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Research Article

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ABSTRACT A coordinate relationship between the activities of two sequential enzymes in the de novo pyrimidine biosynthetic pathway has been demonstrated in human red cells. The two enzymes, orotidylate phosphoribosyltransferase and decarboxylase are responsible for the conversion of orotic acid to uridine-5′-monophosphate. Fractionation of red cells, on the basis of increase of specific gravity with cell age, has revealed that these two enzymes have a marked but equal degree of lability in the ageing red cell. It is postulated that orotidylate phosphoribosyltransferase and decarboxylase form an enzyme-enzyme complex, and that the sequential deficiency of these two enzymes in hereditary orotic aciduria may reflect a structural abnormality in this complex.

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INTRODUCTION

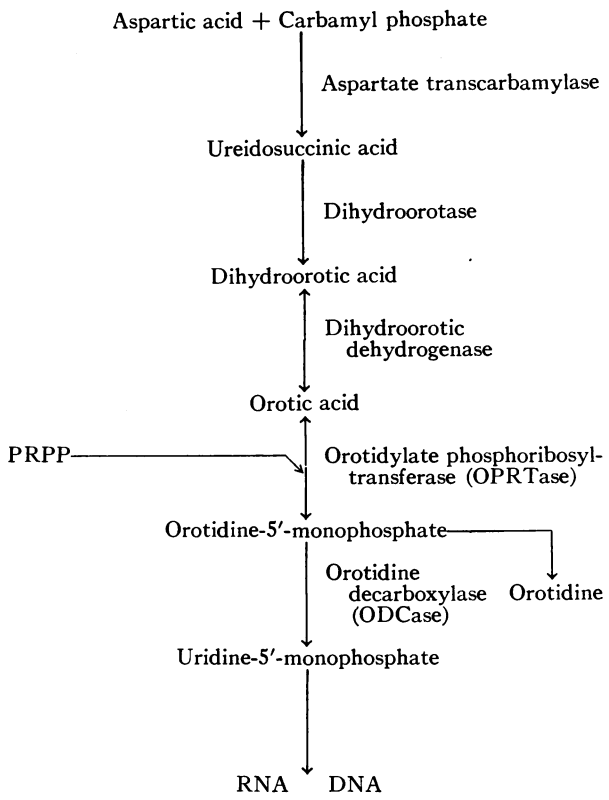
Allopurinol, an inhibitor of xanthine oxidase and *de novo* purine biosynthesis, is now widely used in the control of hyperuricemia (1). Recently, allopurinol has been found to produce an apparent interference with de novo synthesis of the pyrimidine nucleotide, uridine-5′-monophosphate (UMP),[1] resulting in the increased excretion of orotidine and, to a lesser extent, orotic acid (2, 3) (Scheme 1). It was also observed that activity of orotidylate phosphoribosyltransferase (OPRTase) in erythrocytes from patients receiving allopurinol was markedly elevated (2). Possible explanations for the orotidinuria observed after allopurinol administration have come from the in vitro findings that allopurinol ribonucleotide (2, 3) and xanthosine-5′-monophosphate (XMP) (3) are competitive inhibitors of orotidine-5′-monophosphate decarboxylase (ODCase) (Scheme 1).

The phenomenon of allopurinol-induced orotidinuria has a qualitative analogy with an inborn error of UMP synthesis, hereditary orotic aciduria. Although quite

[1] *Abbreviations used in this paper:* ODCase, orotidine-5′-monophosphate decarboxylase; OMP, orotidine-5′-monophosphate; OPRTase, orotidylate phosphoribosyltransferase; POPOP, 1,4-bis(2-(5-phenyloxazolyl)benzene); PPO, 2,5-diphenyloxazole; PRPP, phosphoribosylpyrophosphate; UMP, uridine-5′-monophosphate; XMP, xanthosine-5′-monophosphate.

Scheme 1

De Novo UMP Synthesis



rare, this condition is well documented and patients, usually in infancy, excrete excessive amounts of orotic acid in their urine, fail to thrive, and have a megaloblastic anemia. The anemia responds to the oral administration of uridine, which enables the defect in de novo UMP synthesis to be bypassed. Enzymatically, the defect has been characterized as a sequential deficiency of OPRTase and ODCase (type I) (4), or of ODCase alone (type II) (5).[2] The levels of activity of OPRTase and ODCase in fibroblasts, homozygous for type I orotic aciduria, are less than 1% of normal. However, near normal levels of activity for both of these enzymes develop if the medium in which the cells are cultured, contains certain inhibitors of UMP synthesis. This phenomenon was independent of the presence or absence of the product of the pathway (6, 7). Thus the fundamental defect responsible for the enzyme deficiencies in hereditary orotic aciduria poses a fascinating genetic problem in terms of modern molecular biology.

These findings indicated that a study of the effects of allopurinol on pyrimidine biosynthesis in man could allow further insight into the mechanisms responsible for the enzyme deficiencies in hereditary orotic aciduria. In this paper, we have reported studies on the relationship between the enzymes, OPRTase and ODCase in red cells from both normal individuals and patients receiving allopurinol; we have also studied the in vivo stability of these enzymes in red cells from these two groups. In an endeavor to assess the mechanism of the allopurinol-induced inhibition of UMP synthesis, we investigated the effects of allopurinol administration on patients with hypoxanthine–guanine phosphoribosyltransferase deficiency (the Lesch-Nyhan syndrome) (8).

METHODS

Materials

Orotic carboxyl-^{14}C acid hydrate (3.25 mCi/mmole), orotidine carboxyl-^{14}C 5′-monophosphate (18.4 mCi/mmole), gift of Dr. R. Krooth, Department of Human Genetics, University of Michigan, and adenine-8-^{14}C (6.5 mCi/mmole) were obtained from New England Nuclear Corp., Boston, Mass. Hypoxanthine-8-^{14}C (60.1 mCi/mmole) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. Allopurinol-6-^{14}C (0.272 mCi/mmole) was a gift of Dr. Gertrude Elion of Burroughs Wellcome Research Laboratories, Tuckahoe, N. Y. Allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine) and oxipurinol (4,6-dihydroxypyrazolo[3,4-d]pyrimidine) were provided by Burroughs Wellcome & Co. Inc., Raleigh, N. C.

Orotic acid, hypoxanthine, adenine, and phosphoribosylpyrophosphate (PRPP), ATP, NADP, and glucose-6-phosphate were obtained from (Sigma Chemical Co., St. Louis, Mo.). β-D-ribose-1-phosphate was obtained from Calbiochem, Los Angeles, Calif. Orotidine-5′-monophosphate (OMP) was prepared by the method of Handschumacher (9) (now commercially available from Calbiochem). Partially purified yeast orotidine-5′-monophosphate decarboxylase was prepared by the method of Heppel and Hilmoe (10). Phthallate esters (di-*n*-butyl phthallate and dimethyl phthallate) were obtained from Hopkins and Williams Ltd., Shadwell Heath, Essex, England.

Assay of orotidylate phosphoribosyltransferase (OPRTase) and orotidine-5′-monophosphate decarboxylase (ODCase)

Both enzymes were assayed by the method of Smith, Sullivan, and Huguley (11) with slight modifications. Hemolysates were prepared from venous blood samples collected in either EDTA (ethylene-diamine-tetraacetic acid) or heparinized tubes. After centrifugation, the plasma and buffy coat were removed by suction and the cells washed twice in 0.155 M KCl at 4°C, and resuspended in this solution, reapproximating the original blood volume. The packed red cell volume was determined on a portion of this suspension by the method of Wintrobe (12). The red cells were hemolyzed by freezing in an ethanol-dry ice mixture. OPRTase and ODCase activities for each individual were determined in duplicate on the same hemolysate. Assays were performed on the same day of collection (unless otherwise stated).

OPRTase. The reaction mixture consisted of 0.25 mM orotic carboxyl-^{14}C acid (0.27 mCi/mmole), 0.25 mM phos-

[2] This terminology has been suggested by L. H. Smith, Jr., Department of Medicine, University of California, San Francisco, Calif.

phoribosylpyrophosphate (PRPP), 3 mM $MgCl_2$, and approximately 0.1 U of partially purified yeast ODCase (OPRTase activity less than 1×10^{-4} U) in 0.05 M sodium phosphate buffer pH 7.4, with 0.1 ml of red cell hemolysate, in a final volume of 1.0 ml. (EDTA as an anticoagulant did not effect OPRTase activity.)

ODCase. The reaction mixture consisted of 0.05 mM orotidine-carboxyl ^{14}C 5'-monophosphate (OMP) (0.20 mCi/mmole), in buffer as above, with 0.1 ml of hemolysate, in a final volume of 1.0 ml.

The reactions were carried out in 20 ml scintillation vials sealed with rubber stoppers. Liberated $^{14}CO_2$ was trapped in 0.5 ml of hydroxide of Hyamine (Packard Instrument Co., Downers Grove, Ill.) contained in a small glass tube which was fitted into a slightly larger tube sitting in the vial. The flasks were incubated for 60 min in a Dubnoff metabolic shaker at 37°C and the reaction terminated by the injection of approximately 0.2 ml 0.5 M sulfuric acid through the rubber stopper, the stoppers removed and the glass tube containing the hyamine removed and transferred to a scintillation vial containing 15 ml of a scintillation fluid (toluene, 0.5% 2,5-diphenyloxazole (PPO), 0.05% 1,4-bis(2-[5-phenyloxazolyl])benzene (POPOP) (Packard Instrument Co.), and counted in a Picker Nuclear liquid scintillation (Picker Corp., Cleveland, Ohio) counter with an efficiency of 92%. Enzyme activity was expressed as mμmole CO_2 formed/hr per 0.1 ml packed erythrocytes.

Assay of hypoxanthine-guanine phosphoribosyltransferase (HGPRTase), adenine phosphoribosyltransferase (APRTase), and "Allopurinol phosphoribosyltransferase" (ALLPRTase)

20 μl of hemolysate, prepared by the method of Kelley, Rosenbloom, Henderson, and Seegmiller (13), were incubated with either 0.06 mM hypoxanthine-8-^{14}C (6.78 mCi/mmole), 0.6 mM adenine-8 ^{14}C (0.41 mCi/mmole), or 1.25 mM allopurinol-6-^{14}C [3] (0.11 mCi/mmole) together with 5 mM $MgCl_2$, 0.5 mM PRPP, and 55 mM tris buffer pH 7.4, in a final volume of 0.4 ml. After incubation for 10 min in a water bath at 37°C, the reactions were terminated by the addition of 8 μmoles of neutralized EDTA and immediately frozen in a dry ice–ethanol bath. After thawing, 40 μl portions of the reaction mixtures were spotted on to Whatman no. 1 chromatography paper, together with the appropriate carrier bases and nucleotides. The nucleotide product was separated from base substrate by ascending chromatography in saturated ammonium sulfate, 1 M sodium acetate, isopropanol (80:18:2) for 15 hr. The base and nucleotide carrier spots were identified under ultraviolet light, cut out, and transferred to a scintillation vial containing the same scintillation mixture as used in the OPRTase assay. Conversion of substrate to ribonucleotide product was estimated after counting. Enzyme activity was expressed as mμmoles ribonucleotide produced/mg protein per hour. Protein was determined by the method of Goodwin and Choi (14).

Assay of purine nucleoside phosphorylase. A radiochemical procedure similar to that described for the HGPRTase was used. 50 μl of hemolysate were incubated with either 0.06 mM hypoxanthine-8-^{14}C (6.78 mCi/mmole) or 1.25 mM allopurinol-6-^{14}C (0.11 mCi/mmole), 1 mM ribose-1-phosphate, and 55 mM tris buffer pH 7.4, in a final volume of 0.4 ml for 20 min at 37°C. The reaction was terminated by the addition of 0.1 ml 0.6 M perchloric acid. Nucleoside product was separated from the base by the same chromatographic procedure used in the HGPRTase assay. Deproteinization was necessary to ensure chromatographic resolution. Enzyme activity was expressed as mμmoles of nucleoside produced/mg protein per hour.

Assay of inosine kinase. An attempt to demonstrate the presence of inosine kinase was made by performing the above assay but with the addition of 5 mM ATP and 5 mM $MgCl_2$ to the reaction mixture.

Glucose-6-Phosphate Dehydrogenase (G6PDase) was determined by the method of Marks (15).

Study of "in vivo stability" of enzymes in red cells. The effect of cell age on red cell enzyme activities was assessed by fractionation of blood samples into age-specific groups, utilizing the increase in specific gravity of the red cells as they age in vivo. A modification of the method of Brok, Ramot, Zwang, and Danon (16) was used. Separation of red cells according to their specific gravity was achieved by two phase centrifugation with phthallate esters. Before this procedure the density distribution of the red cells was determined by the method of Danon and Marikovsky (17), using Wintrobe tubes instead of capillary microhematocrit tubes. 25-ml venous blood samples from normal individuals and patients receiving allopurinol were drawn into heparinized tubes. For red cell fractionation, two 10 ml graduated centrifuge tubes were filled with blood which was then overlaid with 2 ml of a phthallate ester of specific gravity 1.094 (produced by mixing appropriate amounts of di-*n*-butyl phthallate of specific gravity 1.046 and dimethyl phthallate of specific gravity 1.191). The tubes were then centrifuged at 1800 *g* at 15°C for 1 hr. This resulted in fractionation of the packed red cells into two approximately equal groups. The plasma and buffy coats were removed by suction. The two upper red cell fractions were pooled and the cells suspended in normal saline, containing 200 mg/100 ml glucose and buffered to pH 7.4 with 0.2 M phosphate buffer to a total volume of 10 ml. 2 ml portions of a phthallate ester mixture of specific gravity 1.090 were layered on to the cell suspension. The lower red cell fractions were similarly treated, using a phthallate ester mixture with a specific gravity of 1.098. The tubes were centrifuged as before. This procedure resulted in four fractions of red cells with specific gravities, less than 1.090, from 1.090 to 1.094, from 1.094 to 1.098, and greater than 1.098, respectively. It was possible to overcome individual variation in fraction size by a further period of centrifugation (10–15 min) using a slight variation in temperature (±3°C). The cell fractions were washed in the buffered glucose saline and resuspended to give an hematocrit of approximately 50%. Enzyme assays (OPRTase, ODCase, HGPRTase, and G6PDase) were performed on the fractions after overnight storage at −15°C.

Quantitation of urinary orotidine and orotic acid. 8-ml urine samples were mixed with 1 g activated charcoal (Norit A, Amend Drug and Chemical Co., Inc., New York), and incubated at 37°C for 1 hr with occasional shaking. The charcoal was then removed by centrifugation and the supernatant retreated in a similar fashion with a further 0.5 g charcoal. The charcoal fractions were pooled and washed once in distilled water. Adsorbed orotidine and orotic acid were recovered by suspending the charcoal in 7 ml of ethanolic ammonia (ethanol, concentrated ammonia, water; 50:2:48) and incubated at 37°C for 3 hr in a stoppered tube. The charcoal was then centrifuged down and reeluted with a further 4 ml of ethanolic ammonia. The pooled supernatants were then evaporated to dryness at 110°C and

[3] This concentration is twice the K_m (0.6 mM) determined by us for allopurinol.

the residue dissolved in 0.5 ml distilled water. A 50 μl portion of this solution was spotted on to a cellulose TLC plate thin-layer chromatography (cellulose MN-300HR, Machery Nagel & Co., Division of Brinkmann Instruments, Westbury, N. Y.). Separation of orotidine and orotic acid was achieved by ascending chromatography for 3 hr using the chromatography system described for the HGPRTase assay. The cellulose areas corresponding to reference spots (identified under ultraviolet light) were scraped from the plate. The orotidine and orotic acid were eluted from the cellulose by treatment with 0.8 ml of 0.01 M hydrochloric acid at 37°C for 3 hr. This elution step was repeated with a further 0.6 ml of the acid and incubation under the same conditions. Orotidine and orotic acid were then quantitated in the acid eluates by the colorimetric reaction of Rogers and Porter (18). The procedure was checked by adding known quantities of orotidine and orotic acid to urine. After charcoal extraction no orotidine or orotic acid could be detected in the extracted urine. Further, the recovery after elution with ethanol-ammonia was quantitative. It was also noted, that in the absence of interfering substances from urine, quantitation of orotidine and orotic acid could be obtained with the colorimetric reaction.

RESULTS

Studies on the relationship between erythrocyte OPRTase and ODCase

The activities of OPRTase and ODCase in hemolysates from 12 normal individuals were found to vary over a 3-fold range. The mean activity of OPRTase was 13.9 ±4.5 mμmoles of orotic acid converted to OMP/hr per 0.1 ml packed erythrocytes, whilst that for ODCase was 29.1 ±9.4 mμmoles of OMP converted to UMP/hr per 0.1 ml packed erythrocytes. However, a linear relationship between OPRTase activity and ODCase activity was observed (Fig. 1). Activities of OPRTase and ODCase were also assayed in hemolysates from patients receiving allopurinol for at least a 4-month period. This group included four patients with a history of gout and hyperuricemia, three patients with HGPRTase deficiency (Table I) and clinical features of the Lesch-Nyhan syndrome. It also included one patient each with hyperuricemia associated with diabetes mellitus, hypertensive renal insufficiency, polycythemia rubra vera, and myelofibrosis. Activities of both OPRTase and ODCase were elevated, from the upper level of the normal group to 127 mμmole/orotic acid converted to OMP/hr per 0.1 ml packed erythrocytes for OPRTase and to 261 mμmoles of OMP converted to UMP/hr per 0.1 ml packed erythrocytes for ODCase. (Enzyme activity levels before therapy were not assessed). Again, the linear relationship between OPRTase and ODCase activity was maintained and was the same as the normal group (Fig. 1). The coefficient of linear correlation (19) was calculated for the relationship between OPRTase and ODCase, and including the data from both groups was 0.99. Regression lines for this relationship were calculated for the control group and allopurinol-treated patients, and were

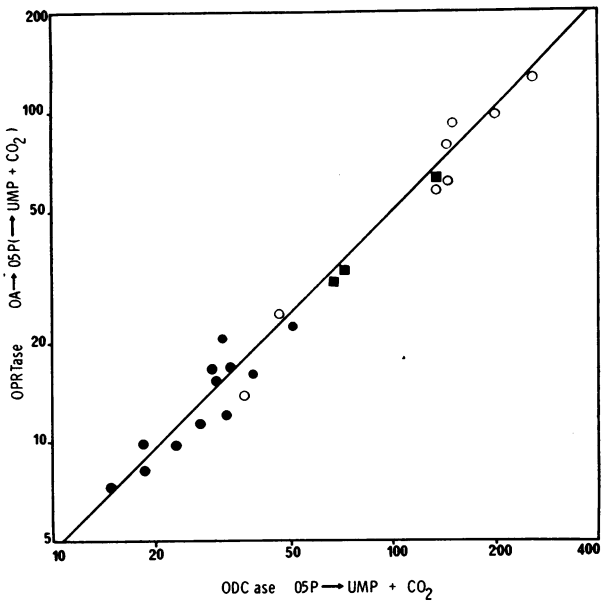


FIGURE 1 Coordinate relationship between OPRTase and ODCase activities in human erythrocytes. Each point represents the mean of duplicate assays for each individual. The closed circles represent normal individuals, the open circles patients receiving allopurinol, and the closed squares patients with HGPRTase deficiency receiving allopurinol. Activities expressed as mμmole CO_2 formed/hr per 0.1 ml packed erythrocytes.

not significantly different (P at least > 0.05) (19). Thus a coordinate relationship between the activity of OPRTase and ODCase in hemolysates was found over an 18-fold range (Fig. 1).

The effect of red cell ageing on this coordinate relationship was investigated. Red cells from seven normal laboratory workers were subjected to two phase centri-

TABLE I

*Levels of Purine Salvage Enzymes in Erythrocytes from Patients with HGPRTase Deficiency**

Enzyme	Normal range ±SD	Patients with HGPRTase deficiency
HGPRTase	71.3 ±9.0 (21)‡	R. W. 0.29 J. W. 0.11 L. B. 0.58
APRTase	15.1 ±3.8 (6)	34.6 ±7.2 (3)
AllPRTase	6.9 ±0.5 (3)	0 (3)
Purine nucleoside phosphorylase		
Hypoxanthine substrate	14.4 ±2.6 (3)	16.4 ±2.4 (3)
Allopurinol substrate	2.8 ±0.4 (3)	3.8 ±0.9 (3)
Inosine kinase	0 (3)	0 (3)

* Enzyme activities expressed as mμmoles product formed/mg protein per hr.

‡ Number of individuals assayed in duplicate.

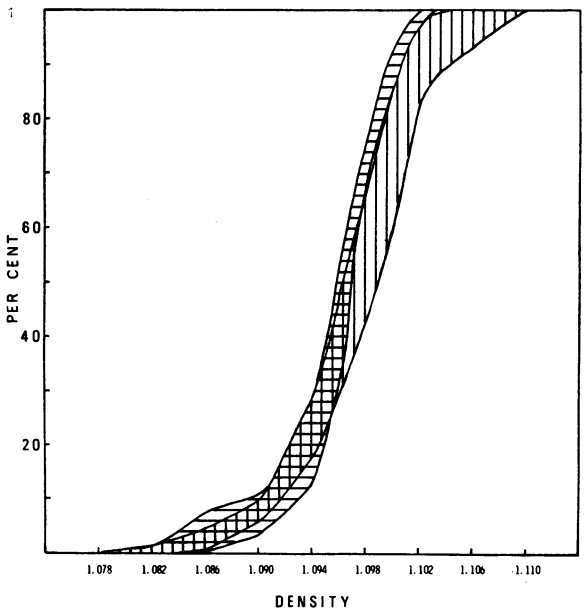


FIGURE 2 Density gradient distribution of red cells. The ordinate represents the percentage of cells lighter than the specific gravity indicated on the abscissa. The vertical hatching describes the curve obtained for seven normal individuals (twice the SD of the mean), whilst the horizontal hatching represents the curve obtained for four patients with primary hyperuricemia receiving allopurinol (twice the SD of the mean).

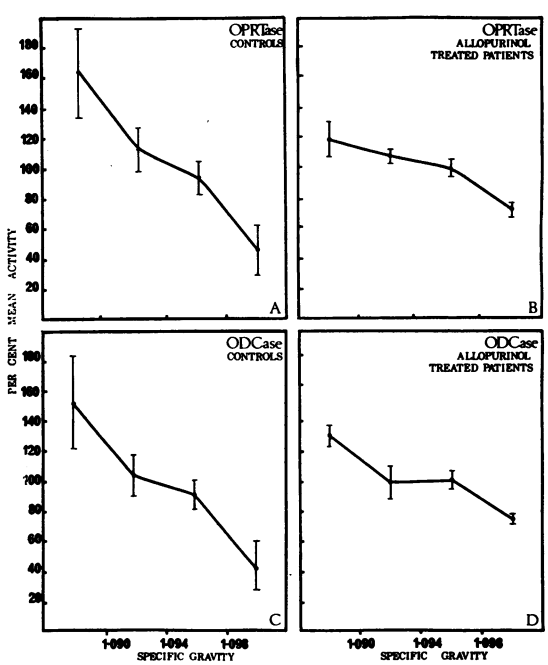


FIGURE 3 The influence of red cell age on OPRTase and ODCase activities in seven normal individuals (A and C) and in four patients with primary hyperuricemia receiving allopurinol (B and D). The vertical lines represent twice the SD of the mean.

fugation on phthallate esters which yielded four approximately equal fractions of differing specific gravity. The density gradient distribution curves obtained for these individuals are illustrated in Fig. 2. Specific activities of OPRTase and ODCase were determined on the lysates of the fractions. The specific activities of HGPRTase and G6PDase were also determined on the lysates from three of these individuals. For each enzyme assayed, the mean of the specific activities of the four fractions from an individual was determined. The activity of the enzyme in each fraction was then expressed as a percentage of this mean. In Figs. 3 A and 3 C the OPRTase and ODCase percentage activities in the red cell fractions from the seven normal individuals are illustrated. A dramatic fall in activity for both of these enzymes was found as the red cell specific gravity increased. In the lightest fraction the activities of OPRTase and ODCase were 160% and 158% respectively, whilst in the heaviest fraction 46% and 48%. No significant difference (P at least > 0.05) could be demonstrated between regression lines calculated for the relationship between specific gravity and OPRTase and ODCase activities respectively.

In Fig. 4 A comparative values for the relative percentage activities of G6PDase in the same fractions from three of these normal individuals are shown. In this case the decrease in activity was much less marked, diminishing from 118% in the lightest fraction to 77% in the heaviest. There was no significant fall in HGPRTase activity with increase in red cell specific gravity (Fig. 5 A). It would appear, on the basis of these findings, that OPRTase and ODCase are significantly more labile (and to an equivalent degree) in vivo when compared with G6PDase, while HGPRTase is relatively stable as the red cell ages.

The in vivo lability of OPRTase and ODCase in patients receiving allopurinol therapy was compared with that of the normal group by red cell fractionation. Blood

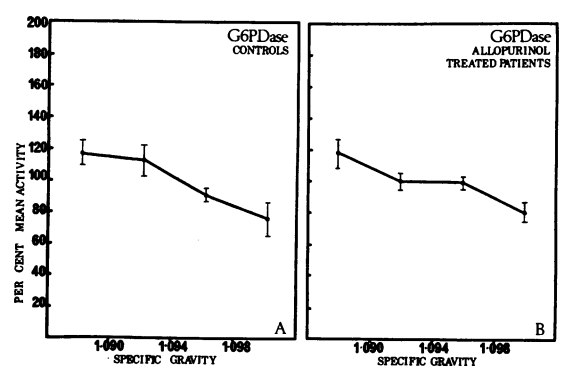


FIGURE 4 The influence of red cell age on G6PDase activity in three normal individuals (A), and three patients with primary hyperuricemia receiving allopurinol (B). The vertical lines represent twice the SD of the mean.

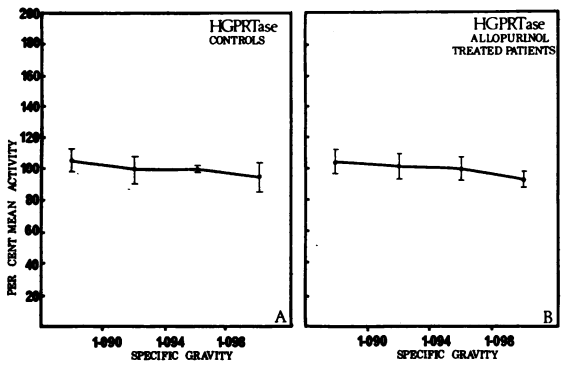


FIGURE 5 The stability of HGPRTase activity in red cells of differing age, in three normal individuals (A), and three patients with primary hyperuricemia (B), receiving allopurinol. The vertical lines represent twice the SD of the mean.

samples from four patients with apparent primary hyperuricemia and gout, who had been receiving allopurinol for at least 4 months, (allowing exposure to the drug throughout the red cell life span), were used. These patients had coordinately elevated levels of OPRTase and ODCase (Table II). Specific activities of OPRTase and ODCase were assessed in the fractions from these patients, and HGPRTase and G6PDase in three of them. The data were treated in a similar manner to that of the control group. A marked diminution in the rate of decrease of both OPRTase and ODCase activities with increase in red cell specific gravity, was found in the allopurinol-treated group. These findings are depicted in Figs. 3 B and 3 D. The OPRTase percentage activity fell from 120 to 73% whilst the ODCase fell from 128 to 74%. Again no significant difference could be found between the regression lines calculated for the relationship between specific gravity and OPRTase and ODCase activities respectively in this group (P at least > 0.05). However, there was a significant difference ($P < 0.001$) between the regression lines determined for the decrease of OPRTase activity for the control group compared with the allopurinol-treated patients. The decline in activity of G6PDase with increase in red cell specific gravity was not significantly different (Fig. 4B) from the control group (P at least > 0.05). Similarly, a change in HGPRTase activity with increase in specific gravity could not be demonstrated in the allopurinol-treated group (Fig. 5B). The specific activities of G6PDase and HGPRTase were the same in unfractionated blood samples from normals and allopurinol-treated patients. Density-gradient distribution curves for this group are illustrated in Fig. 2. There was a smaller percentage of denser red cells in this group. The significance of this observation is not known, but could indicate some shortening of red cell life span.

Studies on patients with HGPRTase deficiency: the effects of allopurinol administration on pyrimidine metabolism

The possibility that allopurinol ribonucleotide is the in vivo inhibitor of UMP synthesis was investigated by studying three patients with HGPRTase deficiency. These patients (R. W., J. W., and L. B.) had less than 1% of the normal red cell HGPRTase activity, and APRTase activity was approximately twice that of a normal group (Table I). In normal hemolysates the rate of formation of allopurinol ribonucleotide was 6.9 ±0.5 mμmoles/mg protein per hr, whilst the hemolysates from the HGPRTase deficient patients completely failed to form any ribonucleotide. The ability of normal hemolysates to form allopurinol ribonucleotide was not impaired by heating at 55°C for 10 min. These findings exclude OPRTase or APRTase as mediators of this reaction.

Despite the failure of the hemolysates from these three patients to form allopurinol ribonucleotide, their erythrocyte OPRTase and ODCase activities were coordinately elevated whilst they were receiving allopurinol therapy (Fig. 2). For one of these patients (L. B.), 24-hr urine collections were made whilst he was receiving allopurinol (600 mg daily), during a 5 day period in which the drug was suspended, and during recommencement of allopurinol therapy at 300 mg daily. A close relationship was found between allopurinol administration and urinary orotidine and orotic acid excretion. This is illustrated by spectrophotometric scans of the patient's urine after colorimetric reaction (18) in Fig. 6, and by quantitation of the daily orotidine and orotic acid excretion (Table III). The patients R. W. and J. W. excreted excessive amounts of orotidine and orotic acid whilst receiving allopurinol. These findings would exclude allopurinol ribonucleotide from consideration as the in vivo inhibitor of ODCase.

The activity of purine nucleoside phosphorylase was normal in the erythrocytes of the three patients with HGPRTase deficiency, and inosine kinase activity could not be demonstrated in either normal or HGPRTase-deficient hemolysates (Table I). In an attempt to assess

TABLE II
OPRTase and ODCase Activities Before Red Cell Fractionation*

	Normal group (7)	Allopurinol treated group (4)
OPRTase	13.1 ±3.5	66.4 ±27.4
ODCase	28.8 ±7.7	148.5 ±59.6

* mμmole CO_2 formed/hr per 0.1 ml packed erythrocytes.

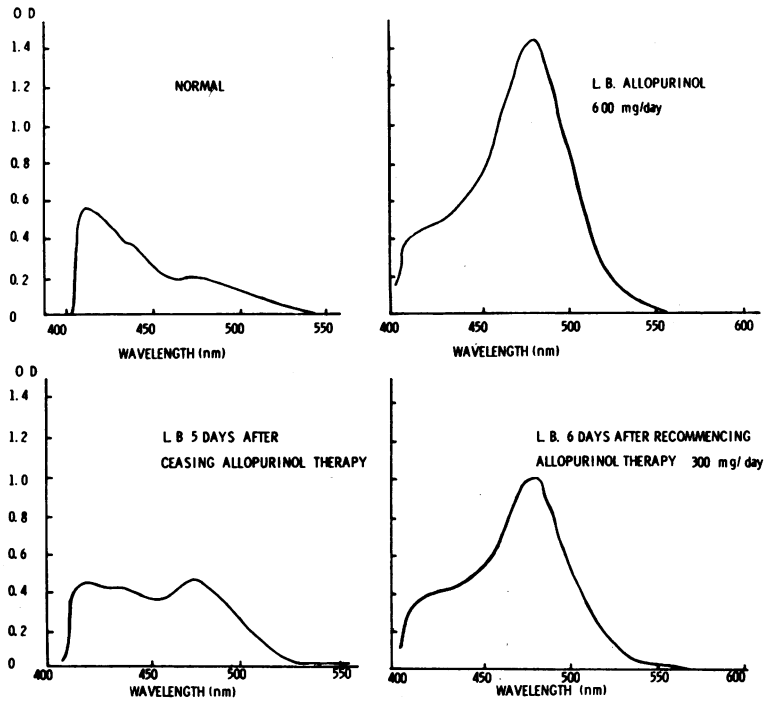


FIGURE 6 Recorded spectra of urine treated according to the method of Rogers and Porter (18). The urine is from an HGPRTase-deficient patient, and demonstrates orotidinuria and orotic aciduria in response to allopurinol administration.

the possible role of allopurinol ribonucleoside as an in vivo inhibitor of ODCase, allopurinol-6-^{14}C was used as the substrate in the purine nucleoside phosphorylase assay. The formation of allopurinol ribonucleoside by erythrocytes deficient in HGPRTase was the same as normal erythrocytes (Table I). However, the incubation of allopurinol and ribose-1-phosphate, or oxipurinol and ribose-1-phosphate with hemolysate did not result in any inhibition of ODCase activity. Thus, it is unlikely that either allopurinol or oxipurinol ribonucleosides were the metabolites responsible for the orotidinuria induced by allopurinol administration. Thus, although neither oxipurinol nor its ribonucleoside are inhibitors of erythrocyte ODCase, the incubation of oxipurinol with PRPP will convert it into an inhibitor of ODCase. This is demonstrated in Table IV, and occurred with both normal hemolysate and hemolysate from HGPRTase-deficient patients. Presumably, then, oxipurinol ribonucleotide is an inhibitor of ODCase.

TABLE III

Urinary Orotidine and Orotic Acid Excretion in Relation to Allopurinol Therapy in Patient L. B. with HGPRTase Deficiency

Allopurinol therapy	Serum uric acid	Urinary orotidine	Urinary orotic acid	Total urinary orotidine and orotic acid
	mg/100 ml	*mg/day*	*mg/day*	*mmoles/day*
600 mg/day	5.2	137.5	24.2	0.51
5 days after ceasing allopurinol	13.3	32.3	8.7	0.14
6 days after recommencing allopurinol 300 mg/day	9.2	115.5	14.2	0.39

In view of the apparent similarity between the metabolic derangement brought about by allopurinol administration and the inborn error of purine metabolism, xanthinuria, we studied the excretion of orotidine and orotic acid in a patient with this condition (20). A portion of a 24 hr urine collection was reacted by the colorimetric procedure of Rogers and Porter; (18) however the spectrophotometric scan was normal. The activity of this patient's erythrocyte OPRTase was 24.4 mμmoles orotic acid converted to OMP/hr per 0.1 ml packed erythrocytes, and the activity of ODCase was 55.8 mμmoles OMP converted to UMP/hr per 0.1 ml-packed erythrocytes. These activities were at the upper limit of the normal range, and the coordinate relationship was maintained.

TABLE IV

Synthesis of Oxipurinol Ribonucleotide, in the Absence of HGPRTase, and Its Inhibition of Erythrocyte ODCase

The following were incubated in the presence of 0.1 ml of hemolyzed red cells, under the conditions described for the assay of ODCase. The results are the mean of duplicate determinations.

	5×10^{-6} M ^{14}C-OMP	2.5×10^{-4} M PRPP	1×10^{-3} M Oxipurinol	Decarboxylation OMP → UMP	Inhibition
				cpm	%
Normal hemolysate	+	−	−	4702	0
	+	+	−	4565	0
	+	−	+	4680	0
	+	+	+	1579	66
HGPRTase-deficient hemolysate (L. B.)	+	−	−	5620	0
	+	+	−	5715	0
	+	−	+	5650	0
	+	+	+	2884	49
HGPRTase-deficient hemolysate (R. W.)	+	−	−	5931	0
	+	+	−	5397	0
	+	−	+	5986	0
	+	+	+	3450	42

DISCUSSION

This investigation has revealed a coordinate relationship between the activities of two sequential enzymes of the de novo pyrimidine pathway in human red cells. The two enzymes, OPRTase and ODCase, have a pronounced but equal degree of in vivo lability in the ageing red cell. Furthermore, the activities of the enzymes are coordinately increased in patients receiving allopurinol, and this increase appears to be due, at least in part, to an in vivo stabilization of both OPRTase and ODCase in the red cell. These observations may be of significance to the control of enzyme activity in human cells, and to the elucidation of the nature of the genetic defect in hereditary orotic aciduria.

Postulated mechanisms for the regulation and genetic control of enzyme activity have been largely based on models developed in the study of microorganisms, especially that of Jacob and Monod (21). Such models envisage transcriptional control of the expression of groups of structural genes (operons) by regulator genes. Beckwith, Pardee, Austrian, and Jacob (22) have demonstrated that in *Escherichia coli* the gene loci determining the structure of the terminal four enzymes leading to UMP synthesis (dihydroorotase, dihydroorotate dehydrogenase, OPRTase, and ODCase) are linked on the chromosome. Under conditions of repression and derepression, the activities of these four enzymes were found to respond coordinately, and presumably their structural genes form an operon, under control of a regulator gene (22). In human cells, the pyrimidine pathway is apparently delineated into smaller groups of concurrently responding enzymes. Wuu and Krooth (23) have shown that while addition of inhibitors of UMP synthesis to the medium, in which normal human fibroblasts are being cultured, leads to increased activity of OPRTase and ODCase, there was little or no effect on the preceding two enzymes in the pathway (dihydroorotase and dihydroorotate dehydrogenase). This is consistent with our findings for OPRTase and ODCase in red cells and with the observation that dihydroorotic dehydrogenase activity is not detectable in the mature human red cell (4). By analogy with the de novo pyrimidine synthetic pathway in *E. coli,* Smith, Huguley, and Bain (4) have postulated that the structural genes coding for OPRTase and ODCase form an operon, and that the sequential deficiency of these two enzymes represents a defect in a regulator or control mechanism.

However, the physical organization of OPRTase and ODCase in the mammalian cell differs markedly from that of the unicellular organism. In yeast, OPRTase and ODCase can be readily purified as independent enzyme moieties (10) whilst studies in mammalian tissues suggest that these two enzymes may exist as an aggregate. Appel has copurified OPRTase and ODCase 600-fold from cow brain (24). His procedures included ion-exchange chromatography, gel filtration, and sucrose density centrifugation. Electrophoresis of the purified preparation in polyacrylamide gel revealed a single major protein band, which retained 20% of the pre-electrophoresis ODCase activity but had lost OPRTase activity (24). Similarly, Kasbekar, Nagabhushanam, and Greenberg (25) in a study of calf thymus gland, copurified OPRTase and ODCase 600-fold but were able to separate their activities by starch gel electro-

phoresis. This procedure also resulted in varying degrees of loss of OPRTase activity. In an investigation on beef erythrocytes Hatfield and Wyngaarden achieved copurification over a 5000-fold range (26). Such demonstrations of copurification of the two enzyme activities suggest that an enzyme complex of two or more polypeptide chains has been isolated. Concepts of enzyme-enzyme complexes have been reviewed recently by Reed and Cox (27). They noted that assembly of such complexes of enzymes catalyzing sequential reactions was likely to increase the efficiency of the over-all process. The formation of an enzyme-enzyme complex provides a simple explanation for the coordinate relationship between OPRTase and ODCase activities, and their identical in vivo lability.

It is now apparent that in mammalian cells, mechanisms of control of enzyme activity may differ markedly from that in bacterial cells. Mammalian messenger RNA is quite stable, whilst it appears that enzymes may be relatively labile. Thus control of enzyme activity may include, apart from rate of synthesis, control of enzyme turnover, activation, and inhibition (28). On the basis of our red cell fractionation studies at least one mechanism for the increased activity of OPRTase and ODCase in patients receiving allopurinol is enzyme stabilization. Such a phenomenon may be relevant to the findings of Pinsky and Krooth (6, 7) who found that fibroblasts, from heterozygous and homozygous patients with type I hereditary orotic aciduria, develop near normal activities of both OPRTase and ODCase if the medium in which the cells are cultured contains inhibitors of UMP synthesis. It was suggested that these inhibitors cause the cells to accumulate higher levels of intermediates in the pyrimidine pathway and that one of these intermediates augments enzyme activity. On the basis of these and other findings, Krooth has postulated that the primary defect in hereditary orotic aciduria (type I) may be the inappropriate excretion of one or more pyrimidine intermediates. Thus the cells might not accumulate enough intermediate to maintain adequate levels of the final two enzymes in the sequence, OPRTase and ODCase (29). We would like to offer an alternate interpretation, that the defect in hereditary orotic aciduria may represent a structural abnormality in an enzyme complex. Perhaps the addition of inhibitors of UMP synthesis to the culture medium of the mutant cells may stabilize an abnormally labile enzyme complex, allowing the activity levels to rise near normal. This hypothesis could also account for the presence of orotidylate phosphoribosyltransferase in type II hereditary orotic aciduria (5).

The extreme in vivo lability of erythrocyte OPRTase and ODCase is in marked contrast with the stability of HGPRTase. Presumably OPRTase and ODCase are residual reminders of the capacity of the developing erythroblast for de novo UMP synthesis. The lability of these two enzymes may reflect their fundamental structural characteristics, however an alternate, although not exclusive possibility, is that they lack interaction with substrate in the ageing red cell. In contrast, the possibility that the red cell has an important role in purine metabolism, largely as a transport vehicle, has been stressed by Murray, Elliott, and Atkinson (30). The red cell appears to take up purine bases, formed by de novo synthesis in the liver, then releases them, largely as hypoxanthine, for salvage utilization in other tissues. The transport form of the purines in the red cell is apparently the ribonucleotide (30), and thus the constant supply of substrate may partly explain the stability of HGPRTase. It has been shown that APRTase has a short half-life in the circulating red cell (31, 32). In patients with HGPRTase deficiency, APRTase levels have been consistently elevated and this has been confirmed in our studies (Table II). This elevation reflects prolongation of the half-life of APRTase, apparently due to stabilization by increased erythrocyte PRPP levels (32).

We have tacitly assumed that the orotidinuria induced by allopurinol administration and the elevated OPRTase and ODCase levels in erythrocytes reflect a common process; presumably the accumulation of an inhibitor of ODCase. A basic problem in the interpretation of our data, and of much work in this field, is that one is dependent on the correlation of findings, made in either hemolysates or fibroblasts in culture, with what is often a metabolic process in the liver. Recent in vitro work has revealed that allopurinol ribonucleotide (2, 3) and xanthine monophosphate (XMP) (3) are inhibitors of erythrocyte ODCase. Our findings that erythrocytes from patients with HGPRTase deficiency fail to convert allopurinol-6-^{14}C to its nucleotide, and similar observations by Kelley and Wyngaarden (33) with respect to fibroblasts suggest that presumably these patients fail to convert allopurinol to its ribonucleotide in vivo. Hence orotidinuria after allopurinol administration in these patients makes it unlikely that allopurinol ribonucleotide is the in vivo inhibitor. Although no documentation is available, it is feasible that XMP could accumulate after inhibition of xanthine oxidase. This would accord with the findings of Pomales, Elion, and Hitchings who demonstrated that allopurinol increases the conversion of xanthine-^{14}C into guanylic acid of DNA in mice; presumably via formation of XMP (34). Should XMP be an in vivo inhibitor of ODCase, then it is feasible that variations in tissue XMP levels could modify de novo UMP synthesis. Although inconclusive and not a valid comparison to the administration of allopurinol, a xanthinuric patient studied did not have orotidinuria,

however the erythrocyte OPRTase and ODCase levels were at the upper limit of our normal range. Obviously further studies in such patients may be of value in elucidating the mechanism of the interference of pyrimidine metabolism by allopurinol.

An alternate, and more likely mechanism, is that oxipurinol ribonucleotide is an in vivo inhibitor of ODCase. Our data (Table IV) suggest that this compound is formed in the absence of HGPRTase; and this could account for the orotidinuria following allopurinol administration to patients with HGPRTase deficiency. Two investigations reported in the literature provide strong background support for this mechanism. In 1964, Hatfield and Wyngaarden (26) purified an enzyme, from beef erythrocytes, capable of synthesizing (3 ribosylxanthine)-5′-phosphate. The position of the ribosyl group in this ribonucleotide corresponds to that of the pyrimidine ribosyl compounds. The enzyme also mediated the synthesis of pyrimidine ribonucleotides, including OMP, from base and PRPP. This enzyme is presumably a form of OPRTase, and the common denominator of the substrates is a 2,4 diketo construction of the pyrimidine portion of the ring structure (see Fig. 7). Krenitsky, Elion, Strelitz, and Hitchings (35) in 1967 isolated, from the urine of patients receiving allopurinol, ribonucleoside derivatives of both allopurinol and oxipurinol. The attachment of the ribosyl group to allopurinol was at nitrogen atom 1 (corresponding to nitrogen atom 9 as is found in most natural purine ribonucleosides, see Fig. 7), whilst the ribosyl group attached to oxipurinol was at nitrogen atom 7. This corresponds to nitrogen atom 3 in xanthine, and thus 7-ribosyl–oxipurinol would be analogous to 3-ribosyl-xanthine. Krenitsky et al. (35) were able to synthesize 7-ribosyl-oxipurinol using uridine phosphorylase and concluded that oxipurinol is metabolized as a pyrimidine analogue in vivo.

On the basis of the data in Table IV, we feel that oxipurinol, with a 2,4-diketo pyrimidine ring, has been converted to a ribonucleotide, and is analogous to (3-ribosyl-xanthine)-5′-phosphate. As this occurred in the absence of HGPRTase, the most likely phosphoribosyltransferase responsible is OPRTase. Presumably, then, oxipurinol represents an analogue of orotic acid, and as a "pseudo-substrate" of OPRTase is responsible for the in vivo stabilization of OPRTase and ODCase.

It would be of interest to establish whether or not, in vivo, xanthine is a substrate for OPRTase, as suggested by the work of Hatfield and Wyngaarden (26). This would offer a possible mechanism for control of de novo UMP synthesis by interreaction with purine metabolism. The recognition of the now known inborn errors of purine and pyrimidine metabolism, and the wide use of metabolic analogs such as allopurinol offer

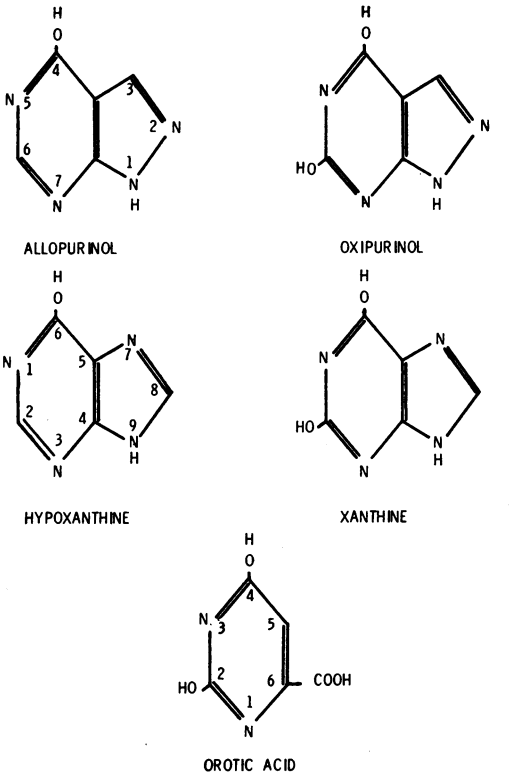


FIGURE 7 Structural formulae of allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine), oxipurinol (4,6-dihydroxypyrazolo[3,4-d]pyrimidine), hypoxanthine, xanthine, and orotic acid.

systems for study which may provide considerable insight into the regulation of enzyme activity in human cells.

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