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ALLOPURINOL TRANSPORT IN HUMAN ERYTHROCYTES

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Abstract—The mechanism of allopurinol [4-hydroxypyrazolo(3,4-*d*)pyrimidine] transport into human erythrocytes was investigated with an inhibitor stop assay. Allopurinol transport could be resolved into two components: (1) a saturable system and (2) a non-saturable process, which most likely represents non-facilitated diffusion. Allopurinol transport had a K_m of 268 μmol/L and a V_{max} of 28 pmol/μL intracellular volume/sec; the non-saturable component was 0.0195/sec. Mutual inhibition studies showed that the competitive K_i values of hypoxanthine and adenine on allopurinol transport were 120 and 3 μmol/L, respectively. These K_i values as well as the IC_{50} values of 100–150 μmol/L for hypoxanthine and 3–10 μmol/L for adenine were similar to the corresponding transport K_m values of these bases, which are 128 and 8 μmol/L, respectively. The K_i of allopurinol on hypoxanthine transport was 274 μmol/L and thus nearly identical to its K_m. Thus in erythrocytes the uricostatic agent allopurinol is an alternative substrate for the purine transport system, but lacks the exceptional high affinity it has for xanthine oxidase. This could explain the paradoxical clinical side effect of allopurinol, namely that it can provoke an attack of gout. Theophylline, a methylated purine, inhibited allopurinol transport with an IC_{50} of 200–400 μmol/L. Oxypurinol [4,6-dihydroxypyrazolo(3,4-*d*)pyrimidine], the main metabolite of allopurinol, also inhibited allopurinol transport with an IC_{50} of 20–40 μmol/L. This is noteworthy, since allopurinol and oxypurinol do not share the same transport system in the kidney.

Allopurinol [4-hydroxypyrazolo(3,4-*d*)pyrimidine] is used in the treatment of hyperuricemia and the prevention of gout [1, 2]. Its uricostatic action is due to its effective inhibition of xanthine oxidase. Oxypurinol [4,6-dihydroxypyrazolo(3,4-*d*)pyrimidine], the main metabolite of allopurinol, also inhibits xanthine oxidase and is more relevant clinically because of its longer biological half-life [3, 4]. Since allopurinol must permeate the plasma membrane to be effective or metabolized to oxypurinol, it was of interest to characterize the transport system by which allopurinol enters cells. Previous studies have established that purines share a common transport system in human erythrocytes [5–7]. Allopurinol and oxypurinol are purine analogs; thus the possibility that they too enter cells by the same mechanism was investigated. The results of the present study confirm this hypothesis.

MATERIALS AND METHODS

Preparation of human red blood cells. Human blood was obtained from outdated blood bank supplies (University Hospital, University of Vienna). The erythrocyte suspension was prepared by extensive washing to remove endogenous purines as described previously [7].

Transport studies. Zero-trans influx was assayed as described previously [6] and as used in our laboratory to characterize in detail the kinetic properties of hypoxanthine and adenine transport in erythrocytes [7]. Erythrocytes (20 μL, 50% hematocrit) were placed in a 1.5 mL Eppendorf tube and the incubation was started by rapid addition of 80 μL labeled substrate in saline at 25°. Unlabeled substrates were always premixed with, and thus added at the same time as, the labeled compounds. Influx was stopped by addition of 700 μL of a 20 mmol/L papaverine solution that had been precooled in a freezing mixture to about −2°. Within 10 sec 0.2 mL silicone oil was added, and the cells separated from the assay medium by immediate centrifugation through the oil layer. Papaverine inhibited allopurinol transport completely and instantaneously: the amount of substrate associated with the cell pellets at zero-time was the same for hypoxanthine and allopurinol, and matched the extracellular volume values obtained with [^{14}C]-inulin.

Time measurements. Incubation times were determined using an electronic clock (Breitenbach and Heller, Vienna, Austria) with a precision of 0.01 sec. The clock was started and stopped by micro switches attached to the plunger of the pipettes used to dispense the start and stop solutions.

Sample processing. After aliquots for radioactivity measurements were taken from the supernatant, the remainder was removed by suction and the tubes rinsed with water. The cell pellet was hemolysed by adding 450 μL H_2O, and extracted with 50 μL perchloric acid (0.5 M final concentration). After centrifugation the radioactivity in the clear supernate was measured by liquid scintillation counting in a Packard CA2000 counter (Packard Instruments) with a constant counting error of 2%.

Data analysis. Cell volume and the extracellular space of cell pellets were determined with ^3H_2O and [^{14}C]inulin [8]. Initial rates of transport were determined by fitting the exponential Eqn 1 to the time courses of influx of labeled substrate using non-linear least-squares regression [9]:

$$S_{in,t} = S_{in,\infty}[1 - \exp^{-kt}]. \quad (1)$$

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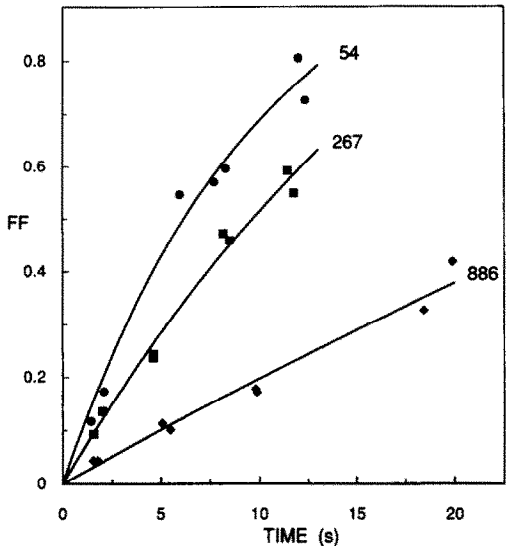
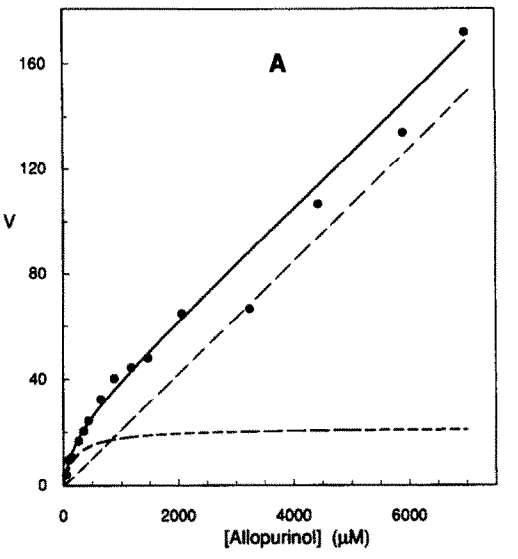


Fig. 1. Time-courses of allopurinol influx. Human erythrocytes were incubated at 25° for the indicated times with [^{3}H]allopurinol. Fractional filling (FF) is the ratio of the intracellular concentration at time t to the equilibrium concentration. Time-courses for the substrate concentrations 54, 267 and 886 μmol/L are shown. Initial rates of transport are the tangents to the curves at time zero, and were determined as described under Materials and Methods.



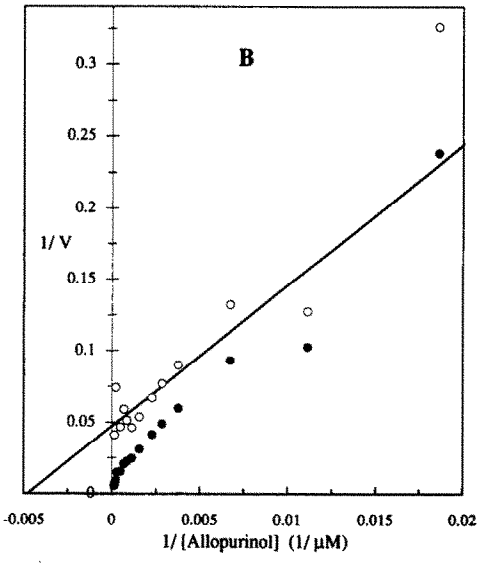


Fig. 2. Concentration dependence of allopurinol transport. Calculation of the kinetic parameters was carried out by a non-linear regression using Eqn 2 as described in Materials and Methods. (A) Plot of initial rates versus concentration. The solid line is the calculated fit to the experimental data (●). The dotted lines were calculated for the saturable and non-saturable components. (B) Lineweaver–Burk plot of the data shown in panel A. The open symbols represent the saturable component and were obtained by subtraction of the non-saturable component from the experimental values. The solid line is a transformation of the hyperbolic curve from panel A, and not a linear regression of the corrected initial rates.

Initial rates of transport were calculated from the slopes at $t = 0$ sec. The kinetic parameters V_{max}, K_m and the non-facilitated influx rate constant were determined by non-linear least squares regression analysis of the initial rate data using Eqn 2 [7]. The K_i values were determined in an analogous fashion using Eqn 3:

$$d[S]/dt = V_{max} \cdot [S]/(K_m + [S]) + \text{constant} \cdot [S] \quad (2)$$

$$d[S]/dt = V_{max} \cdot [S]/(K_m \cdot (1 + [I]/K_i) + [S]) + \text{constant} \cdot [S]. \quad (3)$$

Non-linear least-squares regression was carried out using subroutine CURVFIT which is a FORTRAN implementation of Marquard's algorithm as described by Bevington [10].

Chemicals. [G-^{3}H]Adenine, [2-^{3}H]hypoxanthine, ^3H_2O and [^{14}C]inulin were purchased from Amersham (U.K.); [G-^{3}H]allopurinol from Moravek Biochemicals (Brea, CA, U.S.A.). Papaverine-HCl and unlabeled nucleobases were obtained from Sigma (St Louis, MO, U.S.A.); silicone oil AR200 from Wacker Chemie (Munich, Germany). Ready Solve scintillation cocktail was from Beckman Instruments (Fullerton, CA, U.S.A.)

RESULTS

Time dependence of allopurinol influx

The rate of allopurinol transport was assayed for concentrations ranging from 40 to 7000 μmol/L. Time-courses for concentrations below, at and above the K_m, are shown in Fig. 1. Initial rates of transport were estimated as described in Materials and Methods by determining the slope at $t = 0$ sec. No correction for metabolism was necessary, since TLC analysis [11] showed that under experimental conditions less than 1% of allopurinol was

Table 1. Kinetic parameters of allopurinol and nucleobase zero-trans influx

Permeant	K_m (μmol/L)	V_{max} (pmol/sec/μL)	Constant (10^{-3}/sec)	K_i (μmol/L)
Allopurinol*	268 ± 9	28 ± 3	19.5 ± 3	274‡
Hypoxanthine†	128 ± 5	13 ± 1	3.3 ± 0.1	120§
Adenine†	8.3 ± 1.8	1.8 ± 0.4	14.6 ± 4	3‖

* Values (except K_i values) represent the means ±SD of four independent experiments.
† Values obtained in our laboratory [7].
‡ K_i on hypoxanthine transport (Fig. 3B).
§ K_i on allopurinol transport (data from an experiment analogous to Fig. 3A).
‖ K_i on allopurinol transport (Fig. 3A).

Table 2. Inhibition of allopurinol transport

Inhibitor	IC_{50} (μmol/L)
Adenine	3–10
Hypoxanthine	100–150
Oxypurinol	20–40
Theophylline	200–400

Dose–response studies were performed by determining the initial rates of [^{3}H]allopurinol (100 μmol/L) zero-trans influx in the presence of six different concentrations of inhibitor. The ranges indicate in each instance the concentrations which bracket the 50% inhibition mark.

metabolized over a 10-min incubation period (data not shown).

Concentration dependence of allopurinol influx

The relationship between initial rates of allopurinol influx and allopurinol concentration is shown in Fig. 2A. Allopurinol influx was interpreted to be the result of two components, one saturable and one exhibiting no saturability. The dotted lines represent each of these components individually. The kinetic parameters were estimated by fitting Eqn 2 directly to the initial rates. The saturable hyperbolic system had a K_m of 268 μmol/L and a V_{max} of 28 pmol/μL intracellular volume/sec. The non-saturable rate constant was 19.5×10^{-3}/sec (Table 1).

Figure 2B shows a Lineweaver–Burk diagram of the saturable component, which was obtained after numerical subtraction of the fitted linear component from the influx values. The experimental data are plotted as closed circles; the same data after subtraction of the non-saturable component are plotted as open circles. The solid line is a replot of the fitted saturable component from Fig. 2A, and not a least-squares regression of the corrected data.

Effect of nucleobases on allopurinol transport

To characterize the substrate specificity of the allopurinol transport system, the effect of various purines was tested. Inhibition of allopurinol transport was assayed in dose–response studies (Table 2).

Kinetic analysis of the relationship between nucleobase and allopurinol transport

Since the dose–response studies showed that purine nucleobases inhibited the transport of allopurinol, further studies were performed. The nature of the inhibition was characterized using a range of allopurinol concentrations and various concentrations of nucleobases. The K_i values were similar to the respective K_m values. Both adenine (Fig. 3A) and hypoxanthine (data not shown) were found to inhibit the influx of [^{3}H]allopurinol in a competitive manner. The apparent "mixed-type" inhibition at high inhibitor concentrations is frequently observed, and does not disprove competitive inhibition [12].

Allopurinol was a competitive inhibitor of hypoxanthine transport (Fig. 3B). Summaries of the kinetic parameters derived from these and related experiments are presented in Table 2. Figure 3A and B show Lineweaver–Burk diagrams of the inhibition of allopurinol transport by adenine, and the inhibition of hypoxanthine transport by allopurinol, respectively. As in Fig. 2B the solid lines are replots of the fitted saturable component of transport, and not a least-squared regression of the reciprocal data.

Effect of oxypurinol and theophylline on allopurinol influx

Oxypurinol, the main metabolite of allopurinol, and like allopurinol an effective inhibitor of xanthine oxidase, was tested for inhibition of allopurinol transport in dose–response experiments (Table 2). Theophylline, a methylated purine used widely in the treatment of asthma, was similarly tested (Table 2).

DISCUSSION

The mechanism of allopurinol transport into human erythrocytes was investigated. The structural relationship between allopurinol and purines suggested the use of an inhibitor stop method with papaverine as the chemical stopper [6]. Papaverine that had been used to inhibit purine permeation also inhibited allopurinol transport completely and instantaneously: cell-associated allopurinol at zero-time was exclusively extracellular, as determined by

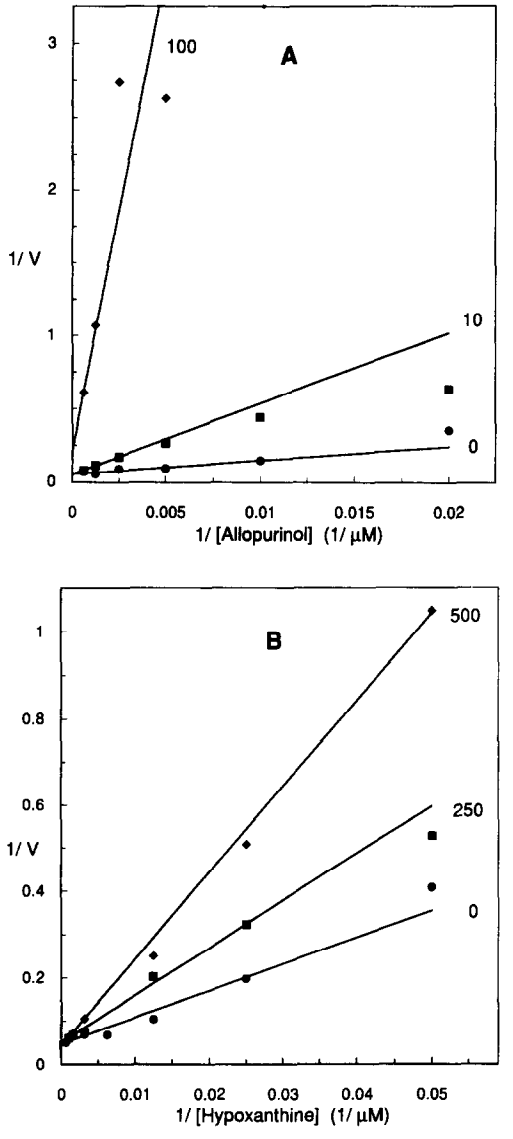


Fig. 3. Mutual inhibition of allopurinol and purines on each other's transport. Calculation of initial rates and kinetic parameters was carried out by a non-linear regression of Eqn 3 as described in Materials and Methods. (A) Lineweaver–Burk plot of the initial rates of allopurinol transport in the absence and in the presence of 10 and 100 μmol/L adenine. (B) Lineweaver–Burk plot of the initial rates of hypoxanthine transport in the absence and in the presence of 250 and 500 μmol/L allopurinol.

comparison to cell-associated inulin under similar experimental conditions. Kinetic transport analysis was not complicated by enzymatic transformation, since allopurinol was not metabolized.

The concentration dependence of allopurinol influx can be resolved into two components: (1) a saturable hyperbolic component and (2) a non-saturable linear component, presumably non-facilitated diffusion. Thus allopurinol transport conforms to a pattern that has been established previously for purine transport [6, 7]. Our mutual inhibition studies show that allopurinol and purines are competitive inhibitors of the transport of each other, since their K_i and K_m values are very similar. These lines of evidence suggest that purines and allopurinol share a common membrane transport system. While in the case of xanthine oxidase allopurinol has a K_i of $1.9–7 \times 10^{-7}$ mol/L, an order of magnitude higher than the K_m of xanthine ($3–4 \times 10^{-6}$ mol/L) [4, 13, 14], in the case of the membrane carrier allopurinol and purines compete with each other on roughly equal terms.

Another substrate for this transport system, which was not examined in our study since it is well documented in the literature, is undissociated uric acid [15–18]. As a consequence, allopurinol may interfere with uric acid transport and slow down uric acid redistribution from the plasma to the cells when given in the therapy of hyperuricemia. This effect is in addition to allopurinol's well-established role in inhibiting uric acid synthesis. Commencement of allopurinol treatment would thus lead to a transient increase in serum uric acid levels by inhibiting its membrane transport to the surrounding tissues. The uricostatic action, on the other hand, will take longer to have an effect, since it depends on intracellular allopurinol inhibition of the formation of uric acid. Thus our findings supply a possible explanation for a well-known clinical side effect of allopurinol: initiation of allopurinol treatment may provoke an attack of gout in hyperuricemic patients [19, 20]. However, pharmacokinetic data do not seem to support this hypothesis on first site. Allopurinol plasma levels do not rise above 15 μmol/L with the standard oral dosage of 300 mg/day [4, 21]. Given the K_m value of 268 μmol/L that we measured, the plasma levels are not high enough to have a physiological significant effect on uric acid transport into tissues. On the other hand, oxypurinol that acts with an IC_{50} of 20–40 μmol/L may well inhibit uric acid transport *in vivo*, since standard oral doses of allopurinol result in serum oxypurinol levels of about 65 μmol/L [4].

Theophylline has been shown to cause hyperuricemia in asthmatic patients [22]. Here therapeutic serum levels of about 100 [23] to 200 μmol/L [22] are reached, which are below the IC_{50} value of about 200–400 μmol/L that we measured. Along with the effect on transport, the slight inhibition of hypoxanthine–guanine phosphoribosylpyrophosphate transferase by theophylline reported by Morita [22] could act synergistically to raise serum uric acid levels.

Erythrocytes seem to have a common transport system for undissociated uric acid, purines, allopurinol, and oxypurinol. This finding was also surprising, since these substances are handled differently in the kidney: here oxypurinol and charged uric acid are reabsorbed in the proximal tubule by the anion exchange carrier, while allopurinol, undissociated uric acid and other purines are simply excreted [3, 21, 24]. Further studies are required to elucidate the transport of uric acid in various tissues.

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REFERENCES

1. Elion GB, The purine path to chemotherapy. *Science* **244**: 41–47, 1989.
2. Rundles RW, The development of allopurinol. *Arch Intern Med* **145**: 1492–1503, 1985.
3. Elion GB, Yü T-F, Gutman AB and Hitchings GH, Renal clearance of oxipurinol, the chief metabolite of allopurinol. *Am J Med* **45**: 69–77, 1968.
4. Elion GB, Allopurinol and other inhibitors of urate synthesis. In: *Handbook of Experimental Pharmacology, Vol. 51, Uric Acid* (Eds. Kelley WN and Weiner IM), pp. 485–514. Springer, Berlin, 1978.
5. Plagemann PGW, Woffendin C, Puziss MB and Wohlhueter RM, Purine and pyrimidine transport and permeation in human erythrocytes. *Biochim Biophys Acta* **905**: 17–29, 1987.
6. Domin BA, Mahony WB and Zimmerman TP, Purine nucleobase transport in human erythrocytes. Reinvestigation with a novel "inhibitor-step" assay. *J Biol Chem* **263**: 9276–9284, 1988.
7. Kraupp M, Marz R, Prager G, Kommer W, Razavi M, Baghestanian M and Chiba P, Adenine and hypoxanthine transport in human erythrocytes: distinct substrate effects on carrier mobility. *Biochim Biophys Acta* **1070**: 157–162, 1991.
8. Wohlhueter RM, Marz R, Graff JC and Plagemann PGW, A rapid-mixing technique to measure transport in suspended animal cells: applications to nucleoside transport in Novikoff rat hepatoma cells. *Methods Cell Biol* **20**: 211–236, 1978.
9. Marz R, Wohlhueter RM and Plagemann PGW, Purine and pyrimidine transport and phosphoribosylation and their interaction in overall uptake by cultured mammalian cells. A re-evaluation. *J Biol Chem* **254**: 2329–2338, 1979.
10. Bevington PR, *Data Reduction and Error Analysis for the Physical Sciences*. McGraw-Hill, New York, 1969.
11. Kolassa N, Roos H and Pfleger K, Separation of purine derivatives by thin-layer chromatography on silica gel plates suitable for metabolic studies. *J Chromatogr* **66**: 175–177, 1972.
12. Deves R and Krupka RM, Testing transport systems for competition between pairs of reversible inhibitors. Inhibition of erythrocyte glucose transport by cytochalasin B and steroids. *J Biol Chem* **255**: 11870–11874, 1980.
13. Elion GB, Taylor TJ and Hitchings GH, Kinetics of the inhibition of xanthine oxidase by allopurinol and alloxanthine. *Fed Proc* **25**: 748, 1966.
14. Massey V, Komai H, Palmer G and Elion GB, On the mechanism of inactivation of xanthine oxidase by allopurinol and other pyrazolo[3,4-*d*]pyrimidines. *J Biol Chem* **245**: 2837–2844, 1970.
15. Lassen UV, Kinetics of uric acid transport in human erythrocytes. *Biochim Biophys Acta* **53**: 557–569, 1961.
16. Lassen UV and Overgaard-Hanse K, Hypoxanthine as inhibitor of uric acid transport in human erythrocytes. *Biochim Biophys Acta* **57**: 111–117, 1962.
17. Lassen UV, Hypoxanthine transport in human erythrocytes. *Biochim Biophys Acta* **135**: 146–154, 1967.
18. Lucas-Heron B and Fontenaille C, Urate transport in human red blood cells. Activation by ATP. *Biochim Biophys Acta* **553**: 284–294, 1979.
19. Grahame R and Scott JT, Clinical survey of 354 patients with gout. *Ann Rheum Dis* **29**: 461–468, 1970.
20. McInnes GT, Lawson DH and Jick H, Acute adverse reactions attributed to allopurinol in hospitalised patients. *Ann Rheum Dis* **40**: 245–249, 1981.
21. Berlinger WG, Park GD and Spector, The effect of dietary protein on the clearance of allopurinol and oxypurinol. *N Engl J Med* **313**: 771–776, 1985.
22. Morita Y, Nishida Y, Mamatani N and Miyamoto T, Theophylline increases serum uric acid levels. *J Allergy Clin Immunol* **74**: 707–712, 1984.
23. Forth W, Henschler D and Rummel W (Eds.) *Allgemeine und Spezielle Pharmakologie und Toxicologie*, 5th Edn. Bibliographisches Institut, Mannheim, Germany, 1987.
24. Park GD, Berlinger WG, Spector R, Kitt TM and Tsalikian E, Sustained reductions in oxipurinol renal clearance during a restricted diet. *Clin Pharmacol Ther* **41**: 616–621, 1987.