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Oxonate-Induced Hyperuricemia and Orotic Aciduria in Mice (40002)

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Hyperuricemia and uricosuria are the most striking characteristics of gout. Painful inflammation in the joints (tophi) caused by deposits of urate classify this disease as a form of arthritis. The formation of renal calculi and progressive renal failure are also common complications (1, 2). In most mammalian species the enzyme uricase catalyzes further oxidation of uric acid to the more soluble allantoin. However, in humans this enzyme is lacking and probably accounts for the difficulty in handling large purine loads.

The presence of uricase activity in experimental animals has complicated their use as models for studying gout. In 1965, Fridovich (3) examined various chemical compounds bearing a structural resemblance to the pyrimidine portion of the purine ring of uric acid and xanthine as possible inhibitors of *in vitro* uricase activity. These studies revealed that potassium oxonate (KOx; 2,4-dihydroxy-6-carboxy-1,3,5-triazine) was a potent inhibitor of *in vitro* hog liver uricase. *In vivo* experiments with rats fed a diet supplemented with 5% KOx were shown to produce a profound hyperuricemia and uricosuria (4). These changes in blood and urine urate were further increased when the diet was supplemented with an additional 1% uric acid. Hyperuricemic nephropathy was first demonstrated in rats fed 5% KOx plus 1% uric acid (5). Subsequent experiments revealed that a diet containing 2% KOx supplemented with 3% urate was sufficient to induce the hyperuricemic nephropathy (6, 7). Several studies using dogs, rabbits, pigs, and mice have reported the inhibitory effects of KOx on purine metabolism (8–11).

The present studies were conducted to more clearly define the effects of graded dietary concentrations of KOx with or without urate supplementation upon hyperuricemia, uricosuria, and orotic aciduria. Comparisons are made between these data and published data on the rat fed KOx.

Materials and methods. Forty male, albino ARS/ICR[1] mice (19–25 g) were adapted for 3 days to a semipurified casein diet suspended in 2% agar gel. Diets consisted of 50% water and 50% dry mix. The dry mix was composed of 15% vitamin-free casein, 0.3% methionine, 5% corn oil, 5% mineral salt mix,[2] 1% vitamin mix,[3] 49.1% cornstarch, and 24.6% sucrose.

All animals were randomly assigned after the adaptation period to one of eight treatment groups. The experimental diets consisted of KOx incorporated at concentrations of 0, 1, 3, and 5%, with or without 1% supplemental uric acid. Oxonate and urate were added at the expense of a 2:1 proportion of cornstarch and sucrose. Food and water were provided *ad libitum*. Weight changes were determined periodically throughout the 15 days of experimental feeding. Mice were identified by ear notching and housed five to a cage, except for purposes of urine collection. During urine collections, animals were housed individually in stainless steel metabolism cages. Food intakes were determined in individually housed animals.

KOx was either purchased commercially[4] or synthesized accordingly to the method of Brandenberger (12). Chemical purity was established by thin-layer chromatography using an *n*-butanol:acetic acid:water solvent system (5:1:2) and by chemical analysis for carbon and nitrogen. The commercial KOx had the following composition: C, 24.66;

[1] ARS/Sprague-Dawley, P. O. Box 4220, Madison, Wisc. 53711.

[2] Rogers and Harper salt mixture No. 170760, Teklad Test Diets, 2826 Latham Drive, Madison, Wisc. 53713.

[3] Vitamin fortification mix No. 40060, Teklad Test Diets, 2826 Latham Drive, Madison, Wisc. 53713.

[4] Oxonate, potassium salt, A grade No. 500111, Calbiochem, 10933 N. Torrey Pines Road, La Jolla, Calif. 92037.

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H, 1.24; N, 21.60. KOx synthesized in our laboratory was found to contain C, 24.38; H, 1.33; and N, 21.31. Synthesized KOx was tested *in vitro* for inhibition of uricase activity. The synthesized oxonate was found to be a potent competitive inhibitor of uricase with a K_I similar to that previously reported (3).

Acidified urine was collected from three randomly selected mice from each treatment on Days 7 and 14 of feeding. Urinary urea, ammonia, creatinine, and orotic acid were analyzed according to the methods of Foster and Hochholzer (13), Chaney and Marbach (14), Folin and Wu (15), and Stajner *et al.* (16), respectively. Urinary uric acid was determined by the method of Archibald (17) and confirmed by high-pressure liquid chromatography (hplc).[5] Blood obtained by heart puncture was also analyzed for urate by hplc.[5]

At the time of sacrifice, spleens, livers, and kidneys were weighed and stored at −20° for subsequent analysis. Portions of kidney and liver were homogenized in 0.4 *M* KCl and centrifuged at 3730*g* for 10 min. Tissue uric acid content was analyzed (17). Uricase activity of these tissues was determined spectrophotometrically as described by Fridovich (3). Tissue protein was determined colorimetrically using bovine serum albumin as a standard (18).

Statistical methods. All data were analyzed statistically by analysis of variance, and comparisons were made by using Duncan's multirange test (19). A *P* value of 0.05 or less was considered statistically significant.

Results. Growth data are presented in Table I. There were no significant alterations in growth of mice fed 1 or 3% KOx. However, growth was severely inhibited by 5% KOx. Although animals consuming the 5% KOx diet grew slightly with time, their growth was still severely inhibited at the end of the feeding period. Uric acid supplementation alone did not significantly influence growth. However, uric acid supplementation to diets containing oxonate depressed growth more severely than diets containing only oxonate. During the first week there was a 50% depression in growth with 1% dietary KOx plus 1% urate. Growth was further inhibited with 3 and 5% oxonate supplemented with uric acid. Similar growth data was obtained after 2 weeks of feeding.

Feed intake was significantly inhibited by supplementation of the diet with 5% KOx (Table I). Animals receiving supplemental uric acid showed further reductions in feed intake. Some adaptation to the diets appears to have occurred during the feeding period since intakes increased with time. However, feed consumption by mice consuming 3 and 5% KOx plus urate remained significantly depressed throughout. The differences in feed consumption are not of sufficient magnitude to account entirely for the severe depressions or inhibition of growth observed with supplementation of KOx. Increasing the dietary content of oxonate resulted in a marked reduction in feed efficiency. This reduction in feed efficiency was further decreased in mice fed supplemental uric acid. Animals consuming the 3% plus, and the 5% plus or minus, urate diets exhibited polyuria (Table I). These animals consumed approximately 200% more water than animals receiving the other treatments.

Plasma urate was increased approximately 250% by supplemental oxonate after 7 days of feeding (Table II). Urate supplementation did not significantly influence the effect of KOx during this time. Longer feeding of the urate-supplemented diets accentuated the hyperuricemia.

Creatinine excretion varied considerably, and no significant differences were detectable. Metabolites were expressed per unit of creatinine to normalize the data and provide a more controlled basis for comparison. Urinary uric acid excretions were consistent with plasma urate concentrations (Table II). Oxonate feeding alone increased the excretion of uric acid by 48 and 112% for 3 and 5% KOx, respectively. After the second week of feeding these diets, uric acid excretion was increased only 22 and 20%, respectively. Supplementation with uric acid increased urinary uric acid excretion 36, 205, and 200% in mice fed 1, 3, and 5%, respec-

[5] Milner, J. A., and Perkins, E. G., Uric acid determination in biological fluids by high-pressure liquid chromatography. Submitted for publication.

TABLE I. EFFECT OF DIETARY OXONATE AND URATE ON GROWTH, FEED INTAKE, AND URINARY EXCRETION IN THE MOUSE.[a]

Dietary supplementation		Growth[b]		Feed intake[c] (g/day)		
Oxonate (%)	Urate (%)	7 days	14 days	7 days	14 days	Urine volume[d] (ml)
0	0	6.6 ± 0.6[1,2]	10.0 ± 0.9[1]	8.9 ± 1.5[1,2]	11.3 ± 1.0[1]	1.4 ± 0.3[1]
0	1	8.0 ± 0.8[1]	10.5 ± 0.5[1]	11.6 ± 0.0[1]	11.8 ± 0.3[1]	1.2 ± 0.4[1]
1	0	4.5 ± 0.6[2]	7.5 ± 1.0[1,2]	10.2 ± 1.5[1,2]	11.0 ± 2.5[1]	2.1 ± 0.2[1,2]
1	1	3.8 ± 0.6[2]	5.0 ± 0.8[2,3]	7.9 ± 0.9[2]	9.6 ± 1.0[1]	1.7 ± 0.3[1]
3	0	7.0 ± 0.8[1,2]	8.7 ± 0.6[1,2]	8.4 ± 0.6[1,2]	10.9 ± 1.1[1]	1.8 ± 0.4[1]
3	1	−0.4 ± 1.9[3]	1.8 ± 3.8[3,4]	2.3 ± 0.4[3]	4.4 ± 4.5[2]	3.8 ± 1.3[2]
5	0	−1.4 ± 1.0[3]	−0.6 ± 0.5[4]	4.0 ± 1.3[3]	8.0 ± 0.3[1]	6.8 ± 2.0[3]
5	1	−3.8 ± 1.2[3]	−4.5 ± 0.5[5]	2.5 ± 0.2[3]	5.2 ± 2.2[2]	3.6 ± 0.8[2]

[a] Mean ± SEM without a common superscript differ, $P < 0.05$. Superscripts are denoted by the numbers 1-5.

[b] Body weight change in grams after 7 and 14 days of experimental feeding.

[c] Grams of diet = 50% dry matter + 50% H_2O.

[d] Day 7 of feeding.

TABLE II. PLASMA CONCENTRATIONS AND DAILY EXCRETION OF URATE IN MICE FED OXONATE WITH OR WITHOUT URIC ACID.[a]

		Plasma		Urinary	
Oxonate (%)	Urate (%)	Day 8 (mg%)	Day 15 (mg%)	Day 7 (μg/μg of creatinine)	Day 14 (μg/μg of creatinine)
0	0	1.8 ± 0.1[1]	2.0 ± 0.3[1]	2.0 ± 0.2[1]	2.3 ± 0.1[1]
0	1	2.9 ± 0.4[1]	4.1 ± 1.0[2]	3.0 ± 0.6[1]	3.6 ± 0.5[1]
1	0	4.9 ± 0.5[2,3]	1.4 ± 0.2[1]	1.7 ± 0.1[1]	2.4 ± 0.1[1]
1	1	6.0 ± 0.9[3]	6.4 ± 0.5[3]	4.0 ± 0.2[1,2]	4.1 ± 1.1[1,2]
3	0	4.7 ± 1.4[2,3]	4.1 ± 0.3[2]	3.0 ± 0.8[1]	2.8 ± 0.2[1]
3	1	4.4 ± 0.5[2,3]	5.1 ± 0.8[3]	9.1 ± 1.4[2]	5.8 ± 2.6[1,2]
5	0	4.1 ± 0.1[2]	3.5 ± 0.3[2]	4.2 ± 0.4[1,2]	2.8 ± 0.3[1]
5	1	4.8 ± 0.3[2,3]	5.7 ± 0.9[3]	8.9 ± 1.4[2]	8.9 ± 1.5[2]

[a] Means ± SEM without a common superscript differ, $P < 0.05$. Superscripts are denoted by the numbers 1-3.

tively. The increase in uricosuria persisted throughout the feeding period. Uric acid excretion was 15, 63, and 119% for the 1, 3, and 5% oxonate plus urate diets, respectively, after 14 days of feeding.

Weights of spleen, kidney, and liver were not significantly altered by any dietary treatment. Neither liver urate or uricase activity were affected significantly by the dietary regimen. However, kidney uric acid increased with increasing oxonate and was further increased by supplemental urate (Table III). One animal consuming the 5% oxonate plus urate diet had grossly visible kidney urate deposits. Kidney uricase activity tended to be depressed by increasing oxonate and further depressed by supplemental urate (Table III).

Urea excretion expressed per gram of N consumed tended to increase with supplemental oxonate and urate after 2 weeks of feeding (Table IV). Urinary ammonia excretion (Table IV) was slightly depressed by oxonate and further depressed by urate supplementation. Orotic acid excretion in mice fed the KOx diets is shown in Table IV. Orotate excretion was increased 10-fold in animals consuming 1% oxonate after Week 2. Further increases were seen with increasing oxonate. Urate supplementation of the diet did not affect orotate excretion. Specificity of the method for orotic acid was checked with large concentrations of oxonate, allantoin, and uric acid. These compounds produced negligible results.

Discussion. Chronic hyperuricemia and renal diseases are classic symptoms of patients with gout. Chronic renal disease has

TABLE III. EFFECT OF DIETARY OXONATE AND URATE ON LIVERS AND KIDNEYS OF MICE.[a]

Dietary supplementation		Organ weights (mg/g body weight)		Uric acid concentration (μg/g of tissue)		
Oxonate (%)	Urate (%)	Liver	Kidney	Liver	Kidney	Uricase[b] Kidney
0	0	52.2 ± 2.0[1]	15.6 ± 1.0[1]	78.9 ± 14.4[1]	17.8 ± 3.1[1]	66.9 ± 14.8[3]
0	1	54.8 ± 0.8[1]	16.0 ± 0.9[1]	54.7 ± 4.0[1]	21.1 ± 3.4[1]	40.0 ± 7.3[1, 2, 3]
1	0	53.3 ± 3.3[1]	15.2 ± 0.8[1]	63.2 ± 14.7[1]	25.3 ± 3.4[1, 2]	39.1 ± 7.2[1, 2, 3]
1	1	53.3 ± 3.5[1]	17.1 ± 0.6[1]	88.2 ± 20.5[1]	37.5 ± 9.0[1, 2]	28.6 ± 2.8[1]
3	0	48.0 ± 2.5[1]	17.1 ± 0.8[1]	85.9 ± 18.6[1]	29.2 ± 3.2[1, 2]	51.0 ± 11.9[1, 2, 3]
3	1	45.3 ± 4.6[1]	16.0 ± 0.4[1]	72.4 ± 12.4[1]	32.1 ± 7.3[1, 2]	30.3 ± 7.7[1, 2]
5	0	49.7 ± 3.7[1]	15.4 ± 0.7[1]	93.2 ± 21.8[1]	36.1 ± 7.6[1, 2]	31.8 ± 5.2[1, 2]
5	1	44.8 ± 2.2[1]	15.6 ± 0.4[1]	80.3 ± 17.4[1]	51.0 ± 10.4[2]	61.2 ± 8.1[2, 3]

[a] Means ± SEM without a common superscript differ, $P < 0.05$. Superscripts are denoted by the numbers 1–3.

[b] Nanomoles of uric acid per hour per milligram of protein.

TABLE IV. EFFECT OF DIETARY OXONATE AND URATE ON URINARY METABOLITES.[a, b]

Dietary supplementation		Urea-N (mg/gram of N consumed)	NH_3-N (mg/mg of creatinine)	Orotate (μg/mg of creatinine)
Oxonate (%)	Urate (%)			
0	0	432 ± 55[1]	6.7 ± 1.1[1]	304 ± 27[1]
0	1	254 ± 44[1]	3.4 ± 0.2[1, 2]	135 ± 16[1]
1	0	232 ± 69[1]	2.8 ± 0.4[1, 2]	3,752 ± 1,089[2]
1	1	427 ± 73[1]	2.2 ± 0.0[2]	3,589 ± 228[2]
3	0	359 ± 77[1]	3.0 ± 0.4[1, 2]	6,617 ± 953[2]
3	1	943 ± 78[1]	2.8 ± 0.1[1, 2]	7,653 ± 869[2]
5	0	251 ± 10[1]	4.1 ± 1.8[1, 2]	10,347 ± 2,845[2]
5	1	580 ± 146[1]	1.8 ± 0.8[2]	9,275 ± 1,921[2]

[a] Means ± SEM without a common superscript differ, $P < 0.05$. Superscripts are denoted by the numbers 1 and 2.

[b] Day 14 of feeding.

been observed in approximately 40% of the patients suffering from gout. Difficulty in finding a suitable model for gout have hindered the study of this disease. Recently, potassium oxonate has proven to be a valuable *in vivo* tool for examination of hyperuricemia and nephropathy in the rat (4–6, 20).

Rats have been shown to tolerate relatively large dosages of KOx for long periods of time (4, 5, 21, 22). However, the fetus may be more susceptible to toxic effects of oxonate. Gralla and Crelin (11) and Gralla (23) reported that KOx was toxic to the developing fetus when fed to pregnant rats or mice at a dietary concentration of 3%. KOx in quantities up to 1 g/day for 28 days has not resulted in toxicity in the rat although these animals exhibited a hyperuricemia and uricosuria (4). Uric acid supplementation accentuates the pharmacological actions of oxonate (4).

Oxonate alone (5%) dramatically affects feed intake or growth in the mouse. However, neither are appreciably affective in the rat (4, 6). Kox also affects feed efficiency in the mouse. Johnson *et al.* (4) reported a 50% reduction in growth and a 20% reduction in feed intake in rats consuming 5% KOx plus 1% urate diets. The mouse shows a far greater reduction in growth and feed intake at lower dietary concentrations of oxonate plus urate. These data indicate that the mouse may be much more susceptible to KOx than is the rat. The lethality of KOx in the mouse is unknown.

Blood urate concentrations of rats fed oxonate supplemented with uric acid vary considerably (4, 6, 7). Primarily, these variations may be explained by the duration of feeding and the percentages of oxonate and uric acid incorporated into the diets. Gralla and Crelin (11) reported that control nonpregnant female mice receiving chow had mean serum urate concentrations of 3.9

mg%. After 3 days on a chow diet supplemented with 3% KOx, these same animals were found to have a mean serum urate concentration of 7.0 mg%. We observed control mice on a semipurified diet to have a mean serum urate concentration of 2.0 mg% after 2 weeks. Mice consuming 3% KOx had a mean serum urate concentration of 4.1 mg% after the same period. The differences in control serum urate concentrations may be explained by the presence of dietary purines in the chow diets. Oxonate alone appears to increase blood urate concentrations more effectively in the mouse than in the rat.

Urinary uric acid excretion also varies markedly. In this respect, KOx appears to have a greater effect in inducing uricosuria in the rat than in the mouse. Rats show a 600% increase in urinary uric acid excretion with 5% dietary KOx (4). A 20-fold increase in urinary urate was noted in rats consuming 5% KOx plus 1% uric acid supplemented to a chow diet. No significant increase in urinary uric acid excretion after 2 weeks was seen in mice consuming KOx alone, and only a three-fold increase was observed in mice consuming 5% KOx plus 1% urate. The differences between diets may again partially explain these variances. Rats fed a chow diet excrete approximately 2.1 mg of uric acid/24 hr (4). In our experiment the control mice excreted 1.0 mg of uric acid/24 hr.

The study of hyperuricemic nephropathy has possibly been the most significant benefit of the oxonate model. Waisman and his associates (6, 22) concluded that there is a high correlation between the morphology of the nephropathy of oxonic acid-induced hyperuricemia and gout. Urate deposits in the urinary tracts of rats have not been reported if fed oxonate without supplemental urate. However, in several studies, rats fed oxonate supplemented with uric acid developed hyperuricemia with secondary renal effects within 2 weeks (4–6). Feeding mice 5% KOx plus 1% urate for 2 weeks increased kidney uric acid concentrations approximately 300% above animals not consuming oxonate or urate. In the presence of uric acid, KOx induced gross accumulation of urate in only one of the animals. Prolonged feeding probably would have induced deposits in the mouse similar to those previously reported for the rat.

The present experiment shows that dietary KOx has a tremendous effect on pyrimidine metabolism. Previously, Cihak and Sorm (24) showed that mice injected with KOx (5-aza-orotic acid) had decreased utilization of orotic acid for the synthesis of liver nucleic acids which was paralleled by an increased orotic aciduria. *In vitro* studies with extracts from rat and mouse liver indicate that KOx may induce an orotic aciduria by inhibiting orotate phosphoribosyltransferase and ortidylic acid decarboxylase activities (24–26). The present experiment shows that dietary KOx also leads to dramatic alterations in *in vivo* pyrimidine metabolism. These findings may well prove dietary KOx will serve as a useful tool for *in vivo* examination of purine and pyrimidine synthesis, their catabolism, and their interrelationship.

More extensive examination of detoxification of KOx in the mouse vs the rat may help explain the differences between these two animals. Our results indicate the mouse is more sensitive to the toxic effects of oxonate. However, the less severe hyperuricemia and uricosuria suggest a dramatic difference in the handling of KOx in the mouse compared to the rat.

Summary. The effects of dietary supplementation of various concentrations of KOx with or without uric acid were examined in the growing mouse. Many of the effects of KOx were accentuated at lower concentrations when uric acid was also supplemented to the diet. Hyperuricemia was caused by oxonate alone as well as by 1% KOx plus 1% uric acid. Oxonate plus uric acid induced an uricosuria. Kidney uric acid content and uricase activity were significantly altered by oxonate with or without urate supplementation. Oxonate in some ways alters pyrimidine metabolism and leads to an orotic aciduria in the mouse. Oxonate may prove to be a valuable tool in the study of the metabolism of purines and pyrimidines as well as their interrelationship.

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