THE URICOSURIA AND OROTIC ACIDURIA INDUCED BY 6-AZAURIDINE *

By HAROLD J. FALLON,† EMIL FREI, III, JEROME BLOCK AND
J. EDWIN SEEGMILLER

(*From the laboratories of the Chemotherapy Service, General Medicine Branch of the National Cancer Institute and the Arthritis and Rheumatism Branch of the National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.*)

The pyrimidine analog 6-azauridine (6-AZUR) interrupts the major pathway for the *de novo* biosynthesis of pyrimidine nucleotides after its biological conversion to 6-azauridine-5'-phosphate (6-AZUMP). 6-AZUMP is a competitive inhibitor of the enzyme which decarboxylates orotidylic acid (see Figure 1) (1, 2). 6-AZUR inhibits bacterial reproduction as well as tumor growth in animals (3–6). The latter effect has recently led to its use in the experimental treatment of human leukemia and solid tumors (7, 8).

Therapy with 6-AZUR in man, conducted at

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† Present address: Department of Medicine, University of North Carolina, Chapel Hill, N. C.

the National Cancer Institute, was occasionally complicated by a pronounced crystalluria. The crystals were separated into two components by ion-exchange chromatography and were identified by their ultraviolet absorption spectra as uric and orotic acid. The appearance of orotidine in tissues and orotic acid and orotidine in the urine of mice receiving 6-AZUR has been previously noted (1, 3) and may be explained by the metabolic block produced by the drug. These compounds have also been noted in the urine of humans receiving 6-AZUR or 6-azauracil by several investigators (9–12). More detailed study of the urinary excretion pattern of orotic acid and orotidine in response to 6-AZUR administration in the human forms one part of this investigation.

KEY
1) ATP = Adenosine Triphosphate
2) FAD = Flavin Adenine Dinucleotide
3) DPN = Diphosphopyridine Nucleotide
4) PRPP= Phosphoribosyl Pyrophosphate
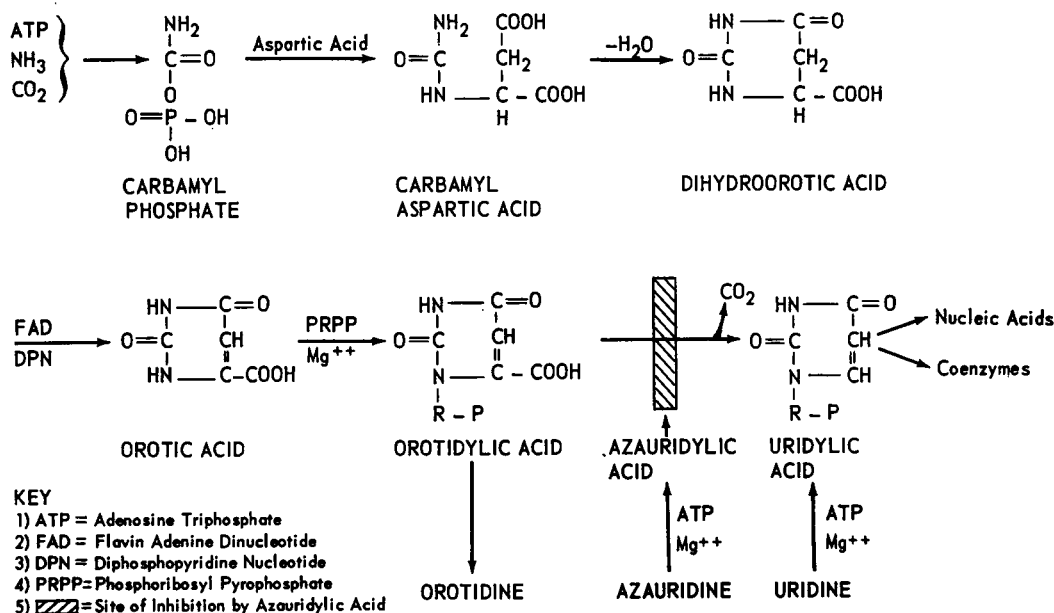5) ▨▨ = Site of Inhibition by Azauridylic Acid

FIG. 1. *De novo* SYNTHESIS OF PYRIMIDINE NUCLEOTIDES. The main pathway leads from carbamyl-phosphate to orotidylic acid and then by decarboxylation to uridylic acid. A less important pathway for most mammalian tissues involves the conversion of uridine to uridylic acid. Azauridylic acid is formed from azauridine by a kinase reaction and inhibits the decarboxylation step.

TABLE I

The changes in serum and urinary urate of representative patients receiving Azauridine. Similar changes have occurred in all patients studied

Patient Diagnosis *	Urine Urate		Serum Urate		Duration of Therapy	Dose of 6-AZUR
	Average Before Therapy	First Day on Therapy	Average Before Therapy	Last Day on Therapy		
	Mg/24 hrs.	Mg/24 hrs.	Mg %	Mg %	Days	Mg/Kg/day
CML	798	2,953	9.5	2.4	20	120
CLL	552	949	4.2	2.6	17	120
CML	750	2,070	10.5	3.1	3	120
CML	1,170	2,145	5.9	2.6	5	60
P.V.	487	1,278	8.6	3.4	4	120
CLL	292	835	6.8	4.2	2	120

* CML — Chronic Myelogenous Leukemia
CLL — Chronic Lymphocytic Leukemia
P.V. — Polycythemia Vera.

The presence of urate crystalluria led to the demonstration of a decline in the serum urate levels and an increased urinary excretion of uric acid during therapy with 6-AZUR. This finding was totally unanticipated, and an attempt was made to determine whether the uricosuria induced by 6-AZUR resulted from a primary renal effect or was related to its metabolic effect and the accompanying orotic aciduria.

METHODS

Patients with nonterminal malignant disease who were to receive intravenous 6-AZUR therapy were placed on diets essentially free of purines and pyrimidines including orotic acid [1] for at least 5 days prior to and throughout their study period. Patients received 6-AZUR in four 2-hour infusion periods daily for 3 to 20 days and were maintained on a constant intravenous infusion of 5 per cent dextrose and water during the remainder of the day. For 12 to 24 hours prior to 6-AZUR or orotic acid administration patients received 1 to 1.5 L of 5 per cent dextrose in water as a control period to correct for any possible uricosuric effect of the large volumes of fluid used for the administration of 6-AZUR. All urine was refrigerated during collection with toluene as a preservative and aliquots were frozen for the subsequent determination of the daily excretion of orotic acid, orotidine and 6-AZUR. Creatinine and uric acid were determined on fresh urine specimens after warming to assure that any crystals were dissolved.

The effect of infusion of 6-AZUR on the renal clearance of inulin and urate was also determined in three patients in order to better define the mechanism of action

of the drug. In addition, urate excretion during the infusion of orotic acid and orotidine was studied.

Preparation of infusions. 6-AZUR obtained from the Cancer Chemotherapy National Service Center was prepared in isotonic sodium bicarbonate at a concentration of 1 g per 10 ml and sterilized by filtration through Millipore filters (0.45 μ). Appropriate quantities of this material were than added to 150 ml of 5 per cent dextrose and water for intravenous infusion during a 2-hour period.

Orotic acid (California Corporation for Biochemical Research) was dissolved in 5 per cent dextrose and water at the concentration of 2 mg per ml by adjusting to pH 7 with sodium hydroxide. This concentration was found to be stable for several months at room temperature after sterilization by filtration (*vide supra*). More concentrated solutions tended to crystallize at room temperature. This material was infused without further dilution.

Orotidine (obtained through the courtesy of R. E. Handschumacher) was administered as the sodium salt in a concentration of 1 mg per ml of 5 per cent dextrose and water.

Analytical methods. The uric acid concentration of serum and urine was determined by the spectrophotometric method of Kalckar (14) as modified by Liddle, Seegmiller and Laster (15). Addition of orotic acid or 6-AZUR to urine did not inferfere with urate determinations by this method. Serum and urine creatinine was determined by the method of Bonsnes and Taussky (16). Inulin was determined as described by Walser, Davidson and Orloff (17).

The pyrimidine compounds were determined by a spectrophotometric method after separation by ion-exchange chromatography.[2] One-ml aliquots of urine, di-

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[1] Milk- and milk product-free (13).

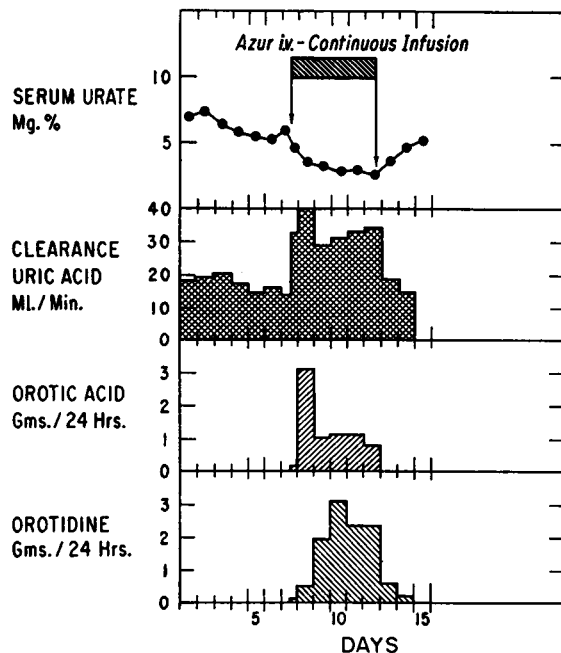[2] Based on method of R. E. Handschumacher.

Fig. 2. INCREASE IN URINARY CLEARANCE OF URIC ACID DURING CONTINUOUS 6-AZAURIDINE INFUSION. The excretion pattern of orotic acid and orotidine shows the rapid peak in orotic acid excretion and the presence of orotidine in the urine for 48 hours following cessation of 6-azauridine.

luted 1 : 10 with 0.05 M ammonium formate, were passed through 5 ml of Dowex 1-X10 formate 200-400 mesh in 1 × 30 cm columns. The resin was washed with boiled distilled water for 6 to 8 hours both before and after application of the urine to reduce the background optical density. Ten-ml fractions of eluate were collected during successive elution with 30 ml of 0.05 N formic acid, 30 ml of 0.1 N formic acid, 10 ml of water and 150 ml of 0.3 N ammonium formate, pH 5, at a flow rate of 0.5 ml per minute. Known pyrimidine compounds were added to normal control urine and identified in the eluate by their ultraviolet absorption spectra as determined in a

Cary recording spectrophotometer. 6-AZUR was found in fractions 1-3, orotidine in 9-11, and orotic acid in 15-19.

The pattern of elution was determined for each urine sample studied by measurement of the optical density of each fraction at 266 and 279 mμ in a Unicam S. P. 500 spectrophotometer. The quantity of pyrimidine present in each sample was calculated from the optical density at the appropriate wave length of pooled fractions using the following molar extinction coefficients: E_{262} 6-AZUR, 6,100; E_{266} orotidine, 10,000; E_{279} orotic acid, 7,580. A small correction in this value was made for any optical density contributed by other substances present in the urine prior to drug therapy or resulting from the action of formate on the resin bed itself.

Concentrations of these compounds above 5 mg per 100 ml were accurately determined by these methods, although the presence of each could usually be noted in concentrations as low as 2 mg per 100 ml.

Renal clearance studies. All clearance studies were performed on catheterized subjects by the technique of Smith (18). Clearance studies consisted of three 20-minute control periods, three 20 to 30-minute periods during infusions of 6-AZUR and two 20 to 40-minute collections after discontinuing the infusion. Urine was then collected in 4-hour fractions after removal of the catheter and corresponding plasma samples obtained for determination of urate and creatinine clearance.

RESULTS

Uricosuria during 6-AZUR therapy. The uricosuric effect of 6-AZUR observed during therapy of six patients with chronic leukemia on whom daily 24-hour urate and creatinine clearances were obtained is shown in Table I. The urinary excretion of uric acid was increased two- to threefold and the serum urate levels were reduced by 40 to 75 per cent during therapy with 6-AZUR. Figure 2 illustrates the twofold increase in uric acid clearance, the decrease in serum urate levels and the concomitant appearance in the urine of orotic

TABLE II

Relation of uric acid excretion to dose of Azauridine

Dose 6-AZUR (mg/24 hrs)	none	70	700	1,400	2,800	450
24-hr. urate (mg)	378	350	469	408	675	744
Serum urate (mg %)	6.68	6.57	6.28	6.05	5.57	4.86
* C creatinine (ml/min)	124	117	126	85	136	139
C uric acid (ml/min)	3.9	3.7	5.0	4.6	8.1	10.6
Ratio $\frac{Cua}{C\ creat.}$ × 100	3.2	3.2	4.0	5.4	5.9	7.6
Orotic Acid (mg/24 hrs)	0	19	42	33	81	65
Orotidine (mg/24 hrs)	0	0	52	205	299	221

* C = clearance

TABLE III

Clearance studies during the acute infusion of azauridine *

		Clearance periods†	Plasma uric acid	U.V./min	Clearance U.A.	Clearance inulin	$\frac{C_{uA}}{C_{In}} \times 100$	Orotic acid†
			mg %	mg/min	ml/min	ml/min		mg
Malignant melanoma F, 42	AZUR 4.6 g	1	3.06	0.587	19.2	145	13.2	0
		2	3.23	0.653	20.2	147	13.7	0
		3	3.38	0.582	17.2	128	13.4	0
		4	3.19	0.538	16.9	119	14.2	0
		5	3.15	0.693	22.0	133	16.5	0
		6	3.28	0.780	23.8	141	16.9	0
		7	3.21	0.769	23.9	131	18.2	0
		8	3.03	0.815	26.9	124	21.7	3
Chronic myelogenous leukemia M, 31	AZUR 4.8 g	1	6.58	0.927	14.09	144	9.75	0
		2	6.87	0.986	14.36	129	11.14	0
		3	6.74	1.211	17.97	150	11.98	0
		4	6.66	1.462	21.95	144	15.17	0
		5	6.32	1.761	27.87	161	17.25	0
		6	6.43	1.796	27.93	151	18.51	0
		7	6.30	1.806	28.67	152	18.85	0
		8	6.28	2.011	32.01	162	19.69	0
Polycythemia vera M, 68	AZUR 5 g	1	10.50	0.656	6.25	85.1	7.3	0
		2	10.98	0.673	6.13	81.7	7.5	0
		3	10.88	0.723	6.65	85.5	7.8	0
		4	11.10	0.805	7.25	88.2	8.2	0
		5	11.14	0.911	8.18	75.8	10.8	0
		6	11.04	1.406	12.74	88.0	14.5	0
		7	11.06	1.474	13.33	77.3	17.2	9
		8	10.66	1.478	13.86	82.0	16.9	17

* The following abbreviations are used: F = female; M = male; U.V. = urine concentration times volume; U.A. = uric acid; C_{uA} = uric acid clearance; and C_{In} = inulin clearance.

† Each period was 20 minutes.

‡ Absence of orotic acid denotes <2.5 mg%; orotidine was absent (<5 mg%) from all urines.

acid and orotidine in quantities as large as 3 g daily as a result of the daily infusion of 4.2 g of 6-AZUR. This patient showed no therapeutic response to treatment with 6-AZUR, and therefore a pronounced dissolution of leukemic tissue in response to chemotherapy probably did not contribute appreciably to his increased urate excretion.

The effects of gradually increased daily doses of 6-AZUR on various parameters of urate excretion are shown in Table II. Infusion of 2,800 mg of 6-AZUR per day produced a definite uricosuria. Lower doses did not cause the urate retention often seen with uricosuric drugs (19).

Clearance studies during 6-AZUR infusion. The magnitude and duration of the effects of 6-AZUR on urate and inulin clearance were simultaneously determined. A prompt increase in uric acid clearance and urate/inulin clearance ratios without alteration in inulin clearance was noted after 6-AZUR infusion (Table III). Orotic acid did not appear in the urine until at least 90 minutes after the start of 6-AZUR infusion and was un-

accompanied by orotidine for the duration of the clearance studies.

The time relationship of uricosuric response to the 6-AZUR infusion and to the urinary excretion of orotic acid (Figure 3) suggested that the uricosuria induced by 6-AZUR could be resolved into two distinct components. The prompt initial uricosuric response to 6-AZUR infusion was unassociated with orotic acid excretion, while the maximal uricosuric effect occurred simultaneously with the greatest excretion of orotic acid. These findings led to an investigation of the uricosuric effect of orotic acid.

Uricosuria induced by orotic acid. The intravenous infusion of 1 g of orotic acid during a 12-hour period in two patients was found to result in a two- to threefold increase in uric acid excretion rate and a 50 per cent decrease in serum urate (Figure 4). The absolute increase in the rate of uric acid excretion (in milligrams per hour) closely paralleled the rate of orotic acid excretion (Figure 4). An infusion of 100 mg of orotic acid in a 12-hour interval produced no uricosuria in a
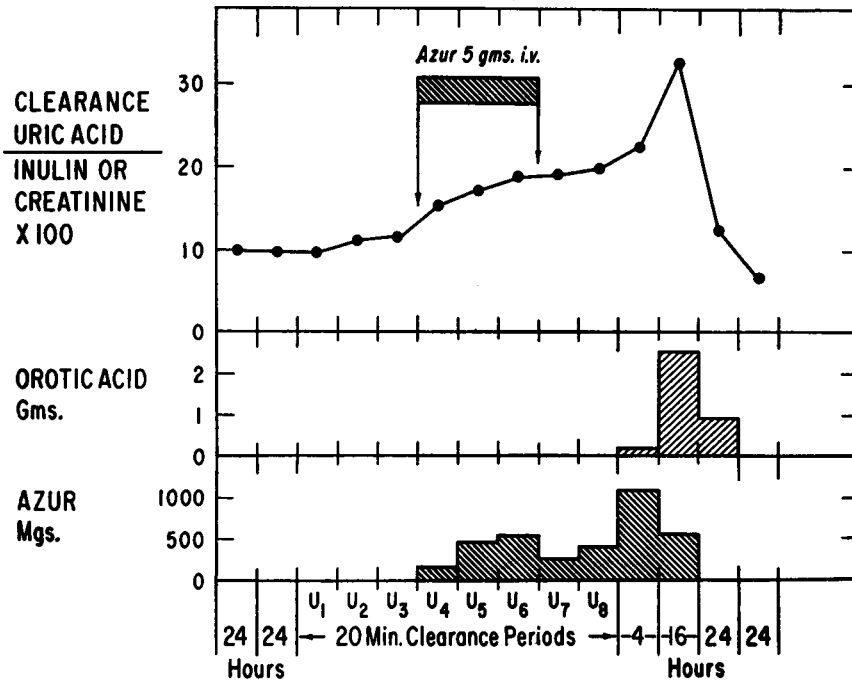
FIG. 3. RELATIONSHIP OF INCREASED URIC ACID CLEARANCE TO THE URINARY EXCRETION OF 6-AZAURIDINE AND OROTIC ACID. Maximal uricosuria occurs simultaneously with maximal orotic aciduria. This patient had polycythemia vera (see Table III).
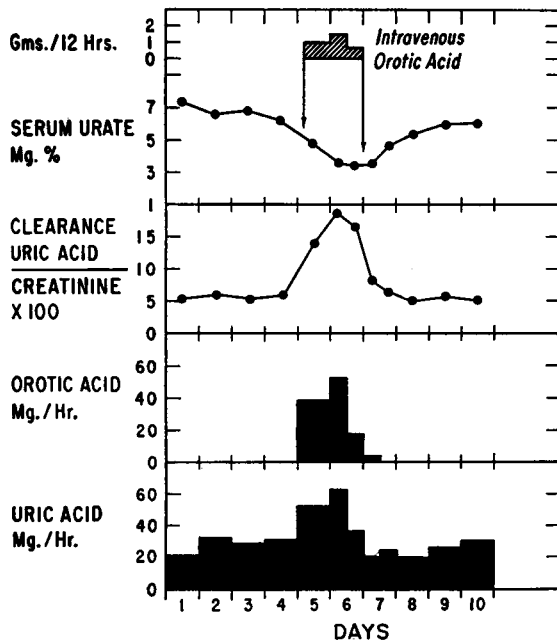

FIG. 4. URICOSURIA PRODUCED BY OROTIC ACID INFUSION. Prompt uricosuria occurs which closely follows, in magnitude, the amount of orotic aciduria.

patient who developed a prompt uricosuria when receiving 2 g per 12 hours.

The urinary recovery of intravenously administered orotic acid was 26 per cent during infusion of 1 g per 12 hours but increased to 48 per cent when the infusion rate was doubled. When orotic acid was given orally in amounts varying from 500 mg to 5 g accompanied by 1 to 2 g sodium bicarbonate to elevate gastric pH, there was no detectable urinary orotic acid and no uricosuria. The intravenous infusion of 660 mg of orotidine to one patient over a 12-hour period produced no increase in urate excretion.

The orotic aciduria and orotidine excretion induced by 6-AZUR. The nature of the major metabolic alteration produced by 6-AZUR was investigated by measuring the urinary excretion pattern of orotic acid and orotidine in patients receiving 6-AZUR. Orotic acid appeared in the urine 90 minutes to 4 hours after the start of 6-AZUR administration, but orotidine did not appear until 1 to 4 hours after the onset of orotic acid excretion (Figure 5).

The largest quantities of orotic acid and oroti-dine were excreted by patients receiving large amounts of 6-AZUR (120 to 200 mg per kg) and especially by those with chronic myelogenous leukemia. The maximum excretion of orotic acid occurred within 3 days and then declined to a constant level (Figure 2). By contrast the maximal excretion of orotidine appeared later and continued at a high level throughout 6-AZUR administration. Furthermore, orotidine persisted in the urine for 24 to 72 hours after cessation of both 6-AZUR and orotic acid excretion.

There was no observed correlation of orotic acid or orotidine excretion with the antileukemic effects of 6-AZUR. Also, variations in the degree of inhibition of the decarboxylation of orotic acid, as measured by an *in vitro* isotopic assay method (8), in intact leukemic cells, was not well correlated with the magnitude of orotic aciduria.

DISCUSSION

Metabolic alterations. The metabolic alterations resulting from the administration of 6-AZUR to the human are best interpreted as resulting from its inhibition of the decarboxylation of oro-tidylic acid (Figure 1). Further evidence that this is the site of action of 6-AZUR *in vivo* is the demonstration that intact leukocyte preparations obtained from patients receiving 6-AZUR show a depressed ability to decarboxylate orotic acid (8). *In vitro* studies have shown that 6-AZUR must be phosphorylated by a kinase reaction (requiring ATP and Mg^{++}) in order to function as a competitive inhibitor of this reaction (1, 2). Uri-dine is in turn a competitive inhibitor of this phosphorylation step *in vitro* (1) as well as an alternate precursor of uridylic acid, and therefore was restricted in the diets of patients receiving 6-AZUR therapy.

Accumulation of orotic acid has been noted in a variety of biological systems. It appears in certain mutant bacteria grown on a minimal pyrimidine media (20), in a rare congenital disorder (orotic aciduria) in man (21), presumably caused by a depressed activity of orotic acid pyro-phosphorylase or orotidylic decarboxylase or both (see Figure 1) (22), and as a result of 6-AZUR administration in both bacteria and animals (1, 4, 9, 10). Feedback control by pyrimidine nucleo-

tides of the production of orotic acid has been described in mutant bacteria by Yates and Pardee (23, 24), in Ehrlich ascites cells (25) and in congenital orotic aciduria in the human by Huguley, Bain, Rivers and Scoggins (21).

In mutant bacteria, increased production of as-partate-carbamyltransferase occurs in response to "pyrimidine starvation." The most potent inhibitors of this increased enzyme production are compounds derived from uracil (24), and in addition, cytidine 5'-phosphate is a competitive inhibitor of the same reaction (23). In one case of congenital orotic aciduria the increased orotic acid excretion was significantly depressed by oral administration of a cytidylic-uridylic acid mixture, which suggests a feedback inhibition by pyrimidine nucleotides of orotate synthesis (21) in man.

The finding of increased levels of activity of the first three enzymes of *de novo* pyrimidine biosynthesis (aspartate-carbamyltransferase, dihydro-orotase and dihydro-orotic dehydrogenase; see Figure 1) in the white cells of untreated leukemic patients (26) may explain the prompt and massive excretion of orotic acid in leukemic patients receiving 6-AZUR. The possibility also exists that "pyrimidine starvation" (23) induced by 6-AZUR further increases the production of orotic acid by releasing the feedback inhibition which normally regulates pyrimidine biosynthesis. This possibility is currently under investigation.
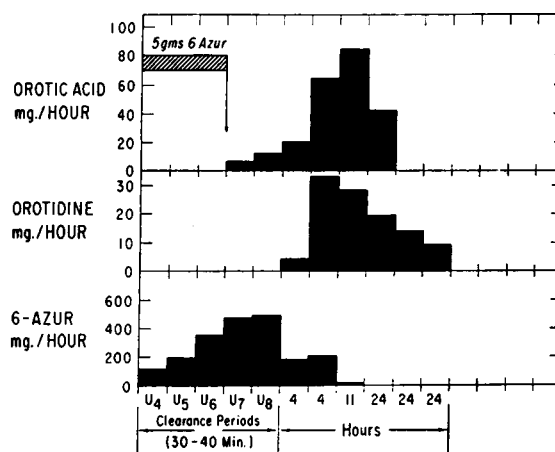

FIG. 5. EXCRETION PATTERN OF OROTIC ACID AND OROTI-DINE AFTER 6-AZUR INFUSION. The excretion of orotic acid precedes orotidine and the former disappears more rapidly from the urine. The urinary excretion of 6-AZUR is also noted. The patient had polycythemia vera (see Table III).

Orotidylic acid is the presumed source of the large quantity of orotidine excreted in the urine of patients receiving 6-AZUR, since *in vitro* studies in mammalian tissues, by isotope incorporation methods and assay of the conversion of orotidine to orotic acid, strongly suggest that orotic acid is not a direct precursor of orotidine (1). The consistent appearance in the urine of orotic acid before that of orotidine and the gradually increasing orotidine excretion associated with a decline in orotic acid excretion do suggest, however, that an accumulation of orotic acid may lead to an increase in its conversion to orotidylic acid and then by dephosphorylation to orotidine. In this fashion orotic acid would be an indirect precursor of orotidine by means of its conversion to orotidylic acid. The *in vitro* equilibrium of the conversion of orotic acid to orotidylic acid is strongly in favor of orotic acid formation and, in addition, orotidylic acid is an inhibitor of the enzyme orotidylic acid pyrophosphorylase (27). In order to form large amounts of orotidine, significant production of orotidylic acid is necessary and would be expected to require a large accumulation of orotic acid. Other interpretations of this data exist, however, and the information now available does not prove the primacy of increased *de novo* orotic acid production as an explanation of the pattern of orotic aciduria and orotidine excretion induced by 6-AZUR.

Uricosuria. Increased uric acid excretion is a frequent occurrence during the treatment of leukemia (28, 29) and occasionally results in urate renal blockade. It is usually associated with hyperuricemia and the rapid destruction of leukemic tissue (30, 31). 6-AZUR therapy resulted in a uricosuric response in patients without leukemia or solid tumor as well as in leukemic patients, regardless of clinical response.

The data presented strongly suggest that both 6-AZUR and orotic acid are capable of producing a uricosuric effect. The correlation of maximal uricosuria with maximal orotic aciduria raised the possibility that orotic acid was the sole mediator of the uricosuria seen during 6-AZUR therapy, since orotic acid infusions alone produced increased urate excretion. The prompt uricosuric response to 6-AZUR infusion, which preceded the appearance of orotic acid in the urine, is evidence that 6-AZUR itself has uri-

cosuric properties. Furthermore, infusion of small quantities of orotic acid, which produced detectable amounts in the urine, had no significant uricosuric effect. This finding argues against the possibility that the initial uricosuria induced by 6-AZUR represented a response to quantities of orotic acid which were undetectable by our analytical methods. It is conceivable that the uricosuric action of 6-AZUR could also be mediated by an accumulation of orotic acid within the tubular epithelial cells but there is at present no evidence to support such a concept. In any event it appears that the initial uricosuric effect of 6-AZUR is not dependent on the excretion of orotic acid, and the increased urate clearance seen in patients receiving 6-AZUR results from a summation of the effects of both compounds.

The magnitude of the uricosuric response produced by 6-AZUR or orotic acid infusion resembles that of such moderately active agents as probenecid (32), adrenal steroids (33) and phenylbutazone (34, 35). The administration of each of these compounds usually results in uric acid clearances of 20 to 40 ml per minute in comparison with a normal clearance of 5 to 10 ml per minute. No attempt has been made, however, to produce a maximal uricosuric effect with 6-AZUR or orotic acid because of the potential hazard of orotic acid crystalluria. Neither of these new uricosuric agents has practical value, at the present, in the treatment of hyperuricemia because intravenous administration is necessary for their action. When administered orally, 6-AZUR is partially converted to 6-azauracil (36, 37), a compound which produces severe neurotoxicity in man (38). Oral administration of as much as 5 g per day of orotic acid produced no uricosuria and no orotic acid could be found in the urine.

Urate excretion by the kidney is interpreted by Gutman and Yü (39) to consist of glomerular filtration, nearly complete tubular reabsorption and active tubular secretion. Most uricosuric compounds are acids (zoxazolamine is an exception) (40) and are presumed to act primarily by inhibition of tubular reabsorption of urate. The urate-retaining properties of salicylates and phenylbutazone in low dosage are interpreted as resulting from inhibition of tubular secretion of urate (39). 6-AZUR and orotic acid have not produced this low-dose effect in our studies. 6-AZUR and

orotic acid are both organic acids which are actively secreted by the avian kidney (37, 41) and therefore have some characteristics in common with other uricosuric agents. In addition, two other possible explanations of their mode of action deserve consideration. There are certain structural similarities (Figure 6) (keto groups at corresponding positions on the pyrimidine ring and anionic form at physiological pH) which suggest that these compounds may compete for an enzymatic transfer mechanism that is based on these factors. The observed close quantitative relationship of orotic acid excretion to increases in uric acid excretion may be further evidence that these structural analogs of uric acid may compete for the same transport mechanism involved in urate reabsorption. Evidence has been obtained that urate secretion may also be influenced by the pyrimidine analog, 6-azathymine. We have confirmed the report (42) that this compound produces a urate retention as shown by diminished urate/creatinine clearance ratios.

Although it is possible that the uricosuric effect of 6-AZUR results directly from its inhibition of pyrimidine nucleotide synthesis in the renal tubules, there is at present no evidence to support such a concept. A possible role of uridine nucleotides in phosphate transport by the kidney has been suggested by de Verdier (43). No such role for pyrimidine containing coenzymes has as yet been proposed for other transport systems.

SUMMARY

1. Administration of 6-azauridine (6-AZUR) intravenously to patients with nonterminal malignancies resulted in the prompt appearance in their urine of orotic acid and, somewhat later, of orotidine. The appearance of these compounds, also noted by others, provides evidence that 6-AZUR produces the same block in pyrimidine biosynthesis *in vivo* in the human that has previously been noted *in vitro* and in other mammals. The pattern of urinary excretion of these compounds has been discussed with reference to a possible feedback control of pyrimidine biosynthesis in man.

2. The uricosuria induced by 6-AZUR administration resulted in part from the direct action of 6-AZUR and also from the uricosuric action of the orotic acid produced in response to the meta-
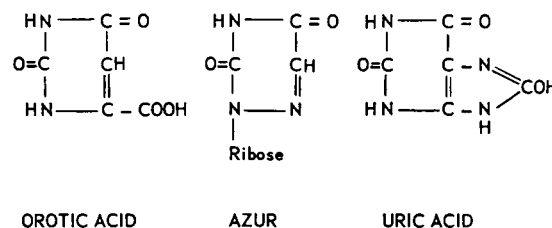

OROTIC ACID   AZUR   URIC ACID

FIG. 6. STRUCTURAL FORMULAS OF OROTIC ACID, 6-AZAURIDINE AND URIC ACID.

bolic block of pyrimidine biosynthesis. The necessity of administering these compounds by the intravenous route prevents their practical use in the management of gout. The chemical structures of 6-AZUR and orotic acid show certain points of similarity to uric acid and they may represent a new class of uricosuric agents based on structural analogs of uric acid. The role of pyrimidines in renal transport mechanisms is, at present, uncertain.

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