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Metabolic Inertness of Orotidine in Man and the Rat. (28769)

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(Introduced by N. I. Berlin)
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Orotidine is the pyrimidine nucleoside moiety of orotidylic acid, an intermediate in the biosynthesis of uridylic acid(1). It is found in elevated amounts in the urine of patients during 6-azauridine administration (2) and in the culture medium of a mutant of Neurospora crassa(3) but otherwise has not been described as occurring at more than trace levels in natural sources.

The only method presently available for quantitating pyrimidine production involves the assumption that the sole route for catabolism of orotic acid is by means of its initial conversion to uridylic acid(4). While conversion of orotic acid to orotidine and subsequent catabolism of orotidine would not change the formal validity of this method measuring pyrimidine ring synthesis, it would mean that the rate of pyrimidine ring biosynthesis would be an overestimate of utilizable pyrimidine synthesis, that is of synthesis of uridylic acid and its derivatives. Conversely, since no more than traces of orotidine normally occur in human urine(5), the conversion of orotic acid to orotidine could account for very little of the disposal of isotope from administered radioactive orotic acid, unless there are enzyme systems capable of catabolizing orotidine. Since it has been suggested that the capacity to metabolize orotidine might be significant in a pyrimidine biosynthetic pathway of lower organisms(6), it was considered necessary to exclude the possibility of such a pathway in humans. Finally, recent efforts to evaluate the effect of the metabolic antagonist 6-azauridine on pyrimidine metabolism in man(7) have been based on the assumption that orotidine excretion, following administration of this drug, is equal to orotidine production. Orotidine has not been found to be a substrate for phosphorylases and has been reported not to dilute the flow of isotope into pyrimidines in liver slices(8). However, because of the above considerations it was felt desirable to test the capacity of mammals to metabolize this substance using isotopic techniques and administering only tracer amounts of the compound.

Methods. C^{14} orotidine labeled in the 7 position was prepared from commercial C^{14} orotic acid as follows: A yeast enzyme preparation containing orotidylic acid pyrophosphorylase and decarboxylase was prepared according to the method of Lieberman *et al* (9). Orotidylic acid was synthesized by placing into a reaction vessel 10 ml of the yeast enzyme containing 14 units of orotidylic acid pyrophosphorylase per ml, 88 μc of orotic acid-7-C^{14} (specific activity 4.4 μc/μmole),* 20 μmoles 5 phosphoribosyl 1-pyrophosphate,† 20 μmoles 6-azauridine-5′ phosphate,‡ 400 μmoles $MgCl_2$, 1,800 μmoles NaF and 8,000 μmoles of Tris pH 8.0 in a final volume of 200 ml. This was allowed to react at 24°C for 10 minutes and the reaction stopped with perchloric acid. The acid soluble supernate was neutralized with KOH, brought to pH 8-9 with NH_4OH and placed on a 3 cm diameter, 40 ml bed volume Dowex-1-formate column. The column was eluted with a linear gradient of increasing concen-

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‡ Calbiochemical Corp., Los Angeles, Calif.

tration produced by 1 liter distilled water and 1 liter of 0.4 M ammonium formate. After 1500 ml of eluate, which removed all the orotic acid, the eluting fluid was changed to 1 M ammonium formate which eluted the orotidylic acid in the first 100 ml. The peak was desalted by diluting to 1 liter with water, alkalinizing with NH_4OH and placing it on a 1 cm diameter, 10 ml bed volume Dowex-1-formate column. The column was eluted with 0.2 N HCl until the orotidylic acid peak came off. This peak was titrated to pH 8 to 9 with NH_4OH and placed on a 4 mm diameter 2 ml bed volume Dowex-1-chloride column. This was eluted with a linear gradient of increasing concentration produced by 200 ml distilled water and 200 ml 0.4 N HCl. The orotidylic acid eluted as a single symmetrical peak between 40 and 60 ml of eluate. This was titrated to pH 7.0 with KOH to give a final concentration of 0.05 M KCl. The yield of orotidylic acid was 10% and its identity was established by its ultraviolet absorption spectrum(9), its migration with authentic orotidylic acid in high voltage electrophoresis in a 0.05 M ammonium formate pH 3.5 buffer, and by complete conversion of its radioactivity to carbon-14 carbon dioxide following incubation with purified yeast orotidylic acid decarboxylase(10). The specific activity of the product was 4.6 μc/μmole. The orotidylic acid was diluted with 0.15 M tris buffer pH 8.0 and incubated with 25 lambda of commercial *E. coli* alkaline phosphatase§ for 45 minutes at 37°C. Orotidine was isolated by placing the reaction mixture on an 11 × 1.1 cm Dowex-1-chloride column and eluting with a gradient established by placing 800 cc of water into the mixing chamber and 0.1 N hydrochloric acid into the reservoir. The orotidine solution emerging from the column was neutralized with sodium hydroxide and sterilized by filtration.

Two patients with malignant neoplastic disease were given a dose of 2.5 μc 7-C^{14} orotidine in 0.9% saline solution, intravenously, over a period of 30 minutes. All urine was collected in increasing interval periods following completion of infusion, and mid-period serum specimens were taken. Respiratory carbon dioxide was collected at intervals for the first 8 hours after isotope administration, using previously described techniques(4).

Each of four 250-300 g NIH stock white rats received 0.4 μc of C^{14}-orotidine, administered intraperitoneally along with 5 ml of normal saline. The animals were placed in metabolic cages and urine collections continued for 48 hours with ½ hour interruption for feeding at 24 hours.

Total radioactivity in urine was determined by placing 10 ml aliquots of serial dilutions of the urine on 2″ diameter aluminum planchets and counting on a low-background gas flow β-counter (background approximately 1 count per minute). Urine counts were carried out to ± 7% statistical accuracy and serum to ± 12%. Standards were simultaneously counted. The identity of labeled material in patients' urine was verified by adding 3 μmoles of non-radioactive orotidine to urine containing 150,000 disintegrations per minute of radioactivity. This was then chromatographed on Dowex-1 chloride as in the preparation of the orotidine. The material from the orotidine peak was lyophilized and the dried residue chromatographed by descending paper chromatography on Whatman No. 1 paper in 2 dimensions. The solvent for the first dimension was isobutyric acid, water, concentrated ammonia, 100:55.8:4.6(11) and for the second dimension n-butanol, acetic acid, water, 4:1:1(11). Location of radioactivity was determined by autoradiography with sheets of X-ray film and location of ultraviolet absorbing substances were determined by scanning with a mineral-lite ultraviolet lamp. Serum radioactivity (Table II) and creatinine content were measured at the mid-point of half-hour intervals of urine collections. Other determinations were rejected because of low count rates in serum. Serum orotidine was estimated by deproteinization of serum with 0.6 N $HClO_4$, and chromatography of the acid soluble material after KOH neutralization, employing Dowex-1-formate as previ-

§ Worthington Biochemical Corporation, Freehold, New Jersey.

ously described. In a control experiment, 79% of 0.20 μmoles of orotidine added to 5 ml of plasma was recovered.

A variety of incubations were performed with labeled orotidine and various fractions of rat liver homogenates, all in 0.02 tris buffer, pH 8.0, using various combinations of adenosine triphosphate, $MgCl_2$, lactic acid, reduced nicotine-adenine dinucleotide, reduced nicotine-adenine dinucleotide phosphate, and glucose-6-PO_4 in 3 ml volumes of 0.15 M tris pH 7.8 using standard enzymologic techniques. The homogenate was centrifuged in sequence for 10 minutes at 800 *g*, for 20 minutes at 15,000 *g* and then for 90 minutes at 100,000 *g* average in the Spinco model L ultracentrifuge. The supernate at each stage was tested for its ability to degrade orotidine. Control experiments in this laboratory have shown complete conversion of uracil, thymine and thymidine to unidentified products with at least some of these conditions.

Incubations were performed in Warburg flasks containing an internal central well with 200 μliter 2 N potassium hydroxide and having half ml of 6 normal perchloric acid in the side arm. After 30 minutes incubation at 37°C, the acid was tipped into the enzyme solution and the closed flask held at 37°C another 30 minutes. Released radioactive carbon dioxide was determined by counting an aliquot of the KOH in a liquid scintillation spectrometer. The identity of radioactivity in the remaining acid soluble portion was examined by the above described methods.

Results. Recoveries of urinary radioactive orotidine following its administration are presented in Table I. It may be seen that essentially quantitative recovery was obtained in both man and the rat. No sample of respiratory CO_2 contained significant amounts of radioactivity (<0.01% of the dose in 10 minutes). All of the urinary radioactivity behaved on Dowex-1 chloride as free orotidine and after paper chromatography of the lyophilized peak essentially all the radioactivity was seen as overlying the U-V absorbing orotidine spot. Traces of radioactivity coincided with traces of ultraviolet absorbing material that presumably were due to degradation of the orotidine during the processing of the urine. Most of the radioactivity in the orotidine was cleared into the urine within one to 2 hours in the 2 patients studied.

TABLE I. Recovery of Radioactive Orotidine in Urine.

Recipient	Time after inj	Cumulative recovery, %
Patient I	30 min	5.3
	60 "	21.8
	3 hr	54.7
	6 "	86.6
	12 "	97.5
Patient II	45 min	23
	90 "	43.5
	8 hr	84.5
	18 "	86
	50 "	96
Rat I	24 hr	119
II	24 "	103
III	48 "	90
IV	48 "	64

Approximate data for renal clearance of orotidine, estimated by total blood and urine radioactivity determinations performed during these studies are presented in Table II. This is compared with 24-hour clearance of orotidine determined on a patient while receiving 6-azauridine therapy. The renal clearance data (Table II) indicate figures for orotidine clearance approaching creatinine clearance, and do not suggest tubular excretion or vigorous tubular reabsorption of this substance. No correction was made for possible binding of orotidine to precipitated plasma protein, but no significant binding was detected in equilibrium dialysis experiment.

The results of the *in vitro* incubations were entirely negative and in no case was any radioactive carbon dioxide released or any detectable derivative formed from the orotidine. The conclusion of this study is that exogenous orotidine represents a non-metabolizable end product in man and the rat. The fact that there is no more than a trace of orotidine in human urine is an indication of the relative effectiveness of orotidylic acid decarboxylase as compared with the action of non-specific phosphatases at steady state levels of orotidylic acid in normal subjects.

TABLE II. Renal Clearance of Orotidine.

	Urine flow, ml/min	Serum orotidine, cpm/ml	Urine orotidine, cpm/ml	Orotidine clearance, ml/min	Creatinine clearance, ml/min
Patient I*	7.7	50	340	53	125
" II†	3.8	39.5	784	73	157
	8.3	14.5	276	139	131

Patient III‡	Serum orotidine, μmoles %	Serum orotidine (corrected for 79% recovery), μmoles %	Urine orotidine, μmoles/min	Orotidine clearance, ml/min
Day 1 of therapy	1.5	1.9	1.6	85
Day 2	2.7	3.4	4.2	125
Day 3	3.2	4.0	4.9	120
Day 6	2.6	3.3	3.2	116

* 61-yr-old man with chronic lymphocytic leukemia.
† 57-yr-old man with chronic lymphocytic leukemia.
‡ 60-yr-old man with chronic myelogenous leukemia receiving 6-azauridine therapy.

Summary. 7-C^{14}-labeled orotidine was prepared and administered parenterally to men and rats. The compound was recovered quantitatively in the urine and cleared from the plasma at rates approaching the glomerular filtration rate.

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Potassium Concentration in the Small Intestine of Guinea Pigs. (28770)

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The efflux of potassium from the small intestine of the guinea pig has been demonstrated *in vitro* to be influenced by certain drugs(1,2,3,4). These observations appear to lend themselves to adaptation as a possible bioassay of analgesic drugs, as reported by Benjamin(4). In our own investigation, it became apparent that the relationship between drug activity and potassium efflux varied considerably among isolated strips taken from different segments of the small intestine. Moreover, we were acutely aware of differences between adjacent strips. Such irregularities would appreciably alter or mask the effects of drugs and confuse rather than clarify the influence of drugs on potassium

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