaternary structure of the orotate phosphoribosyltransferase–orotidylic acid decarboxylase complex consequent to interaction with a nucleotide derivative of oxipurinol. We have confirmed the stabilization of both enzyme activities accompanying inhibition of orotidylic acid decarboxylase and suggest that the increased enzyme activities resulting from incubation of intact cells with oxipurinol may reflect this stabilization. Lymphoblasts thus represent a convenient model *in vitro* in which virtually all the effects of oxipurinol on pyrimidine metabolism observed in previous studies *in vivo* (11, 13, 14, 16) and in tissue culture (12) can be investigated and related to one another.

MATERIALS AND METHODS

Materials. [7-^{14}C]Orotic acid (1.5 mCi/mmole), [6-^{14}C]orotic acid (51.6 mCi/mmole), [7-^{14}C]OMP (1.05 mCi/mmole), [5-^{3}H]-uridine (28 Ci/mmole), and Liquifluor phosphor were obtained from New England Nuclear Corporation. Dimagnesium PP-ribose-P was purchased from P-L Biochemicals; cycloheximide, from Nutritional Biochemicals; chymotrypsinogen, creatine kinase, and catalase, from Worthington; and unlabeled OMP, from Calbiochem. Sephadex G-150 and G-25 were purchased from Sigma. Oxipurinol was a gift of Dr. Gertrude B. Elion, Burroughs-Wellcome. Auto-Pow medium and fetal calf serum were obtained from Flow Laboratories. All other chemicals were purchased from commercial sources and were of the highest grade generally available.

Cell cultures. Lymphoblast cell line WI-L_2, obtained from Dr. Richard Lerner of Scripps Clinic and Research Foundation, is a long-term diploid line of human lymphoblasts derived from the spleen of an individual with congenital spherocytic anemia (19). These cells show a normal karyotype, and characteristics of their purine metabolism have been studied (20). A lymphoblast cell line, UM-11 (21), derived from an individual with severe hypoxanthine-guanine phosphoribosyltransferase deficiency (Lesch-Nyhan syndrome) (22), was a gift of Dr. Arthur Bloom, University of Michigan. Previous studies of the purine metabolism in this cell line have demonstrated marked purine overproduction, increased intracellular PP-ribose-P concentration, and severe deficiency of the enzyme hypoxanthine-guanine phosphoribosyltransferase (20). For certain experiments a lymphoblast cell line (Ag^r9 Cl3 SC1), selected by Dr. G. Nuki and Dr. J. Lever from WI-L_2 by resistance to 8-azaguanine and found to have a severe deficiency of hypoxanthine-guanine phosphoribosyltransferase, was also used (23). All lymphoblast lines were grown in suspension culture in Auto-Pow medium with 10% fetal calf serum, 50 units/ml of penicillin, and 50 μg/ml of streptomycin at 37° in 5% CO_2 in air. Under these conditions the cells grow to a maximum density of approximately 1.6–2.0 $\times$ 10^6/ml. For the present experiments cells were harvested during the logarithmic phase (0.8–1.2 $\times$ 10^6 cells/ml).

Fibroblast cell lines derived from skin biopsy specimens obtained from normal individuals and patients with severe hypoxanthine-guanine phosphoribosyltransferase deficiency were grown and propagated in monolayers as previously described (24) in Eagle's minimal essential medium with added nonessential amino acids, 10% fetal calf serum, and antibiotics in the concentrations noted above. Experiments were carried out on cells grown to confluence on the surface of glass roller bottles and removed by treatment with 0.25% trypsin.

Preparation of cell extracts. Lymphoblasts in suspension culture were centrifuged at 1800 $\times$ g for 5 min and washed three times in cold Dulbecco's phosphate-buffered NaCl solution. Cells were resuspended at a density of 4 $\times$ 10^7/ml in 0.02 M potassium phosphate buffer, pH 7.4; they were then frozen and thawed three times in liquid nitrogen. After centrifugation for 20 min at 48,000 $\times$ g, the supernatant layer was dialyzed at 4° for 120 min against 0.02 M potassium phosphate buffer, pH 7.4.

Fibroblast extracts were prepared in an identical fashion following trypsin digestion and resuspension at 2–4 $\times$ 10^7 cells/ml.

Estimation of rate of pyrimidine synthesis.

The incorporation of [6-^{14}C]orotic acid or [^{3}H]uridine into cold acid-insoluble material was used to assess the effects of oxipurinol on pathways of pyrimidine nucleotide synthesis (12). To cultures of lymphoblasts in growth medium, appropriate concentrations of oxipurinol were added. After incubation of the cells at 37° for the appropriate period of time, [^{14}C]orotic acid or [^{3}H]uridine (diluted with nonlabeled uridine to give a final specific activity of 560 mCi/mmole) was added to the cell suspension at a final concentration of 20 μM, and incubation was continued for 60 min. Three volumes of ice-cold phosphate-buffered NaCl were added to stop the incubation. Following centrifugation at 1800 × *g*, the cell pellet was washed three times with 2.5 ml of ice-cold 10% TCA, resuspended in 1.25 ml of 1% sodium dodecyl sulfate–10% TCA, and placed at 0° for 20 min. The suspension was then filtered through a 0.45 μ Millipore filter, which was washed with 5% TCA, dried in air, and counted in Liquifluor phosphor in a Beckman LS-233 liquid scintillation counter. For these studies counting efficiencies were 62% for ^{14}C and 1.5% for ^{3}H.

Enzyme assays. Orotate phosphoribosyltransferase and orotidylic acid decarboxylase activities were assayed in accordance with the methods described by Fox, Wood, and O'Sullivan (13), except that the final [^{14}C]-OMP concentration in the orotidylic acid decarboxylase assay was 0.1 mM and dialyzed lymphoblast or fibroblast extract replaced hemolysate. In the orotate phosphoribosyltransferase assay the product of the reaction was converted to uridylic acid by addition of 0.1 unit of yeast orotidylic acid decarboxylase, partially purified by the method of Heppel and Hilmoe (25). This preparation contained less than 0.06% orotate phosphoribosyltransferase activity relative to orotidylic acid decarboxylase activity. In each assay the appropriate [7-^{14}C]labeled substrate yielded $^{14}CO_2$ which was collected in 0.1 ml of Hyamine hydroxide (Packard) contained in center wells (Kontes Glass) suspended from rubber gaskets mounted on 17 × 100 mm polypropylene tubes. Collection of CO_2 was maximized by stopping the reactions with 0.1 ml of 5.0 N H_2SO_4 injected into the bottom of the test tubes. After 30 min of incubation at 37°, the center wells were removed from the tubes and clipped directly into scintillation vials to which 10 ml of Bray's solution were added. Radioactivity was then directly measured in a Beckman liquid scintillation counter. Generation of $^{14}CO_2$ was proportional for both enzymes to time of incubation up to 60 min, and to protein concentration, from 150 to 400 μg in the assays.

Other methods. Protein determinations were carried out by the method of Lowry *et al.* (26). Hypoxanthine-guanine phosphoribosyltransferase assays were performed in lymphoblast extracts as described by Wood, Becker, and Seegmiller (20), and in fibroblast extracts, as described by Fujimoto and Seegmiller (27).

RESULTS

Inhibition of pyrimidine synthesis. Incorporation of [6-^{14}C]orotic acid into cold acid-insoluble material is markedly inhibited by incubation of intact lymphoblasts with oxipurinol. As shown in Fig. 1A and B, respectively, the inhibition of pyrimidine synthesis is maximal at 0.5–1 mM oxipurinol and is evident within 90 min of addition of oxipurinol to the medium. Incubation of intact lymphoblasts with similar concentrations of oxipurinol has no effect on the incorporation of [^{3}H]uridine into cold acid-insoluble material. Inhibition of pyrimidine nucleotide synthesis by oxipurinol or its metabolic products is localized by these experiments to the sequence of two reactions resulting in the synthesis of UMP from orotic acid. In the presence of 0.5 mM oxipurinol in the growth medium the growth rate of WI-L_2 lymphoblasts is diminished approximately 20% although normal maximal cell density is achieved.

Activities of orotate phosphoribosyltransferase and orotidylic acid decarboxylase. The activities of both enzymes involved in the conversion of orotic acid to UMP are measurable in lymphoblasts and fibroblasts grown in tissue culture, although the specific enzyme activities of both enzymes are higher in lymphoblast lines (Table 1). As previously reported in erythrocytes (13), coordination of orotate phosphoribosyltransferase and orotidylic acid decarboxylase activities is

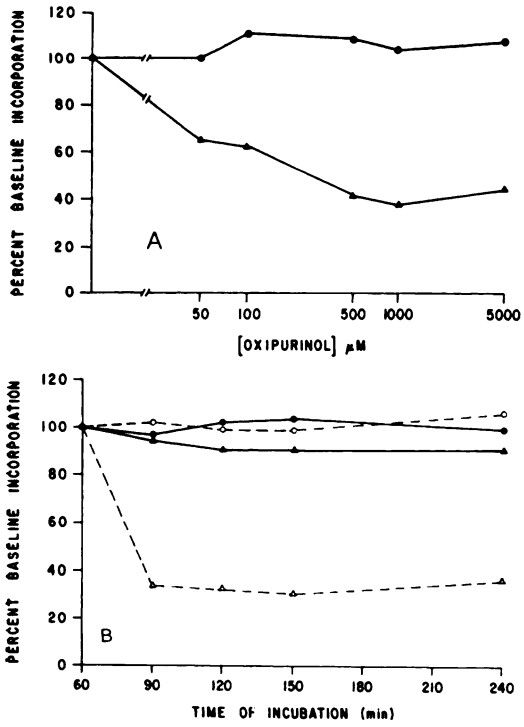

FIG. 1. *Inhibition of pyrimidine synthesis in human lymphoblasts produced by oxipurinol*

After incubation of 1×10^6 intact cells at 37° in 1.0 ml of growth medium with or without oxipurinol, either [6-^{14}C]orotic acid (1 μCi, 20 nmoles) or [^{3}H]uridine (11.2 μCi, 20 nmoles) was added, and the incubation was continued for 60 min. The rate of pyrimidine synthesis was estimated as described in MATERIALS AND METHODS.

A (upper). Effect of oxipurinol concentration on the rate of pyrimidine synthesis. Time of incubation prior to isotope addition was 3 hr. ●—●, [^{3}H]uridine; ▲—▲, [^{14}C]orotic acid. The baseline incorporation of [^{3}H]uridine was 6200 cpm, and of [^{14}C]orotic acid, 2100 cpm.

B (lower). Time course of oxipurinol effect on pyrimidine synthesis. The oxipurinol concentration was 0.5 mM. Times on the abscissa indicate total periods of incubation with medium in the presence or absence of oxipurinol. O- - -O, [^{3}H]uridine, no oxipurinol; ●——●, [^{3}H]uridine, oxipurinol added; ▲——▲, [^{14}C]orotic acid, no oxipurinol; △- - -△, [^{14}C]orotic acid, oxipurinol added. The baseline incorporation of [^{3}H]uridine was 5700 cpm, and of [^{14}C] orotic acid, 2300 cpm.

present in both cell types. In contrast to the increased activities of orotate phosphoribosyltransferase and orotidylic acid decarboxylase described in erythrocytes of children with severe deficiency of hypoxanthine-guanine phosphoribosyltransferase (28), both lymphoblasts and fibroblasts derived from individuals with severe hypoxanthine-guanine phosphoribosyltransferase deficiency possess activities of both enzymes comparable to those seen in cells with normal hypoxanthine-guanine phosphoribosyltransferase activity (Table 1). Lymphoblasts selected by spontaneous resistance to 8-azaguanine and also severely deficient in hypoxanthine-guanine phosphoribosyltransferase likewise have activities of these enzymes in the range of the normal cell lines.

Incubation of intact lymphoblasts with oxipurinol results in dose-dependent increases in activity of both orotate phosphoribosyltransferase and orotidylic acid decarboxylase (Fig. 2A). The increases in enzyme activities are coordinate and are demonstrable within 2 hr of incubation with oxipurinol. Prolongation of the period of incubation up to 9 hr (Fig. 2B) fails to increase the activities beyond those achieved by 3 hr. Prior incubation of cells with 5.0 μM cycloheximide (a concentration of this inhibitor of protein synthesis which markedly inhibits [^{14}C]-leucine incorporation into hot TCA-insoluble counts in lymphoblasts[4]) does not prevent the increased enzyme activities resulting from incubation with oxipurinol (Table 1).

When lymphoblasts are incubated in growth medium with 0.5 mM oxipurinol, similar increases in enzyme activities occur within 6 hr and persist to at least 24 hr. Normal and hypoxanthine-guanine phosphoribosyltransferase-deficient fibroblasts grown to confluence in the presence of 0.5 mM oxipurinol show increases in the activities of both enzymes similar in magnitude to those seen in lymphoblasts (Table 1) and in fibroblasts incubated with azauridine (29).

Inhibition of orotidylic acid decarboxylase. Within 2 hr of incubation of intact lymphoblasts with oxipurinol an inhibitor of orotidylic acid decarboxylase, but not of orotate phosphoribosyltransferase, is detectable in the cells. Inhibition can be demonstrated by comparing the enzyme activity at 0.01 mM OMP with that at 0.1 mM OMP as substrate (Table 2). The inhibitor can be generated in cell-free lymphoblast extracts

[4] M. H. Hershfield, unpublished observations.

TABLE 1

Activities of orotate phosphoribosyltransferase and orotidylic acid decarboxylase in normal and hypoxanthine-guanine phosphoribosyltransferase-deficient human lymphoblast and fibroblast cell lines, and effect of incubation with oxipurinol

Cells	Cell line	Hypoxanthine-guanine phosphoribosyltransferase activity	Enzyme activities			
			Orotate phosphoribosyltransferase		Orotidylic acid decarboxylase	
			Control	Oxipurinol	Control	Oxipurinol
		nmoles/hr/mg protein	*nmoles/hr/mg protein*		*nmoles/hr/mg protein*	
Lymphoblasts						
Normal	WI-L_2	413 (389–429)	41 (34–48)	55 (52–58)[a]	69 (60–81)	89 (77–104)[a]
	WI-L_2		42	57[b]	61	92[b]
Hypoxanthine-guanine phosphoribosyltransferase-deficient	UM-11	<6	35		68	
	Ag^r9 Cl 3 SC1	<4	33		59	
Fibroblasts						
Normal	Bas	216	11	19[c]	17	28[c]
	Car	194	8		17	
Hypoxanthine-guanine phosphoribosyltransferase-deficient	Rog	2	13	22[c]	17	23[c]
	Ros	1	11		18	

All activity measurements were made in duplicate. Numbers in parentheses represent the ranges of at least three separate duplicate sets of determinations.

[a] Cells incubated for 3–6 hr with 0.5 mM oxipurinol in Dulbecco's phosphate-buffered NaCl solution

[b] Cells incubated for 1 hr with 5.0 μM cycloheximide prior to addition of 0.5 mM oxipurinol.

[c] Cells incubated with 0.5 mM oxipurinol in growth medium (as described in MATERIALS AND METHODS) to confluence (4 days).

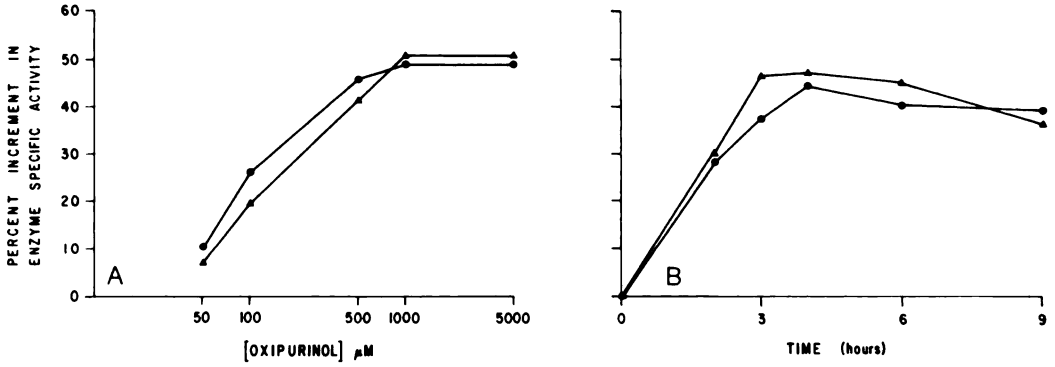

FIG. 2. *Increments in human lymphoblast (WI-L_2) orotate phosphoribosyltransferase and orotidylic acid decarboxylase activities resulting from incubation with oxipurinol*

Intact lymphoblasts were incubated at 37° in Dulbecco's phosphate-buffered NaCl solution with or without oxipurinol. At the appropriate time the incubation was stopped by addition of ice-cold medium, and cell extracts were prepared and activities assayed using 150–400 μg of protein in the assays as described in MATERIALS AND METHODS. ▲——▲, orotate phosphoribosyltransferase; ●——●, orotidylic acid decarboxylase.

A (left). Effect of oxipurinol concentration on enzyme activities. Time of incubation was 2 hr. The baseline specific enzyme activities were 42 and 66 nmoles/hr/mg of total protein for orotate phosphoribosyltransferase and orotidylic acid decarboxylase, respectively.

B (right). Time course of oxipurinol effect on enzyme activities. The oxipurinol concentration was 0.5 mM. Specific enzyme activities were 40 and 63 nmoles/hr/mg of total protein for orotate phosphoribosyltransferase and orotidylic acid decarboxylase, respectively, at zero time, and changed less than 10% during the course of the experiment in cells unexposed to oxipurinol.

TABLE 2

Inhibition of orotidylic acid decarboxylase activity in intact lymphoblasts and lymphoblast cell extracts incubated with oxipurinol

Intact cells were incubated in Dulbecco's phosphate-buffered NaCl solution, with additions as noted, for 2 hr prior to preparation of extracts and assay of orotidylic acid decarboxylase activity as described in MATERIALS AND METHODS. The decarboxylase activity was consistently higher in extracts of cells incubated intact with oxipurinol when determined in the standard enzyme assay using 0.1 mM OMP. Cell extracts from freshly harvested cells were prepared as described in the text but then were incubated with the indicated additions for 1 hr before enzyme assay.

Cells	OMP[a]	Oxipurinol (0.5 mM)	PP-ribose-P (0.5 mM)	Orotidylic acid decarboxylase activity	Inhibition
	mM			*nmoles/hr/mg protein*	%
Intact cells					
WI-L_2	0.1	+	−	71	
	0.01	+	−	37	48
	0.1	−	−	40	
	0.01	−	−	45	−13
Cell extracts					
WI-L_2	0.1	−	−	64	
	0.01	−	−	64	
	0.01	+	−	66	−3
	0.01	−	+	62	3
	0.1	+	+	55	14
	0.01	+	+	8	87
Ag^r9 Cl3 SC1	0.1	−	−	61	
	0.1	+	+	57	6
	0.01	+	+	8	87

[a] Concentration of orotidylic acid in standard enzyme assay.

only in the presence of both oxipurinol and PP-ribose-P and competes with OMP for binding to orotidylic acid decarboxylase, although an inhibitory constant cannot be calculated without knowledge of the concentration of the inhibitor. Generation of an inhibitor of orotidylic acid decarboxylase is not dependent on hypoxanthine-guanine phosphoribosyltransferase activity, since lymphoblasts severely deficient in this latter enzyme produce an inhibitor with similar characteristics when incubated with oxipurinol (Table 2).

Stabilization of orotate phosphoribosyltransferase and orotidylic acid decarboxylase. The activities of orotate phosphoribosyltransferase and orotidylic acid decarboxylase are dependent on the enzyme concentration used in the standard assay (Fig. 3A). However, when lymphoblasts are incubated with oxipurinol, extracts of these cells demonstrate increased stability at low enzyme concentration (Fig. 3A). In addition, both orotate phosphoribosyltransferase and orotidylic acid decarboxylase from cells incubated with oxipurinol demonstrate increased stability to heat activation at 57° (Fig. 3B). The time course of development of stabilization and the concentration of oxipurinol necessary for the development of this stabilization of enzyme activity in intact cells appear to be identical with the corresponding requirements for the production of the inhibitor of orotidylic acid decarboxylase activity. Furthermore, the factors responsible for both inhibition and stabilization are apparently generated simultaneously in an oxipurinol- and PP-ribose-P-dependent reaction in cell-free lymphoblast extracts. Under the latter conditions, however, no increase in specific activity of either enzyme has been observed, even when the substrate concentrations are increased to overcome potential competition by inhibitors.

Neither the increased stability to thermal inactivation nor the loss of concentration

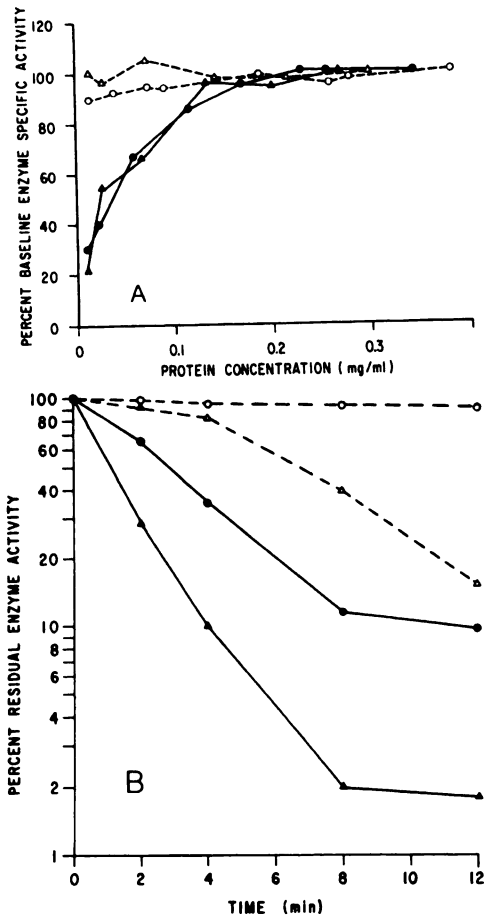

FIG. 3. *Effect of incubation with oxipurinol on stability of orotate phosphoribosyltransferase and orotidylic acid decarboxylase activities*

Intact lymphoblasts were incubated for 2 hr at 37° in Dulbecco's phosphate-buffered NaCl solution with or without 0.5 mM oxipurinol. Incubations were terminated by addition of ice-cold medium, and extracts were prepared as described in MATERIALS AND METHODS △- - -△, orotate phosphoribosyltransferase, no oxipurinol; ▲——▲, oxipurinol added; O- - -O, orotidylic acid decarboxylase, no oxipurinol; ●——●, oxipurinol added. The baseline specific enzyme activities were: for orotate phosphoribosyltransferase, no oxipurinol, 38 nmoles/hr/mg of total protein; oxipurinol added, 56; orotidylic acid decarboxylase, no oxipurinol, 73; oxipurinol added, 100.

A (upper). Dependence of enzyme activities on final protein concentration in assay. B (lower). Thermal inactivation of enzyme activities at 57°.

dependence of orotate phosphoribosyltransferase and orotidylic acid decarboxylase activities after exposure to oxipurinol is reversed by 2 hr of dialysis in 0.02 M phosphate buffer, pH 7.4. Similarly, inhibition of orotidylic acid decarboxylase activity is fully demonstrable following dialysis of extracts from intact cells incubated with oxipurinol. These findings suggest that the factor or factors giving rise to both stabilization and inhibition are either tightly bound to the enzyme complex, poorly dialyzable, or of relatively high molecular weight. In experiments in which extracts were chromatographed on Sephadex G-25 columns prior to studies of thermal stability or concentration dependence of both enzymes or the presence of an inhibitor of orotidylic acid decarboxylase, stabilization and inhibition were lost at least partially in all cases (Table 3). Moreover, despite variation in the magnitude of reversal of these effects, in each experiment both stabilization and inhibition were lost, and to comparable degrees. A low molecular weight compound (such as a nucleotide derivative of oxipurinol, which may be only slowly dialyzable but more rapidly removed from the enzyme complex by gel filtration) could readily account for both effects.

Gel filtration studies. Incubation of intact lymphoblasts with oxipurinol results in an alteration in the elution profile of the enzyme activities when extracts of these cells are chromatographed on Sephadex G-150 (Fig. 4). Orotidylic acid decarboxylase and orotate phosphoribosyltransferase activities are eluted together in identical fractions whether oxipurinol is present or not, but in either case the latter activity is markedly diminished following gel filtration. Activities of orotate phosphoribosyltransferase and orotidylic acid decarboxylase in extracts from oxipurinol-treated cells are eluted in a single peak corresponding to a molecular weight of approximately 108,000. These same activities from untreated lymphoblasts are eluted later in the gel profile at a point corresponding to a molecular weight of approximately 41,000. Enzyme from cells treated with oxipurinol and eluted in the earlier peak show slight but definite inhibi-

TABLE 3

Reversal by Sephadex G-25 filtration of enzyme stabilization and orotidylic acid decarboxylase inhibition resulting from incubation of human lymphoblasts with oxipurinol

Lymphoblast extracts prepared as described in MATERIALS AND METHODS were incubated in Dulbecco's phosphate-buffered NaCl with and without 0.5 mM oxipurinal and 0.5 mM PP-ribose-P.

Extracts	Sephadex G-25 filtration	Orotate phosphorlbosyltransferase (OPRT) and orotidylic acid decarboxylase (ODC) stabilization				Orotidylic acid decarboxylase inhibition[c]
		Concentration dependence[a]		Thermal stability[b]		
		OPRT	ODC	OPRT	ODC	
		% activity		% activity		%
Control	−	58	61	2	12	2
	+	50	53	4	13	2
Oxipurinol-treated	−	98	92	43	99	85
	+	76	73	27	54	16

[a] Percentage of activity demonstrable with 0.05 mg of protein in the assay relative to activity with 0.15–0.40 mg of protein.

[b] Percentage of activity demonstrable after 8 min at 57°.

[c] Decrement in activity demonstrable with 0.01 mM OMP in the assay relative to activity with 0.1 mM OMP.

tion and greater stability of orotidylic acid decarboxylase activity relative to the enzyme activity from untreated cells eluted later in the profile (Table 4).

DISCUSSION

The effects of allopurinol and its congeners on purine metabolism have constituted the major objects of interest in this family of chemical compounds. The recent awareness that allopurinol derivatives induce to a small degree (9, 10) in man a chemical state found to a greater extent in the disease hereditary oroticaciduria (30, 31) has now directed attention to the effects of these drugs on pyrimidine metabolism. Orotidinuria and, to a lesser extent, oroticaciduria are constant accompaniments of allopurinol and oxipurinol administration in man (9, 10). Evidence that the abnormal accumulation of orotidine and orotic acid in the urine of patients treated with these drugs reflects a block in pyrimidine metabolism has come from studies showing a diminished conversion of carboxyl-labeled orotic acid to $^{14}CO_2$ in expired air of patients treated with allopurinol or oxipurinol (14). In addition, in human fibroblasts, the incorporation of [6-^{14}C]orotic acid into nucleic acids is diminished by incubation with allopurinol (12). Our demonstration that oxipurinol incubation results in diminished incorporation of orotic acid, but not of uridine, into acid-insoluble material of human lymphoblasts confirms the drug-related blockade of pyrimidine synthesis in these cells and localizes the inhibitory effects of oxipurinol metabolites to the two steps involved in the conversion of orotic acid to UMP.

Evidence that inhibition of orotidylic acid decarboxylase activity constitutes the site of inhibition of pyrimidine synthesis has come from a variety of studies (9–15). First, the major excretory product following administration of allopurinol or oxipurinol is orotidine rather than orotic acid (9, 10). Second, studies of chemically synthesized oxipurinol 1-ribonucleotide and oxipurinol-7-ribonucleotide (15) indicate that these compounds are potent inhibitors of orotidylic acid decarboxylase activity. Recently high-pressure liquid chromatographic analysis of the nucleotide content of rat liver following administration of allopurinol has revealed the existence of these two nucleotides of oxipurinol (5). The results of our experiments with human lymphoblasts, as well as prior studies of human erythrocytes (13, 14, 16) and fibroblasts (12) and of rat erythrocytes and liver (11), indicate that the generation of an inhibitor of orotidylic acid decarboxylase activity is dependent on PP-

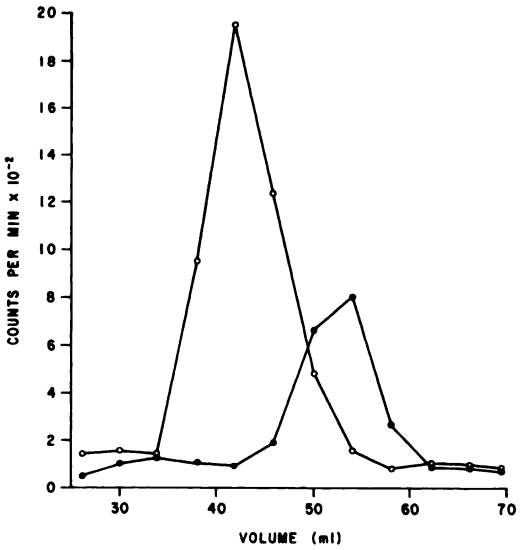

FIG. 4. *Elution profile of human orotidylic acid decarboxylase before* (●——●) *and after* (○——○) *4 hr of incubation of intact cells with 0.5 mM oxipurinol*

Cell extracts were prepared as described in MATERIALS AND METHODS, except that final cell density was 6 × 10^7/ml prior to freezing and thawing. Equal amounts of protein in dialyzed extracts were applied to matched Sephadex G-150 columns (33 cm × 2.3 cm^2) equilibrated with 0.02 M potassium phosphate buffer, pH 7.4. The enzymes were eluted at 7 ml/hr, and 2-ml fractions were collected. Enzyme activities were determined as described in the text, using 100–250 μg of protein in the assays. Void volumes of the columns matched within 0.25 ml as determined with blue dextran. Molecular weights of the earlier and later peaks of eluted orotidylic acid decarboxylase activity were estimated to be 108,000 and 41,000, respectively, by calibration of the columns with chymotrypsinogen, creatine kinase, and catalase. The activity of orotate phosphoribosyltransferase (not shown) was eluted in the same fractions as orotidylic acid decarboxylase in each case, but was markedly reduced following gel filtration.

ribose-P and oxipurinol. Apparently this inhibitor is responsible for inhibition of orotidylic acid decarboxylase following administration of allopurinol or oxipurinol both *in vivo* and *in vitro*.

The molecular basis of the oxipurinol ribonucleotide inhibition of orotidylic acid decarboxylase activity may be an alteration in the structure of the complex containing orotate phosphoribosyltransferase and orotidylic acid decarboxylase activities. Our findings in human lymphoblasts confirm those of Brown *et al.* (11) in rat liver in the demonstration of altered gel mobility of orotate phosphoribosyltransferase and orotidylic acid decarboxylase activities following incubation with oxipurinol. In addition to the altered quaternary structure resulting from exposure to oxipurinol, inhibition of orotidylic acid decarboxylase but not orotate phosphoribosyltransferase activity is demonstrable, as is stabilization of both enzyme activities. These findings suggest that one or both oxipurinol ribonucleotides alter the structure of the enzyme complex to produce an aggregate of about 3 times the molecular weight, inhibition of orotidylic acid decarboxylase activity, and stabilization of both activities to inactivation. The time course of the effects resulting from incubation of lymphoblasts with oxipurinol indicates that inhibition of orotidylic acid decarboxylase and stabilization of both enzymes are probably synchronous events, neither of which is readily reversible by dialysis but both of which are at least partially reversible by filtration on Sephadex G-25. These findings support a common molecular basis for orotidylic acid decarboxylase inhibition, the stabilization of both enzyme activities, and the hypothesized increase in degree of aggregation of the orotate phosphoribosyltransferase–orotidylic acid de-

TABLE 4

Characteristics of orotidylic acid decarboxylase activity from oxipurinol-treated and untreated lymphoblasts following Sephadex G-150 filtration

Characteristics of the Sephadex G-150 column are described in Fig. 4.

Cell source of extract	Elution volume of activity peak	Enzyme inhibition[a]	Concentration dependence[b]
	ml	%	%
Control	55	0	32
Oxipurinol-treated	44	26	71

[a] Decrement in activity demonstrable with 0.01 mM OMP in the assay relative to activity with 0.1 mM OMP.

[b] Proportion of activity demonstrable with 0.01 mg of protein in the assay relative to activity with 0.10 mg of protein.

carboxylase complex resulting from oxipurinol incubation.

Increased activities of both enzymes have been reported to follow the administration of allopurinol or oxipurinol (13, 16) to humans and rats (11). We have observed a coordinate increase in both enzyme activities, amounting to somewhat less than 200%, following the incubation of intact lymphoblasts or fibroblasts with oxipurinol (Table 1). The rapidity with which this increased enzyme activity is observed in lymphoblasts and its apparent persistence in the face of inhibition of protein synthesis by cycloheximide suggest that the increased activity may reflect greater stability of the enzyme activities demonstrated *in vitro* rather than increased new enzyme synthesis. Since the enzyme activities reach maximal values within about 3 hr and remain constant thereafter, a diminished rate of enzyme catabolism within the intact cell also seems unlikely. Stabilization of the enzyme activities to partial inactivation during extraction from the cell could explain these findings, although other explanations cannot be excluded by these data. For example, a previously inactive, high molecular weight, more stable form of the enzyme complex could be activated by oxipurinol or one of its metabolites. While this latter mechanism would require the normally active species of the enzyme complex to be inactivated simultaneously, distinction between these two possibilities awaits demonstration of interconvertibility between the two molecular weight forms of the enzyme complex as predicted by the former mechanism.

Activities of orotate phosphoribosyltransferase and orotidylic acid decarboxylase are comparable in normal lymphoblasts and in lymphoblasts severely deficient in the enzyme hypoxanthine-guanine phosphoribosyltransferase. These findings also extend to fibroblasts and peripheral blood leukocytes (28) and stand in contrast to the increased activities of these enzymes reported in erythrocytes from patients with severe hypoxanthine-guanine phosphoribosyltransferase deficiency (28). Similar increases in adenine phosphoribosyltransferase (EC 2.4.-2.7) (32) and inosinic acid dehydrogenase (EC 1.2.1.14) (33) have been reported in hypoxanthine-guanine phosphoribosyltransferase-deficient erythrocytes but not in other tissues examined for these enzymes (33, 34).

The relationship among the increased molecular weight of the orotate phosphoribosyltransferase–orotidylic acid decarboxylase complex, the stabilization of enzyme activity, and the inhibition of orotidylic acid decarboxylase activity provides an interesting insight into the pharmacological effects of allopurinol and its derivatives, which appear to alter purine and pyrimidine simultaneously, although by different mechanisms. The extent to which similar molecular mechanisms may underlie the pharmacological effects of other drugs will undoubtedly be of interest. The present studies demonstrate the suitability of human lymphoblast lines in tissue culture for extending these studies and suggest that such a system may be useful in the study of other drug effects in man.

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