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MECHANISM OF ALLOPURINOL-MEDIATED INHIBITION AND STABILIZATION OF HUMAN OROTATE PHOSPHORIBOSYLTRANSFERASE AND OROTIDINE PHOSPHATE DECARBOXYLASE

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Abstract—Allopurinol ribonucleotide and oxipurinol-7-ribonucleotide appeared to be strong inhibitors of orotidine phosphate decarboxylase in human hemolysates. The enzyme exhibited bimodal kinetics. The ribonucleotides of allopurinol and oxipurinol caused an inhibition of orotate phosphoribosyltransferase, which appeared to be due to accumulation of OMP. Inhibition by OMP was competitive with respect to phosphoribosylpyrophosphate with a K_i value of 11 μM. The inhibition of ODC and OPRT activity may cause the increased urinary excretion of orotidine and orotic acid, respectively, observed after allopurinol therapy. Values measured for OPRT activity in intact erythrocytes and in hemolysates agreed very well. Therefore OPRT activity does not decrease during cell lysis and extraction. Hypoxanthine-guanine phosphoribosyltransferase deficiency as well as allopurinol therapy led to a marked increase in OPRT and ODC activities in human hemolysates. In lysates from leukocytes only a slight increase of ODC activity was observed, while OPRT activity did not differ significantly from the controls. *In vitro* incubations of hemolysates demonstrated a considerable increase of the stability of OPRT by addition of OMP or PRPP and of ODC by addition of OMP, PRPP, UMP and the ribonucleotides of allopurinol and oxipurinol. These findings suggest that the apparent increase of OPRT and ODC activity after allopurinol therapy is due to stabilization of the enzymes during the life span of the erythrocytes.

Allopurinol inhibits the final enzyme of purine metabolism in man, xanthine oxidase (EC 1.2.3.2.) and is an effective agent for treatment of hyperuricemia [1]. Allopurinol therapy also interferes with pyrimidine metabolism as indicated by an increased excretion of orotidine and orotic acid in urine [2, 3]. Subsequent investigations have established that allopurinol and its major metabolite oxipurinol inhibit pyrimidine biosynthesis in cultured human cells by interfering with the conversion of orotic acid to UMP [4, 5]. The increased excretion of orotidine has been ascribed to inhibition of orotidine-5′-monophosphate decarboxylase (EC 4.1.1.23) by ribonucleotides of allopurinol and oxipurinol. The increase in excretion of orotic acid suggests that inhibition of orotate phosphoribosyltransferase (EC 2.4.2.10) may also occur but the exact mechanism has not yet been elucidated.

In addition it was observed that activities of OPRT and ODC in erythrocytes from patients receiving allopurinol were markedly elevated. This phenomenon has been attributed to enzyme stabilization *in vivo* [7] or enzyme activation [8]. The apparent increase in activity might also be due to stabilization of the enzymes during cell lysis and extraction rather than stabilization *in vivo* [5, 9].

In the present study we have examined the mechanisms responsible for enzyme inhibition on the one hand and apparently increased enzyme activities on the other hand. In these studies we used both normal blood cells and cells obtained from patients deficient in hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) activity. Our results suggest that allopurinol-induced inhibition of ODC activity is associated with inhibition of OPRT activity by accumulated OMP. In addition, evidence is presented that the apparent increase in OPRT and ODC activities is probably due to stabilization of the enzymes during the life span of the erythrocyte.

MATERIALS AND METHODS

Materials. Phosphoribosylpyrophosphate tetrasodium salt was purchased from Boehringer, Mannheim. Orotic acid monosodium salt and orotidine-5′-monophosphoric acid trisodium salt were obtained from Sigma Chemical Co., St. Louis, Mo. (U.S.A.). Allopurinol, oxipurinol and allopurinol ribonucleotide were gifts from Dr. G. B. Elion, Burroughs–Wellcome Co., Research Triangle Park, N.C. (U.S.A.). Oxipurinol-7-ribonucleotide was generously provided by

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† *Abbreviations used*: OPRT: orotate phosphoribosyltransferase; ODC: orotidine-5′-phosphate decarboxylase; HGPRT: hypoxanthine-guanine phosphoribosyltransferase; PRPP: phosphoribosylpyrophosphate; OMP: orotidine-5′-phosphate; PEI-cellulose: polyethyleneimine-cellulose.

Henning Chemie & Pharmawerk, Berlin, West Germany. Dextran T 500 and Sephadex G-25 (coarse) were obtained from Pharmacia, Uppsala, Sweden. [Carboxyl-^{14}C]orotic acid (42.4 mCi/m-mole), [carboxyl-^{14}C] orotidine-5′-monophosphate (36.9 mCi/m-mole) and [6-^{14}C]orotic acid (49.1 mCi/m-mole) were obtained from New England Nuclear Corp., Dreieichenhain, West Germany, as well as omnifluor and aquasol. Aluminum sheets (20 × 20 cm) precoated with 0.1 mm of polyethyleneimine-cellulose were purchased from Merck, Darmstadt, West Germany. The other chemicals were of the highest quality commercially available.

Blood samples. Venous blood samples were obtained from three gouty patients receiving allopurinol (300 mg/day) for at least 6 months, from three children with HGPRT-deficiency who have been on allopurinol medication (200 mg/day) for several years and from a gouty adult (E.F.) with partial deficiency of HGPRT (1 per cent of normal value) who also has received the drug (300 mg per day) for several years. Two children with HGPRT-deficiency exhibited all symptoms of the Lesch–Nyhan syndrome including automutilation [10] while the third one (P.K.) had no severe neurological lesions. Adult volunteers served as control group.

Preparation of cells and cell extracts. In order to separate leukocytes from erythrocytes by differential sedimentation, dextran was added to the blood samples [11]. After the erythrocytes had settled, they were washed twice with Tris-buffered saline (pH 7.4). The cells were used intact or lysed by rapidly freezing and thawing twice. Leukocytes were centrifuged at 1500 rpm and remaining erythrocytes were removed by a hypotonic shock for 60 sec after which isotonicity was restored. Leukocytes were washed with phosphate-buffered saline and lysed either by prolonged hypotonic shock or by sonication. Lysates of leukocytes were immediately assayed for enzyme activities while hemolysates could be stored at −20° for several weeks without detectable loss of OPRT and ODC activity.

Analytical procedures. The actual PRPP content of stock solutions was determined enzymatically using [8-^{14}C]adenine and adenine phosphoribosyltransferase partially purified from human erythrocytes [12]. The reaction was stopped by boiling for 2 min and AMP was separated from adenine by thin-layer chromatography on PEI-cellulose [13]. Enzyme activities were estimated with radiochemical methods using ^{14}C-labeled compounds. Determination of radioactivity was performed in a Packard 3380 liquid-scintillation spectrophotometer with external standardization. Protein was determined according to Lowry *et al* [14]. Specific enzyme activities are expressed in nmoles of product formed in 1 hr per mg of protein at 37° under the assay conditions specified below. All enzyme assays were checked on linearity with respect to enzyme concentration and time.

Assay of orotate phosphoribosyltransferase. Assay I. Incubation mixtures contained 0.05 M Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 0.7 mM PRPP, 0.3 mM [carboxyl-^{14}C]orotic acid (0.15 mCi/m-mole) and enzyme protein in a total volume of 0.6 ml. Reactions were carried out in scintillation vials sealed with rubber caps. The $^{14}CO_2$ evolved was trapped in 0.2 ml of a mixture of ethyleneglycol ethanolamine (2 : 1, v/v) present in a small polypropylene tube fitted in a slightly larger tube. After incubation for 10–60 min with shaking, OPRT activity was stopped by injection of 0.2 ml 0.25 M neutralized EDTA. Because ODC does not require Mg^{2+}-ions, decarboxylation can be allowed to proceed for another hour. Injection of 0.2 ml 5 M perchloric acid removed all $^{14}CO_2$ from the reaction mixture within 1 hr. The small tube containing trapped $^{14}CO_2$ was transferred to a scintillation vial with 10 ml of toluene methanol (2 : 1, v/v) containing 4 g omnifluor per litre. Blanks contained no enzyme protein.

This assay was also employed when OPRT activity was determined in intact erythrocytes. A 25% suspension of erythrocytes in phosphate buffered saline containing 0.1% glucose and 0.1% $MgCl_2$ was preincubated for 1 hr resulting in intracellular PRPP synthesis. [Carboxyl-^{14}C]orotic acid was added to 500 μl of this suspension to reach a final concentration of 0.3 mM (sp. act. 0.15 mCi/m-mole). During a 30-min incubation $^{14}CO_2$ production was trapped as described above. Reaction was terminated by injection of perchloric acid. Preliminary experiments had shown that intracellular orotic acid concentration almost instantaneously equals the medium concentration.

Assay II. In this assay unreacted orotate is separated from the products which are synthesized from orotate by OPRT and any consecutive enzyme which may be present [15]. The reaction mixture contained 40 mM Tris HCl (pH 7.4), 0.1 mM [6 ^{14}C]orotic acid (49.1 mCi/m-mole), 5 mM $MgCl_2$, 1.1 mM PRPP and enzyme protein in a final volume of 50 μl. The mixture was incubated for 30 min in small polypropylene tubes. Reaction was terminated by immersing the tubes in boiling water for 2 min. Carrier orotic acid and uridine were added and precipitated protein removed by centrifugation for 10 min at 4500 g in a Misco centrifuge (Microchemical Specialities Co., Berkeley, California, U.S.A.). Ten-μl samples of the supernatant were spotted on PEI-cellulose thin-layer plates. Development was accomplished with 0.2 M LiCl (saturated with boric acid and adjusted to pH 4.5)–ethanol (1 : 1, v/v). Chromatograms were dried at room temperature. The spots were visualized under u.v. light and cut out. In order to circumvent any self absorption of radioactivity, compounds were eluted prior to counting by shaking with 1.0 ml of 0.1 M HCl–0.2 M KCl for 40 min in scintillation vials. Ten ml of aquasol was added and radioactivity measured. Blanks were obtained by immersing the tubes in boiling water for 2 min prior to incubation. The conversion of substrate into products was calculated by comparing net radioactivity in products derived from orotate with the total amount of radioactivity present. A higher PRPP concentration than in assay I was necessary since the higher protein concentration employed seemed to be associated with an increase of enzymatic breakdown of PRPP.

Assay of orotidine-5′-phosphate decarboxylase. Cell lysates, appropiately diluted with 50 mM Tris HCl buffer (pH 7.4) were incubated with 0.1 mM [carboxyl-^{14}C]orotidine-5′-monophosphate (0.2 mCi/m-mole) for 10–60 min in a final volume of 0.55 ml. Reaction was terminated by injection of 0.2 ml 5 M perchloric

acid. Production of $^{14}CO_2$ was measured as described for OPRT, assay I.

Stabilization studies. Hemolyzed red cells were diluted with 9 vol of Tris-HCl buffer (50 mM, pH 7.4) and incubated for 16 hr at 37°. To avoid bacterial contamination penicillin-G (100 U/ml) and streptomycin (100 μg/ml) were present in the incubation vessels. When it was necessary to remove inhibitors of OPRT or ODC before enzyme assay lysates were passed through Sephadex G-25 columns after incubation. 10 mM Tris-HCl (pH 7.4) was used for suspending the Sephadex powder as well as for eluting the protein from the column. The enzymes were eluted in the void volume together with hemoglobin.

RESULTS

Activities of orotate phosphoribosyltransferase and orotidine-5'-phosphate decarboxylase in circulating blood cells. OPRT and ODC activities were elevated in hemolysates from the allopurinol-treated group when compared with the control group (Table 1). This increase in enzyme activity was seen with both the HGPRT-deficient patients and the gouty patients. Enzyme activities were also measured in HGPRT-deficient subject P. K. before allopurinol therapy was started. Values averaged 1.0 nmole/hr per mg protein for OPRT, and 1.7 nmole/hr per mg protein for ODC activity.

In lysed leukocytes OPRT activity of the allopurinol-treated patients was not higher than in controls. ODC activity was slightly higher than in 6 control subjects.

OPRT activity was also measured in intact erythrocytes after preincubation in a PRPP-generating system. For control subjects, OPRT activities in intact erythrocytes and in lysed erythrocytes agreed very well (Table 2). However, OPRT assay in intact erythrocytes from allopurinol-treated patients resulted always in lower values when compared with corresponding hemolysates.

Effect of allopurinol and its metabolites on activity of orotidine-5'-phosphate decarboxylase. Allopurinol and oxipurinol were found to have no effect on ODC activity in hemolysates. However when a hemolysate was preincubated with allopurinol or oxipurinol (1 mM) in the presence of 1 mM PRPP compounds were synthesized which strongly inhibited ODC activity. Longer periods of preincubation resulted in stronger inhibition of ODC. The inhibitors formed during the preincubation period were shown to be competitive with respect to orotidine-5'-monophosphate (Fig. 1). The inhibitory agents are presumably allopurinol ribonucleotide and oxipurinol-7-ribonucleotide. These compounds appeared to be competitive inhibitors of ODC (Fig. 2). Oxipurinol-7-ribonucleotide (K_i value 0.06 μM) was a much stronger inhibitor of ODC than the former compound (K_i value 5 μM).

The synthesis of allopurinol-1-ribonucleotide is catalyzed by HGPRT, since no inhibition of ODC was observed when allopurinol and PRPP were incubated with HGPRT-deficient lysate. HGPRT activity did not appear necessary for an inhibitory oxipurinol ribonucleotide to be formed (Table 3). Xanthosine monophosphate was reported to be a competitive inhibitor of ODC activity [3, 6]. It was also inhibitory in our assay system. However, preincubation of xanthine and PRPP with hemolysate did not result in a detectable inhibition of the ODC activity.

We found bimodal kinetics for the ODC activity in hemolysates. At (unphysiologically) high concentrations of substrate the ODC enzyme shows higher apparent values for both K_m and V_{max} (Fig. 3). At

Table 2. Orotate phosphoribosyltransferase activity in intact and lysed human erythrocytes

Subject		Intact cells	Hemolysate
Controls			
	G.H.	0.22*	0.19
	W.T.	0.22	0.22
	W.G.	0.14	0.12
Allopurinol-treated			
	H.v.S.	0.59	1.09
	P.K.	0.47	1.23
	Re.W.	0.81	1.49
	Ru. W.	0.84	1.87

* Enzyme activity was measured by assay I and is given in nmoles/hr per mg protein.

Table 1. Orotate phosphoribosyltransferase and orotidine monophosphate decarboxylase activities in lysates of human leukocytes and erythrocytes

Subject	Leukocytes OPRT	Leukocytes ODC	Erythrocytes OPRT	Erythrocytes ODC
Control group (n=6)	1.66 ± 0.53*	5.09 ± 1.32	0.19 ± 0.05	0.34 ± 0.10
Allopurinol treated				
HGPRT-deficient				
E.F.	0.88	6.70	0.72	1.14
P.K.	3.28	8.39	1.23	2.42
Re.W.	1.00	7.76	1.49	2.94
Ru.W.	1.39	7.04	1.87	2.76
Gouty				
E.v.d.W.	2.06	6.55	0.51	0.70
H.v.S.	1.26	5.34	0.54	1.60
H.V.	1.08	6.30	0.31	0.57

* Enzyme activity ± S.D. in nmoles/hr per mg protein.

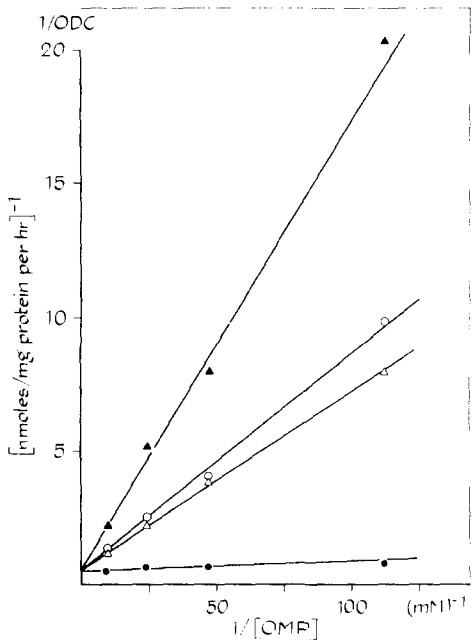

Fig. 1. Inhibition of ODC in hemolysate after incubation with allopurinol or oxipurinol in the presence of 1 mM PRPP. Control (●), allopurinol during 30 min (○), oxipurinol during 30 min (△), oxipurinol during 60 min (▲).

OMP concentrations lower than about 30 μM a K_m value of 1.7 $\pm$ 0.4 μM (mean $\pm$ S.D., 4 determinations) was found. Values for high K_m averaged 33 μM. Similar kinetics were observed when the enzyme concentration was 4-fold higher. This finding of bimodal kinetics does not affect the K_i values reported above for oxipurinol-7-ribonucleotide and allopurinol ribonucleotide. These values were determined at the low concentration range of OMP.

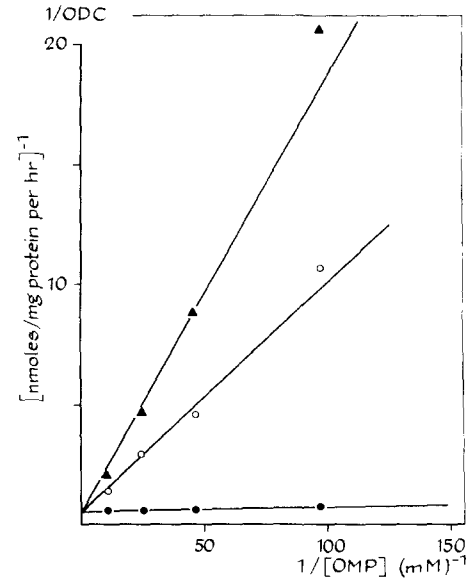

Fig. 2. Inhibition of ODC in hemolysate by ribonucleotides of allopurinol and oxipurinol. Control (●), 1.1×10^{-5} M oxipurinol-7-ribonucleotide (▲), 5×10^{-4} M allopurinol-ribonucleotide (○).

Table 3. Relative orotidine monophosphate decarboxylase activity in hemolysates after preincubation with allopurinol and oxipurinol

Preincubation conditions	Normal	HGPRT-deficient
PRPP	100*	100†
Allopurinol + PRPP	42	100
Oxipurinol + PRPP	42	12

* 100% value was 0.7 nmoles/hr per mg protein.
† 100% value was 2.8 nmoles/hr per mg protein.
Time of preincubation was 60 min, compounds were added in a concentration of 1 mM. OMP concentration during assay was 0.1 mM.

Effect of allopurinol and its metabolites on activity of orotate phosphoribosyltransferase. Unless otherwise indicated all results described in this section have been obtained using assay II for OPRT assay. Allopurinol has no inhibitory effect on OPRT activity at saturating concentrations of orotic acid and PRPP. Even at concentrations of PRPP which are suboptimal for OPRT we found no inhibition of OPRT activity by allopurinol. Under these conditions hypoxanthine strongly inhibits OPRT activity (Table 4). This inhibition is probably due to consumption of PRPP in a HGPRT-catalyzed reaction since no inhibition was found with HGPRT-deficient lysate. Inhibition is nearly absent when PRPP concentration is high.

A small but significant inhibition of OPRT activity was found when allopurinol-1-ribonucleotide or oxipurinol-7-ribonucleotide was present in the assay mixture. Inhibition was stronger at lower PRPP concentrations and also increased with longer incubation periods. This type of inhibition could be explained by assuming that the presence of ribonucleotides of allopurinol and oxipurinol causes an accumulation of an inhibitor. Because ODC activity is inhibited by ribonucleotides of allopurinol and oxipurinol (see above) OMP might accumulate during their presence. This compound was therefore studied with regard to possible inhibition of OPRT activity.

When studying the effect of OMP on OPRT, care must be taken to prevent utilization of this compound. Some dephosphorylation of OMP was found to occur in hemolysates but this reaction was of

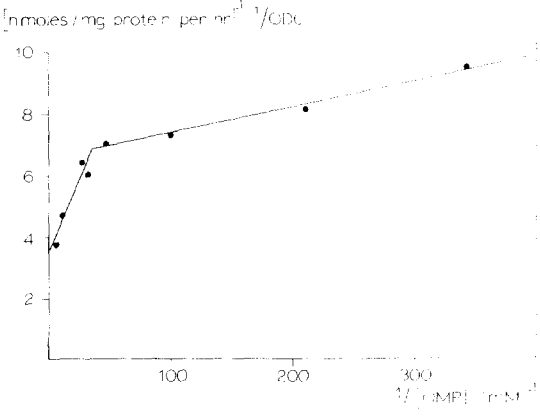

Fig. 3. Biphasic kinetics of ODC in human hemolysate.

Table 4. Relative orotate phosphoribosyltransferase activity in hemolysates in the presence of allopurinol and hypoxanthine

Addition \ PRPP (mM)	Normal 0.5	Normal 2.0	HGPRT-deficient 0.5	HGPRT-deficient 2.0
Hypoxanthine (1 mM)	39	82	103	104
Allopurinol (1 mM)	107	94	112	102

Control values (set at 100%) were 0.19, 0.20, 1.1 and 1.2 nmoles/hr per mg protein, respectively. Results are the mean of two experiments.

minor importance at the low concentrations of OMP used in these studies. Decarboxylation of OMP is much more of a problem since ODC activity in hemolysates is twice as high as OPRT activity. In order to prevent a decrease in the OMP concentration during the OPRT assay due to ODC activity, allopurinol ribonucleotide (1 mM) was added to all reaction mixtures (including controls) when studying the effect of OMP. Under these conditions OMP appeared to be a potent inhibitor of OPRT activity. Inhibition is competitive with respect to PRPP (Fig. 4). When the K_i values is calculated from the Lineweaver–Burk plots, a value of 13 μM is obtained. However this value depends on the PRPP concentration. Because of the known instability of PRPP [16] the values of the PRPP concentrations may be to some extent inaccurate, which affects the calculated value of K_i. Therefore the K_i value for OMP was also determined according to the Dixon method [17], using various concentrations of inhibitor and two different concentrations of PRPP. This method yielded a K_i value of 11 μM (Fig. 5).

Several nucleotides appeared to be inhibitors of OPRT (Table 5). Assay I for OPRT was used in these experiments. Prior to testing the effect of these nucleotides on OPRT activity, their non-interference with the ODC reaction was ascertained. Inhibition was competitive with respect to PRPP for all inhibitors tested, except TTP. This compound strongly inhibits OPRT activity even at a high concentration of PRPP.

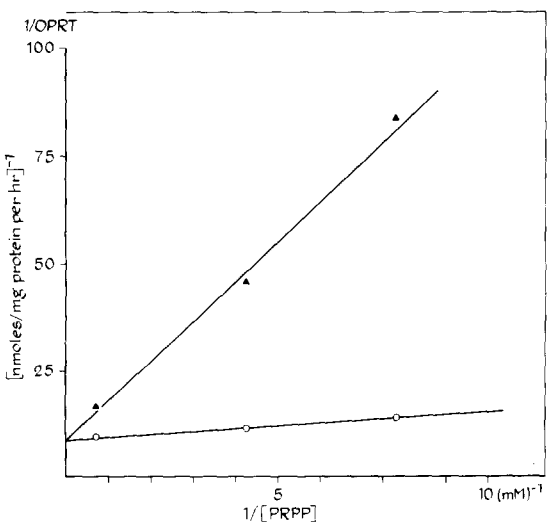

Fig. 4. Inhibition of OPRT in hemolysate by OMP. Control (○), 2×10^{-4} M OMP (▲).

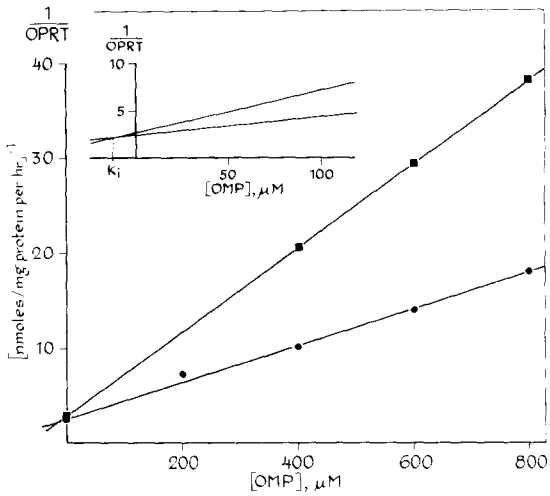

Fig. 5. Determination of K_i value of OMP for OPRT from hemolysate according to Dixon [17]. OPRT activity was measured with assay II (see Methods). Allopurinol ribonucleotide (1 mM) was present to prevent decarboxylation of OMP. Concentration of PRPP was 1 mM (●) or 0.5 mM (■).

Stabilization studies. When hemolysates were diluted with Tris–HCl buffer (pH 7.4) and incubated for 16 hr at 37°, both OPRT and ODC activity decreased strongly. The extent of this decrease was very different for various hemolysates (Table 6). This great variability is reflected in the variability of the values found after incubation in the presence of stabilizing compounds, since these values were calculated by comparison with their respective control values. The presence of 0.1 mM allopurinol ribonucleotide or oxipurinol-7-ribonucleotide appeared to stabilize ODC activity, but no effect on OPRT activity was observed. Allopurinol ribonucleotide had similar effects at 1 mM, but no stabilization could be measured with 1 mM oxipurinol-7-ribonucleotide. As a matter of fact, OPRT and ODC activities were under these conditions even lower than in controls without any addition. Stabilization of ODC occurred also when UMP

Table 5. Effect of nucleotides on orotate phosphoribosyltransferase activity in hemolysates

Addition	Relative OPRT activity
None	100
TTP	14 ± 1
AMP	48 ± 2
GMP	54 ± 1
ATP	62 ± 1
IMP	71 ± 2
TMP	75 ± 2
ADP	76 ± 1
CMP	89 ± 4
cAMP	100 ± 5

Mean and deviation from the mean of two experiments are given as percent of the control. Concentration of nucleotides was 5 mM, and concentration of PRPP was 0.2 mM.

Table 6. Relative activity of OPRT and ODC in hemolysates after incubation for 16 hr at 37° in the presence of nucleotides*

Addition	No. expts.	OPRT	ODC
None		100	100
UMP (1 mM)	2	115 (112–118)	236 (219–253)
OMP (0.1 mM)	2	119 (113–124)	204 (181–226)
OMP (1 mM)	6	182 (157–259)	508 (208–1033)
Oxipurinol ribonucleotide (0.1 mM)	3	92 (80–100)	258 (179–348)
Allopurinol ribonucleotide (0.1 mM)	3	94 (87–100)	305 (187–528)
Allopurinol ribonucleotide (1 mM)	3	91 (72–107)	318 (273–404)

* Enzyme activities after incubation without addition were set at 100%. Values between brackets indicate the range observed with different hemolysates. Before incubation relative OPRT activity was 1233 (672–2667, n=10) and relative ODC activity was 623 (396–1255, n=10).

was present at a concentration of 1 mM, but was not detectable at 0.1 mM. Again, no stabilizing effect on OPRT was observed.

OPRT activity, as well as ODC activity was stabilized by OMP at 1 mM. Stability of both enzymes was not significantly influenced by any of the following compounds at 1 mM concentration: orotic acid, allopurinol, oxipurinol, TMP or TTP. OPRT and ODC activities in hemolysates incubated for 5 hr in Tris–HCl buffer were only 6 and 30 per cent, respectively (mean of 2 experiments) of the enzyme activities before incubation. When the incubation was performed in phosphate buffer (50 mM, pH 7.4) instead of Tris–HCl buffer, both OPRT and ODC activity appeared much more stable. Activities were 95 and 81 per cent of the starting value, respectively (mean of 2 experiments). Complete stabilization of OPRT and ODC activities (110 and 96 per cent respectively of the starting value, 2 experiments) was observed in Tris–HCl buffer during the 5-hr period when at 30 min intervals PRPP was added to a final concentration of 0.4 mM.

DISCUSSION

The effects of allopurinol on OPRT and ODC activities include enzyme inhibition on one hand leading to increased excretion of orotic acid and orotidine [2, 3] and an apparent increase of enzyme activities in erythrocytes on the other hand [7, 8] (Table 1). The observed increase in orotidine excretion was ascribed to inhibition of the ODC enzyme [3, 7]. Our experiments sustain this explanation. Allopurinol ribonucleotide and xanthosine-5′-monophosphate are potent inhibitors of ODC activity [3, 4]. We found no inhibition of ODC after preincubation of hemolysates with xanthine and PRPP, despite the fact that XMP inhibited ODC activity. However, xanthine is a much poorer substrate for HGPRT than is allopurinol [18]. Allopurinol ribonucleotide is readily formed from allopurinol and PRPP, and strongly inhibits ODC activity (Fig. 1, 2). Since HGPRT activity is necessary for the formation of this inhibitor (Table 3) allopurinol-mediated inhibition of ODC activity cannot be ascribed to allopurinol ribonucleotide in HGPRT-deficient patients. Several studies [5–7] have stressed the importance of oxipurinol metabolites as inhibitors of ODC. Oxipurinol is converted to an inhibitor of ODC activity by HGPRT-deficient hemolysate in the presence of PRPP (Table 3). Oxipurinol-7-ribonucleotide may be responsible for inhibition of ODC in HGPRT-deficient patients treated with allopurinol [6]. Oxipurinol ribonucleotides may be more important *in vivo* with regard to ODC inhibition than is allopurinol ribonucleotide. This is suggested by comparison of their concentrations in rat liver after allopurinol administration [19] and of their K_i values. The K_i value of oxipurinol-7-ribonucleotide for the ODC enzyme of human hemolysate (0.06 μM) is similar to the values reported for yeast and rat liver [6]. For allopurinol ribonucleotide the observed K_i value was slightly higher than the values for the enzyme from erythrocytes [3], yeast and rat liver [6].

Several nucleotides inhibit OPRT activity (Table 5) but have no effect on ODC activity of human hemolysates. CMP, GMP and AMP were reported to inhibit cow brain ODC activity [20] while GMP, AMP and IMP did not affect ODC activity in human hemolysates [3]. K_i values for GMP, AMP and IMP were all determined to be greater than 100 μM with the enzyme from yeast [6]. UMP inhibition of rat liver ODC has been reported [21] but this was not confirmed by other investigators [22].

The ODC enzyme from rat liver [6], yeast [6] and human fibroblasts [23] exhibits bimodal kinetics, as in human hemolysate (Fig. 3). The K_m value at low OMP concentrations (1.7 μM) is close to the values reported for erythrocytes [3], rat liver [6], fibroblasts [23] and yeast [6]. The K_m value at high concentrations of OMP (33 μM) was much higher than in rat liver [6] and yeast [6]. Very recently a triphasic Lineweaver–Burk plot was found with partially purified ODC from human erythrocytes [24].

Competitive inhibition of OPRT by allopurinol has been suggested [2] as an explanation for the observed increase in excretion of orotic acid during allopurinol therapy, but our results do not support this hypothesis. Since OMP might accumulate during allopurinol therapy by inhibition of ODC activity, the competitive inhibition of OPRT activity by OMP may be a probable cause for the excretion of orotic acid. Inhibition by OMP of OPRT activity in bakers' yeast was previously reported [25, 26] but the mechanism of inhibition was not clarified. Recently a similar competitive inhibition was also demonstrated in rat liver [27]. Because tissue concentrations of PRPP are

probably much below the K_m value of PRPP for the OPRT enzyme [28, 29], competitive inhibition of OPRT by OMP may be of physiological significance.

Several other nucleotides inhibit OPRT activity in hemolysates (Table 5) and in other systems. UMP inhibition of OPRT was found with rat liver [27] but not with the enzyme from rat hepatoma cells [30] and baker's yeast [26]. The enzyme from yeast was inhibited by CMP and GMP, but not by adenine nucleotides or uridine nucleotides [26]. IMP, XMP and GMP inhibited only slightly the activity of a pyrimidine phosphoribosyltransferase from murine leukemia cells while CMP, TMP and AMP were ineffective [31].

OPRT activity may also be inhibited by depletion of PRPP. A decrease of PRPP concentration has been reported in human red cells following a single dose of allopurinol [32]. No such decrease was found in human fibroblasts [4] when allopurinol concentration was 0.1 mM which is much higher than the plasma levels in man [33]. Allopurinol had no detectable effect under conditions at which hypoxanthine caused a marked inhibition of OPRT by PRPP depletion (Table 4). This is consistent with the high K_m value of allopurinol (1 mM) for HGPRT when compared with the natural substrate hypoxanthine (2.4 μM) [18]. Since allopurinol has also a short half-life [34] depletion of PRPP does not appear to be the mechanism responsible for allopurinol-induced orotic aciduria.

The apparent increase of erythrocyte OPRT and ODC activities after allopurinol therapy (Table 1) has been attributed to stabilization *in vivo* [7], to enzyme activation [8], and most recently to stabilization of these enzymes during cell lysis and extraction [5, 9]. Our results do not support this last suggestion. Measurement of OPRT activity in intact and lysed erythrocytes of controls reveals that these activities agree very well (Table 2). In intact erythrocytes from allopurinol-treated patients the production of $^{14}CO_2$ from [carboxyl-^{14}C]orotic acid is consistently lower than in lysates. This can be attributed to inhibition of ODC activity by ribonucleotides of allopurinol and/or oxipurinol. Assay II was not appropriate for measuring OPRT activity in intact erythrocytes, because of technical complications. Besides, this assay is also sensitive to indirect inhibition by OMP due to the presence of inhibitors of ODC.

Decrease of ODC activity during incubation at 37° is partially prevented by OMP and also by allopurinol ribonucleotide, UMP and oxipurinol-7-ribonucleotide (Table 6). Because these latter compounds compete with OMP at the ODC enzyme, the stabilizing effect might be called pseudosubstrate stabilization. A similar *in vitro* stabilization of ODC has been found with the enzyme purified from yeast [6]. An increased thermal stability of ODC activity was present in lymphoblasts after incubation with oxipurinol [5] and in rat liver after administration of allopurinol [35]. An increased heat stability of ODC from cow brain was found in the presence of competitive inhibitors of the enzyme [20]. The occurrence of biphasic kinetics for the ODC enzyme from hemolysate (Fig. 3) might be associated with a change in quaternary structure brought about by OMP [31]. All findings suggest that both OMP and inhibitors of ODC which are competitive with OMP may change the quaternary structure of the ODC enzyme and thereby affect the stability and activity of the enzyme. The inhibition of the enzyme activities observed after incubation with 1 mM oxipurinol ribonucleotide can be attributed to the tight binding of this compound to the enzyme protein during the gel filtration. The same phenomenon was found with lymphoblast extracts [5].

Stabilization of OPRT was observed in the presence of OMP which appeared to be a competitive inhibitor of OPRT with respect to PRPP. TTP, an inhibitor of OPRT which is not competitive with regard to PRPP, has no stabilizing effect. PRPP itself gives complete protection of both OPRT and ODC activities when loss of this instable compound is compensated for by addition of fresh PRPP at regular intervals. The stabilizing effect of phosphate on OPRT and ODC activities is probably mediated by PRPP since phosphate is an activator of PRPP synthesis in hemolysates [36]. Stabilization by PRPP could account for the apparent increase of both OPRT and ODC activities measured in the HGPRT-deficient subject P. K. before allopurinol therapy was started. Erythrocyte PRPP concentrations are elevated in HGPRT-deficient patients [37]. A similar increase of OPRT and ODC activities was reported previously [38] in HGPRT-deficient patients without allopurinol therapy. It was suggested from thermal inactivation studies that the increase was not due to stabilization by PRPP. Because of the instability of PRPP these studies do not seem very suitable to investigate a possible stabilization by PRPP.

The ODC activity in leukocytes (Table 1) is slightly higher than that reported earlier [8]. Values for OPRT activity in human leukocytes have not been reported previously. The absence of a striking increase of OPRT and ODC activities in leukocytes after allopurinol administration is consistent with the theory of enzyme stabilization because of the short life-span of these cells.

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REFERENCES

1. R. W. Rundles, J. B. Wyngaarden, G. H. Hitchings and G. B. Elion, *A. Rev. Pharmac.* **9**, 345 (1969).
2. R. M. Fox, D. Royse-Smith and W. J. O'Sullivan, *Science, N.Y.* **168**, 861 (1970).
3. W. N. Kelley and T. D. Beardmore, *Science, N.Y.* **169**, 388 (1970).
4. W. N. Kelley, T. D. Beardmore, I. H. Fox and J. C. Meade, *Biochem. Pharmac.* **20**, 1471 (1971).
5. M. A. Becker, K. F. Argubright, R. M. Fox and J. E. Seegmiller, *Molec. Pharmac.* **10**, 657 (1974).
6. J. A. Fyfe, R. L. Miller and T. A. Krenitsky, *J. biol. Chem.* **248**, 3801 (1973).
7. R. M. Fox, M. H. Wood en W. J. O'Sullivan, *J. clin. Invest.* **50**, 1050 (1971).
8. T. D. Beardmore, J. S. Cashman and W. N. Kelley, *J. clin. Invest.* **51**, 1823 (1972).
9. W. Grobner and W. N. Kelley, *Biochem. Pharmac.* **24**, 379 (1975).

10. S. P. M. v.d. Zee, E. J. P. Lommen, J. M. F. Trijbels and E. D. A. M. Schretlen, *Acta paediat. scand.* **59,** 259 (1970).
11. J. P. Blass, J. Avigan and B. W. Uhlendorf, *J. clin. Invest.* **49,** 423 (1970).
12. J. F. Henderson and M. K. Y. Khoo, *J. biol. Chem.* **240,** 2349 (1965).
13. P. Reyes, *Analyt. Biochem.* **50,** 35 (1972).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
15. P. Reyes and M. Guganig, *Analyt. Biochem.* **46,** 276 (1972).
16. J. G. Flaks, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan) Vol. VI, p. 473. Academic Press, New York (1963).
17. M. Dixon, *Biochem. J.* **55,** 170 (1953).
18. T. A. Krenitsky, R. Papaioannou and G. B. Elion, *J. biol. Chem.* **244,** 1263 (1969).
19. D. J. Nelson, C. J. L. Buggé, H. C. Krasny and G. B. Elion, *Biochem. Pharmac.* **22,** 2003 (1973).
20. S. H. Appel, *J. biol. Chem.* **243,** 3924 (1968).
21. W. A. Creasey and R. E. Handschumacher, *J. biol. Chem.* **236,** 2058 (1961).
22. J. Pausch, D. Keppler and K. Decker, *Biochim. biophys. Acta* **258,** 395 (1972).
23. T. E. Worthy, W. Grobner and W. N. Kelley, *Proc. natn. Acad. Sci. USA* **71,** 3031 (1974).
24. G. K. Brown, R. M. Fox and W. J. O'Sullivan, *J. biol. Chem.* **250,** 7352 (1975).
25. I. Lieberman, A. Kornberg and E. S. Simms, *J. biol. Chem.* **215,** 403 (1955).
26. K. Umezu, T. Amaya, A. Yoshimoto, *J. Biochem.* **70,** 249 (1971).
27. A. Čihak, *Collect. Czechoslov. chem. Commun.* **39,** 3782 (1974).
28. A. J. Clifford, J. A. Riumallo, B. S. Baliga, H. N. Munro and P. R. Brown, *Biochim. biophys. Acta* **277,** 443 (1972).
29. I. H. Fox and W. N. Kelley, *Ann. intern. Med.* **74,** 424 (1971).
30. N. J. Hoogenraad and D. C. Lee, *J. biol. Chem.* **249,** 2763 (1974).
31. P. Reyes and M. E. Guganig, *J. biol. Chem.* **250,** 5097 (1975).
32. I. H. Fox, J. B. Wyngaarden en W. N. Kelley, *New Engl. J. Med.* **283,** 1177 (1970).
33. G. B. Elion and D. J. Nelson in *Purine metabolism in man* (Eds O. Sperling, A. de Vries and J. B. Wyngaarden) p. 639. Plenum Publishing Co., New York (1974).
34. G. B. Elion, A. Kovensky, G. H. Hitchings, E. Metz and R. W. Rundles, *Biochem. Pharmac.* **15,** 863 (1966).
35. G. K. Brown, R. M. Fox and W. J. O'Sullivan, *Biochem. Pharmac.* **21,** 2469 (1972).
36. A. Hershko, A. Razin and J. Mager, *Biochim. biophys. Acta* **184,** 64 (1969).
37. M. L. Greene and J. E. Seegmiller, *Arthritis Rheum.* **12,** 666 (1969).
38. T. D. Beardmore and W. N. Kelley, *J. lab. clin. Med.* **78,** 696 (1971).