Uricase Inhibition in the Rat by *s*-Triazines: An Animal Model for Hyperuricemia and Hyperuricosuria (33791)

W. J. Johnson, B. Stavric, and A. Chartrand

Food and Drug Research Laboratories, Department of National Health and Welfare, Tunney's Pasture, Ottawa, Canada

Uric acid in most mammalian species is converted to allantoin by uricase, an enzyme found predominantly in the liver. Uricase is lacking in man and the higher apes, and in these species uric acid rather than the more water-soluble allantoin is the urinary end-product of purine metabolism. This absence of uricase in man is undoubtedly the result of a mutational event in the anthropoid phylogeny, and the occurrence of gout uniquely in man leads to the inference that the lack of uricase is a necessary antecedent condition for the disease, the fundamental abnormal phenomenon of which is hyperuricemia.

To our knowledge, there has never been an appropriate animal model for research in this area of medicine. We have attempted to produce such a model by blocking the activity of hepatic uricase in the laboratory rat by means of selective inhibitors of the enzyme.

Symmetrical triazines comprise a large group of chemical compounds bearing a structural resemblance to the pyrimidine portion of the purine ring system of uric acid and xanthine. Indeed, Fridovich has shown that certain *s*-triazines are potent competitive inhibitors of uricase (1) and of xanthine oxidase (2) *in vitro*. But unlike uric acid, the *s*-triazines do not serve as substrates for uricase and hence their destruction by this enzyme is prevented. It was of interest, therefore, to determine whether a nontoxic member of the *s*-triazine group could be found which would effectively block uricase activity *in vivo* when administered orally, as indicated by its effect on blood and urine levels of uric acid and allantoin. The results of this investigation are described here. A preliminary report was presented recently (3).

Materials and Methods. Beef kidney uricase and milk xanthine oxidase were obtained from Worthington Biochemical Corporation. Uricase inhibition assays *in vitro* were carried out essentially as described by Fridovich (1) using a Beckman recorder attached to a Beckman DB-G spectrophotometer. The same equipment was used for studying *in vitro* inhibition of xanthine oxidase. All assays of xanthine oxidase activity were carried out at pH 7.5 in 0.03 *M* sodium phosphate buffer with hypoxanthine as substrate. The *s*-triazine inhibitors were added to the reaction vessels as a solution in sodium phosphate buffer, pH 7.5. The rate of formation of urate from hypoxanthine was determined by measuring increase in absorbancy at 290 mμ.

Oxonic acid was prepared as the potassium salt as described by Brandenberger (4). Analysis: Calculated for $C_4H_2O_4N_3K$:C, 24.61; H, 1.04; N, 21.53. Found: C, 24.63; H, 1.29; N, 21.00. Allantoxaidine was prepared from the di-ammonium salt of oxonic acid (5, 6). All batches of purified oxonate and allantoxaidine were assayed for *in vitro* uricase inhibitory activity (1). Other chemicals, including compounds shown in Table I, were obtained from commercial sources and were used without further purification.

Male Wistar rats (175–200 g) were randomized into control and experimental groups. They were fed pulverized commercial rat chow and had free access to water. In long-term studies the test compounds were incorporated into the diet. All rats were kept in separate metabolic cages; and food intake, weight gain, and urine volume were recorded daily. Plasma and urine uric acid was determined by Archibald's method (7), following protein precipitation by the procedure of Buchanan *et al.* (8). Kidneys were individually homogenized in 0.5% lithium carbonate to dissolve any crystalline uric acid which may have been present (9); the lithium carbonate extracts were assayed for uric acid content (7, 8). Urinary allantoin was deter-

TABLE I. Inhibition of Uricase and Xanthine Oxidase by *s*-Triazines *in Vitro*.

Compound tested		Molar conc for 50% inhibition	
Common name	Chemical name	Uricase[c]	Xanthine oxidase
Oxonic acid[b]	2,4-Dihydroxy-6-carboxy-1,3,5-triazine	8×10^{-7}	—[a]
Allantoxaidine	2-Hydroxy-1,3,5-triazine-4-one	5×10^{-6}	—[a]
Cyanuric chloride	2,4,6-Trichloro-1,3,5-triazine	1×10^{-5}	—[a]
Cyanuric fluoride	2,4,6-Trifluoro-1,3,5-triazine	5×10^{-5}	—[a]
Cyanuric acid	2,4,6-Trihydroxy-1,3,5-triazine	5×10^{-4}	—[a]
Trithiocyanuric acid	2,4,6-Trithiol-1,3,5-triazine	1×10^{-5}	1×10^{-4}
Trichloromelamine	N^2,N^4,N^6-Trichloro-2,4,6-triamino-1,3,5-triazine	5×10^{-5}	1×10^{-6}
Violuric acid	5-Hydroxyiminobarbituric acid	3×10^{-5}	5×10^{-5}

[a] No inhibition at 10^{-3} *M*; experimental conditions are described under "Materials and Methods."

[b] The same activity was obtained whether oxonic acid was added as the free acid, potassium salt or ammonium salt. The terms "oronic acid" and oxonate are used interchangably throughout this paper.

[c] The following compounds were not inhibitory at 10^{-3} *M*: *s*-triazine; melamine; propazine; atrazine; atrazide, tris-(2-hydroxyethyl)-*s*-triazine-2,4,6-(1*H*,3*H*,5*H*)-trione; 2,4-diamino-6-phenyl-*s*-triazine; 2-amino-4-morpholino-*s*-triazine; trialyl-*s*-triazine-2,4,6(1*H*,3*H*,4*H*)-trione; 2-amino-4-(*p*-chloroanilino)-*s*-triazine (insoluble at 10^{-3} *M*, no inhibition at 10^{-5} *M*).

mined according to Young and Conway (10).

Procedures and Results. A total of 18 *s*-triazines were tested for *in vitro* inhibition of uricase and xanthine oxidase activity. The compounds found to have the highest activity as uricase inhibitors are listed in Table I. The results shown for oxonic acid and allantoxaidine are in agreement with those reported by Fridovich (1). On the basis of their high potency as uricase inhibitors and comparatively insignificant activity as xanthine oxidase inhibitors, oxonic acid and allantoxaidine were selected for *in vivo* trials. It should be noted that valuable as xanthine oxidase inhibition *in vivo* might be in another setting, it was inimical to our purpose in the present situation.

In vivo *experiments.* Preliminary trials with graded doses indicated that 250 mg/kg was the optimum dose level for intraperitoneal injection. Thus, injection of 250 mg/kg of potassium oxonate into rats gave rise to a plasma urate level of 16.5 μg/ml (175% increase) 2 hr after injection; the plasma urate level after a 500 mg/kg dose was 16.1 μg/ml. Although similar results were obtained with allantoxaidine it was decided to use oxonate for further studies. The effect of multiple

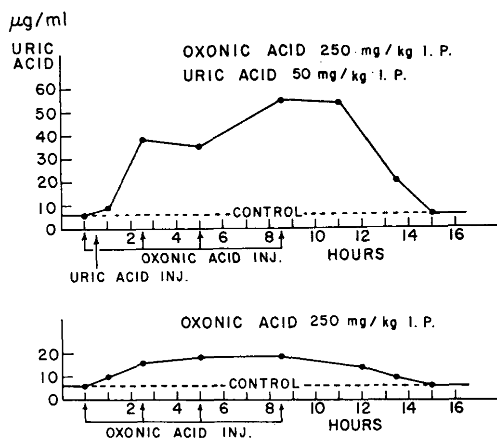

FIG. 1. Effect of 4 consecutive ip injections of 250 mg/kg of oxonic acid (as potassium salt) on blood plasma level of uric acid in rats. Upper graph: 50 mg/kg of uric acid was injected 30 min after first injection of oxonate. Blood samples were taken from the tail vein for uric acid determination at hours indicated by dots on the graph. Each point represents the average plasma urate concentration of 5 rats.

injections of oxonate at 2.5-hr intervals on plasma urate is shown in Fig. 1. At the time of the third injection plasma urate was at its maximum of 2.5 times the control. When a single injection of 50 mg/kg uric acid was

given 0.5 hr after the first oxonate injection (upper graph) the plasma urate level increased sharply to reach a peak of 12 times the control. No significant increase in plasma urate was observed when 50 mg/kg uric acid was injected into rats untreated with oxonate. These experiments demonstrate that oxonate is an effective inhibitor of uricase *in vivo*, but also indicate that it is rapidly inactivated or excreted, since normal levels of plasma urate are rapidly attained upon cessation of oxonate administration. When 5% of the normal rat diet was replaced by oxonate the plasma urate increased gradually until it reached a peak of 3 times the control (Fig. 2). Urinary allantoin dropped to 50% of normal within the first 24 hr and continued at this level of output for the duration of the experiment. Excretion of uric acid in the urine increased 4-fold in the first 24 hr and followed a pattern roughly parallel to the plasma urate level.

Supplementing the oxonate diet with 1% uric acid resulted in a further marked increase in plasma urate concentration (Fig. 3) and urine uric acid output (Fig. 4). Plasma urate rose sharply to reach a peak 6 times the control level on the ninth day of treatment, at which level it remained for the duration of the experiment. The addition of 1% uric acid alone to the normal diet, which provided an intake of approx. 200 mg of uric acid/rat/day, produced no appreciable effect on plasma urate (Fig. 3). Similarly, when both oxonate and uric acid were present in the diet a remarkably greater effect on urinary uric acid output was observed than when either was present individually (Fig. 4). Thus, urine output of uric acid in the group of rats consuming the oxonate–uric acid diet was 10 times the control within the first 24 hr followed thereafter by a steady rise to 46.6 mg/24 hr (22 times the control level) by day 20.

The high plasma and urinary urate levels observed in the presence of dietary oxonate plus uric acid suggested the possibility that, in the course of kidney tubular concentration of urine, the limit of uric acid solubility

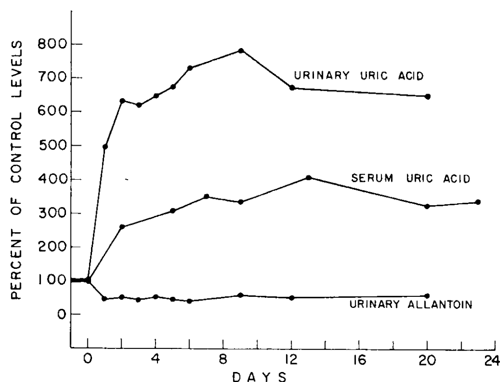

FIG. 2. Effect of dietary (5%) oxonate on serum and urinary uric acid and urinary allantoin in rats. The control group were fed pulverized rat cubes; the experimental group received the same diet containing 5% oxonate in admixture. Each point on the graph represents average values of 5 rats.

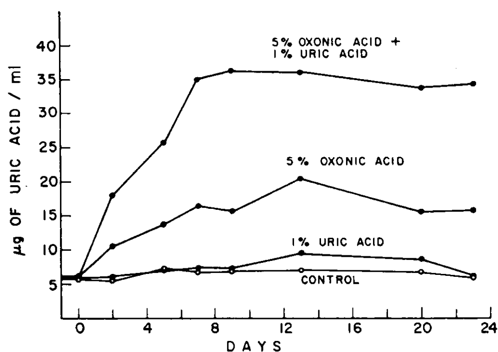

FIG. 3. Effect of dietary oxonate on plasma urate levels in rats. The rats were divided into 4 groups, as represented by each line on the graph. The control group were fed pulverized rat cubes. The other groups were fed the same diet with additions as indicated on the graph. Each point represents the average plasma urate concentration of 5 rats.

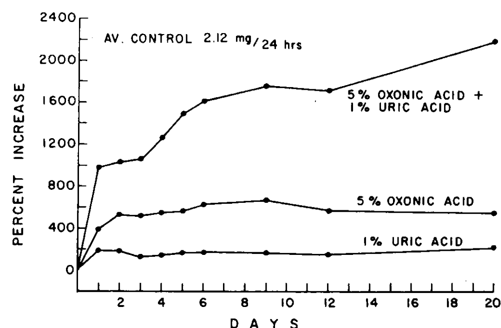

FIG. 4. Effect of dietary oxonate on urinary uric acid output. The conditions were the same as those in Fig. 3.

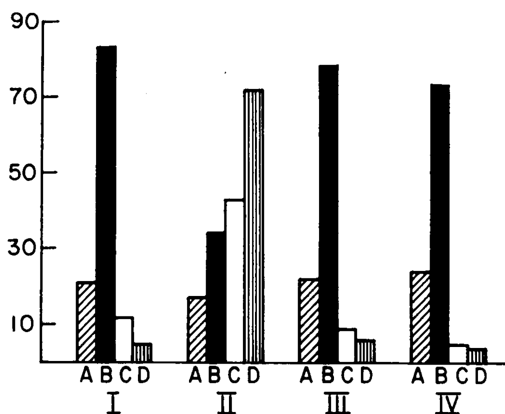

FIG. 5. Food intake, wt gain, urine volume, and kidney content of uric acid of 4 groups of rats employed to provide the data in Figs. 3 and 4. Group IV (Controls) were fed pulverized rat cubes. Groups I, II, and III were fed the same diet to which had been added: 5% oxonate, 5% oxonate plus 1% uric acid, and 1% uric acid, respectively. (A), food intake (g/rat/day); (B), wt gain (g/rat/23 days); (C), 24-hr urine volume (ml/rat/twenty-third day); (D), kidney uric acid content (mg/100 g of wet wt/twenty-third day). Each bar represents the average value of 5 rats from each group.

might be exceeded, in which case uric acid would precipitate in the kidney tubules. This was indeed found to be the case. As shown in Fig. 5 (Column D) the kidney uric acid content of Group II (oxonate and uric acid) was 72 mg/100 g of wet weight of kidney on day 23, as compared with 5 mg of uric acid/100 g in the other three groups. No uric acid was found when the diluted lithium carbonate extracts of homogenized kidney were treated with uricase prior to uric acid determination. It was apparent on gross observation of the dissected kidney of the Group II rats that uric acid was present in solid form. This was confirmed by examination of a photomicrograph of stained frozen section of kidney taken under polarized light which showed multiple granular birefringent crystals concentrated mainly within the collecting tubules. It should be noted that these effects were produced by a dietary intake of 200 mg of uric acid/day, but only when oxonate was also present in the diet as uricase inhibitor. In contrast, Grimes (11) found no concretions of uric acid in the kidneys of rats fed 1 g of uric acid/day for 21 days.

Oxonate and uric acid, when present individually in the diet, had no appreciable effect on food intake or weight gain, and produced a slight increase in urine volume (Fig. 5). When these substances were fed in combination they depressed food intake by 20% (Fig. 5IIA) decreased weight gain by more than 50% (Fig. 5IIB), and increased urine volume to 8–9 times the control (Fig. 5IIC). As an explanation for the latter, the possibility of interference by uric acid with antidiuretic hormone (ADH) function at the renal level was considered, but this has not been investigated further. Oxonate appears to have a slight diuretic action (Fig. 5IC), which is in keeping with the known diuretic activity of *s*-triazines (12), but the diuretic effect of oxonate can only partially account for the large urine volume of the rats in Group II (Fig. 5). It should be noted that, vis-a-vis the self-mutilation associated with the Lesch-Nyhan syndrome (13), our hyperuricemic rats displayed no tendency towards self-mutilation or aggression towards others.

Discussion. It is evident from the data presented in this paper that oxonic acid is an effective inhibitor of uricase activity *in vivo*. When given by the intraperitoneal or oral route it increased plasma and urine concentrations of uric acid and decreased the excretion of urinary allantoin. No studies have been carried out as yet on its metabolism or excretion characteristics. Feeding experiments indicate that it is well absorbed by mouth. Since the activity of a single dose is short-lived, and frequent injections (2.5-hr intervals) are required for sustained uricase inhibitory activity, it is apparently metabolized or excreted quite rapidly. Its low toxicity, however, permits the administration of large doses: an oral intake of 1 g/day for 28 days produced no obvious ill effects, except when uric acid was also administered.

The ideal uricase inhibitor would be one which was irreversible and noncompetitive, so that its activity would be independent of high levels of uric acid and effective inhibition could be attained at low dosage levels. It

is hoped that this ideal may be realized by appropriate substitution on the *s*-triazine ring.

Hyperuricemia and hyperuricosuria are associated with a number of human diseases and systemic disorders. These include primary gout, diabetes mellitus, psoriasis, glycogen storage disease, sarcoidosis, the Lesch-Nyhan syndrome, and leukemia. In the human, hyperuricemia and hyperuricosuria lead to deposition of urate within the kidney tubules and interstitial tissues. This condition is simulated in the oxonate-treated rat as described herein. A more detailed description of the nephropathy will be presented in a separate paper (14). We hope that after further refinement of experimental conditions our animal model will serve as a valuable research tool in this area of medical research.

Summary. Oxonic acid and allantoxaidine were potent *in vivo* inhibitors of uricase. Oxonic acid, when fed to rats or injected intraperitoneally, produced a marked increase in blood plasma urate levels and urinary uric acid output with a concomitant decrease in urinary allantoin excretion. Addition of 5% oxonic acid plus 1% uric acid to the diet resulted in a 22-fold increase in urinary uric acid by day 23 of treatment with deposition of uric acid in the kidney tubules. The oxonic acid-treated rat may serve as a useful animal model for the study of hyperuricemia and hyperuricosuria.

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