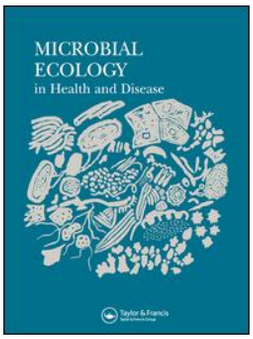

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Intestinal Colonisation of Laboratory Rats by Anaerobic Oxalate-degrading Bacteria: Effects on the Urinary and Faecal Excretion of Dietary Oxalate

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Oxalobacter formigenes, an anaerobic bacterium that degrades oxalate to CO_2 and formate, colonises the intestinal tracts of man and other animals. In this study, the large intestines of laboratory rats were experimentally colonised with a strain of *O. formigenes* to examine effects of these bacteria on the fate of dietary oxalate. When rats ($n=6$) were fed a standard rat diet plus 2 per cent sodium oxalate, urinary oxalate excretion was not significantly changed following inoculation and colonisation with *O. formigenes*. There was a consistent trend towards less oxalate excretion in faeces of rats after they became colonised with *O. formigenes*, but differences between colonised and non-colonised states were not significant. In an isotope recovery study, when rats were orally dosed with [^{14}C]oxalate, the percentage of ^{14}C in expired CO_2 from three colonised rats was 10-fold greater than from three non-colonised rats. Although ^{14}C excretion in faeces was decreased three-fold in the group of colonised rats, ^{14}C activity in the urine of colonised and non-colonised rats was not significantly different. Thus, although *O. formigenes* colonised and degraded oxalate in the rat intestinal tract, under conditions of these experiments, this colonisation did not markedly influence urinary oxalate excretion.

KEY WORDS—Oxalic acid; *Oxalobacter formigenes*; Intestinal bacteria; Oxalate degradation; Oxalate balance.

INTRODUCTION

Oxalic acid is present either as the free acid or as the anion in many plants ingested by humans and animals.[26] In mammals, dietary oxalate that is absorbed from the intestinal tract is not metabolised and is excreted unchanged in the urine.[21] Increased urinary oxalate excretion may arise when dietary oxalate intake is increased or when oxalate absorption is increased due to intestinal disease or resection.[9,25,28] Urolithiasis, a disease characterised by the formation of urinary tract stones, affects 5–10 per cent of the human population. Approximately 70 per cent of these stones contain calcium oxalate as a major component.[8,31] While the effects of diet and intestinal absorption on oxalate excretion have been extensively studied,[21] relatively little is known about bacterial degradation of oxalate in the mammalian intestinal tract and its influence on the absorption and excretion of dietary oxalate.

Oxalobacter formigenes, an anaerobic bacterium that utilises oxalate as a major source of carbon and energy (converts oxalate to CO_2 and formate), is responsible for oxalate degradation in the mammalian intestinal tract.[3,15] Rates of oxalate degradation in the rumen and in the large bowel of some non-ruminant herbivores increase dramatically when dietary oxalate intakes are increased due to selection of increased numbers of gastrointestinal *O. formigenes*.[1,4–6] Evidence now exists that humans also adapt to diets high in oxalate through increases in colonic populations of *O. formigenes*.[17] Selection of *O. formigenes* in the rumen benefits host animals by limiting the absorption and, thus, toxicity of dietary oxalate.[4,12] Whether intestinal *O. formigenes* provides similar benefits to non-ruminant mammals, where the anaerobic colonisation site is the hindgut rather than the forestomach, remains to be resolved.

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The intestinal tracts of wild rats,[14] other rodents,[32] herbivores[1] and humans are colonised by oxalate-degrading bacteria. Laboratory rats, however, appear to be unique in that most animals from commercial suppliers do not harbour intestinal *O. formigenes*.[14] As laboratory rats have frequently been used as models in studies on the metabolism and excretion of oxalate,[16,18,20–22] the present study was conducted to investigate whether or not colonisation by oxalate-degrading bacteria influences the metabolic fate of dietary oxalate and is thus a factor that needs to be considered with the rat model.

MATERIALS AND METHODS

Animals and diets

Outbred, male Sprague–Dawley rats (300–400 g) from a single commercial colony (Harlan Sprague–Dawley, Inc., Madison, WI, USA) were used. Previous studies indicated that *O. formigenes* was not present in the gastrointestinal tracts of these rats.[13,14] Each rat was housed in a metabolic cage (area 450 cm^2; Nalge/Sybron Corp., Rochester, NY, USA) that allowed complete separation of urine and faeces. The control diet was ground (3-mm sieve) Teklad LM-485 mouse/rat diet (Teklad, Winfield, IA, USA) and contained, by wet weight, 0·13 per cent oxalic acid, as determined by gas chromatography.[14] The oxalate diet was the control diet with 2 per cent sodium oxalate added (1·47 per cent oxalic acid). Diets and water were provided *ad libitum*.

Cultural techniques

O. formigenes strain OxCR6, isolated from caecal contents from laboratory rats that were naturally colonised by intestinal oxalate-degrading bacteria,[14] was used as inoculum.[13] Cultural detection and enumeration of *O. formigenes* and measurements of rates of oxalate degradation in caecal and colonic contents of rats were as described.[14] Similar techniques were used to measure faecal rates of oxalate degradation. Faeces, collected for up to 12 h from a rat, were diluted 10-fold (wt/vol) in anaerobic diluent[13] and homogenised prior to analysis. Concentrations of *O. formigenes* and rates of oxalate degradation were determined from measurements in triplicate and duplicate tubes, respectively.

Oxalate balance studies

In two experiments, rats (three per experiment) were initially fed the control diet. Following 24 h urinary and faecal collections, rats were switched to the oxalate diet; after 6 d, 24 h urinary and faecal collections were again obtained from each rat. Next, each oxalate-fed rat received 1 ml of a suspension containing $2{\cdot}5 \times 10^{10}$ viable *O. formigenes* cells. This inoculum was administered intragastrically with a feeding needle. Urinary and faecal collections (24 h) were obtained from each rat at 3, 4 and 5 and at 10, 11 and 12 d after inoculation in experiments 1 and 2, respectively (Table 1). Feed intake was measured during each 24 h collection period.

Isotope studies: recovery of ^{14}C from sodium [^{14}C]oxalate

From a separate group of six rats, three rats that had been fed the oxalate diet while they were being maintained in conventional plastic shoebox-type cages were inoculated with *O. formigenes* (using methods as described above). Twenty-five days after the inoculation, each rat was dosed intragastrically with 1 ml of a sodium [^{14}C]oxalate solution (50 mM; 2·56 KBq/mol; New England Nuclear Corporation, Boston, MA, USA) and was placed in a metabolic cage that was contained in a plastic box. Room air was pulled through the box (1 l/min) and then through a series of three CO_2 traps containing 300, 150 and 150 ml of 6 N NaOH. Rats were given access to water, but not feed, during the 24 h periods after dosing when expired CO_2, faeces, and urine were collected. Three rats which were treated as above, except they were not inoculated with *O. formigenes*, were used as controls.

A 2 ml volume of each CO_2 trap was injected through a rubber port into 25 ml of an acidified solution[10] in a closed vessel that also held a scintillation vial containing 2 ml of phenethylamine. $^{14}CO_2$ released by the acid was trapped in the phenethylamine during 8 h on a shaking water-bath at 37°C. Radioactivity was measured by liquid scintillation counting (LS-9800; Beckman Instruments, Inc., Palo Alto, CA, USA) with 10 ml of Biofluor (New England Nuclear). Counting efficiency was monitored by external standardisation (H-number) and was measured by adding [^{14}C]toluene (Amersham Corp., Arlington Heights, IL, USA).

Analytical techniques

Urine was collected in tubes containing 1–2 ml of 12 N HCl. Prior to analysis, urine samples were diluted two- to four-fold in deionised water and the pH was adjusted to 2–3 with 6 N NaOH. Oxalic acid

Table 1. The effects of diet and inoculation with *O. formigenes* on urinary and faecal excretion of oxalic acid from laboratory rats

Expt*	Treatment†	Oxalic acid intake (mg/24 h)	Oxalate degradation rate (μmol/g/h)‡	Oxalic acid excretion (mg/24 h)§	
				Urine	Faeces
1	Control diet	28·1	ND	0·6[a] (2·1)	17·2[a] (61·0)
	Oxalate diet				
	Uninoculated	317·5	0·9	2·9[b] (0·9)	216·8[b] (68·1)
	Inoculated	316·2	8·3	2·9[b] (0·9)	185·4[b] (56·6)
2	Control diet	27·5	1·8[a]	0·6[a] (2·2)	23·9[a] (86·8)
	Oxalate diet				
	Uninoculated	302·1	1·8[a]	3·0[b] (1·0)	236·5[b] (78·7)
	Inoculated	265·8	9·8[b]	2·6[b] (1·0)	201·1[b] (75·6)

ND = Not determined.
[a,b]For each experiment, means within a column with different superscript letters are different ($P < 0·05$).
*Three rats per experiment. In experiment 1, urinary and faecal collections (24 h) were obtained at 3, 4 and 5 d after inoculation and at 10, 11 and 12 d after inoculation in experiment 2.
†Treatments: control diet, Teklad LM-485 mouse/rat diet; oxalate diet, control diet with 2 per cent sodium oxalate added; innoculated, rats fed the oxalate diet and inoculated with *O. formigenes* strain OxCR6.
‡Expressed on a per gram (dry weight) of faeces basis.
§Values are the means of data from three rats (two to five measurements per rat). Values in parentheses are oxalic acid excretion expressed as a percentage of oxalic acid intake.

in diluted, pH-adjusted urine was measured by an enzymatic assay.[11] In this assay, oxalic acid was extracted from urine by adsorption onto aluminium hydroxide and then eluted with 0·2 N NaOH. The efficiency of oxalic acid extraction was determined by adding [^{14}]oxalic acid to diluted, pH-adjusted urine samples. Extraction efficiency averaged 83 per cent; all values were corrected for the percentage of [^{14}C]oxalic acid not extracted.

In experiment 1 (Table 1), collected faeces were oven dried at 55°C. In all other experiments, to reduce the microbial breakdown of oxalate, faeces were collected in tubes containing 10–15 ml of 1·2 N HCl and dried by lyophilisation. Dried faeces were extracted with 1 ml of 3 N HCl per 0·1 g (dry weight) of faeces for 3 h at 40°C. All faecal extracts were clarified by centrifugation at 1790 ***g*** and by filtration (pore size, 0·45 μm). Oxalic acid in clarified faecal extracts was measured by gas–liquid chromatography of the dibutyl ester.[14] The recovery of oxalic acid added to faeces was 98 per cent. Dependent data in Table 1 were analysed using a paired *t*-test while independent data in Table 3 were analysed using a two-way analysis of variance and *t*-test.[33]

RESULTS

When the diet of rats was changed from the control diet to the oxalate diet, the amount of oxalate excreted in the urine increased five-fold, from 0·6 to 3·0 mg/24 h. When expressed as a percentage of the daily intake of oxalate, however, urinary oxalate excretion decreased from 2·2 to 1·0 per cent when rats were switched from the control diet to the oxalate diet.

Following inoculation of oxalate-fed rats with *O. formigenes*, urinary oxalate excretion, expressed as a percentage of the daily intake of oxalate, remained essentially unchanged while faecal oxalate excretion in each of the six rats decreased (but not significantly; $P > 0·10$) by 9·5 and 3·1 per cent in experiments 1 and 2, respectively. No significant trends were noted for urinary or faecal excretion of oxalate when examined with respect to length of time (3–5 or

Table 2. Rates of oxalate degradation and concentrations of *O. formigenes* in caecal and colonic contents from inoculated laboratory rats

Rat*	Segment of intestinal tract†	Oxalate degradation rate (μmol/g/h)‡	Concentration of *O. formigenes* (log_{10}/g)§
1	Caecum	32·6	7·60
	Colon	40·8	8·26
2	Caecum	86·0	8·82
	Colon	53·9	8·88
3	Caecum	6·3	6·63
	Colon	5·9	6·96

*Inoculated rats fed oxalate diet described in Table 1 (experiment 1) were sacrificed 57–68 d after inoculation.
†Contents from each gut segment were diluted 10-fold and homogenised before analysis.
‡Expressed on a per gram (dry weight) of intestinal contents basis. Each value is the mean of duplicate tubes.
§Expressed on a per gram (dry weight) of intestinal contents basis. Counts were from colonies producing clear zones in D agar[16] after 7 d of incubation. Each value is the mean of triplicate tubes.

10–12 d) after inoculation (data not shown). Total recovery of dietary oxalate averaged 74·4 per cent (range 61·7–87·0 per cent) from control rats fed the oxalate diet and 68·1 per cent (range 53·2–79·6 per cent) from inoculated rats. Following inoculation with *O. formigenes*, five-fold or greater increases in rates of oxalate degradation by microbes in faeces (Table 1) indicate that the inoculated rats were colonised by *O. formigenes*. Results of cultural counts indicating that population densities of *O. formigenes* in caecal and colonic contents from inoculated rats ranged from 6·63 and 8·82 and from 6·96 to 8·88 log_{10} viable cells/g (dry weight), respectively (Table 2), confirmed that these animals were indeed colonised.

Following the intragastric administration of sodium [^{14}C]oxalate, a group of rats that had been inoculated with *O. formigenes* excreted approximately 10-fold more ^{14}C as expired CO_2 than uninoculated rats (Table 3). This finding was in agreement with data indicating that the proportion of ^{14}C activity that was excreted in faeces of inoculated rats was only about one-third of that excreted in the faeces of uninoculated rats. However, even with the greater *in vivo* degradation of [^{14}C]oxalate by *O. formigenes* in the large intestinal tracts of

Table 3. Distribution of carbon-14 from [^{14}C]oxalate in expired CO_2, faeces and urine from laboratory rats

	Distribution of ^{14}C (% of oral dose/24 h)†			
Treatment*	Expired CO_2	Faeces	Urine	Total
Control	3·9[a]	46·5[a]	5·6	56·0
Inoculated	44·7[b]	16·3[b]	6·7	67·7

*Three rats per treatment. Treatments: control, rats fed the oxalate diet; inoculated, rats fed the oxalate diet and inoculated with *O. formigenes*. Rats were analysed 25 d after inoculation.
†Each rat was dosed intragastrically with 1 ml of sodium [^{14}C]oxalate (50 mM; 2·59 KBq/μmol) solution. Expired CO_2, faeces and urine were collected for 24 h.
[a,b]Means within a column with different superscript letters are different ($P<0·05$).

inoculated rats, the percentage of ^{14}C activity excreted in the urine of inoculated and uninoculated rats did not differ significantly ($P>0·1$; Table 3).

DISCUSSION

A protective function for toxin-degrading intestinal microbes is not difficult to envisage if the microbes colonise forestomach fermentative sites (e.g. the rumen) where degradative reactions can occur before dietary toxins are exposed to absorptive tissues. When the colonisation site is in the hindgut, a protective role seems less obvious. In the case of oxalate, the proximal small bowel appears to be the major absorptive site,[27,29] but the colon is also an absorptive tissue.[18]

Although Barber and Gallimore[7] demonstrated in 1940 that microbes in human faeces were able to degrade oxalate, knowledge about the nature of bacteria responsible for oxalate degradation and about rates of oxalate degradation by these microbes is recent and limited in scope. The first report of isolation of an oxalate-degrading bacterium from human faeces was in 1986.[2] All isolates from humans were similar to *O. formigenes* strains isolated from the rumen[15] and from the faeces of pigs and other animals.[3] Concentrations of these bacteria in human faeces were as high as 10^7 per gram; however, not all attempts to culture them from different individuals were successful.[2] Doane *et al.*[17] confirmed the presence of *O. formigenes* in faecal samples from humans but they also reported that three of eight humans in their study were not colonised by oxalate-degrading bacteria. Although

urinary oxalate excretion was consistently higher in persons that were not colonised, this difference was significant during only one of three test periods. Doane and coworkers[17] also reported that humans colonised by intestinal oxalate-degrading bacteria excreted significantly less faecal oxalate than subjects that were not colonised. Workers from other laboratories have now confirmed that *O. formigenes*-like bacteria are present in human faeces[24] and in samples obtained during colonoscopy from the caecum and sigmoid colon.[34] The possibility that oxalate-degrading bacteria in the human gastrointestinal tract may play a role in determining the fate of dietary oxalate and thus in calcium oxalate urolithiasis, and that this activity might be manipulated, has not yet been fully examined.

Laboratory rats have been extensively used as models for study of oxalate metabolism. This use can, however, be questioned since most laboratory rats from various commercial suppliers differ from most humans, from wild rodents,[14,32] and from most other animals that have been examined,[1] in that laboratory rats are not generally colonised by oxalate-degrading bacteria.[14]

The experiments described here were conducted to assess whether or not oxalate-degrading bacteria play a significant role in regulation of the fate of dietary oxalate in laboratory rats where the colonisation site for the oxalate degrader is the large intestine.[14] We propose that our results are relevant to use of the laboratory rat as a model as well as to comparisons between humans that are, or are not, colonised by oxalate-degrading bacteria. Since oxaluria is a critical determinant for calcium oxalate urolithiasis, our main interest was in determining whether or not colonisation by *O. formigenes* would influence urinary oxalate excretion.

During oxalate balance studies, the amount of oxalate excreted in the urine of rats fed the control diet averaged 0·6 mg/24 h (Table 1). This value is in agreement with other reported values for urinary oxalate excretion from rats fed similar diets.[16,23,30]

One reason that a relatively small proportion of the dietary oxalate in the present experiments was absorbed and excreted in the urine may be that the diet contained 0·93 per cent calcium, by wet weight (as stated by the supplier). High levels of calcium can reduce the absorption of dietary oxalate[21] and the molar ratio of calcium to oxalate in the oxalate diet was nearly 1·5 to 1. High calcium levels could also limit oxalate degradation by *O. formigenes* and account for similarly high levels of faecal oxalate in both colonised and non-colonised animals fed the oxalate diet (Table 1). With rats fed [^{14}C]oxalate, however, there were major differences in ^{14}C excreted in the faeces, possibly because rats were not given feed during the 24 h collection period. Under these conditions, there would be less calcium to bind the [^{14}C]oxalate and, thus, more soluble [^{14}C]oxalate available for microbial degradation.

Other information supporting a role for gastrointestinal microbes in determining the fate of dietary oxalate includes data showing that germ-free rats excreted more urinary and faecal oxalate than conventional rats and that the frequency of urinary calculi (composed of calcium oxalate and calcium citrate) in germ-free rats was reduced significantly when they were colonised with the intestinal microbiota of conventional rats.[19] Whether this colonisation introduced *O. formigenes* was not examined. In another study, elevated rates of degradation of oxalate by bacteria in the intestinal tract of guinea-pigs were correlated with lower rates of oxalate absorption and urinary oxalate excretion.[6] Reports that laboratory rats excreted approximately 5 per cent of an oral dose of oxalate as CO_2[20] are similar to findings in the present study where uninoculated rats excreted 3·9 per cent of an intragastric dose of [^{14}C]oxalate as respiratory $^{14}CO_2$. These values are in contrast to our results with rats inoculated with *O. formigenes* when 44·7 per cent of the ^{14}C from oxalate was recovered as $^{14}CO_2$ (Table 3). Thus, although colonisation of rats with *O. formigenes* led to a major shift of the route of excretion of labelled carbon in oxalate from faeces to expired carbon dioxide, this did not lead to a significant reduction in the amount of ^{14}C from oxalate excreted in the urine.

Rates of oxalate degradation in caecal contents from rats colonised by *O. formigenes* in these experiments were of the same magnitude as rates measured in samples of caecal contents from guinea-pigs and rabbits[1] or from naturally, or purposely colonised, laboratory rats fed diets high in oxalate.[13,14] In addition caecal and colonic rates of oxalate degradation generally reflected the culturable numbers of *O. formigenes* (Table 2); this relationship also occurred in ruminal contents of sheep.[12] Caecal concentrations of *O. formigenes* observed in the present study were similar to those reported previously for rats colonised by *O. formigenes* strain OxCR6[13] and to the concentrations of *O. formigenes* in the caecal sample from which strain OxCR6 was originally isolated.[14] *O. formigenes* OxCR6 does not colonise the small intestine of adult laboratory rats.[13]

Our results give little support to the hypothesis that urinary oxalate excretion in rats might be altered by colonisation with *O. formigenes*. We believe, however, that different results would be obtained with less calcium in the diet and that it would be premature to conclude that the present information can be extrapolated to other diets or to humans or other animals that are colonised in hindgut sites by anaerobic oxalate-degrading bacteria.

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