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Microbial degradation of oxalate in the gastrointestinal tracts of rodents and ruminants

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Iowa State University, 1988

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Microbial degradation of oxalate in the gastrointestinal tracts of rodents and ruminants

by

Steven Lee Daniel

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
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GENERAL INTRODUCTION

Through their interactions with toxic substances, intestinal microbes play an critical role in the health of the host animal. One such substance, oxalate, is indeed degraded by microbial populations in the rumen and in the large bowel of man and other herbivores. Oxalate is ubiquitous in nature, occurring in many of the plants consumed by mammals. Foods such as nuts, chocolate, tea, and coffee contain high levels of oxalate. Oxalate, absorbed from the gut and synthesized endogenously, is excreted in the urine (Fig. 1). Hyperoxaluria leading to urolithiasis may arise when dietary oxalate or endogenous oxalate synthesis is increased. Hyperoxaluria may also be caused by increases in the intestinal absorption of oxalate as a result of intestinal disease or resection. Urolithiasis, a disease characterized by the formation of urinary tract stones, affects 5 to 10% of the human population. Approximately 70% of urinary stones contain calcium oxalate as a major constituent. Although the effects of diet, intestinal absorption and endogenous synthesis on oxalate excretion have been extensively studied, there is a paucity of information about microbial decomposition of oxalate in the gut of mammals and its influence on oxalate metabolism and excretion.

A new species of anaerobic bacteria, Oxalobacter formigenes, which uses oxalate as a major carbon and energy source, is responsible for intestinal oxalate degradation. Increased dietary oxalate leads to increased rates of oxalate degradation in gastrointestinal contents from ruminants and nonruminant herbivores probably because of the

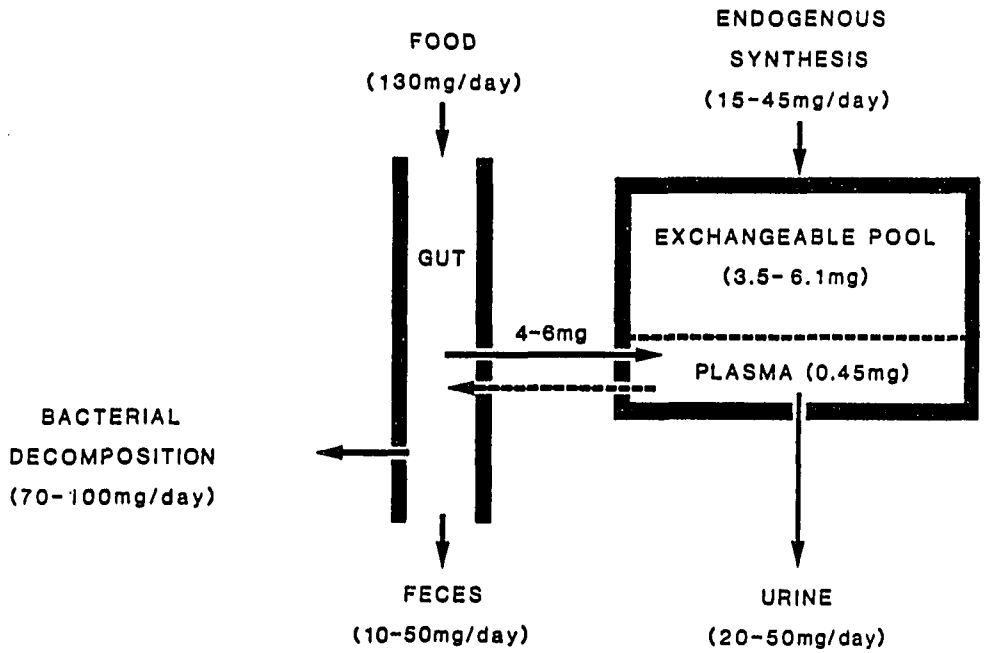

Figure 1. The metabolism of oxalic acid in humans (Hodgkinson, 1977)

selective enrichment of intestinal O. formigenes. Attempts to confirm this, however, have been hindered by the inability to enumerate O. formigenes in intestinal contents.

Unlike most mammals, laboratory rats apparently do not have intestinal oxalate-degrading bacteria. Since laboratory rats are frequently used as models representative of monogastric animals in studies of oxalate metabolism, the apparent absence of oxalate-degrading bacteria in rats is significant. In most studies, the possibility that these bacteria may influence intestinal absorption and urinary and fecal excretion of dietary oxalate has been neglected.

Therefore, the purposes of this study were to determine: the presence or absence of anaerobic oxalate-degrading bacteria in the intestinal tracts of both laboratory rats obtained from different commmercial suppliers and wild rats; whether or not anaerobic oxalate-degrading bacteria from the intestinal tracts of a variety of animals and from human feces can colonize the intestines of laboratory rats; and the effect of different amounts of dietary oxalate on concentrations of anaerobic oxalate-degrading bacteria in the rumen of sheep.

Dissertation Format

This dissertation is presented in the alternate thesis format and includes three manuscripts. The first two are to be submitted for publication in Applied and Environmental Microbiology and the third in FEMS Microbiology Ecology. An overall literature review is presented

first, followed by the three manuscripts. A general summary and discussion follow the final manuscript.

LITERATURE REVIEW

Properties of Oxalate

Oxalic acid (HOOC-COOH) or ethanedioic acid, molecular weight 90.04, is the simplest of the dicarboxylic acids. It is a fairly strong corrosive organic acid with dissociation constants (pK_a's) of 1.23 and 3.83. Oxalic acid crystallizes from aqueous solutions as either a mono-, di- or trihydrate; the dihydrate is the most commonly encountered form (Hodgkinson, 1977). Oxalic acid forms a variety of compounds: neutral and acid salts with alkali metals (K, Li, Na), rare earth metals (Ce, La, Th), and ammonia; a monoamide (oxamic); a diamide (oxamide); and mono- and diesters (Hagler and Herman, 1973a).

The free acid is moderately soluble in water, very soluble in ethanol and glycerol, and insoluble in benzene and chloroform. Most salts of oxalic acid, excluding the alkali metal and ammonium salts, are relatively insoluble. One of the more common and least soluble salts of oxalic acid is calcium oxalate. In the physiological pH range, calcium oxalate readily precipitates out of aqueous solutions; however, precipitation can be inhibited by inorganic pyrophosphate, various divalent metals (Mg, Fe, Cu, Zn), sulfate, citrate, and lactate (Williams and Smith, 1972).

Oxalic acid and its soluble salts are classified as systemic poisons. Low concentrations of oxalic acid inhibit a number of mammalian enzymes, including lactate dehydrogenase and pyruvate kinase (Beutler et al., 1987; Hodgkinson, 1970; Laker, 1983).

Oxalate in Nature

Oxalic acid is ubiquitous in nature, occurring in many of the plants consumed by man and animals. Hodgkinson (1977) listed the oxalate content of over 80 foods and beverages. Beer, fish, meats, cereals, and fresh fruit all contain very little oxalate. Moderate amounts of oxalate (45-225 mg/100 g of fresh material) are present in coffee, chocolate, and parsley. High amounts of oxalate (greater than 225 mg/100 g of fresh material) are present in spinach, rhubarb, nuts, cocoa, and tea leaves. Ramasastri (1983) reported that certain condiments and spices, such as poppy and coriander seeds, tumeric, and cloves, may also contain exceptionally high levels of oxalate (greater than 1200 mg/100 g of fresh material). Likewise, in many pasture plants and grasses consumed by animals, the oxalate content is often high (Libert and Franceschi, 1987); it can represent up to 30% of the total dry weight of Halogeton glomeratus (halogeton) (Dye, 1956). In most plants, oxalic acid is present as water-soluble and insoluble salts, instead of the free acid. The water-soluble oxalates consist of sodium, potassium, and ammonium salts, whereas the insoluble oxalates are calcium and to a lesser extent magnesium salts (Oke, 1969).

The concentration of oxalate in plants is influenced by many factors, including climate, soil composition, type of fertilizer used, season, plant species and age. Unreliable methods for oxalate analysis may also account for discrepancies in the reported oxalate concentrations of certain plants.

Measurement of Oxalate

Perhaps one of the most frustrating and limiting factors in the study of oxalate metabolism is the lack of reliable methods for measuring oxalate in foodstuffs and biological fluids, especially urine. Although numerous methodologies have been published, most are time-consuming, technically demanding, and lack precision and reproducibility. One purpose of this review was to evaluate the methods used to measure oxalate in urine and plant material and some of the problems associated with these methods.

Urine

Methods for measuring oxalate in urine can be divided into two categories, those that require the preliminary separation of oxalate from interfering substances and those that measure oxalate directly. A frequently used method involves the precipitation of oxalate from urine as the insoluble calcium salt (Hodgkinson and Williams, 1972). One inherent problem with this method of separation is incomplete precipitation of oxalate. Since calcium and phosphate concentrations, as well as urine pH, affect oxalate precipitation, [^{14}C]oxalate is often used to correct for the incomplete precipitation of urinary oxalate (Mazzachi et al., 1984). In another method, oxalate is removed from urine with solvents such as tri-n-butyl phosphate, diethyl ether, and trialkylamines (Pegon and Vallon, 1981; Zarembski and Hodgkinson, 1965). Major disadvantages of solvent extraction include incomplete extraction and lack of specificity for oxalate. A third method,

ion-exchange chromatography, is probably the least used method of the three for preliminary separation of oxalate. Anion exchange resins, Lewatit MP 7080, Dowex AG 2-X8, Carbopack B, and DEAE-Sephadex, have been used successfully; however, inconsistent recovery of oxalate and time-consuming procedures preclude this method of oxalate separation for routine laboratory usage (Di Corcia et al., 1982; Johansson and Tabova, 1974; Olthuis et al., 1977; Sims et al., 1981). More recently, an octadecyl-silane bonded-phase packing (Sep-Pak C_{18} cartridge) has been used for initial clean-up of urine samples (Larsson et al., 1982). Although this preparative procedure does not separate oxalate from the urine, it does remove non-polar compounds which may interfere with subsequent analysis.

After separation, oxalate can be quantified by a wide variety of methods: potassium permanganate titration (Hallson and Rose, 1974); enzymatic degradation (Obzansky and Richardson, 1983); fluorimetry (Zarembski and Hodgkinson, 1965); colorimetry (Hodgkinson and Williams, 1972); isotope dilution (Prenen et al., 1983); electron capture chromatography (Moye et al., 1981); gas-liquid chromatography (GLC) (Yanagawa et al., 1983); and high-performance liquid chromatography (HPLC) (Imaoka et al., 1983). In addition to the limitations associated with the preliminary separation of oxalate, all of the above techniques have characteristic shortcomings. For example, during the process of derivatization for chromatographic analysis, oxalate may be produced from a variety of oxalogenic compounds present in urine (Moye et al., 1983). This results in the overestimation of urinary oxalate

concentrations.

For routine laboratory use, it would be advantageous to use techniques which measure urinary oxalate directly without preliminary separation. One of these techniques is enzymatic analysis. Currently, two enzymatic methods are available: decarboxylation by oxalate decarboxylase with measurement of CO_2 or formate (Costello et al., 1976; Vadgama et al., 1984) and oxidation by oxalate oxidase with measurement of CO_2 or hydrogen peroxide (Buttery et al., 1983; Crider and Curran, 1984; Kasidas and Rose, 1987; Kohlbecker et al., 1979; Kohlbecker and Butz, 1981). Although these enzymatic methods are highly sensitive and specific for oxalate, the enzymes involved are expensive and are inhibited by nitrate, phosphate, and sulfate ions present in normal urine (Goldsack et al., 1984; Robertson and Rutherford, 1980). In addition, enzymatic analyses generally underestimate oxalate concentrations in urine by 10 to 20%. Besides enzymatic analysis, additional methods for measuring oxalate directly in unprocessed urine include HPLC (McWhinney et al., 1986; Murray et al., 1982), isotachophoresis (Tschope et al., 1981), and conductimetry (Classen and Hesse, 1987; Menon and Mahle, 1983; Santos and Baldwin, 1987). The practicality of the latter two methods has yet to be determined.

In a recent report by Zerwekh et al. (1983), six different methodologies for the determination of urinary oxalate were compared. These methods included colorimetry, a modified colorimetric procedure, HPLC, GLC, enzymatic degradation, and ion chromatography with

conductimetric detection. They concluded that, of the six oxalate assay methods evaluated, no single method was superior and the method of choice for each laboratory will depend upon the number of samples to be analyzed and the availability of reagents and equipment.

Plant material

Permanganate titration (Baker, 1952), colorimetry (Zarembski and Hodgkinson, 1962), atomic absorption spectrophotometry (Abaza et al., 1968), thin-layer chromatography (Roughan and Slack, 1973), enzymatic methods (Bengtsson, 1967), and GLC (Roughan and Slack, 1973; Rumsey et al., 1966) have been used to quantitate oxalate in plant material. Besides being complicated and time consuming, these methods were often inaccurate because of incomplete extraction of oxalate from plant material, the destruction of oxalate by excessive drying of plant material at high temperatures, and the production of oxalate from oxalogenic substances during extraction. Simple, rapid and accurate GLC and HPLC methods have been recently developed which minimize the destruction and oxalogenic production of oxalate in plant material (Ohkawa, 1985; Wilson et al., 1982).

Oxalate Metabolism in Mammals

Dietary intake

There is a paucity of information concerning the daily dietary intake of oxalate by man and animals. Estimates of the oxalate content in a typical Western adult diet have ranged from 70 to 980 mg/day (Hagler and Herman, 1973b). In developing countries, where a

significant portion of the diet consists of leafy vegetables, daily intakes of oxalate may reach 2000 mg/day (Menon and Mahle, 1982). The daily intake of oxalate by laboratory rats, fed a commercially prepared diet, averages approximately 18 mg/day (Hodgkinson, 1978). Pack rats and sand rats may consume, in the wild, up to 500 mg of oxalate per day in their diets (Shirley and Schmidt-Nielsen, 1967). Reliable estimates of the normal daily oxalate intake by foraging ruminants have apparently not been published. This information is probably difficult to obtain because of the wide range of oxalate concentrations present in the variety of pasture plants and grasses consumed by the grazing animal.

High levels of oxalate in the diet can seriously restrict the utilization of dietary calcium by forming insoluble salts in the intestine (Weaver et al., 1987). Pingle and Ramasastri (1978) reported that oxalate in green leafy vegetables not only impaired the utilization of calcium from the vegetables but also decreased calcium absorption from the rest of the diet. In studies with laboratory rats, calcium bioavailability in diets containing low levels of calcium and high levels of oxalate was reduced, causing slower growth rates, decreased bone ash, and decreased calcium deposition in the body (Kelsay, 1985). Similarly, when equids were fed diets supplemented with increased quantities of oxalate, calcium retention in the body was severely reduced because of the inhibition of calcium absorption (McKenzie et al., 1981; Swartzman et al., 1978). These findings, together with the work of Blaney et al. (1981a), provided evidence that

a calcium deficiency syndrome, called nutritional secondary hyperparathyroidism or osteodystrophia fibrosa, which occurs in horses grazing certain oxalate-rich tropical grasses in Australia, is a direct result of inhibition of the absorption of dietary calcium by oxalate. Subsequent studies suggested that the majority of the calcium in tropical grasses is already present in an insoluble form as calcium oxalate crystals, rendering it completely unavailable to equids and decreasing its availability to ruminants by 20% (Blaney et al., 1981b; Blaney et al., 1982; McKenzie and Schultz, 1983). Likewise, the calcium present in calcium oxalate crystals of alfalfa is poorly utilized by cattle (Ward et al., 1979), lambs, cockerels (Ward et al., 1982), and exotic ruminants (Harbers et al., 1980). The poor utilization of calcium from calcium oxalate crystals in plants may be attributed to the relative insolubility of calcium oxalate and the encapsulation of calcium oxalate crystals in the parenchymal sheath cells of plants (Hans et al., 1984; Ward et al., 1979). Since sheath cells and the surrounding vascular bundles are fairly resistant to both microbial degradation in the rumen and to postruminal digestion, a significant portion (up to one-third) of the calcium in these enclosed calcium oxalate crystals is unavailable to the animal and eliminated from the body in the feces (Ward et al., 1979; Ward and Harbers, 1982). Thus, in ruminants and monogastric animals, three factors ultimately determine the utilization of calcium from calcium oxalate crystals in plants: the pH of the gastrointestinal tract, especially the stomach; digestion of plant material in the gut by microbial and mammalian

enzymes; and the mechanical action of the intestinal tract (Ward and Harbers, 1982).

Intestinal absorption

Dietary oxalate is poorly absorbed from the gastrointestinal tract of man and laboratory animals. In man, approximately 2-7% of the dietary oxalate is normally absorbed from the healthy gastrointestinal tract (Archer et al., 1957; Brinkley et al., 1981; Earnest et al., 1974; Prenen et al., 1984). When large quantities of oxalate were given to normal subjects in the fasting state or without additional food, there was a 5-fold increase in the absorption of oxalate from test meals (Chadwick et al., 1973; Tiselius et al., 1981). Enhanced absorption was probably a result of the administration of oxalate in the test meals as the sodium salt and a reduction in the level of substances in the gut which complex with oxalate. Studies with laboratory rats have shown that 15 to 25% of the oxalate consumed in conventional diets is absorbed by the rat intestine (Bannwart et al., 1979; Hodgkinson, 1978). Although information is limited, these findings suggest that when expressed on a percentage basis, the amount of dietary oxalate absorbed by laboratory rats from their intestine is more than that absorbed by humans.

The location of oxalate absorption in the human gastrointestinal tract has been estimated mainly on the basis of studies in various animal models. Schwartz and associates (1980), using everted sacs of rat intestine, demonstrated that regional differences in oxalate uptake existed within the rat colon. In these experiments, the rate of

oxalate uptake in the proximal colon was more than twice that observed in the distal portion. Through the use of macroautoradiography, Hagmaier et al. (1980) followed the absorption and anatomical distribution of exogenous [^{14}C]oxalate in rats. Within 30 min of ingestion, most of the labeled oxalate had been absorbed from the intestine and excreted into the bladder, indicating that the small intestine and possibly the stomach, but not the colon, may be sites of oxalate absorption. Utilizing a different technique (isolated intestinal segments), Madorsky and Finlayson (1977) and Saunders et al. (1975) reported that, although oxalate absorption occurred throughout the entire rat intestine, the rate of oxalate absorption was greatest in the jejunum and least in the colon. In contrast, when similar experiments were performed with intestinal segments from Rhesus monkeys, Sidhu et al. (1984) found that the rate of oxalate uptake was similar in the jejunum, ileum, and colon, suggesting an absence of regional differences in the monkey intestine for oxalate absorption. Recently, a number of studies on humans have been performed in an effort to better define the location of intestinal oxalate absorption (Erickson et al., 1984; Prenen et al., 1984; Tiselius et al., 1981). In these studies, intestinal oxalate absorption was examined in healthy subjects by first administering [^{14}C]oxalate in test meals and then monitoring the excretion pattern of carbon-14 in the urine. As indicated by the relative amount of labeled carbon appearing in the urine during a 24-h collection period, maximum absorption occurred within 1-8 h after isotope ingestion. Very little, if any, isotope was

excreted after 10 h. Since an ordinary meal consumed by man requires approximately 10 h to travel from stomach to ileum (Read et al., 1980), these results suggest that under normal physiological conditions, the proximal small bowel is the major site of dietary oxalate absorption in man and that colonic absorption of oxalate is minimal.

The mechanism of intestinal oxalate transport was first studied by Binder in 1974. He observed that oxalate transport in the small and large intestines of laboratory rats was not energy-dependent, saturable, or carrier-mediated. The rate of oxalate absorption was directly proportional to oxalate concentration. These findings were confirmed in rats by Schwartz et al. (1980) and in guinea pigs and monkeys (Farooqui et al., 1983; Sidhu et al., 1984). Results of the studies cited above suggested that oxalate was absorbed mainly across the intestinal mucosa by passive diffusion. Under certain in vitro conditions, however, active transport of oxalate may occur in the intestine. Pinto and Paternain (1978) measured oxalate uptake by brush border cells from the human small intestine and detected an oxalate-binding protein that facilitated oxalate mobility. The oxalate-binding protein was isolated from the cytosol cellular fraction of the brush border cells; its oxalate-binding activity was greatly enhanced by the presence of magnesium and calcium. Freel et al. (1980) and Hatch et al. (1981) examined the transport of oxalate across isolated, short-circuited preparations of rabbit and rat colonic mucosa in both the presence and absence of calcium. Only when calcium was present in the incubation solution was net oxalate absorption observed.

They concluded that free calcium was required to maintain the normal junctional integrity of colonic epithelial tissue and prevent the development of a "leaky" epithelial membrane which would have otherwise obscured the net absorptive transport of oxalate. This may explain why Binder (1974) and Schwartz et al. (1980), who used calcium-free solutions to avoid the formation of calcium oxalate, failed to demonstrate active transport of oxalate. Thus, two different oxalate transport processes may operate in the mammalian intestine, depending upon the luminal concentration of oxalate (Pinto and Paternain, 1978). At high concentrations, intestinal oxalate absorption may occur by a passive diffusion process, whereas at low concentrations, a carrier-mediated active transport system may be functional. However, before their physiological significance can be defined, further research is needed. Intestinal oxalate absorption directly affects the bioavailability of oxalate and the luminal concentration of calcium ions. Brinkley et al. (1981) examined the bioavailability of oxalate from seven "oxalate-rich" foods. Of the food items tested, only oxalate from spinach was readily absorbed. They concluded that the majority of dietary oxalate is not available for absorption because of the presence of oxalate-binding components, especially calcium, in foods. Calcium oxalate not only appears to be poorly absorbed by the human intestine, but also the amount of oxalate absorbed is inversely proportional to dietary levels of calcium (Archer et al., 1957; Zarembski and Hodgkinson, 1969). In contrast to this concept, Prenen et al. (1984) recently reported that calcium oxalate is absorbed to the

same extent as soluble oxalate by the human small intestine.

Endogenous biosynthesis

Depending upon the diet and physiological conditions, approximately 0.4 mg of oxalate per kilogram body weight per day are endogenously produced in man, whereas laboratory rats synthesize about 1.0 mg of oxalate per kilogram body weight per day (Hodgkinson, 1977; Ribaya-Mercado and Gershoff, 1984). In most mammals, including man and laboratory rats, the liver plays a significant role in the biosynthesis of oxalate (Richardson and Farinelli, 1981). Recently, Ribaya and Gershoff (1982) suggested that the intestinal mucosa (enterocytes) provides an additional source of endogenously derived oxalate, especially in laboratory rats. The majority of endogenous oxalate is synthesized in the mammalian liver from ascorbate, glyoxylate, and glycolate (Fry and Richardson, 1979; Williams and Smith, 1968). Ascorbate and glyoxylate metabolism account for 90% of the endogenous oxalate formed in man (Laker, 1983; Smith, 1980). The remainder consists of oxalate produced from glycolate metabolism and a variety of endogenous and exogenous sources, including dietary carbohydrates, especially galactose, which increases endogenous oxalate production in laboratory rats (Hagler and Herman, 1973a; Ribaya-Mercado and Gershoff, 1984). The pathways of oxalate biosynthesis are summarized in Figure 2.

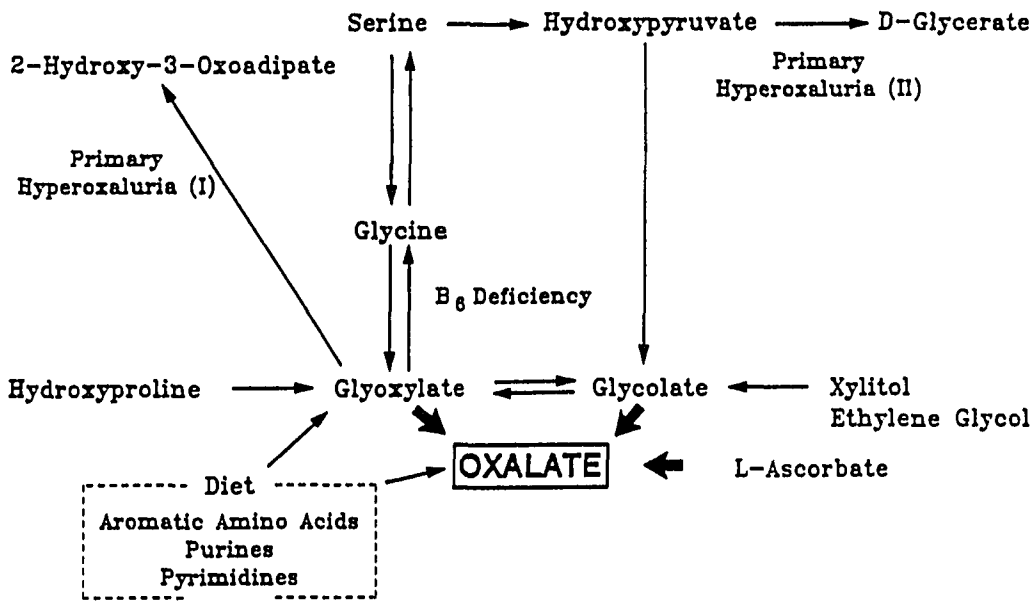

Figure 2. Pathways of endogenous oxalate biosynthesis in humans (Hodgkinson, 1977)

Ascorbate is a direct precursor of oxalate in man, monkeys, and guinea pigs (Baker et al., 1966; Hellman and Burns, 1958). By using isotopic techniques, it was established that 25-65% of the ascorbate in the body is converted non-enzymatically to oxalate and that oxalate is derived from the first two carbon atoms of ascorbate. The mechanism for this conversion is poorly understood (Baker et al., 1962; Baker et al., 1966). The consumption of large doses of ascorbate can increase urinary oxalate excretion in both man and laboratory animals (Farooqui et al., 1983; Schmidt et al., 1981). However, in recent studies, a direct correlation between excessive intake of ascorbate and hyperoxaluria could not be substantiated. Tsao and Salimi (1984) reported no significant increase in either plasma oxalate levels or oxalate excretion in normal subjects when mega doses (gram quantities) of ascorbate were consumed. By using trained monkeys as a model for ascorbate metabolism in man, Tillotson and McGown (1981) investigated the relationship between ascorbate intake and the amounts of urinary ascorbate metabolites derived from [1-^{14}C]ascorbic acid. The percentage of carbon-14 that appeared as oxalate in the urine was inversely proportional to the level of ascorbate supplemented in the diet. This suggested that oxalate production from ascorbate was directly influenced by the ascorbate nutritional status of the animal. Evidence presented here and by Moser and Hornig (1982) suggested that the metabolic mechanism for converting ascorbate to oxalate is easily saturated and may not represent a functional means of disposing of excess ascorbate from the body.

Glyoxylate, considered the major precursor of oxalate in mammals, is formed primarily from glycine, 1-keto-3-hydroxyglutarate, and glycolate (Liao and Richardson, 1973). Glycine is metabolized to glyoxylate by two direct mechanisms, oxidative deamination by glycine oxidase (D-amino acid oxidase) and vitamin B_6-dependent transamination with keto acids, and indirectly via serine and glycolate (Williams and Smith, 1972). Although by using isotopic experiments it was shown that only 0.5% of the available glycine is converted to endogenous oxalate in man (Elder and Wyngaarden, 1960), the relatively large amounts of glycine available make it an important progenitor of oxalate. The second precursor, 1-keto-3-hydroxyglutarate, is a product of hydroxyproline catabolism. Hydroxyproline, an amino acid found in gelatin and connective tissues proteins (collagen, elastin), is catabolized through a series of oxidation reactions and a vitamin B_6-dependent transamination to 1-keto-3-hydroxyglutarate, the latter being cleaved enzymatically to pyruvate and glyoxylate (Ribaya and Gershoff, 1981). When laboratory rats were fed diets supplemented with hydroxyproline, not only was endogenous oxalate synthesis increased, but the amount of oxalate formed was directly related to the vitamin B_6 nutritional status of the animal (Ribaya and Gershoff, 1979; Ribaya and Gershoff, 1981). However, in man, evidence does not support hydroxyproline or 1-keto-3-hydroxyglutarate as important precursors of either glyoxylate or oxalate (Williams and Smith, 1968). Glycolate, is converted to glyoxylate by glycolic acid oxidase (Hagler and Herman, 1973a). In mammals, glycolate originates from the diet, mainly green

leafy vegetables, and by production from endogenous glycolaldehyde, hydroxypyruvate, serine, tyrosine, ethanolamine, and phenylalanine (Gambardella and Richardson, 1978; Rofe et al., 1986; Talwar et al., 1984). The relative significance of dietary glycolate as an important oxalate precursor in rats has recently been the focus of several investigations. Talwar et al. (1985) reported that when rats were fed sodium glycolate, a 2-fold increase in urinary oxalate excretion was obtained. Although only .3-5% of dietary glycolate was metabolized to oxalate in normal rats (Harris and Richardson, 1980), this rate of conversion is greatly enhanced by vitamin B_6 deficiency (Runyan and Gershoff, 1965).

Glyoxylate is converted to oxalate by lactate dehydrogenase, glycolic acid oxidase, and xanthine oxidase. The latter is the least significant of the three (Menon and Mahle, 1982). Unlike lactate dehydrogenase, which is present throughout the tissues of the body, glycolic acid oxidase is mainly limited to the liver in man and laboratory rats (Varalakshmi and Richardson, 1983). Both enzymes catalyze the oxidation of glyoxylate to oxalate, whereas glycolic acid oxidase also catalyzes the oxidation of glycolate to oxalate via glyoxylate (Richardson and Tolbert, 1961). In addition, glycolate may be oxidized directly to oxalate by glycolate dehydrogenase, an enzyme recently discovered in the liver of man and rats (Fry and Richardson, 1979; Gambardella and Richardson, 1978). By using isolated perfused rat livers, Gambardella and Richardson (1978) and Liao and Richardson (1973) found that oxalate formation in the liver was the direct result

of glycolic acid oxidase and glycolate dehydrogenase activities and did not involve lactate dehydrogenase. Studies with hepatectomized rats were conducted to determine whether the liver enzymes, glycolic acid oxidase and glycolate dehydrogenase, or non-liver lactate dehydrogenase, participated in the in vivo synthesis of endogenous oxalate. Hepatectomy caused a significant reduction in the biosynthesis of oxalate from glycolate and a shift in the oxidation of glyoxylate to oxalate from glycolic acid oxidase to another enzyme, presumably extrahepatic lactate dehydrogenase (Farinelli and Richardson, 1983; Varalakshmi and Richardson, 1983). These findings and those of Richardson and Farinelli (1981) suggest that during normal physiological conditions, when glyoxylate and glycolate are present at low levels, glycolic acid oxidase and glycolate dehydrogenase are responsible for the majority of endogenous oxalate synthesis in man and the laboratory rat. When these two liver enzymes are saturated with substrate or not functioning properly, however, the amount of endogenous oxalate produced by extrahepatic lactate dehydrogenase is greatly increased.

Catabolism and excretion

Catabolism When [^{14}C]oxalate was intravenously administered to healthy humans, 90-100% of the isotopically labeled oxalate was excreted unchanged in the urine within 48 h and there was little labeling of respiratory CO_2 (Elder and Wyngaarden, 1960; Hodgkinson and Wilkinson, 1974). In contrast, when [^{14}C]oxalate was parenterally administered to laboratory rats, only 50-70% of the label was recovered

in the urine; the remaining activity was found in the muscle, bone, and feces (Curtin and King, 1955; Jones et al., 1981; Weinhouse and Friedmann, 1951). Less than 1.0% of the radioactive oxalate was converted to $^{14}CO_2$. Thus, the oxalate present in the tissues of man and rats, regardless of whether it is endogenously derived or absorbed from the gut, appears to be an end-product. However, Murthy et al. (1981) recently reported that oxalate was catabolized in the liver, intestine, kidney, and blood of the guinea pig by the enzyme, oxalate decarboxylase. A partial characterization of this enzyme revealed a pH optimum of 4.0 and no cofactor requirement in the crude tissue homogenate. Before the significance of this enzyme can be realized, further studies must be performed on the isolation, characterization, and distribution of oxalate decarboxylase among mammalian species.

Excretion With most mammals, unabsorbed dietary oxalate is excreted from the body in the feces. Oxalate, both metabolically derived and absorbed from the intestine, is eliminated from the body almost exclusively in the urine. In addition, oxalate may be eliminated from the body via nonrenal excretion, which involves the secretion of small amounts of oxalate into the intestine via the bile and enterocytes (Dobson and Finlayson, 1973; Ribaya and Gershoff, 1982). The oxalate secreted into the gut is subsequently eliminated from the body in the feces. This form of excretion may account for the presence of carbon-14 in the feces of laboratory rats parenterally injected with [^{14}C]oxalate (Hodgkinson, 1978; Shirley and Schmidt-Nielsen, 1967; Weinhouse and Friedmann, 1951). Even though the

magnitude of nonrenal excretion and its relative contribution to the overall elimination of oxalate from the body has yet to be fully investigated in man and rats, preliminary evidence suggests that the amount of oxalate excreted via this route is insignificant in most mammals. However, a recent report on the presence of calcium oxalate gallstones in a premature neonate suggests that oxalate secretion into the bile is significant when certain predisposing conditions exist (Wolf et al., 1982).

In humans, the amount of oxalate excreted in the urine increases during childhood until the age of 14, whereupon adult levels, approximately 4.5 mg per kilogram body weight per day, are achieved (Gibbs and Watts, 1969; Hodgkinson, 1977). Of this amount, endogenous oxalate synthesis and intestinal absorption of dietary oxalate account for 85 and 15%, respectively. In comparison, laboratory rats excrete about 9.0 mg of oxalate per kilogram body weight per day, of which 70% is from endogenous production and 30% is derived from the diet (Hodgkinson, 1978). Thus, when expressed on a similar basis, laboratory rats not only excrete greater quantities of oxalate in their urine than man, but also the contribution of dietary oxalate to urinary oxalate excretion is significantly greater, a noteworthy difference on the physiology and metabolism of oxalate between man and rat.

The amount of oxalate excreted in the urine of man and animals is obviously modulated by three main factors: diet, intestinal absorption, and endogenous synthesis. The next section will discuss how these factors may contribute to increased oxalate excretion

(hyperoxaluria) and calcium oxalate urolithiasis in mammals.

Calcium Oxalate Urolithiasis

Urolithiasis refers to the pathological formation of calculi (stones) in the urinary tract of mammals. In Western Europe and the United States, 5-10% of the normal adult population is afflicted with urinary calculi (Rose, 1982). Urinary calculi also occur in dogs (Klausner et al., 1981), horses (Andrews, 1971), cattle (Huntington and Emerick, 1984), sheep (Nottle, 1982), and laboratory rats (Kuhlmann and Longnecker, 1984).

In humans, the majority (70-80%) of urinary calculi are composed primarily of calcium oxalate monohydrate (whewellite), calcium oxalate dihydrate (weddellite), or both (Smith, 1980). Secondary components include magnesium ammonium phosphate (struvite), calcium phosphate (apatite), cystine, urate, and silica (Churchill, 1983). However, the overall mineral composition of urinary stones tends to vary considerably among mammalian species.

Urinary calculi formation is affected by the ability of factors in urine to inhibit urolith formation, factors which act as stone initiators, and factors that affect the supersaturation of urine with respect to the precipitating salt (e.g., calcium oxalate). The inhibitory activity of urine may be credited to pyruvate, citrate, magnesium, pyrophosphate, and certain high molecular weight compounds (glycosaminoglycans, acidic peptides); initiating activity is restricted to a Tamm-Horfall mucoprotein (Anasuya, 1983; Ogawa et al.,

1986; Scurr et al., 1981). The supersaturation of urine with a particular insoluble salt is dependent upon the urine volume and pH and the urinary concentration of such stone components as phosphate, urate, magnesium, calcium, and oxalate (Anasuya, 1983; Churchill, 1983). Although each of these factors determines to some degree whether or not urinary calculi will develop, increased oxalate excretion is the single most important risk factor for stone formation, because of its dramatic influence on calcium oxalate saturation in urine (Robertson et al., 1981b). Since most normal urine is nearly saturated with calcium oxalate, hyperoxaluria may ultimately lead to supersaturation and the subsequent precipitation of calcium oxalate in urine, which in turn provides the nucleus for calculi formation.

Hyperoxaluria with associated calcium oxalate urolithiasis is frequently caused by a variety of clinical disorders in man and animals. In most instances, these disorders can be classified as to whether the resulting hyperoxaluria is caused by an increase in either dietary oxalate intake, intestinal oxalate absorption, or endogenous oxalate synthesis.

Increased dietary intake

Oxalate intoxication The ingestion of plants of high oxalate content by grazing livestock (sheep, cattle, horses) can result in oxalate intoxication, producing such symptoms as depression, hypocalcemia, alkalosis, and oxalate crystals in the kidney and rumen wall (James, 1972). These symptoms are uncommon in other mammalian species. In normal man and laboratory animals, the occasional

consumption of oxalate-rich foods does result in increased urinary oxalate excretion; however, the resulting hyperoxaluria only rarely leads to calcium oxalate urolithiasis, suggesting additional factors are necessary for stone development (Finch et al., 1981; Hodgkinson, 1978). With laboratory rats, for example, special semi-synthetic diets, either low in phosphorous or supplemented with glycine and hydroxyproline, or increased oxalate ingestion associated with vitamin B_6 deficiency, is required to induce oxalate urolithiasis (Coburn and Packett, 1962; Lyon et al., 1966; Ribaya and Gershoff, 1982).

Increased intestinal absorption

Enteric (secondary) hyperoxaluria Hofmann et al. (1970) and Smith et al. (1970) first reported the association between calcium oxalate urolithiasis, hyperoxaluria, and ileal resection. Now, in addition to ileal resection, numerous gastrointestinal disorders are associated with enteric hyperoxaluria. Some of these include regional enteritis, pancreatic exocrine insufficiency, bacterial overgrowth, ileal and celiac diseases, and jejunoileal bypass for morbid obesity (Smith, 1980). Associated with each of these disorders is an increased frequency of urolithiasis; for example, among jejunoileal bypass patients, the prevalence of calcium oxalate stones ranges from 11 to 32%, and renal complications account for 20% of all deaths related to bypass surgery (Clayman and Williams, 1979; O'Leary et al., 1974).

Three mechanisms were originally proposed for the etiology of enteric hyperoxaluria. One hypothesis was that enteric hyperoxaluria was caused by a disruption of bile acid metabolism (Hofmann et al.,

1970; Smith et al., 1970). Since bile acid malabsorption is often associated with these intestinal disorders, it was believed that the excessive deconjugation of bile salts released large amounts of glycine into the intestinal lumen and enteric bacteria converted the deconjugated glycine to glyoxylate. After absorption, the glyoxylate was metabolized to oxalate, which is then excreted in the urine producing hyperoxaluria. Secondly, Smith et al. (1972) proposed that, since glycine is required for bile acid conjugation, the malabsorption of bile salts caused a significant reduction in hepatic glycine levels. This glycine deficiency would in turn enhance the hepatic production of glycine from various precursors including glyoxylate, of which some was converted to oxalate. Thirdly, Earnest et al. (1972) suggested that enteric hyperoxaluria may be due to an acquired hepatic defect in the metabolism of glyoxylate. This defect apparently resulted in increased formation of oxalate from glyoxylate.

Although these proposed etiological mechanisms for enteric hyperoxaluria appear quite attractive, additional observations have made their validity uncertain. Chadwick et al. (1973) and Hofmann et al. (1973) obtained negligible conversion of orally administered cholyl-[^{14}C]glycine to urinary [^{14}C]oxalate in resected-ileal patients, suggesting that the glycine moiety of conjugated bile salts is not a major precursor of urinary oxalate during enteric hyperoxaluria. In addition, Earnest et al. (1974) reported that, if enteric hyperoxaluria is like Type I primary hyperoxaluria (see below), the increased synthesis of glyoxylate would not only increase oxalate but also

glycolate excretion in the urine. However, glycolate excretion appeared to be normal during enteric hyperoxaluria, indicating that an abnormality in glyoxylate metabolism is not the major cause of enteric hyperoxaluria. Therefore, based on these observations, none of the proposed mechanisms adequately explains why hyperoxaluria is often associated with certain enteric disorders.

Chadwick et al. (1973) and Earnest et al. (1974) provided convincing evidence that increased absorption of exogenous oxalate was the major cause of hyperoxaluria secondary to enteric disease. In these studies, patients with enteric hyperoxaluria not only absorbed and excreted 2 to 4 times more dietary oxalate than normal subjects, but also the severity of this type of hyperoxaluria was effectively reduced, but not abolished, by a low-oxalate diet. These original findings have been further confirmed by other investigators (Nordenvall et al., 1981; Stauffer et al., 1973; Tiselius et al., 1981).

In patients with enteric hyperoxaluria, the large intestine is considered the site of increased oxalate absorption. By measuring the urinary excretion of orally administered [^{14}C]oxalate, Earnest et al. (1974) and Dobbins and Binder (1977) found that the intestinal absorption of oxalate was significantly increased in patients with extensive ileal resection and intact colons, but normal in patients with an ileal resection and ileostomy or colectomy. Similar results were reported by Modigliani et al. (1978), who, in addition to Earnest et al. (1974), also demonstrated that a direct correlation existed between fecal fat malabsorption (steatorrhea) and urinary oxalate

excretion in patients with intact colons and hyperoxaluria secondary to ileal resection or disease. Hyperoxaluria has also been associated with steatorrhea in each of the gastrointestinal disorders listed above. Thus, regardless of the cause, steatorrhea and the colon play an important role in the etiology of enteric hyperoxaluria.

Based on in vitro studies in humans and in animal models, two theories have been proposed to explain the increased colonic absorption of oxalate which occurs during enteric hyperoxaluria. The first, commonly called the solubility theory, suggests that, since steatorrhea is common in patients with enteric hyperoxaluria, large amounts of unabsorbed fatty acids decrease the intraluminal concentration of calcium by forming calcium soaps (Chadwick et al., 1973; Dobbins and Binder, 1976; Earnest et al., 1975). The decrease in available calcium thereby allows more oxalate to remain in a soluble and absorbable form. The second or permeability theory suggests that, because enteric disorders often cause both fat and bile acid malabsorption, the colon may be exposed to excessive concentrations of fatty acids (oleate, ricinoleate) and dihydroxy bile salts (chenodeoxycholate, deoxycholate). Unlike the small intestine, the presence of these malabsorbed fatty acids and bile salts nonspecifically increases colonic mucosal permeability to oxalate in man (Earnest, 1977; Fairclough et al., 1977) and laboratory rats (Dobbins and Binder, 1976; Kathpalia et al., 1984; Saunders et al., 1975; Schwartz et al., 1980). The increased colonic permeability to oxalate subsequently causes increased oxalate absorption and hyperoxaluria. Even though fatty

acids and bile salts both participate in the pathogenesis of hyperoxaluria, further studies are needed to determine the relative contributions of the oxalate solubility and colonic permeability theories to increased oxalate absorption associated with enteric hyperoxaluria.

The elimination of hyperoxaluria secondary to enteric disease is extremely difficult, even when patients are given controlled diets low in oxalate content (Earnest et al., 1974). Hofmann et al. (1983) suggested that a significant proportion of urinary oxalate may originate from sources other than preformed food oxalate in patients with enteric hyperoxaluria, especially when dietary oxalate is restricted. In their study, two sources of oxalate (other than dietary oxalate) were postulated: the production of oxalate by bacteria in the intestine or in tissues (liver, intestinal mucosa) and an oxalogenic substance associated with foods rich in protein and creatinine. These additional sources of oxalate may account for the apparent difficulty in abolishing secondary hyperoxaluria by a low-oxalate diet alone.

Besides hyperoxaluria, additional abnormalities, related to the general disturbance of intestinal absorption in patients with enteric disease, may be equally important in the pathogenesis of calcium oxalate urolithiasis. Some of these abnormalities or risk factors include: decreased urine volume due to water malabsorption; reduced ionic strength of urine because of electrolyte malabsorption; magnesium and citrate depletion; reduced urine pH; and reduced urinary sulfate and pyrophosphate resulting from protein malabsorption (Bambach et al.,

1981; Nordenvall et al., 1983; Smith et al., 1980). Since citrate, magnesium, phosphate, pyrophosphate, and sulfate participate in both the supersaturation and inhibitor activity of urine, decreases in their urinary concentrations would reduce complexation and promote the formation of calcium oxalate crystals in urine.

Stone disease Among the normal human population, there exists a group that suffers from recurrent idiopathic calcium oxalate urolithiasis (stone disease). Within this stone-forming group, both intestinal oxalate absorption and urinary oxalate excretion are slightly, but significantly, increased when compared to normal subjects (Brown et al., 1987; Robertson et al., 1978; Tiselius et al., 1981; Zarembski and Hodgkinson, 1969). In addition, Broadus and Thier (1979) found that approximately 50% of the stone-formers exhibited hypercalciuria.

Although the mechanism of increased intestinal oxalate absorption is currently unknown, Erickson et al. (1984) and Marangella et al. (1982) suggested that hyperoxaluria is secondary to calcium hyperabsorption in patients with idiopathic calcium oxalate stone disease. Based on the fact that oxalate absorption may be influenced by intestinal calcium concentrations, Erickson et al. (1984) postulated that hyperabsorption of calcium would cause a decrease in the intraluminal concentration of calcium, thereby allowing more oxalate to remain soluble and available for absorption. However, this hypothesis fails to elucidate the etiology of hyperoxaluria in normocalciuric stone-formers, suggesting that additional factors, besides the

hyperabsorption of dietary calcium, are involved in increased intestinal oxalate absorption associated with stone disease.

Robertson et al. (1978) showed that the abnormal excretion of one or more urinary constituents (eg. oxalate, calcium, and urate) by idiopathic stone-formers contributes to the formation of urinary calculi. Numerous environmental and metabolic "risk" factors have been identified which may influence the excretion of these potential stone-forming urinary constituents and the probability of urinary calculi formation. These risk factors include: reduced fluid intake (decreased urine volume); affluence; sedentary lifestyle; climate (increased temperature decreases urine volume, increased exposure to sunlight increases calcium absorption through the action of vitamin D); age (the peak age of stone occurrence is 35 for men and 30 for women); sex (the male/female ratio of stones is 2 to 3:1); and a high oxalate and/or animal protein diet (Hagler and Herman, 1973c; Robertson et al., 1981a). Thus, various epidemiological and pathophysiological factors, besides hyperoxaluria secondary to oxalate hyperabsorption, may participate in the pathogenesis of recurrent stone disease.

Pyridoxine deficiency Although investigators have clearly demonstrated that endogenous oxalate biosynthesis in mammals is increased during pyridoxine (vitamin B_6) deficiency (Gershoff, 1964), only recently has the intestinal absorption of dietary oxalate been shown to be influenced by the pyridoxine in the body. Farooqui et al. (1981) used laboratory rats and demonstrated that intestinal oxalate absorption was enhanced by 80% during acute vitamin B_6 deficiency. The

intestinal hyperabsorption of oxalate was apparently a result of the induction of a biphasic oxalate transport system: a saturable, sodium-independent transport process and a linear nonsaturable passive-diffusion process. Further studies by Farooqui et al. (1984) revealed that the sialic acid, cholesterol, and protein contents of the brush border membrane were severely reduced in vitamin B_6-deficient rats. Alterations in the chemical composition of the microvillus membrane may, therefore, be responsible for both the development of a two-component transport system and the increase in intestinal oxalate absorption observed in vitamin B_6-deficient rats. Whether or not pyridoxine deficiency produces similar responses in humans is unknown.

Increased endogenous synthesis

Pyridoxine deficiency Both endogenous oxalate formation and urinary oxalate excretion in man and laboratory animals are inversely related to the level of vitamin B_6 in the diet (Gershoff, 1964; Ribaya and Gershoff, 1981). Vitamin B_6 supplementation reduces the degree of hyperoxaluria in man and is often used in the treatment of idiopathic calcium oxalate stone disease, even though its mechanism of action is largely unknown (Smith, 1980). Vitamin B_6 deficiency does reduce the activity of most aminotransferases, including glyoxylate transaminase, an enzyme that catalyzes the transamination of glyoxylate to glycine and therefore controls the pool of glyoxylate in the body (Hagler and Herman, 1973b). Thus, during pyridoxine deficiency, glyoxylate oxidation to oxalate may be enhanced due to the increased pool of available glyoxylate. However, studies by Runyan and Gershoff (1965)

demonstrated that vitamin B_6-deficient rats synthesized and excreted significantly more oxalate from glycolate, ethylene glycol, and ethanolamine than from glyoxylate, when compared to control animals. Contrary to earlier beliefs, these findings suggest that glycolate oxidation rather than glyoxylate oxidation is stimulated during vitamin B_6 deficiency. Glycolate is converted to oxalate by the liver enzymes, glycolic acid oxidase, lactate dehydrogenase, and glycolate dehydrogenase. Since glycolate oxidation to oxalate by glycolic acid oxidase involves the formation of glyoxylate as a metabolic intermediate (Richardson and Tolbert, 1961) and because glyoxylate oxidation is not stimulated by a deficiency in vitamin B_6, glycolic acid oxidase does not appear to be the enzyme stimulated during pyridoxine deficiency. Regarding lactate dehydrogenase, vitamin B_6 deficiency has little affect on either the hepatic or extrahepatic activity of this enzyme in laboratory rats (Murthy et al., 1982). Based on these observations and the recent findings of Varalakshmi and Richardson (1983), glycolate dehydrogenase, which oxidizes glycolate directly to oxalate, is now considered to be the enzyme solely responsible for the increase in endogenous oxalate synthesis and resulting hyperoxaluria observed in vitamin B_6 deficiency. Further studies are required to determine the process by which vitamin B_6 deficiency influences the activity of glycolate dehydrogenase and whether or not similar effects can be demonstrated in humans.

Primary hyperoxaluria Primary hyperoxaluria is a rare genetic disorder that usually manifests itself in early childhood (Morris et

al., 1982). This inherited syndrome is characterized by hyperoxaluria and calcium oxalate nephrolithiasis which frequently results in chronic renal failure and death (Williams and Smith, 1968). Two forms of primary hyperoxaluria have been described: Type I (glycolic aciduria) and Type II (L-glyceric aciduria). In Type I hyperoxaluria, the more common form, there is a defect in 2-oxoglutarate:glyoxylate carboligase, an enzyme which converts glyoxylate to 2-hydroxy-3-oxoadipate (Smith, 1980). In the absence of this enzyme, excessive amounts of oxalate and glycolate are produced and excreted in the urine. In the rarer Type II, or L-glyceric aciduria, there is a deficiency in D-glycerate dehydrogenase, an enzyme catalyzing the interconversion of hydroxypyruvate to D-glycerate (Laker, 1983). Due to the deficiency in D-glycerate dehydrogenase, accumulated hydroxypyruvate is reduced to L-glycerate by lactate dehydrogenase, causing excessive urinary excretion of L-glycerate (not a normal urine constituent). Oxalate excretion also increases, while glyoxylate and glycolate excretion are normal (Williams and Smith, 1972). Currently, there are three hypotheses for the origin of this hyperoxaluria. The first suggests that the reduction of hydroxypyruvate to L-glycerate by lactate dehydrogenase causes an increase in the NAD:NADH ratio (Williams and Smith, 1971). To regenerate NADH, glyoxylate oxidation to oxalate by lactate dehydrogenase is enhanced. Secondly, hydroxypyruvate may be metabolized to glycolate and then to oxalate by glycolate dehydrogenase (Fry and Richardson, 1979; Gambardella and Richardson, 1978). Finally, hydroxypyruvate may be converted to

oxalate by a non-enzymatic process recently described by Raghavan and Richardson (1983). Further studies are needed to verify which mechanism(s) is truly responsible for Type II hyperoxaluria.

Increased intake of oxalate precursors Increased consumption of certain oxalate precursors by man and laboratory rats may lead to hyperoxaluria. Precursors known to produce hyperoxaluria include glycolate, glycine, ascorbate, ethylene glycol, xylitol, and methoxyflurane. Since the role of glycolate, glycine, and ascorbate as oxalate precursors has been previously discussed, this section will focus on ethylene glycol, xylitol, and methoxyflurane.

Ethylene glycol Ethylene glycol is widely used as an industrial solvent and an antifreeze agent. When accidentally ingested, ethylene glycol is in part excreted unchanged in the urine, metabolized to carbon dioxide, and oxidized to oxalate via glycolaldehyde and glyoxylate (Smith, 1980). Thus, ethylene glycol, which itself is not toxic, becomes toxic when converted to oxalate in mammalian tissues. Ingestion of large quantities of ethylene glycol may lead to severe hyperoxaluria, resulting in calcium oxalate nephrolithiasis, renal failure, and death (Hagler and Herman, 1973d).

Xylitol Intravenous xylitol is often administered as a source of calories during parenteral nutrition. Mammalian tissues metabolize xylitol to oxalate and oxalate precursors. Thus, the infusion of xylitol can lead major adverse reactions, including severe hyperoxaluria and the deposition of calcium oxalate crystals in renal, vascular, and cerebral tissues (Thomas et al., 1976). This suggests

that the metabolism of xylitol by mammalian tissues generates oxalate or oxalate precursors which can contribute to endogenous oxalate. However, not all patients receiving xylitol develop oxalosis or hyperoxaluria. Attempts to induce xylitol oxalosis in laboratory rats have not been successful, suggesting that additional factors, besides xylitol breakdown, may be required for oxalate crystal deposition (Hannett et al., 1977; Hauschildt et al., 1976; Rofe et al., 1977).

Methoxyflurane Following the administration of methoxyflurane, a general anesthetic, postoperative patients often exhibit increased excretion of urinary oxalate followed by acute renal failure (Frascino et al., 1970). Since oxalate and fluoride are both produced from the metabolism of methoxyflurane by mammalian tissues, these two metabolic end-products have been implicated as the causative agents of methoxyflurane-induced hyperoxaluria and oxalosis (Wilson et al., 1972).

Oxalate Degradation in the Gastrointestinal Tract

As illustrated in previous sections, studies abound on the physiopathological effects of diet, intestinal absorption, and endogenous synthesis on oxalate metabolism and excretion in mammals. Information concerning the microbial decomposition of oxalate in the gastrointestinal tract of mammals, however, is limited. This paucity of information is somewhat surprising, since the decomposition of exogenous oxalate by intestinal oxalate-degrading microbes may decrease the amount of dietary oxalate which is available for absorption and

thus the amount of oxalate which is excreted in the urine. The purpose of this section will be to discuss the role that gastrointestinal microbes play in the degradation and detoxification of oxalate consumed by the host animal and their possible influence on the absorption and urinary excretion of dietary oxalate.

Adaptation of the ruminal microflora to oxalate

The destruction of oxalate by the ruminal microflora was first suggested by Talapatra et al. (1948) and later confirmed by Morris and Garcia-Rivera (1955). Subsequently, it was demonstrated that cattle and sheep adapted to high-oxalate diets tolerated levels of oxalate that would otherwise be toxic or lethal to unadapted animals (Dobson, 1959; James et al., 1967; Watts, 1957). This resistance or adaptation to the toxic effects of oxalate was attributed to an increase in the ability of microbes to detoxify (degrade) ingested oxalate in the rumen; however, neither the actual rates of oxalate degradation in ruminal contents, the time required for oxalate adaptation, nor the specific microbes involved in oxalate degradation were determined. By using the production of labeled CO_2 from [^{14}C]oxalate to measure oxalate degradation, Allison et al. (1977) determined the *in vitro* rates of oxalate degradation in ruminal contents of sheep before, during, and after adaptation to diets supplemented with increasing quantities of halogeton (12 to 15% oxalic acid). During a 3- to 4-day adaptation period, ruminal rates of oxalate degradation increased from less than 0.05 μmol/ml per hour to more than 0.2 μmol/ml per hour. Further tests with ruminal contents from adapted sheep demonstrated

that a significant portion of the oxalate-degrading capacity was associated with ruminal fractions containing bacteria rather than protozoa. Oxalate degradation was inhibited by oxygen and by neomycin and penicillin. In subsequent studies by Allison et al. (1981), a similar pattern of adaptation to increased quantities of oxalate was also demonstrated in an in vitro continuous culture system that contained a mixed bacterial population established with an inoculum of ruminal contents from an unadapted sheep. In this oxalate-adapted continuous culture system, oxalate degradation rates were often 10 to 100-fold greater than rates measured in the original inoculum. Furthermore, when the rumen of an unadapted sheep was inoculated with contents from an oxalate-adapted culture and then challenged with a lethal dose of oxalate, the inoculated animal tolerated a quantity of oxalate that would have been lethal to a sheep not previously exposed to oxalate (Allison and Reddy, 1984). These authors postulated that inoculation with an oxalate-adapted mixed ruminal population apparently prevented oxalate intoxication by replacing the requirement for gradual adaptation to oxalate by unadapted sheep. These observations, together with the traditional view that obligate anaerobes tend to be the most functional bacteria in the healthy rumen, support the hypothesis that obligately anaerobic, oxalate-degrading bacteria, members of the normal ruminal microflora, are responsible for the degradation of oxalate in the rumen and that adaptation to dietary oxalate involves the selection of an increased proportion of these oxalate-utilizing bacteria. However, initial attempts to isolate and enumerate these anaerobic

oxalate-degrading bacteria from ruminal contents of an adapted sheep were not successful (Allison et al., 1977).

Microbial degradation of oxalate in the nonruminal gut

The degradation of dietary oxalate by microflora of the gastrointestinal tract is not restricted solely to the rumen and its resident microbes. The microbial degradation of oxalate also occurs in the large bowel of humans and a variety of nonruminant herbivores.

Humans Barber and Gallimore (1940) presented the first evidence that oxalate could be degraded by human fecal bacteria. The microbial destruction of oxalate in feces was independent of the presence of atmospheric oxygen, occurring under both aerobic and anaerobic environments and was inhibited when fecal material was heated to 80°C. This suggested that a facultatively or obligately anaerobic, non-sporeforming microorganism may be responsible for oxalate degradation in human feces. No attempts were made, however, to isolate and characterize the specific microbe(s) involved, and little research on the oxalate-degrading capacity of human intestinal microflora was published for 40 years. In 1981, the original findings of Barber and Gallimore (1940) were essentially reconfirmed in a report by Hagmaier and associates (1981). The latter workers demonstrated that microbes present in human feces possessed the capacity to destroy oxalate. Allison et al. (1986) measured the rates of conversion of [^{14}C]oxalate to $^{14}CO_2$ during *in vitro* incubations of fecal samples from healthy humans who consumed normal uncontrolled diets. Oxalate degradation rates varied considerably between subjects, ranging from 0.1 to 4.8

μmol/g (wet weight) of feces per hour. These measurements provided the first real estimates of oxalate degradation rates by mixed bacterial populations under conditions which emulated those of the human bowel. Since the diets were not controlled, the wide range of oxalate degradation rates observed in these studies was attributed to possible differences in dietary oxalate intake between subjects, although additional factors may have also been involved. Allison and associates also determined the rates of oxalate degradation in fecal samples from individuals with enteric dysfunction caused by previous jejunoileal bypass surgery for morbid obesity. In contrast to normal subjects, oxalate degradation rates from jejunoileal bypass patients were negligible or extremely low, suggesting an absence or reduced concentration of colonic oxalate-degrading bacteria. Based on the above observations, these authors proposed that the reduced colonic rates of oxalate degradation observed in individuals with altered intestinal function due to enteric disease or resection may enhance oxalate availability in the colon and thereby contribute to increased absorption of dietary oxalate and hyperoxaluria, both of which are associated with these enteric abnormalities. Further studies are warranted before this "microbial" theory can be employed along with the solubility and permeability theories to explain the etiological basis of enteric hyperoxaluria.

Nonruminant herbivores

Swine and horses Studies on experimental oxaluria and oxalate toxicity in swine (Bruce and Bredehorn, 1961; Wilson and

Harvey, 1977) and oxalate-balance studies in horses (McKenzie et al., 1981) led to the suggestion that the gastrointestinal microflora present in these animals may be responsible for the destruction of considerable amounts of oxalate in the ingesta. Allison and Cook (1981) measured the *in vitro* rates of oxalate degradation in cecal contents of swine and the rectal contents of a horse. Oxalate degradation rates by mixed bacterial populations in gastrointestinal contents from both horses and swine increased significantly when dietary oxalate levels were gradually increased and returned to original pre-adaptive values when oxalate supplementation to the diet was eliminated. Thus, in both swine and horses, the addition of oxalate to the diet appears to elicit a response analogous to that observed with ruminal microbes in sheep and cattle adapted to high oxalate diets (Allison et al., 1977).

Guinea pigs and rabbits In an effort to study the *in vivo* decomposition of dietary oxalate in animals, Hagmaier et al. (1981) measured the exhalation of $^{14}CO_2$ by guinea pigs orally dosed with [^{14}C]oxalate. Within 24 h, 70-75% of the labeled dose of oxalate had been excreted as $^{14}CO_2$. Thus, it appears that the main route of metabolism and excretion of exogenous oxalate in the guinea pig is by respiratory exhalation as CO_2. However, whether or not the destruction of exogenous oxalate was by oxalate-degrading microbes in the gut or *in vivo* mammalian metabolism after absorption was not specifically demonstrated. Allison and Cook (1981) confirmed the destruction of exogenous oxalate by oxalate-degrading microbes in the gut of guinea

pigs, as well as rabbits, by gradually adapting these animals to laboratory diets supplemented with increasing quantities of halogeton (contained 14.7% oxalate) and then measuring the in vitro rates of oxalate degradation in cecal contents from these animals. Mean cecal rates of oxalate degradation from both guinea pigs and rabbits fed high-oxalate diets were significantly greater (10-fold) than cecal rates from animals fed diets without oxalate supplementation. Thus, cecal oxalate-degrading microbes were similar to ruminal microbes in their response to the increased availability of dietary (exogenous) oxalate.

Rats Desert sand rats and pack rats, which consume high-oxalate diets in the wild, excreted within 24 h approximately 20 and 40%, respectively, of an orally administered dose of calcium [^{14}C]oxalate as respiratory $^{14}CO_2$. In contrast, laboratory rats excreted less than 1.0% as labeled CO_2 (Shirley and Schmidt-Nielsen, 1967). Hagmaier et al. (1981) noted a similar response by laboratory rats orally dosed with sodium [^{14}C]oxalate instead of calcium oxalate. In this study, laboratory rats expired only 5% of the labeled oxalate as $^{14}CO_2$. Bannwart et al. (1979) found that approximately 98% of an oral dose of sodium [^{14}C]oxalate was distributed in the urine (25%) and feces (73%) of laboratory rats. Respiratory CO_2 was not directly measured, however, since only 2% of the carbon-14 administered as oxalate was not recovered, little of the dietary oxalate was destroyed in vivo. Since neither the oxalate absorbed from the intestine nor that endogenously synthesized is catabolized to any significant extent

in laboratory rats, oxalate-degrading microbes appear to be essentially absent from the gut of these rodents. Results of Allison and Cook (1981) support the above findings. They demonstrated that in vitro rates of oxalate degradation by the microflora in cecal contents from laboratory rats were very low or negligible and did not increase when animals were fed diets supplemented with high levels of oxalate. Thus, contrary to most animals, neither the presence nor the selective enrichment of oxalate-degrading bacteria has been documented in the gastrointestinal tract of the laboratory rat.

The apparent lack of oxalate-degrading bacteria in the intestines of laboratory rats may be attributed to dietary factors. Shirley and Schmidt-Nielsen (1967) postulated that, through the standard use of oxalate-deficient diets, laboratory rats have simply lost the capacity (microbes) necessary to metabolize oxalate in gut over the many generations of laboratory breeding. On the other hand, this may explain why wild desert rodents accustomed to high oxalate diets on a daily basis possess microbial populations required to degrade and detoxify dietary oxalate in the gastrointestinal tract. In addition to diet, methods used in the development and maintenance of commercial rat colonies may hinder both acquisition and establishment of oxalate-degrading populations in the gut of laboratory rats. Gustafsson and Norman (1962) observed that the frequency of urinary calculi, composed primarily of calcium citrate and calcium oxalate, was significantly greater in germfree rats than in conventional rats. These authors attributed calculi formation in germfree rats to high levels of calcium

and citrate, low phosphate levels, and the high pH of urine. This urinary excretion pattern was changed to that of conventional rats when germfree rats were colonized with the intestinal flora from conventional rats. Whether colonization introduced oxalate-degrading bacteria and/or effected urinary oxalate excretion in germfree rats was not determined; however, this study illustrates the potential influence that intestinal bacteria may have on the absorption and urinary excretion of dietary constituents, including the development of urinary stones in the host animal.

Conventional laboratory rats are often used as models in studies of oxalate absorption and excretion and most studies have neglected the possible influence that intestinal oxalate-degrading microbes could have on oxalate metabolism. Future research should thus be directed towards determining whether or not oxalate-degrading microbial populations from different sources (e.g., wild rats, human feces) can colonize the gastrointestinal tracts of laboratory rats and the effects that these microbial populations have on the absorption of dietary oxalate, on urinary oxalate concentrations, and on the incidence of urinary oxalate stone formation. By providing models which more closely simulate human conditions, these studies could furnish better insights into the role that oxalate-degrading bacteria play in the metabolism of oxalate in the human body.

Isolation of intestinal oxalate-degrading bacteria

While a variety of obligately and facultatively aerobic, oxalate-degrading bacteria have been isolated from the rumen of sheep (Muller,

1950; O'Halloran, 1962) and the gastrointestinal tracts of other animals (Chandra and Shethna, 1975; Khambata and Bhat, 1953), it seems improbable that these microorganisms would be functional in the strictly anaerobic environment of the gastrointestinal ecosystem. Few obligately anaerobic bacteria able to degrade oxalate have been described. Desulfovibrio vulgaris subsp. oxamicus, was initially isolated by enrichment on oxamate from the mud of a stream heavily contaminated with waterfowl excrement (Postgate, 1963). An oxalate-degrading Clostridium species isolated from donkey feces was reported by Bhat (1966); however, neither the methods used for isolation nor a description of this organism were adequately given. The first fully documented report of the isolation and description of an obligately anaerobic, oxalate-degrading bacterium from the gastrointestinal tract was that of Dawson et al. (1980a). This isolate (designated OxB), obtained by enrichment culture from the rumen of a sheep, was a gram-negative, nonmotile, slightly curved rod which produced approximately equal amounts of formate and CO_2 during growth on oxalate. Attempts to replace oxalate with a variety of other substrates were not successful, indicating that oxalate is the sole carbon- and energy-yielding substrate utilized. This rather unique substrate requirement suggests that the availability of oxalate in the diet could influence concentrations of intestinal oxalate-degrading bacteria and thus oxalate degradation rates. A substrate-based selection of increased numbers of anaerobic oxalate-degrading bacteria may, therefore, be directly responsible for the increased rates of oxalate degradation

observed in the rumen and large bowel of animals fed gradually increasing quantities of dietary oxalate (Allison et al., 1977; Dawson et al., 1980b).

Methods used for isolation of strain OxB involved very tedious and time-consuming enrichment procedures in a chemostat culture. However, once isolated, OxB was used to develop a direct isolation medium (Allison et al., 1985). This culture medium contained minerals, acetate, 0.1% yeast extract, 7 mM $CaCl_2$, 40 mM sodium oxalate, and 1.5% agar. The presence of calcium oxalate made the medium slightly opaque; colonies of oxalate-degrading bacteria were detected by the production of clear zones around the colonies. Development of this culture medium should facilitate direct isolation and/or enumeration of anaerobic oxalate-degrading bacteria. Recently, Allison et al. (1986) used this culture medium to enumerate anaerobic oxalate-degrading bacteria from human feces. Counts of these organisms ranged from 10^5 to 10^7/g of feces from normal humans consuming uncontrolled diets. Even at the highest concentration, oxalate-degrading bacteria represented less than 0.05% of the "total" culturable fecal flora. Future studies with this culture medium should include assessments of the correlations between viable culture counts of oxalate-degrading bacteria from gastrointestinal contents, including feces, and in vitro rates of oxalate degradation.

Strains of anaerobic oxalate-degrading bacteria have now been isolated from human feces (Allison et al., 1986) and the cecal contents of guinea pigs (Fischer, 1985) and a pig (Allison et al., 1985) by

using the newly developed calcium oxalate medium. Although these isolates are closely related to the rumen isolate, OxB, differences among isolates, based on both serologic reactions and cellular fatty acid profiles, have been observed (Allison et al., 1985). Since these anaerobic oxalate-degrading bacteria encompass a unique group of microorganisms which apparently do not belong to any existing taxonomic group, Allison et al. (1985) proposed that a new genus and species, Oxalobacter formigenes, within the family, Bacteriodaceae, be established to accommodate them. Strain OxB was designated as the type strain for this new taxonomic group. Similar obligately anaerobic, oxalate-degrading bacteria have been recently isolated from freshwater lake sediments (Smith and Oremland, 1983; Smith et al., 1985). In these studies, two different morphotypes of oxalate-degrading bacteria were isolated. The first, a rod-shaped oxalate degrader, was similar to OxB, whereas the second was a spiral-shaped microbe, and thus appears to represent a new group of oxalate-degrading bacteria. These findings extend the known anaerobic habitats of anaerobic oxalate-degrading bacteria and also suggest that sediments, as well as soil, may aid in the inoculation of anaerobic oxalate-degrading bacteria in the rumen and large bowel of man and animals (Smith et al., 1985). However, further studies are needed to determine whether or not these organisms and oxalate-degrading bacteria from the gastrointestinal tract indeed represent the same species.

In summary, obligately anaerobic, oxalate-degrading bacteria are ubiquitous in nature, occurring in the gastrointestinal tracts of most

mammals and other anaerobic habitats such as lake sediments. The development of a direct isolation/enumeration medium will now permit more detailed studies into the ecology and physiology of these bacteria and of their relative contribution to the overall metabolism of oxalate in the mammalian body and in other environmental niches.

SECTION I.

MICROBIAL DEGRADATION OF OXALATE IN THE GASTROINTESTINAL TRACTS OF RATS

Microbial Degradation of Oxalate in the Gastrointestinal Tract of Rats

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ABSTRACT

Rates of oxalate degradation by mixed bacterial populations in cecal contents from wild rats ranged from 2.5 to 20.6 μmol/g (dry weight) per h. The oxalate-degrading activity in cecal contents from three strains of laboratory rats (Long-Evans, Wistar, and Sprague-Dawley) from four commercial breeders was generally lower, ranging from 1.8 to 3.5 μmol/g (dry weight) of cecal contents per h. This activity did not increase when diets were supplemented with oxalate. When Sprague-Dawley rats from a fifth commercial breeder were fed an oxalate diet, rates of oxalate degradation in cecal contents increased from 2.0 to 23.1 μmol/g (dry weight) per h. Obligately anaerobic, oxalate-degrading bacteria, similar to ruminal strains of Oxalobacter formigenes, were isolated from the latter group of laboratory rats and from wild rats. Viable counts of these bacteria were as high as 10^8/g (dry weight) of cecal contents, which was less than 0.1% of the total viable population. This report presents the first evidence for the presence of anaerobic oxalate-degrading bacteria in the cecal contents of rats and represents the first direct measurement of the concentration of these bacteria in the large bowel of monogastric animals. We propose that methods used for the maintenance of most commercial rat colonies often preclude the intestinal colonization of laboratory rats with anaerobic oxalate-degrading bacteria.

INTRODUCTION

Oxalate is degraded by microbial populations in the gastrointestinal tracts of humans (Allison et al., 1986; Barber and Gallimore, 1940), ruminants (Morris and Garcia-Rivera, 1955; Watts, 1957), and certain nonruminant herbivores (Allison and Reddy, 1984).

Oxalate degradation rates by microbial populations from the rumen and the bowel of nonruminants increase dramatically when increasing amounts of oxalate are added to the diet (Allison et al., 1977; Allison and Cook, 1981). Increased rates of oxalate degradation are apparently a result of the selection of obligately anaerobic, oxalate-degrading bacteria (Allison et al., 1981). Dawson et al. (1980a) reported the first isolation of these bacteria from ruminal contents of sheep. Similar bacteria have now been isolated from human feces (Allison et al., 1986); the cecal contents of guinea pigs (Fischer, 1985), swine (Allison et al., 1985); and from lake sediments (Smith et al., 1985). To accommodate this unique group of bacteria, a new genus and species, Oxalobacter formigenes, was established (Allison et al., 1985).

Attempts to demonstrate the presence of oxalate-degrading intestinal microbes in laboratory rats have been unsuccessful (Allison and Cook, 1981; Hagmaier et al., 1981; Shirley and Schmidt-Nielsen, 1967). In the present study, a variety of laboratory rats and wild rats were examined for oxalate-degrading activity in their intestinal contents and for the presence of anaerobic oxalate-degrading bacteria.

MATERIALS AND METHODS

Animals and Diets

Sprague-Dawley rats were obtained from Holtzman Co., Madison, Wis.; King Animal Laboratories, Inc., Oregon, Wis.; Biolab Corp., St. Paul, Minn.; Harlan Sprague-Dawley, Madison, Wis.; and Charles River Breeding Laboratories, Inc., Wilmington, Mass. Long-Evans and Wistar rats were obtained from Charles River Breeding Laboratories, Inc. Wistar rats were also obtained from Harlan Sprague-Dawley, Inc. All rats were 300- to 400-g males. Pairs of rats were housed in plastic cages (53 by 29 cm) containing Softwood Laboratory Bedding (Northeastern Products Corp., Warrensburg, N.Y.) in conventional animal rooms on a 12-h light-dark cycle. Rats were randomly assigned to pelleted control or oxalate diets. The control diet (Teklad 4% fat mouse-rat diet [Teklad, Winfield, Iowa]) contained less than 0.1% oxalic acid, by dry weight, as determined by gas chromatography (Allison et al., 1981). The oxalate diet consisted of the control diet with 4.5% sodium oxalate (Barium and Chemicals, Inc., Steubenville, Ohio) added. The animals were provided diets and water ad libitum for at least 15 days before sacrificing. Feed consumption was the same for both diets.

Wild rats were captured from the area surrounding Ames, Iowa, and were transported to the laboratory within 24 h. Rats from a single collection, two to four animals, were sacrificed and analyzed as a group.

Rats were sacrificed by CO_2 narcosis. Cecal contents from a pair of laboratory rats or from a group of wild rats were pooled in a weighing dish. A 2-g sample was transferred to a Waring blender that contained 18 ml of anaerobic dilution solution (less the $CaCl_2$ [Bryant and Burkey, 1953]) homogenized at high speed for 15 s under CO_2. In certain experiments, the contents of the small and large intestines from laboratory rats were also processed in the same manner.

Cultural Methods

Decimal dilutions of cecal homogenates were made in anaerobic dilution solution, and 0.2-ml portions of each dilution (10^{-4} to 10^{-9}) were inoculated into duplicate roll tubes of enumeration medium. All procedures were performed under strictly anaerobic conditions (Hungate, 1970; Bryant, 1972). A 20 mM oxalate medium employed for the enumeration and isolation of viable anaerobic oxalate-degrading bacteria was designated D agar. D agar contained (per liter): KH_2PO_4, 0.25 g; K_2HPO_4, 0.25 g; $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.025 g; trace metals solution (Pfennig and Lippert, 1966), 20 ml; sodium acetate, 0.82 g; sodium oxalate (Sigma Chemical Co., St. Louis, Mo.), 2.7 g; $CaCl_2 \cdot 2H_2O$, 1.0 g; yeast extract, 1.0 g; resazurin, 0.001 g; agar, 15 g; Na_2CO_3, 4.0 g; and cysteine hydrocholoride$\cdot H_2O$, 0.5 g. Ingredients other than the last two were mixed, and the pH was adjusted to 6.8. After boiling, the mixture was maintained under CO_2 while it was cooled, while sodium carbonate and cysteine were added, and while 5-ml volumes were dispensed into culture tubes (18 by 150 mm). This medium

was opaque because of the presence of calcium oxalate. Clear zones developed around colonies of oxalate-degrading bacteria. D broth was identical to D agar except that calcium and agar were omitted and the culture tubes contained 10 ml of medium.

Medium 10, used for enumeration of "total" viable bacteria in cecal homogenates (Caldwell and Bryant, 1966), has been used for the enumeration of bacteria in human feces (Allison et al., 1986; Eller et al., 1971). In preliminary experiments, we found that colony counts of bacteria from homogenates were greater in medium 10 than in CCA medium (Allison et al., 1979) or modified Balch medium (Miller and Wolin, 1982).

With the aid of a stereoscopic microscope, colonies were counted after 7 to 10 days of incubation at 37°C. Few additional colonies appeared after 10 days. Colonies in D agar that were surrounded by clear zones were picked and streaked on roll tubes of D agar. After 5 to 14 days of incubation, colonies with clear zones were restreaked. Subsequent colonies with clear zones were transferred to D broth. Growth in broth was measured as absorbance at 600 nm against a blank of uninoculated medium by using a Spectronic 70 colorimeter (Bausch and Lomb, Rochester, N.Y.). The calcium precipitation test was used to detect the presence of oxalate (Dawson et al., 1980b).

For electron microscopy, cultures were grown in D broth that contained 100 mM sodium oxalate. After incubation for 18 h, the cells were collected by centrifugation and were prepared for examination by using the procedures of Ritchie and Fernelius (1969).

Analytical Methods

Oxalate degradation rates were estimated from measurements of $^{14}CO_2$ production. Duplicate 1.8-ml portions of a sample plus 0.2 ml of sodium [^{14}C]oxalate (0.1 M, 0.02 μCi/μmol; New England Nuclear Corp., Boston, Mass.) were incubated in rubber-stoppered test tubes (13 by 100 mm) under CO_2 at 38°C for 1 or 2 h. The reactions, including 0-min controls, were stopped by injecting 1 ml of 3 N NaOH through the stopper. $^{14}CO_3^{2-}$ was measured after diffusion of $^{14}CO_2$ from an acidified reaction mixture (Conway, 1962) into phenethylamine (Allison et al., 1977). Radioactivity trapped in phenethylamine was counted in a model LS-9000 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.) with 10 ml of Biofluor (New England Nuclear). Counting efficiency (90%) was monitored by external standardization (H-number) and determined by the addition of [^{14}C]toluene (Amersham Corp., Arlington Heights, Ill.).

Oxalate is decarboxylated to CO_2 and formate by O. formigenes (Allison et al., 1985). However, when sodium [^{14}C]formate (10 mM) was incubated with samples of cecal contents (Allison and Cook, 1981) and feces (this study) from laboratory rats, rates of $^{14}CO_2$ production were at least four times greater than oxalate degradation rates. Therefore, the production of 2 mol of CO_2 per mole of oxalate degraded was assumed for the calculation of oxalate degradation rates. The specific activity of [^{14}C]oxalate in the reaction tubes was corrected to account for soluble oxalate present in the samples.

Intestinal homogenates were clarified by centrifugation at 12,000

x g for 10 min, and soluble oxalate in the supernatants was measured by gas chromatography of the dibutyl ester (Allison et al., 1977; Salanitro and Muirhead, 1975). Reported concentrations are means of measurements from duplicate samples.

For wild rats, duplicate 2- or 3-ml portions of the cecal homogenates were weighed, lyophilized, and weighed again to determine water content. This dried material was subsequently analyzed for total oxalate by the gas chromatographic procedures just described. For laboratory rats, duplicate 4-ml volumes of the homogenates were oven-dried at 55°C until a constant weight was achieved. Results are reported per unit dry weight unless indicated otherwise.

Statistical evaluations of oxalate degradation rates were performed with the Student's t test (Snedecor and Cochran, 1980).

RESULTS

Oxalate Degradation by Contents from the Intestinal Tracts of Laboratory and Wild Rats

In a preliminary series of experiments, contents of the small intestines, ceca, and large intestines from Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.) were examined to determine whether oxalate-degrading activity could be increased by the addition of oxalate to the diet. Mean values for oxalate degradation rates (means ± standard error, six pairs of rats per diet) were 2.1 ± 0.2 and 1.4 ± 0.1 μmol/g per h for samples from the cecum and large intestines, respectively, of rats fed the oxalate diet. The values were 2.7 ± 0.3 and 1.5 ± 0.2 μmol/g per h for cecum and large intestine samples, respectively, for rats fed the control diet. These low rates of $^{14}CO_2$ production from [^{14}C]oxalate were thus not affected by adding oxalate to the diet, and no trends related to length of time on the diets (15, 30, and 60 days) were detected. Oxalate-degrading activity was not observed in any sample of small intestinal contents. Tests to determine factors responsible for the low levels of $^{14}CO_2$ production were performed. Oxalate-degrading activity was not observed when samples of anaerobic dilution solution, control diet, or rat cecal tissue were incubated with [^{14}C]oxalate. After centrifugation of diluted cecal contents at high speed, all the oxalate-degrading activity was recovered in the pellet. Attempts to isolate oxalate-degrading microbes from the cecal contents of these rats, either by enrichment culture in D broth or by

direct isolation on D agar, were unsuccessful. These and other results suggest that the low levels of oxalate-degrading activity were not due to microbes such as O. formigenes, which require oxalate as a source of carbon and energy and are selected by diets high in oxalate.

In a second series of experiments, laboratory rats from different commercial breeders were surveyed for the presence and the selection of oxalate-degrading intestinal microbes. The oxalate-degrading activity was low (1.8 to 3.0 μmol/g per h) in samples of cecal contents from Sprague-Dawley rats from four breeders, Wistar rats from two breeders, and Long-Evans rats from a single breeder, and did not increase when animals were fed the oxalate diet (Table 1). When Sprague-Dawley rats from a fifth breeder (Charles River Breeding Laboratories, Inc.) were fed the oxalate diet, oxalate degradation rates in samples of cecal contents increased from 2.0 to 23.1 μmol/g per h. The latter value is of the same magnitude as rates measured in samples of cecal contents from other laboratory animals (guinea pigs, rabbits) adapted to diets high in oxalate (Allison and Cook, 1981). Tests for the presence of oxalate degraders were also made by inoculating D broth with 10^{-2} to 10^{-3} g (wet weight) of cecal contents from rats fed the oxalate diet (Table 1). Oxalate was degraded within 7 days to a level which could not be detected in D broth that had been inoculated with cecal contents from Sprague Dawley rats from breeder 5. After 21 days of incubation, however, no loss of oxalate was detected in D broth cultures that had been inoculated with cecal contents from any of the other rats (data not shown).

The mean rate of oxalate degradation from samples of cecal contents from five groups of wild rats (16 animals) was 12.2 ± 2.4 μmol/g per h and ranged from 7.4 to 20.6 μmol/g per h. These rates resemble those obtained with samples of cecal contents from Sprague-Dawley rats (Charles River Breeding Laboratories, Inc.) fed the oxalate diet.

In wild rats, total oxalate concentrations varied from 2.1 to 12.6 μmol/g of cecal contents. Of the total oxalate present, soluble oxalate represented anywhere from 0 to 45%. Soluble oxalate concentrations in samples of intestinal contents from laboratory rats fed the control diets were negligible; oxalate was not detected in the supernatant fluid of any of these samples. In samples of intestinal contents from oxalate-fed laboratory rats, soluble oxalate concentrations ranged from 0 to 4 μmol/g of contents (data not shown).

Enumeration, Isolation, and Characterization of Oxalate-Degrading Anaerobes

In initial cultural studies with cecal samples from wild rats, colony formation was inhibited when the medium contained 40 mM sodium oxalate (data not shown). When the oxalate content of the medium was 20 mM (D agar), colony counts of anaerobic oxalate-degrading bacteria from wild rats and from Sprague-Dawley rats (Charles River Breeding Laboratories, Inc.) adapted to a high-oxalate diet ranged from 7.24 to 8.09 $\log_{10}$ per g of cecal contents (Table 2). When the level of yeast extract was increased from 0.1 to 0.3% in D agar, the colony count of

oxalate-degrading bacteria from the cecal contents of these laboratory rats increased nearly twofold (Table 3). An additional threefold increase in the colony count was observed when the $CaCl_2$ concentration was increased from 7 to 14 mM (D3 agar). However, with nearly a sevenfold increase over D agar in the colony count of oxalate-degrading bacteria, D5 agar, which contained 10 rather than 20 mM oxalate, 7 mM $CaCl_2$, and 0.3% yeast extract, was the optimum medium in this study.

Nine oxalate-degrading isolates (OxWR1, OxWR2, and OxWR4 through OxWR10) were obtained from wild rats. Six isolates (OxCR1 to OxCR6) were obtained from Sprague-Dawley rats (Charles River Breeding Laboratories, Inc.). Isolates were gram-negative, non-motile, nonsporeforming, slightly curved rod-shaped cells, occurring singly and in pairs (Fig. 1). Typical cell dimensions were 1.1 to 1.8 μm by 3.1 to 9.4 μm. No significant relationship was observed between cell morphology and culture conditions. All isolates degraded oxalate to CO_2 and formate.

Dawson et al. (1980a) reported that OxB, an oxalate-degrading bacterium isolated from ruminal contents by using enrichment medium that contained 45 mM oxalate, was capable of growth in medium containing oxalate concentrations as high as 111 mM. In the present study, all strains of oxalate-degrading bacteria grew well in D broth (20 mM oxalate). Maximum absorbance typically occurred after about 24 h of incubation. After several passages, these strains were used to inoculate D broth that contained either 40 or 100 mM oxalate. Only one

strain (OxWR1) grew in medium containing 100 mM oxalate, but all strains grew within 7 days in the 40 mM oxalate medium.

None of the oxalate-degrading isolates grew in either medium 10 broth or PYG medium (Holdeman et al., 1977) without oxalate. Medium 10 broth was often inoculated as a test for contamination of oxalate-degrading cultures.

All strains of oxalate-degrading bacteria were obligate anaerobes and did not grow in D broth in which resazurin had turned pink (oxidized) or D broth (minus cysteine and sodium carbonate) prepared under aerobic conditions.

Dawson et al. (1980a) tested a limited number of substrates for their ability to support growth of OxB and found that none could replace oxalate as a growth substrate. In addition, Allison et al. (1985) reported that none of a wide variety of substrates, when present with oxalate, would enhance the growth of OxB. In the present study, a large number of substances were tested as possible growth substrates with strain OxWR1 in D broth, both in the presence and absence of 20 mM oxalate. Growth of OxWR1 was not enhanced or supported by the addition of any of the following filter-sterilized substances at a concentration of 20 mM: acetaldehyde, acrylate, adipate, alanine, aspartate, benzoate, butyrate, citrate, ethanol, ethylene glycol, formamide, formate, fumarate, glutarate, glyceraldehyde, glycerol, glycine, glycolate, glyoxal, glyoxylate, isocitrate, itaconate, ketoglutarate, lactate, malate, maleate, malonate, methanol, oxaloacetate, oxamate, parabanate, phenylpyruvate, phthalate, propionate, pyruvate, serine,

succinate, tartarate, tartonate, or urea. Although both parabanate and dimethyl oxalate supported the growth of strain OxB of O. formigenes (Allison et al., 1985), only dimethyl oxalate supported growth of strain OxWR1.

Antibiotics were tested for their effects on the growth of strains OxCR6 and OxWR1 in D broths that contained 20 and 100 mM oxalate, respectively. Growth of both strains was less than growth in control tubes in the presence of chloramphenicol (12 µg/ml), colistin (2 µg/ml), tetracycline (6 µg/ml). Strain OxWR1 was resistant to kanamycin (6 µg/ml), erythromycin (3 µg/ml), vancomycin (6 µg/ml), rifampin (1 µg/ml), streptomycin (2 µg/ml), penicillin (2 U/ml), carbenicillin (20 µg/ml), and ampicillin (4 µg/ml). Both strains were resistant to cephalothin (6 µg/ml) and neomycin (6 µg/ml); however, only OxWR1 was resistant to clindamycin (1 µg/ml).

DISCUSSION

The results of several studies indicate that oxalate-degrading microbes are few or absent in laboratory rats (Allison and Cook, 1981; Hagmaier et al., 1981; Shirley and Schmidt-Nielsen, 1967). Data presented here provide the first evidence that anaerobic oxalate-degrading bacteria are present in certain laboratory rats and in wild rats and the first direct cultural measurements of the concentrations of these bacteria in the cecal contents from monogastric animals. Of the three strains of laboratory rats from five breeders, only Sprague-Dawley rats from one breeder harbored significant cecal populations of anaerobic oxalate-degrading bacteria (Table 1). A different colony of Sprague-Dawley rats from this same breeder was also tested, and oxalate-degrading bacteria were not detected in these rats. Although the lack of a certain bacterial species among the normal flora inhabiting a specific group of mammals is not a new phenomenon, this is the first report involving oxalate-degrading bacteria. So far, each human, laboratory animal (other than rats), and farm animal that has been tested harbored gastrointestinal oxalate-degrading bacteria (Allison, 1985). Although these bacteria were present in wild rats at numbers as high as 10^8/g (dry weight) of cecal contents (Table 2), they represented less than 0.1% of the total viable count of bacteria that were able to grow in medium 10. A similar ratio was noted between concentrations of anaerobic oxalate-degrading bacteria and the total viable count from human feces (Allison et al., 1986).

All strains of anaerobic oxalate-degrading bacteria isolated from

wild and laboratory rats were similar in morphology and nutrition to the type strain of O. formigenes, strain OxB; to strains isolated from humans and a pig; and to rod-shaped bacteria isolated from lake sediments (Allison et al., 1985; Smith et al., 1985). Of the two rat strains tested for antibiotic sensitivity, both were sensitive to essentially the same antibiotics reported as being effective against strain OxB (K. A. Dawson, Ph.D. dissertation, Iowa State University, Ames, 1979). The only difference was that strain OxWR1 was resistant to clindamycin. Other differences, based on tolerance to oxalate, were noted between strains. Unlike OxB, the growth of most rat strains was inhibited by high oxalate concentrations (100 mM) in the culture medium. Inhibition by high levels of oxalate was also observed with strains isolated from lake sediments (Smith et al., 1985).

The production of small amounts of $^{14}CO_2$ when [^{14}C]oxalate was incubated with contents from the ceca and large intestines of laboratory rats that apparently did not harbor anaerobic oxalate-degrading bacteria is not yet explained (Table 1). However, results of this and other studies do indicate that this oxalate-degrading activity (i) is limited to the particulate fraction of gut contents (Table 2); (ii) is not associated with oxalate degradation in oxalate enrichment cultures or in roll tubes of D agar; (iii) is low, in comparison with oxalate degradation rates found in populations where O. formigenes is present, and does not increase when diets high in oxalate are given (Table 1); (iv) is not affected by antibiotics (cepthalothin, chloramphenicol, tetracycline), gas phase (H_2, O_2, room air), or

temperature (4 or 65°C); only autoclaving (121°C for 15 min) completely destroys this oxalate-degrading activity (S. L. Daniel, Ph.D. dissertation, Iowa State University, Ames, 1988); and (v) is neither proportional to the amount of gut contents nor linear with time (Daniel, Ph.D. dissertation). The above evidence suggests that this oxalate-degrading activity is the result of a nonspecific chemical reaction(s), although the process by which these nonspecific reactions occur remains to be resolved.

The reasons that some but not all laboratory rats harbor oxalate degrading bacteria are unknown. Shirley and Schmidt-Nielsen (1967) postulated that laboratory rats maintained for generations on diets low in oxalate have simply lost the capacity (microbes) for intestinal oxalate degradation. The control diet used here contained only about 0.1% oxalic acid; however, this level of oxalate was sufficient to maintain a population of oxalate-degrading microbes in one group of laboratory rats. Allison and Cook (1981) suggested that laboratory rats lack intestinal oxalate-degrading microbes because of their limited contact with other herbivores. In support of this are preliminary studies showing that laboratory rats inoculated with mixed populations of microbes from wild rats develop populations of cecal microbes that have an increased capacity for oxalate degradation (Daniel et al., 1983). Also, Smith et al. (1985) suggested that sediments and soils may also provide a source of oxalate-degrading organisms. Thus, we propose that procedures used for the establishment (e.g., Caesarean-originated) and maintenance of some commercial rat

colonies limit the introduction and establishment of anaerobic oxalate-degrading bacteria.

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Table 1. Oxalate degradation by the cecal contents from laboratory rats obtained from various commercial breeders

Breeder[a]	Strain[b]	Oxalate degradation rate[c]	
		Control diet	Oxalate diet
Holtzman Co.	SD	2.6 ± 0.3	2.0 ± 0.2
Harlan Sprague-Dawley, Inc.	SD	2.5 ± 0.4	2.1 ± 0.1
	WI	2.3 ± 0.1	1.8 ± 0.0
King Animal Laboratories, Inc.	SD	3.0 ± 0.1	2.6 ± 0.3
Biolab Corp.	SD	2.7 ± 0.2	2.2 ± 0.1
Charles River Breeding Laboratories, Inc.	SD	2.0 ± 0.1	23.1 ± 6.6[d]
	WI	2.3 ± 0.0	1.8 ± 0.2
	LE	1.8 ± 0.1	1.8 ± 0.0

[a]Diets were fed for a minimum of 15 days before rats were sacrificed. Three pairs of rats (one pair of rats per cage) were sacrificed per strain per diet. Cecal contents from a pair of rats were pooled before analysis.

[b]Strains: SD, Sprague-Dawley; WI, Wistar; LE, Long-Evans.

[c]Micromoles of oxalate degraded per gram (dry weight) of cecal contents per hour. Each value is the mean ± standard error of three determinations.

[d]Significantly greater ($P < 0.05$) than control diet.

Table 2. Colony counts of anaerobic oxalate-degrading bacteria from the cecal contents of wild and laboratory rats

Rats[a]	Oxalate degradation rate[b]	Colony count[c]	
		Oxalate[d]	Total[e]
Wild	4.1 ± 0.8	7.78 ± 0.16	11.12 ± 0.08
Laboratory	17.4	7.24	10.71

[a]Three pairs of wild rats and one pair of laboratory rats were tested. Laboratory rats were Sprague-Dawley animals (Charles River Breeding Laboratories, Inc.) that had been fed the oxalate diet for 24 days. Cecal contents from each pair of rats were pooled before analysis.

[b]Micromoles of oxalate degraded per gram (dry weight) of cecal contents per hour. Each value is the mean (± standard error of three determinations for wild rats).

[c]Log_{10}/g (dry weight) of cecal contents.

[d]Colonies producing clear zones in D agar after 7 to 10 days of incubation.

[e]Total viable count in medium 10 (Caldwell and Bryant, 1966).

Table 3. Comparison of medium modifications: colony counts of anaerobic oxalate-degrading bacteria from the cecal contents of laboratory rats[a]

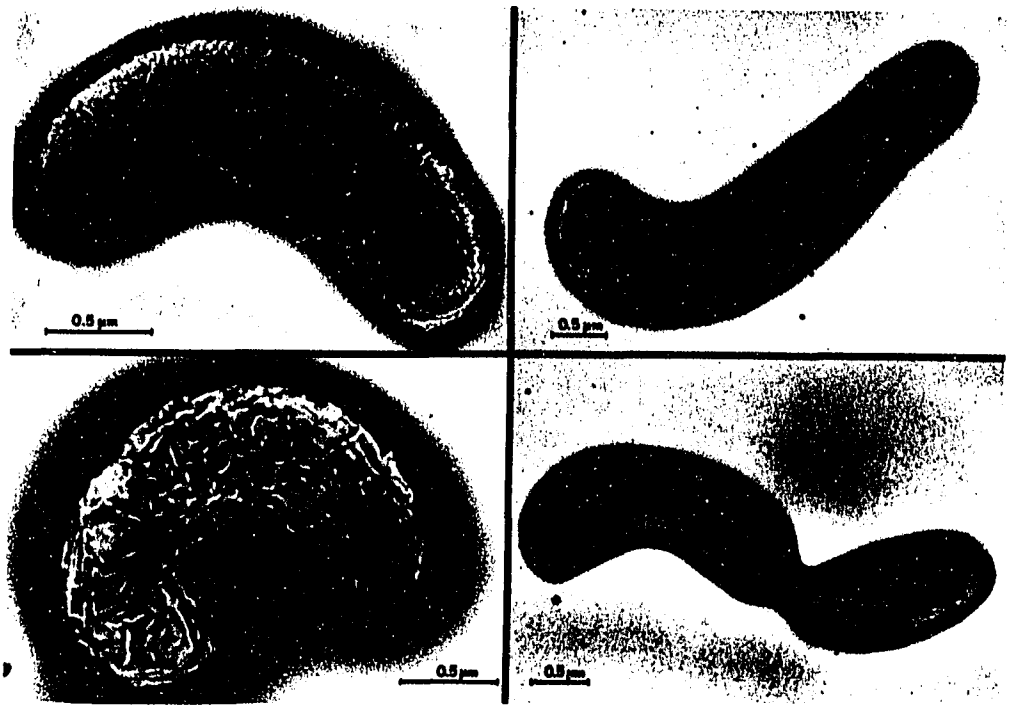
Medium	Sodium oxalate (mM)	Yeast extract (%)	$CaCl_2$ (mM)	Colony count[b]
D[c]	20	0.1	7	7.24
D1	20	0.3	7	7.46
D2	20	0.1	14	7.73
D3	20	0.3	14	7.97
D4	10	0.1	7	7.88
D5	10	0.3	7	8.11

[a]Cecal contents were from the same pair of oxalate-fed Sprague-Dawley rats described in Table 2.

[b]Log_{10}/g (dry weight) of cecal contents. Counts were from colonies producing clear zones after 7 to 10 days of incubation. Each value is the mean of duplicate tubes.

[c]D agar as described in the text was modified as indicated here.

Figure 1. Electron micrographs of an anaerobic oxalate-degrading isolate (OxWR1) from the wild rat, grown for 18 h in D broth that contained 100 mM oxalate. Stained with neutralized 4% phosphotungstic acid. The morphologic diversity commonly seen is illustrated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SECTION II.

INTESTINAL COLONIZATION OF CONVENTIONAL LABORATORY RATS WITH *OXALOBACTER FORMIGENES*

Intestinal Colonization of Conventional Laboratory Rats with *Oxalobacter formigenes*

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Running Title: INTESTINAL COLONIZATION OF RATS WITH *O. FORMIGENES*

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ABSTRACT

Six strains of Oxalobacter formigenes (anaerobic oxalate-degrading bacteria) were examined for their ability to colonize the gastrointestinal tracts of adult laboratory rats. These rats did not harbor O. formigenes. Strain OxCR6, isolated from the cecal contents of a laboratory rat that was naturally colonized by oxalate-degrading bacteria, colonized the ceca and colons of adult rats fed a diet that contained 4.5% sodium oxalate. Five days after rats were inoculated intragastrically with 10^9 viable cells of strain OxCR6, oxalate degradation rates in cecal and colonic contents increased by 19 and 40 times, respectively. Viable counts of strain OxCR6 from these rats averaged 10^8/g (dry weight) of cecal contents. Strain OxCR6 was not detected in the cecal contents of inoculated rats fed diets that contained less than 3.0% sodium oxalate. Strains of O. formigenes isolated from the cecal contents of swine, guinea pigs, and wild rats and from human feces also colonized the ceca of laboratory rats; a ruminal strain failed to colonize the rat cecum.

INTRODUCTION

Bacterial degradation of oxalate occurs in the intestines of humans and animals (Allison, 1985). In the rumen, a selection for increased numbers of oxalate-degrading bacteria occurs with the addition of oxalate to the diet. This selection is based on oxalate availability, and the elevation of numbers of oxalate-degrading bacteria serves to limit the absorption, and, thus toxicity of high levels of dietary oxalate (Allison and Reddy, 1984).

Laboratory rats are widely used as models for the absorption and excretion of oxalate in man (Hodgkinson, 1977); however, there is now evidence that laboratory rats are unique in that most are not colonized by oxalate-degrading bacteria (Allison and Cook, 1981; Hagmaier et al., 1981; Shirley and Schmidt-Nielsen, 1967). How this fact affects conclusions from data obtained with rats is not known because the role of oxalate-degrading bacteria in the colon has yet to be defined. In a survey of commercially available rats, Daniel et al. (1987) discovered that rats from only one of the five commercial breeders tested harbored colonic oxalate-degrading bacteria. Oxalate-degrading isolates from these rats were similar to isolates from the rumen (Dawson et al., 1980); from the bowel of humans and certain nonruminant herbivores (Allison et al., 1985); and from aquatic sediments (Smith et al., 1985). A new genus and species, Oxalobacter formigenes, has been proposed for this unique group of anaerobic bacteria that use oxalate as a major source of carbon and energy (Allison et al., 1985).

Reasons for the absence of O. formigenes in the intestinal tracts

of most laboratory rats are unknown. In this study, we used rats which did not harbor O. formigenes to determine whether different strains of O. formigenes could colonize the intestinal tracts of adult laboratory rats. We also studied some of the conditions (e.g., time, dietary oxalate levels, and strain specificity) required for colonization.

MATERIALS AND METHODS

Animals and Diets

Outbred male Sprague-Dawley rats (250 to 350 g; designated pathogen-free) from a single commercial colony (building 207; Harlan Sprague-Dawley, Inc., Madison, Wis.) were used. Previous studies (Daniel et al., 1987) indicated that O. formigenes was not present in the gastrointestinal tracts of these rats. Pairs of animals were housed in plastic shoebox-type cages and maintained as described previously (Daniel et al., 1987). The control diet (Teklad 4% fat mouse/rat diet; Teklad, Winfield, Iowa) contained less than 0.1% oxalate. Oxalate diets consisted of the control diet plus sodium oxalate at dry weight concentrations of 1.5, 3.0, or 4.5%. All diets were mixed and then pelleted. The animals were provided diets and water ad libitum.

Preparation of O. formigenes Inoculum

O. formigenes strains used were OxB (sheep rumen; Dawson et al., 1980); POxC (pig cecum; Allison et al., 1985); OxK (human feces; Allison et al., 1986); OxGP (guinea pig cecum; Fischer, 1985); OxWR1 (wild rat cecum; Daniel et al., 1987); and OxCR6 (laboratory rat cecum; Daniel et al., 1987).

Strains of O. formigenes were grown under anaerobic conditions at 37°C without agitation in 600-ml side-arm (diameter, 18 mm) flasks that contained 500 ml of prereduced D broth (Daniel et al., 1987). The

inoculum (1 to 1.5%) was grown in culture tubes (18 by 150 mm) of D broth, and cell densities were measured at 600 nm by using a Spectronic 70 colorimeter (Bausch and Lomb, Rochester, N.Y.). Cells at mid- to late exponential phase of growth (A_{600} = 0.10 to 0.14; concentration = 4 x 10^7 to 6 x 10^7 viable cells per ml) were harvested by centrifugation (14,600 x g for 10 min at 4°C), washed once in anaerobic dilution solution (less the $CaCl_2$; Bryant and Burkey, 1953), and resuspended in anaerobic dilution solution to a final concentration of 0.5 x 10^{10} to 1 x 10^{10} viable cells per ml in CO_2-flushed 10- or 25-ml serum bottles.

Inoculation of Rats with *O. formigenes*

In all colonization experiments, rats were switched from the control diet to the 4.5% sodium oxalate diet at least 4 days before inoculation. Both rats in a pair then received 0.5 or 1.0 ml of a cell suspension of a strain of *O. formigenes* administered intragastrically with a slightly curved 19-gauge feeding needle. Uninoculated rats that had been fed the 4.5% sodium oxalate diet or the control diet were included as controls.

Cultural and Analytical Analyses

At the conclusion of a colonization experiment (time intervals after inoculations are given in Results), rats were sacrificed by CO_2 narcosis, and the cecal contents from a pair of rats were pooled before analysis. In some experiments, the contents of the small and large

intestines were each pooled also. Techniques used for the measurement of rates of oxalate degradation and for the cultural detection and enumeration of O. formigenes have been described previously (Daniel et al., 1987). The rate of oxalate degradation and the concentration of O. formigenes in homogenized pooled contents from a pair of rats were determined from measurements in duplicate and triplicate tubes, respectively.

Intestinal tracts were considered colonized when rates of oxalate degradation in intestinal contents were greater than rates measured intestinal contents from uninoculated animals or when viable cells of the O. formigenes strain were recovered from the intestinal contents.

RESULTS

Rates of oxalate degradation in homogenates of cecal and colonic contents from rats fed a 4.5% oxalate diet and inoculated with 5 x 10^9 viable cells of strain OxCR6 increased by 5- to 10-fold at 3 days after inoculation (Table 1). Rates of oxalate degradation from colonized rats were always greater in colonic contents than in cecal contents. Oxalate-degrading activity was not detected in small intestinal contents. Five days after inoculation, rates of oxalate degradation in cecal and colonic contents were 19 and 40 times greater than rates measured in cecal and colonic contents from uninoculated rats, respectively. Based on these results, tests for O. formigenes colonization were subsequently conducted with cecal contents at 5 to 10 days after inoculation. In other studies with inoculated rats, cecal and colonic rates of oxalate degradation remained high for 68 days after inoculation (Daniel et al., 1988a).

Although some of the carbon-14 from [^{14}C]oxalate was recovered as $^{14}CO_2$ after incubation with homogenates of cecal contents from rats fed the control diet (Table 2), this activity did not increase when rats that had not been inoculated with O. formigenes were given the 4.5% sodium oxalate diet. In addition, clear zones of O. formigenes were not observed in tubes of D5 agar inoculated with dilutions that contained 10^{-1} to 10^{-6} ml of cecal contents from these rats. These results support previous conclusions that O. formigenes is absent in the ceca of conventional adult Sprague-Dawley rats from this supplier and that the low-level oxalate-degrading activity observed in cecal

contents from these rats (before inoculation with O. formigenes) was the result of nonspecific chemical or microbial degradation (Daniel et al., 1987). Ten days after rats fed the 4.5% sodium oxalate diet were inoculated with 3 x 10^9 viable cells of strain OxCR6, rates of oxalate degradation averaged 19.1 μmol/g (dry weight) per h and population densities of strain OxCR6 averaged 8.29 $\log_{10}$/g (dry weight) in the cecal contents of colonized rats (Table 2).

Experiments were conducted to determine what effects different levels of dietary oxalate would have on numbers of O. formigenes in the cecal contents of colonized rats. While on the 4.5% sodium oxalate diet, 16 rats (eight pairs) were each inoculated with 3 x 10^9 viable cells of strain OxCR6. Six days after inoculation, two pairs of rats were sacrificed and two pairs each were switched to 3.0, 1.5, and 0% (control) sodium oxalate diets. These diets were fed for 6 days before the rats were sacrificed. Strain OxCR6 was present at a concentration of 10^8 viable cells/g (dry weight) of cecal contents in the ceca of both pairs of rats fed the 4.5% sodium oxalate diet (Table 3). Only one of the two pairs of rats fed the 3% sodium oxalate diet and none of the rats switched either to the 1.5% sodium oxalate diet or the control diet harbored detectable levels of strain OxCR6.

Strains of O. formigenes isolated from the gastrointestinal tracts of a variety of mammals were also tested for their ability to colonize the ceca of conventional laboratory rats. In this experiment, six rats (three pairs) on the 4.5% sodium oxalate diet were each inoculated with 0.7 to 1 x 10^{10} viable cells of a single strain of O. formigenes.

Based on measurements of rates of oxalate degradation, strains POxC, OxK, OxGP, OxWR1, and OxCR6 each colonized the ceca of at least one pair of rats (Table 4). Only strain OxB failed to colonize the ceca of any of three pairs of rats. However, even strain OxCR6, which was isolated from the cecal contents of laboratory rats (Charles River Breeding Laboratories, Inc.), failed to colonize the ceca of one pair of rats.

DISCUSSION

O. formigenes colonized the ceca of adult laboratory rats fed a diet containing 4.5% sodium oxalate. Our evidence for colonization by specific strains of O.formigenes is indirect because we currently have no marker that would enable us to distinguish between strains. We did not, however, observe colonization of any rats in the laboratory that were not purposely inoculated. This includes observations of at least 35 pairs of rats that were not inoculated. Colonization by strain OxCR6 is, however, the most plausible explanation for our findings because large numbers of viable cells of an organism resembling strain OxCR6 were detected in the cecal contents of rats that had been inoculated, whereas none were detected in cecal contents from uninoculated rats. Furthermore, rates of oxalate degradation measured in cecal contents from rats inoculated with strain OxCR6 were markedly greater than cecal rates measured in cecal contents from uninoculated rats (Table 2). These increased rates were similar to those in cecal contents from guinea pigs and rabbits and in naturally colonized laboratory rats that had been adapted to diets high in oxalate (Allison and Cook, 1981; Daniel et al., 1987).

Although strain OxCR6 attained levels of 10^8/g (dry weight) of cecal contents, it made up less than 1.0% of the total viable count of bacteria that grew in medium 10 (Table 2). These concentrations of strain OxCR6 were similar to the concentrations of O. formigenes in the cecal sample from which strain OxCR6 was isolated (Daniel et al., 1987). When the sodium oxalate level in the diet was decreased to

3.0%, the concentration of strain OxCR6 in cecal contents from one pair of colonized rats was about 3% of the concentration found in colonized rats on 4.5% sodium oxalate (Table 3). Strain OxCR6 was not detected by colony counts in the cecal contents from the other pair of rats fed 3% sodium oxalate nor was it detected in cecal contents of inoculated rats fed 1.5% or less sodium oxalate. The concentration of oxalate in the diet, therefore, appeared to influence both colonization by strain OxCR6 and its concentration in cecal contents. Reductions in dietary oxalate also reduced concentrations of O. formigenes in ruminal contents of sheep (Daniel et al., 1988b).

Host specificity has been observed in the colonization by lactobacilli of the gastrointestinal tracts of mice (Tannock and Archibald, 1984). In the present study, of the six strains of O. formigenes tested, five colonized the ceca of adult laboratory rats fed the 4.5% sodium oxalate diet (Table 4). All five of these strains were originally isolated from monogastric mammals. The ruminal strain, OxB, was the only strain from a pregastric intestinal tract site and also the only strain that did not colonize the rat cecum. In addition, rates of oxalate degradation in most cecal contents from rats colonized with rodent strains (OxGP, OxWR1, and OxCR6) were 2 to 12 times greater than the rates measured in cecal contents from rats colonized with swine and human strains (POxC and OxK), suggesting that population levels of rodent strains were greater than swine and human strains in colonized rats. Also in this study, of the five strains that were capable of colonizing the rat cecum, only two (strains OxK and OxGP)

colonized the ceca all pairs of rats tested (Table 4). The regulation of microbial communities in the gastrointestinal tract is complex and, as yet, poorly understood (Savage, 1977). While additional data would be needed for firm conclusions, our results suggest that host specificity and natural exclusion processes by indigenous flora operate to influence the colonization of the rat bowel by given strains of O. formigenes. By administering high concentrations of dietary oxalate, we attempted to overcome barriers to colonization by strains of O. formigenes. However, with some rats, this evidently was insufficient.

We currently do not know why O. formigenes is absent from the intestinal tracts of most laboratory rats. Procedures used by most commercial breeders for the establishment (e.g., Caesarean-originated) and maintenance (e.g., barrier-sustained) of rat colonies may limit the contact of rats with exogenous microorganisms and thus with O. formigenes (Daniel et al., 1987). However, even if conventional adult rats are exposed to O. formigenes, the results of the present study indicate that a laboratory diet that is low in oxalate greatly reduces the chances of O. formigenes becoming established in the intestinal tract. However, the isolation of O. formigenes from a colony of laboratory rats that had been maintained on a diet containing less than 0.1% oxalate suggests the same may not be true of newborn rats (Daniel et al., 1987). An alternate possibility is that other microorganisms present in the laboratory rats that were naturally colonized are important for the establishment of O. formigenes when dietary oxalate is limited, and these microorganisms were not present in rats

inoculated here. Additional studies, possibly with newborn or germ-free rats, may provide insight into the dynamics of intestinal colonization by O. formigenes.

The ability to implant O. formigenes into the indigenous intestinal flora of adult laboratory rats should provide an animal model in which the influence of O. formigenes on the fate of dietary oxalate in mammals can be studied. Studies with these rats may be useful for gaining a better understanding of factors influencing the absorption of dietary oxalate and its regulation and relation to urinary stone formation in humans (Hodgkinson, 1977).

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Table 1. Time course and sites of colonization by O. formigenes in the intestinal tracts of conventional laboratory rats fed a diet with 4.5% sodium oxalate

Day[a]	Oxalate degradation rate[b]	
	Cecum	Colon
0	2.4 ± 0.1	1.3 ± 0.5
3	12.7 ± 5.0	16.8 ± 6.1
5	45.4 ± 0.2	51.5 ± 5.7
7	17.4 ± 0.7	26.4 ± 1.7
10	28.4 ± 13	44.2 ± 7.1

[a]Days after inoculation (two pairs of rats per time period). Rats (one pair per cage) were each inoculated intragastrically with 5×10^9 viable cells of strain OxCR6. Contents of each intestinal segment from a pair of rats were pooled before analysis.

[b]Micromoles of oxalate degraded per gram (dry weight) of intestinal contents per hour. Each value is the mean ± standard error of two determinations. There was no oxalate-degrading activity in the small intestine.

Table 2. Oxalate degradation rates and colony counts of O. formigenes in the cecal contents of conventional laboratory rats

% Sodium oxalate in diet[a]	Inoculated[b]	Oxalate degradation rate[c]	Colony count[d]	
			Oxalate[e]	Total[f]
0	-	2.4 ± 0.2	ND[g]	10.79 ± 0.06
4.5	-	2.0 ± 0.1	ND	10.84 ± 0.02
4.5	+	19.1 ± 3.3	8.29 ± 0.10	10.73 ± 0.05

[a]Three pairs of rats (one pair per cage) per treatment.

[b]Rats were each inoculated intragastrically with 3×10^9 viable cells of strain OxCR6 and were sacrificed 10 days after inoculation. Cecal contents from a pair of rats were pooled before analysis.

[c]Micromoles of oxalate degraded per gram (dry weight) of cecal contents per hour. Each value is the mean ± standard error of three determinations.

[d]Log_{10}/g (dry weight) of cecal contents. Each value is the mean ± standard error of three determinations.

[e]Colonies producing clear zones in D5 agar (Daniel et al., 1987).

[f]Total colony count in medium 10 (Caldwell and Bryant, 1966).

[g]ND, Not detected ($\leq$ 200 CFU/g [dry weight] of cecal contents).

Table 3. Effect of dietary oxalate concentrations on the maintenance of O. formigenes in the cecal contents of colonized laboratory rats

% Sodium oxalate in diet	Pair no.[a]	Colony count[b]	
		Oxalate[c]	Total[d]
4.5	1	8.19	10.45
	2	8.22	10.27
3.0	1	6.72	10.56
	2	ND[e]	10.66
1.5	1	ND[f]	10.74
	2	ND[f]	10.88
0	1	ND[f]	10.63
	2	ND[f]	10.70

[a]Rats (one pair per cage) were each inoculated intragastrically with 2.5×10^9 viable cells of strain OxCR6. Rats were fed the diets for 6 days then sacrificed. Cecal contents from a pair of rats were pooled before analysis.

[b]Log_{10}/g (dry weight) of cecal contents. Each value is the mean of triplicate tubes.

[c]Colonies producing clear zones in D5 agar (Daniel et al., 1987).

[d]Total colony count in medium 10 (Caldwell and Bryant, 1966).

[e]ND, Not detected ($\leq$ 2,000 CFU/g [dry weight] of cecal contents).

[f]ND, Not detected ($\leq$ 200 CFU/g [dry weight] of cecal contents).

Table 4. Rates of oxalate degradation in cecal contents from conventional laboratory rats fed a diet with 4.5% sodium oxalate and inoculated with different strains of O. formigenes

Strain[a]	Source	Oxalate degradation rate[b]		
		Pair no. 1	Pair no. 2	Pair no. 3
Control	-	2.1	2.1	1.8
OxB	Sheep rumen	1.8	2.1	1.8
POxC	Pig cecum	2.1	1.8	8.0
OxK	Human feces	3.3	7.6	8.9
OxGP	Guinea pig cecum	19.5	34.1	25.8
OxWR1	Wild rat cecum	34.6	3.6	1.6
OxCR6[c]	White rat cecum	36.2	25.1	1.7

[a]Three pairs of rats per strain. Rats (one pair per cage) were each inoculated intragastrically with 0.7 to 1 x 10^{10} viable cells of a strain of O. formigenes and were sacrificed 8 days after inoculation. Cecal contents from a pair of rats were pooled before analysis.

[b]Micromoles of oxalate degraded per gram (dry weight) of cecal contents per hour. Each value is the mean of duplicate tubes.

[c]Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.).

SECTION III.

ENUMERATION OF ANAEROBIC OXALATE-DEGRADING BACTERIA IN THE RUMINAL CONTENTS OF SHEEP

Enumeration of Anaerobic Oxalate-Degrading Bacteria in the Ruminal Contents of Sheep

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ABSTRACT

Concentrations of oxalate-degrading anaerobes in the ruminal contents of sheep were determined from counts of colonies producing clear zones on a calcium oxalate medium (D agar with 7 mM $CaCl_2$). Colony counts of oxalate degraders from ruminal samples of a 55-kg sheep fed a diet containing 32% halogeton (4.6% oxalate) averaged 2.6 x 10^6/g (dry weight). When the halogeton level in the diet was reduced to 16%, colony counts of oxalate degraders decreased nearly 300-fold. Oxalate-degrading isolates from this sheep were similar to OxB, the type strain of Oxalobacter formigenes. When a 45-kg sheep was fed diets containing 2.2, 1.5, and 0.8% oxalate, viable counts of oxalate degraders in ruminal samples (enumerated on D agar with 14 mM $CaCl_2$ and 20% filter-sterilized ruminal fluid) represented 0.85, 0.52, and 0.06% of the total viable population, respectively. Similar percentages of oxalate degraders were also observed when a 23-kg sheep was fed diets containing 1.5 or 0.8% oxalate. This report presents the first direct measurements of the concentrations of oxalate-degrading bacteria in the rumen and supports the concept that the availability of oxalate in the diet influences the proportion of oxalate-degrading bacteria in the rumen.

INTRODUCTION

After ruminants have been adapted to diets that contain gradually increased concentrations of oxalate, they are able to tolerate amounts of dietary oxalate that would be toxic to nonadapted animals (James and Butcher, 1972). This adaptation to oxalate is the result of an increase in the rate of oxalate degradation by ruminal microbes (Allison et al., 1977; Morris and Garcia-Rivera, 1955; Watts, 1957). This and other information (Allison et al., 1981; Dawson et al., 1980b) suggested that bacteria are responsible for the degradation of oxalate in the rumen and that ruminant adaptation to oxalate involves substrate-based selection leading to increased concentrations of oxalate-degrading anaerobes.

A method for the enumeration of oxalate-degrading anaerobes was recently developed (Allison et al., 1985). The method utilizes a culture medium which is opaque because of the presence of calcium oxalate; oxalate degraders are detected by the formation of clear zones around the colonies. In the present study, we used this method to estimate numbers of oxalate-degrading anaerobes in the ruminal contents of sheep. Data presented here provide the first estimates of the numbers of oxalate degraders in the rumen and the first comparisons between these numbers, ruminal oxalate degradation rates, and dietary oxalate levels.

MATERIALS AND METHODS

Animals and Diets

Three crossbred sheep (23-, 45-, and 55-kg body weight), prepared with ruminal cannulae, were housed in separate pens. The 23- and 45-kg sheep received daily 0.7 and 1 kg of a ground (6-mm sieve) alfalfa hay cubes diet, respectively. The 55-kg sheep received daily 0.85 kg of a 70% ground alfalfa hay cubes-30% oats diet. Hay cubes and oats contained 0.8 and 0.02% (wt/wt) oxalate, respectively. Sheep were fed at 8:00 a.m. and 4:00 p.m, with half of the daily ration given at each feeding. Feed was usually consumed within 30 min. Water and trace-mineral blocks were provided ad libitum.

Sheep were adapted to a high level of dietary oxalate by adding increasing amounts of ground (4-mm sieve) Halogeton glomeratus (halogeton) over a 45-day period to the ground hay cubes-oats diet or technical-grade sodium oxalate (Barium and Chemicals, Inc., Steubenville, Ohio) over a 7-day period to the ground hay cubes diet. The halogeton contained 13% (wt/wt) oxalate, most of which was present as the soluble sodium salt (Hodgkinson, 1977). Final concentrations of halogeton and sodium oxalate added to the diets were 32 and 2% (wt/wt), respectively. Sheep received these diets for a minimum of 5 days before ruminal samples were collected. Next, halogeton and sodium oxalate levels in the diets were reduced to 16 and 1% (wt/wt), respectively; sheep received these diets for 25 days and were then returned to their respective pre-adaptive diets. With each of these

diets, to minimize possible carryover effects when going from high to low concentrations of dietary oxalate, a transition period of at least seven days was observed before ruminal samples were collected. In previous studies, ruminal rates of oxalate degradation in adapted sheep returned to pre-adapative values within 3 days when halogeton feeding was stopped (Allison et al., 1977).

Sample Collection and Processing

Four to 6 h after the morning feeding, samples of ruminal contents were collected through the ruminal cannula. Preliminary studies indicated that ruminal rates of oxalate degradation were maximal during this time period after feeding. Ruminal samples were blended in a Waring blender at high speed for 1 min under CO_2. An 11-g (wet weight) portion of homogenate was transferred to a second blender that contained 99 ml of anaerobic dilution solution (less the $CaCl_2$; Bryant and Burkey, 1953) and was homogenized at high speed for 1 min under CO_2.

Microbial Analysis

The homogenates were serially diluted (10-fold) in anaerobic dilution solution, and 0.2-ml aliquots of each dilution (10^{-1} to 10^{-8}) were inoculated into triplicate anaerobic roll tubes (Bryant, 1972; Hungate, 1970) of the appropriate melted enumeration medium (48°C). After incubation at 37°C, concentrations of viable anaerobic oxalate-degrading bacteria were determined from counts of individual colonies

producing clear zones in media with opacity due to calcium oxalate. The media used were D agar (Daniel et al., 1987) or a modification of D agar that contained 14 mM $CaCl_2$ and 20% (vol/vol) filter-sterilized ruminal fluid (FSRF; S. L. Daniel, Ph.D. dissertation, Iowa State University, Ames, Iowa, 1988). The FSRF was added to the melted culture medium just prior to inoculation. Medium 98-5 with 0.025% xylose was used for the enumeration of total viable bacteria (Bryant and Robinson, 1961).

Analytical Methods

Oxalate degradation rates were estimated from measurements of $^{14}CO_2$ production during incubation of duplicate 1.8-g (wet weight) portions of a homogenate with sodium [^{14}C]oxalate in rubber-stoppered test tubes (Daniel et al., 1987).

Oxalate concentrations in the hay cubes, oats, and halogeton were determined by gas-liquid chromatography of the dibutyl ester (Daniel et al., 1987). Reported concentrations are the means of duplicate samples.

For dry weight determinations, duplicate 10-ml volumes of a homogenate were oven-dried at 55°C to a constant weight. Unless stated otherwise, oxalate degradation rates and colony counts were expressed on a per gram (dry weight) basis.

RESULTS AND DISCUSSION

Halogeton-Fed Sheep

Medium A (Allison et al., 1985), a roll tube medium containing 40 mM sodium oxalate, was used previously to enumerate anaerobic oxalate-degrading bacteria in human feces (Allison et al., 1986). However, colony formation by anaerobic oxalate-degrading bacteria from the sheep rumen was inhibited in medium A or when D agar was modified to contain 40 mM sodium oxalate (data not shown). High concentrations of oxalate in D agar are also inhibitory to oxalate degraders from the cecal contents of wild rats (Daniel et al., 1987).

When the oxalate concentration in medium D was reduced to 20 mM, the mean viable count of anaerobic oxalate-degrading bacteria in samples of ruminal contents from a sheep fed a diet containing 32% halogeton (4.6% oxalate) was 2.6×10^6/g (Table 1). The anaerobic oxalate-degrading bacteria represented slightly less than 0.01% of the total viable count. When the halogeton level in the diet was reduced from 32 to 16% (2.6% oxalate), ruminal rates of oxalate degradaton and colony counts of oxalate-degrading bacteria decreased by about 3- and 300-fold, respectively (Table 1).

Four strains of anaerobic oxalate-degrading bacteria isolated from the ruminal contents of this halogeton-fed sheep grew readily in D broth that contained either 20 or 40 mM sodium oxalate. These strains were similar in morphology to strain OxB, the type strain of Oxalobacter formigenes (Allison et al., 1985), and to strains isolated

from humans (Allison et al., 1986), rodents (Daniel et al., 1987), and lake sediments (Smith et al., 1985).

When the sheep was returned to the pre-adaptive diet (no halogeton; 0.6% oxalate), no viable oxalate degraders could be obtained from samples of ruminal contents, even though rates of oxalate degradation were measurable (Table 1). This, together with the fact that changes in viable counts of oxalate degraders did not correlate well with changes in ruminal rates of oxalate degradation when the oxalate level in the diet was decreased from 4.6 to 2.6%, suggests that colony counts reported here with D agar underestimated the true concentration of oxalate degraders.

Modification of D Agar

Based on cultural studies with the halogeton-fed sheep, most anaerobic oxalate-degrading bacteria in the rumen, besides having different degrees of tolerance to oxalate, appear to also have different nutrient requirements than the ruminal isolate OxB. Therefore, modifications of D agar that improved colony counts of anaerobic oxalate-degrading bacteria from the cecal contents of rats (Daniel et al., 1987) were tested in an effort to improve the recovery of oxalate-degrading anaerobes from ruminal contents of sheep. When the concentration of $CaCl_2$ in D agar was increased from 7 to 14 mM, the opacity of the medium increased substantially, and the counts of oxalate-degrading bacteria increased nearly 7-fold (Table 2). An additional increase in colony counts was not observed when the level of

yeast extract in D agar with 14 mM $CaCl_2$ was increased from 0.1 to 0.3%; however, with the higher level of yeast extract, the incubation time required for detection of colonies of oxalate degraders decreased. In a second experiment (Table 2), the addition of either 10 or 20% FSRF to D agar with 14 mM $CaCl_2$ and 0.1% yeast extract resulted in increased counts and decreased incubation times. Based on these findings, D agar modified to contain 14 mM $CaCl_2$ and 20% FSRF was used in subsequent studies.

Sodium Oxalate-Fed Sheep

Numbers of oxalate-degrading bacteria in the ruminal contents of two sheep adapted to a diet containing 2.0% sodium oxalate (2.2% total oxalate) were 1.9 x 10^8/g and 5.9 x 10^8/g, respectively (Table 3). These values are 10-fold greater than colony counts of anaerobic oxalate-degrading bacteria from cecal samples of laboratory rats adapted to a diet containing 4.5% sodium oxalate (Daniel et al., 1987).

Overall, rates of oxalate degradation decreased as the level of oxalate in the diet was decreased (Table 3); however, there was not always a direct relationship between oxalate degradation rates and colony counts. When the oxalate level in the diet was reduced to 1.5%, colony counts of oxalate degraders from the 23-kg sheep increased (from 1.9 to 6.8 x 10^8/g), whereas with the same diet change, counts from the 45-kg sheep were not appreciably changed. The colony counts of oxalate degraders from both sheep decreased by 7- to 13-fold when the dietary oxalate level was reduced to 0.8% (pre-adaptive diet).

Measurable quantities of oxalate are present in many plants consumed by ruminants (Libert and Franceschi, 1987; Rumsey et al., 1967). Oxalate is the only substrate known to be used by Oxalobacter formigenes, and survival of the bacterium in the rumen apparently depends on the ruminant's frequent ingestion of oxalate-containing plants. However, oxalate exists in alfalfa mainly as calcium oxalate crystals that are encapsulated in parenchymatous sheaths around vascular bundles (Ward et al., 1979). Since this structure is resistant to ruminal digestion, a major portion of the oxalate in alfalfa escapes bacterial degradation (Ward et al., 1982). In the present study, some of the oxalate in alfalfa must have been available, because oxalate degraders were present at numbers as high as 10^7/g in samples of ruminal contents from sheep fed only ground alfalfa hay cubes (Table 3).

With the exception of the 23-kg sheep fed a diet containing 2.2% oxalate, the proportions of oxalate degraders in the total viable microbial population of the rumen were directly associated with the level of oxalate in the diet (Table 3). Mean viable counts of oxalate degraders from sheep fed a diet containing 1.5% oxalate were 0.52-0.60% of mean total viable counts. When the sheep were returned to the pre-adaptive diet (0.8% oxalate), the percentages decreased about 9-fold to 0.06-0.07%. These proportions are similar to those noted for oxalate-degrading bacteria in human feces (Allison et al., 1986) and wild rat cecal contents (Daniel et al., 1987).

Summary

Previous reports have described the isolation of ruminal oxalate-degrading anaerobes (Dawson et al., 1980a) and the physiological role of these anaerobes (Allison, 1985). The present report describes measurements of the concentrations of anaerobic oxalate-degrading bacteria in the rumen. Our results indicate that sheep harbor significant populations of anaerobic oxalate-degrading bacteria in the rumen, although these populations represent only a small proportion of the total viable population. The concentrations of oxalate degraders in the rumen were, generally, influenced by the level of dietary oxalate. These data support the concept of substrate-based selection of anaerobic oxalate-degrading bacteria in the mammalian gastrointestinal tract (Allison and Cook, 1981; Allison et al., 1977).

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Table 1. The effects of halogeton feeding on rates of oxalate degradation and viable counts of anaerobic oxalate-degrading bacteria in the ruminal contents of a sheep[a]

% Halogeton added to diet (% oxalate)	Oxalate degradation rate[b]	Colony count[c]	
		Oxalate[d]	Total[e]
32 (4.6)	26.4	6.41	10.51
16 (2.6)	9.4	3.91	10.63
0 (0.6)	1.8	-[f]	10.42

[a]A 55-kg sheep fed the ground alfalfa hay cubes-oats diet.

[b]Micromoles of oxalate degraded per gram (dry weight) of ruminal contents per hour. Each value is the mean of data obtained from two samples.

[c]Log_{10}/g (dry weight) of ruminal contents. Each value is the mean of data obtained from two samples.

[d]Colonies with clear zones in D agar (Daniel et al., 1987) after 5 to 10 days of incubation.

[e]Total colony count in medium 98-5 with 0.025% xylose (Bryant and Robinson, 1961) after 10 days of incubation.

[f]-, Colonies with clear zones were not observed after 25 days of incubation.

Table 2. Comparison of medium modifications: colony counts of anaerobic oxalate-degrading bacteria from the ruminal contents of sheep[a]

Expt no.	Yeast extract (%)	FSRF[b] (%)	$CaCl_2$ (mM)	Colony count[c]
1	0.1[d]	-[e]	7	6.83 (42/66)
	0.3	-	7	7.14 (42/66)
	0.1	-	14	7.67 (42/66)
	0.3	-	14	7.28 (21/66)
2	0.1	-	14	8.22 (49/49)
	0.1	10	14	8.42 (28/49)
	0.1	20	14	8.51 (28/49)

[a]Ruminal contents were from oxalate-adapted sheep.

[b]FSRF, Filter-sterilized ruminal fluid.

[c]Log_{10}/g (dry weight) of ruminal contents. Each value is the mean of duplicate or triplicate tubes. Numbers in parentheses are day of incubation that majority of colonies with clear zones appeared/total days of incubation.

[d]D agar (Daniel et al., 1987) was modified as indicated below.

[e]-, Not added to the culture medium.

Table 3. The effects of sodium oxalate feeding on rates of oxalate degradation and viable counts of anaerobic oxalate-degrading bacteria in the ruminal contents of sheep

Sheep[a]	% Sodium oxalate added to diet (% oxalate)	Oxalate degradation rate[b]	Colony count[c]		% of Total[f]
			Oxalate[d]	Total[e]	
23-kg	2 (2.2)	7.5 ± 1.3	8.28 ± 0.12	11.00 ± 0.03	0.19
	1 (1.5)	4.3 ± 0.1	8.83 ± 0.02	11.05 ± 0.03	0.60
	0 (0.8)	1.6 ± 0.3	7.97 ± 0.10	11.14 ± 0.09	0.07
45-kg	2 (2.2)	8.8 ± 1.7	8.77 ± 0.23	10.84 ± 0.06	0.85
	1 (1.5)	7.2 ± 0.5	8.76 ± 0.03	11.04 ± 0.02	0.52
	0 (0.8)	3.9 ± 1.1	7.64 ± 0.40	10.85 ± 0.18	0.06

[a]Sheep were fed a diet of ground alfalfa hay cubes.

[b]Micromoles of oxalate degraded per gram (dry weight) of ruminal contents per hour. Each value is the mean ± the standard error of data obtained from three samples.

[c]Log_{10}/g (dry weight) of ruminal contents. Each value is the mean ± standard error of data obtained from three samples.

[d]Colonies producing clear zones in D agar (Daniel et al., 1987) containing 14 mM $CaCl_2$ and 20% (vol/vol) filter-sterilized ruminal fluid after 42 to 49 days of incubation.

[e]Total colony count in medium 98-5 with 0.025% xylose (Bryant and Robinson, 1961) after 28 days of incubation.

[f]Mean oxalate count divided by mean total count x 100.

SUMMARY AND DISCUSSION

Oxalate is degraded by bacteria in the gastrointestinal tracts of mammals. A new species of obligately anaerobic bacteria, Oxalobacter formigenes, which uses oxalate as a major source of carbon and energy, has been isolated from the rumen and from the bowel of humans and certain nonruminant herbivores. From the results of this dissertation, as well as other studies, it appears that oxalate-degrading anaerobes similar to O. formigenes are responsible for the degradation of dietary oxalate in the intestines of mammals.

Results of studies reported in Section I indicate that, unlike other mammals, most commercially available laboratory rats are not colonized by oxalate-degrading bacteria. Laboratory rats from only one of the five commercial breeders tested harbored cecal oxalate-degrading bacteria. An oxalate-degrading isolate (OxCR6) from these laboratory rats was similar to the ruminal isolate OxB, the type strain of O. formigenes. Although the reasons that some but not all laboratory rats harbor oxalate-degrading bacteria are presently unknown, we propose that procedures used for the establishment and maintenance of most commercial rat colonies often preclude the introduction and multiplication of anaerobic oxalate-degrading bacteria.

Laboratory rats are frequently used as models in studies of oxalate metabolism in humans. The apparent absence of intestinal oxalate-degrading bacteria in rats is significant because most studies have neglected the possible influence that these anaerobes might have on the metabolism of oxalate. In Section II, we demonstrated that

strain OxCR6, as well as other strains of O. formigenes isolated from humans, swine, guinea pigs, and wild rats, could colonize the ceca of adult laboratory rats which did not harbor O. formigenes. After colonization by OxCR6, cecal rates of oxalate degradation increased dramatically. However, colonizaton only occurred when rats were fed diets containing 3 or 4.5% sodium oxalate. Thus, a high level of dietary oxalate was required for colonization by O. formigenes.

The effects of diet, intestinal absorption, and endogenous oxalate synthesis on oxalate excretion by mammals have been studied extensively; however, little is known about bacterial degradation of oxalate in the gastrointestinal tracts of mammals and how it affects urinary or fecal excretion of dietary oxalate. The ability to implant O. formigenes into the indigenous intestinal flora of laboratory rats should provide an animal model in which the influence of oxalate-degrading bacteria on the fate of dietary oxalate in mammals can be studied. Such studies could provide insight into the factors that affect the absorption of dietary oxalate and would thus be pertinent to urolithiasis in humans.

The results of Section III indicate that sheep harbor significant populations of anaerobic oxalate-degrading bacteria in the rumen, although these populations represent only a small proportion of the total viable population. The concentrations of anaerobic oxalate-degrading bacteria in the rumen were generally influenced by the level of dietary oxalate. These data support the concept that increased rates of oxalate degradation in ruminal contents of animals fed diets

containing increasing amounts of oxalate are the result of substrate-based selections of anaerobic oxalate-degrading bacteria. Such selections, through the destruction of oxalate, apparently serve to limit the absorption, and thus toxicity of high levels of dietary oxalate.

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APPENDIX A.

SUPPLEMENT TO SECTION I

Procedure for the Measurement of Rates of Oxalate Degradation

1. Feces and contents of the small intestines, ceca, or large intestines from rats were diluted 10-fold in anaerobic dilution solution (Table A1).

2. Diluted contents were homogenized for 15 s in a Waring blender under CO_2.

3. Duplicate 1.8-ml aliquots of a homogenate were transferred to test tubes (13 by 100 mm) which contained 0.2 ml of sodium [^{14}C]oxalate (0.1 M, 0.02 μCi/μmol). During this transfer, blender jar and test tubes were gassed with CO_2. For a 0-min control, a third 1.8-ml aliquot of the homogenate was transferred to a tube which contained the [^{14}C]oxalate solution and 1 ml of 3 N NaOH.

4. After tubes were stoppered and the contents thoroughly mixed, tubes were placed in a 38°C water bath.

5. After incubation for 1 or 2 h, the reactions (except for controls) were stopped by injecting 1 ml of 3 N NaOH and then mixing the contents of each tube thoroughly. At this point, stopped reactions were stored at 4°C until diffusions were performed.

6. A 0.5-ml aliquot of a stopped reaction was transferred to a 25-ml erlenmeyer flask. These flasks had rubber stoppers with attached plastic hanging buckets (Kontes, Vineland, N.J.). Each bucket contained 0.25 ml of phenethylamine.

7. Two milliliters of the diffusion buffer (Table A2) was added to the flask. The stopper was immediately and carefully secured in place and the flask was transferred to a 38°C water bath.

8. Flasks were incubated (with gentle shaking) for 90 min.

9. After incubation, buckets were cut from the stoppers and placed in scintillation vials which contained 10.0 ml of Biofluor.

10. The radioactivity was counted in a liquid scintillation counter.

Table A1. The composition and preparation of anaerobic dilution solution[a]

Component[b]	Gram per liter
K_2HPO_4	0.225
KH_2PO_4	0.225
$(NH_4)_2SO_4$	0.450
NaCl	0.450
$MgSO_4 \cdot 7H_2O$	0.045
$CaCl_2$	0.023
Resazurin	0.001
Na_2CO_3	4.000
Cysteine·HCl·H_2O	0.500
Distilled H_2O	1000.000 ml

[a]As described by Bryant and Burkey (1953). Final pH = 6.6 to 6.8. In the present study, the $CaCl_2$ was deleted.

[b]After the components were mixed, the solution was maintained under CO_2 while it was boiled, cooled, and dispensed into culture tubes (16 by 150 mm). Tubes were held in a press while they were sterilized by autoclaving (121°C for 15 min, fast exhaust).

Table A2. The composition of the diffusion buffer[a]

Component	Amount
Citric acid·H_2O	78.8 g
NaOH	15.0 g
Absolute ethanol	40.0 ml
Distilled H_2O	q.s. to 1 l

[a]Conway, 1962. Final pH = 3.8.

Table A3. Oxalate degradation by feces from laboratory rats[a]

% Halogeton in the diet[b]	% Oxalic acid in the diet	Oxalate degradation rate[c]
0	0.1	0.3 ± 0.1
10	1.5	0.2 ± 0.1
20	3.0	0.4 ± 0.4

[a]Three male Sprague-Dawley rats (300-400 g; Biolab Corp.) were used in this experiment (one rat per cage).

[b]The control diet (Teklad 4% fat mouse/rat diet; ground through a 1-mm sieve) was supplemented with Halogeton glomeratus (halogeton). The halogeton contained 14.8% oxalic acid. Diets were fed to the rats for at least 8 days before feces were collected.

[c]Micromoles of oxalate degraded per gram (dry weight) of feces per hour. Each value is the mean ± the standard deviation of three determinations (one determination per rat).

Table A4. Oxalate degradation by the contents of the small intestines, ceca, and large intestines from laboratory rats[a]

Day[b]	Intestinal segment[c]	Oxalate degradation rate[d]	
		Control diet	Oxalate diet
15	SI	0	0
	Cecum	3.5 ± 0.6	2.5 ± 0.1
	LI	2.0 ± 0.5	1.4 ± 0.2
30	SI	0	0
	Cecum	2.3 ± 0.4	1.9 ± 0.2
	LI	1.3 ± 0.3	1.2 ± 0.3
60	SI	0	0
	Cecum	2.3 ± 0.4	1.9 ± 0.1
	LI	1.1 ± 0.1	1.5 ± 0.1

[a]Two pairs of Sprague-Dawley rats (one pair of rats per cage) were sacrificed per time period per diet.

[b]Time on each diet before sacrificing.

[c]SI, Small intestine; LI, large intestine. The contents of each intestinal segment from a pair of rats were pooled before analysis.

[d]Micromoles of oxalate degraded per gram (dry weight) of contents per hour. Each value is the mean ± standard error of two determinations.

[e]Not significantly different ($P > 0.1$).

Table A5. Oxalate degradation by anaerobic dilution solution, control diet, cecal tissue, and fractions of cecal contents

Sample[a]	Oxalate degradation rate[b]
ADS	0
ADS + control diet[c]	0
ADS + cecal tissue[d]	0
ADS + cecal contents[e]	1.3
Centrifuged (12,000 x g for 10 min)	
Supernatant fluid	0
Pellet resuspended in ADS	1.9

[a]Samples were diluted 10-fold in anaerobic dilution solution (ADS) (Bryant and Burkey, 1953; less the $CaCl_2$) and were homogenized before analysis.

[b]Micromoles of oxalate degraded per gram (wet weight) of sample per hour. Each value is the mean of duplicate tubes.

[c]The control diet was ground through a 4-mm sieve.

dCecal tissue was prepared by emptying and inverting the ceca from two rats and then rinsing the tissue three times with ADS.

[e]Cecal contents were from a pair of Sprague-Dawley rats that had been fed the control diet for 16 days.

Table A6. Colony counts of anaerobic bacteria from the cecal contents of wild rats: comparison of different media

Medium[a]	Colony count[b]
10	11.12 ± 0.08
CCA	11.09 ± 0.05
Modified Balch	11.06 ± 0.06

[a]Medium: 10 (Caldwell and Bryant, 1966; Table A7); CCA (Allison et al., 1979); and modified Balch (Miller and Wolin, 1982).

[b]Log_{10}/g (dry weight) of cecal contents. Colony counts determined from duplicate tubes incubated for 7 to 10 days. Each value is the mean ± standard error of three determinations. Cecal contents from different groups of wild rats were used in each determination.

Table A7. The composition and preparation of medium 10

Component[a]	Gram per liter
Trypticase	2.000
Yeast extract	0.500
Glucose	0.500
Cellobiose	0.500
Soluble starch	0.500
Hemin	0.001
Volatile fatty acids solution (Table A8)	3.100 ml
Resazurin	0.001
K_2HPO_4	0.225
KH_2PO_4	0.225
$(NH_4)_2SO_4$	0.450
NaCl	0.450
$MgSO_4 \cdot 7H_2O$	0.045
$CaCl_2$	0.023
Resazurin	0.001
Distilled H_2O	996.900 ml
Agar	15.000
Na_2CO_3	4.000
Cysteine·HCl·H_2O	0.500

[a]As described by Caldwell and Bryant (1966). Components other than the last two were mixed and the pH was adjusted to 7. After boiling, the mixture was maintained under CO_2 while it was cooled to 50°C, while sodium carbonate and cysteine were added, and while 10-ml volumes were dispensed into culture tubes (18 by 150 mm). Tubes were held in a press, and the medium was sterilized (121°C for 15 min, fast exhaust).

Table A8. The composition of the volatile fatty acids solution[a]

Acid	Amount (milliliter)
Glacial acetic acid	51.0
Propionic acid	18.0
Butyric acid	12.0
Isobutyric acid	3.0
n-Valeric acid	3.0
Isovaleric acid	3.0
2-methylbutyric acid	3.0

[a]As described by Caldwell and Bryant (1966). The volatile fatty acids solution was stored at 4°C.

Methane Production by the Cecal Contents From Wild Rats

In certain experiments, measurements were made of the quantities of methane in the headspace of reaction tubes used for the determination of oxalate degradation rates. Since these tubes had a negative pressure due to the absorption of CO_2 after injection of NaOH, unopened tubes were equilibrated to atmospheric pressure with helium from a lubricated glass syringe. After equilibration, the headspace (volume approximately 4.5 ml) was sampled through the stopper with a disposable hypodermic needle and syringe. Gas samples (1 ml) were analyzed with a model AD-2000 respiratory gas analyzer (Loenco Inc., Altadena, Calif.) as described by Dawson et al. (1980b). Reported values for methane production represent the mean of duplicate tubes.

Measurements of methane production listed in Table A9 provide the first evidence for the presence of methanogenic bacteria in the cecal contents of wild rats. Rates of methane production varied from 2.7 to 11.9 μmol/g (dry weight) of cecal contents per hour. Results indicated that there was no apparent relationship between rates of methane production and either rates of oxalate degradation or oxalate concentrations (both soluble and total) in the cecal contents of wild rats. No attempt was made to enumerate or isolate the methanogens.

Table A9. Methane production, oxalate degradation, and total oxalate concentrations in the cecal contents from wild rats

Group no.[a]	Total cecal oxalate concn[b]	Oxalate degradation rate[c]	Methane production[d]
1 (2)	11.5 (35)	11.1	4.0
2 (4)	11.0 (5)	20.6	11.9
3 (4)	2.6 (0)	7.4	4.6
4 (3)	12.6 (45)	13.7	2.7
5 (3)	5.6 (29)	8.3	3.3

[a]Indicates different groups of wild rats that were collected. Values in parentheses indicate the number of wild rats per group. The contents of the ceca from a group of wild rats were pooled before analysis.

[b]Micromoles of oxalic acid per gram (dry weight) of cecal contents. Each value is the mean of duplicate tubes. Values in parentheses are the percentages of total oxalate present as soluble oxalate.

[c]Micromoles of oxalate degraded per gram (dry weight) of cecal contents per hour. Each value is the mean of duplicate tubes.

[d]Micromoles of methane produced per gram (dry weight) of cecal contents per hour. Each value is the mean of duplicate tubes.

Sources of Anaerobic Oxalate-Degrading Bacteria Isolated From the Cecal Contents of Wild and Laboratory Rats

Anaerobic oxalate-degrading bacteria were isolated from colonies with clear zones in D agar or D agar containing 40 mM oxalate that had been inoculated directly with 0.2 and 2 or 20 µg of cecal contents from wild rats, respectively. Seven oxalate-degrading isolates were obtained from D agar (OxWR4 through OxWR10) and two from D agar with 40 mM oxalate (OxWR1 and OxWR2).

Anaerobic oxalate-degrading bacteria were isolated from colonies with clear zones in media D2, D3, and D5 that had been inoculated directly with 2 µg of cecal contents from oxalate-fed laboratory rats (Charles River Breeding Laboratories, Inc.). Six oxalate-degrading isolates were obtained from these media: two from D2 agar (OxCR1 and OxCR2); two from D3 agar (OxCR3 and OxCR4); and two from D5 agar (OxCR5 and OxCR6).

The isolation of anaerobic oxalate-degrading bacteria from these media basically followed the procedures described in Materials and Methods, except that agar versions of D2, D3, D5, and D with 40 mM oxalate and broth versions of D3, D5, and D with 40 mM oxalate were used in place of D agar and D broth, respectively.

Table A10. The composition of the trace metals solution used in the preparation of D agar[a]

Component[b]	Gram per liter
EDTA (disodium salt)	0.500
$FeSO_4 \cdot 7H_2O$	0.200
$ZnSO_4 \cdot 7H_2O$	0.010
$MnCl_2 \cdot 4H_2O$	0.003
H_3BO_4	0.030
$CoCl_2 \cdot 6H_2O$	0.020
$CuCl_2 \cdot 2H_2O$	0.001
$NiCl_2 \cdot 6H_2O$	0.002
$Na_2MoO_4 \cdot 2H_2O$	0.003
Distilled H_2O	1000.000 ml

[a]Trace metals solution (Pfennig and Lippert, 1966).

[b]After mixing the components in the order listed above, the solution was stored at 4°C.

APPENDIX B.

SUPPLEMENT TO SECTION II

Table B1. Rates of oxalate degradation in cecal contents from laboratory rats inoculated with large bowel contents from captured wild rats[a]

% Sodium oxalate in the diet[b]	Oxalate degradation rate[c]	
	Control	Inoculated[d]
0	1.0	1.2
1.5	0.9	1.5
3.0	1.0	10.2
4.5	1.0	44.8

[a]One pair of Sprague-Dawley rats (Biolab Corp.) per diet per control or inoculated group.

[b]The control diet (Teklad 4% fat mouse/rat diet) supplemented with sodium oxalate.

[c]Micromoles of oxalate degraded per gram (dry weight) of cecal contents per hour. Each value is the mean of duplicate tubes.

[d]Laboratory rats were inoculated by placing 3.0 ml of a 10-fold dilution of cecal contents from wild rats on a portion of the feed. Rats were sacrificed 14 days after inoculation. Cecal contents from a pair of rats were pooled before analysis.

Table B2. Rates of oxalate degradation in cecal contents from laboratory rats inoculated with *O. formigenes*[a]

% Sodium oxalate in the diet[b]	Oxalate degradation rate[c]	
	Control	Inoculated[d]
0	1.5	1.1
1.5	1.1	0.8
3.0	1.1	0.8
4.5	1.1	3.7

[a]One pair of Sprague-Dawley rats (Biolab Corp.) per diet per control or inoculated group.

[b]The control diet (Teklad 4% fat mouse/rat diet) supplemented with sodium oxalate.

[c]Micromoles of oxalate degraded per gram (dry weight) of cecal contents per hour. Each value is the mean of duplicate tubes.

[d]Rats were inoculated by placing 6×10^{10} viable cells of strain OxWR1 on a portion of the feed. Rats were sacrificed 14 days after inoculation. Cecal contents from a pair of rats were pooled before analysis.

Table B3. Oxalate degradation rates in cecal contents from laboratory rats fed a diet with 4.5% sodium oxalate and inoculated with different strains of O. formigenes[a]

Strain[b]	Source	Oxalate degradation rate[c]
Control	-	1.3
OxB	Sheep rumen	0.9
POxC	Pig cecum	1.2
OxK	Human feces	5.1
OxWR1	Wild rat cecum	4.5

[a]One pair of Sprague-Dawley rats (Biolab Corp.) per strain. Rats were fed Teklad 4% fat mouse/rat diet supplemented with 4.5% sodium oxalate.

[b]Rats were inoculated by placing 4×10^{10} to 2×10^{11} viable cells of the assigned strain of O. formigenes on a portion of the feed. Rats were sacrificed 14 days after inoculation. Cecal contents from a pair of rats were pooled before analysis.

[c]Micromoles of oxalate degraded per gram (dry weight) of cecal contents per hour. Each value is the mean of duplicate tubes.

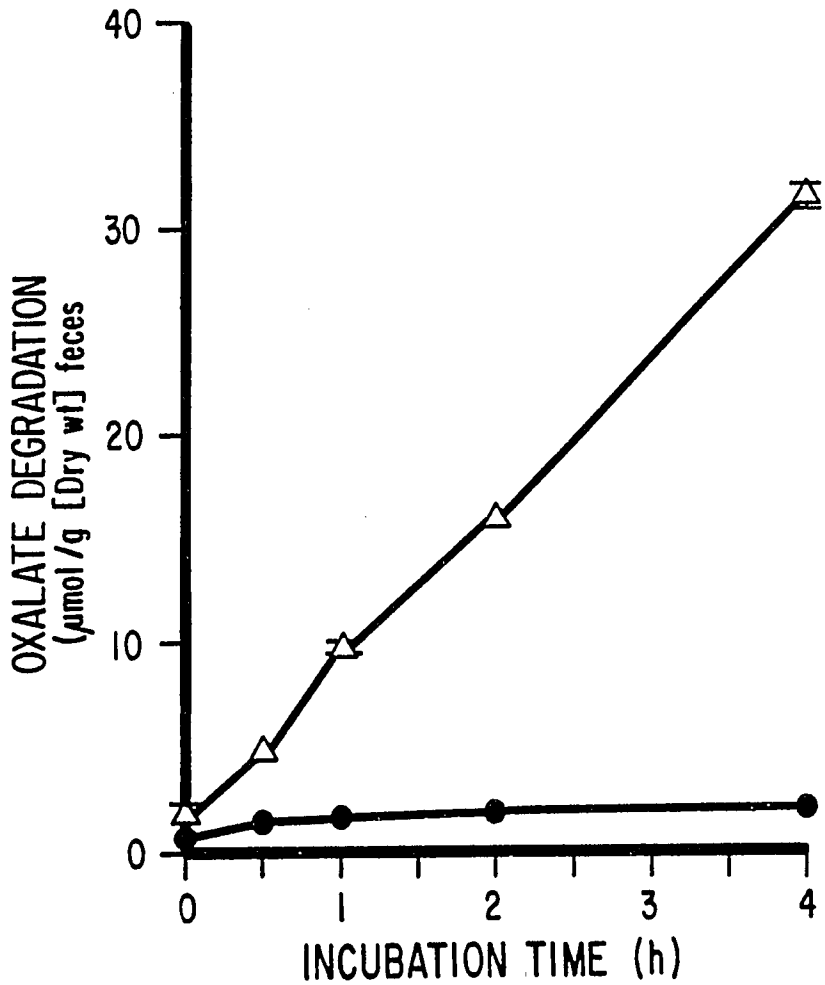

Figure B1. Time course of oxalate degradation by feces from Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.) fed the control diet (Teklad LM-485 mouse/rat diet) (●) and from inoculated rats fed the control diet supplemented with 2% sodium oxalate (Δ). These rats were each inoculated with 2.5×10^{10} viable cells of strain OxCR6. Feces were collected 17 days after inoculation. Each point is the mean ± standard deviation of triplicate tubes

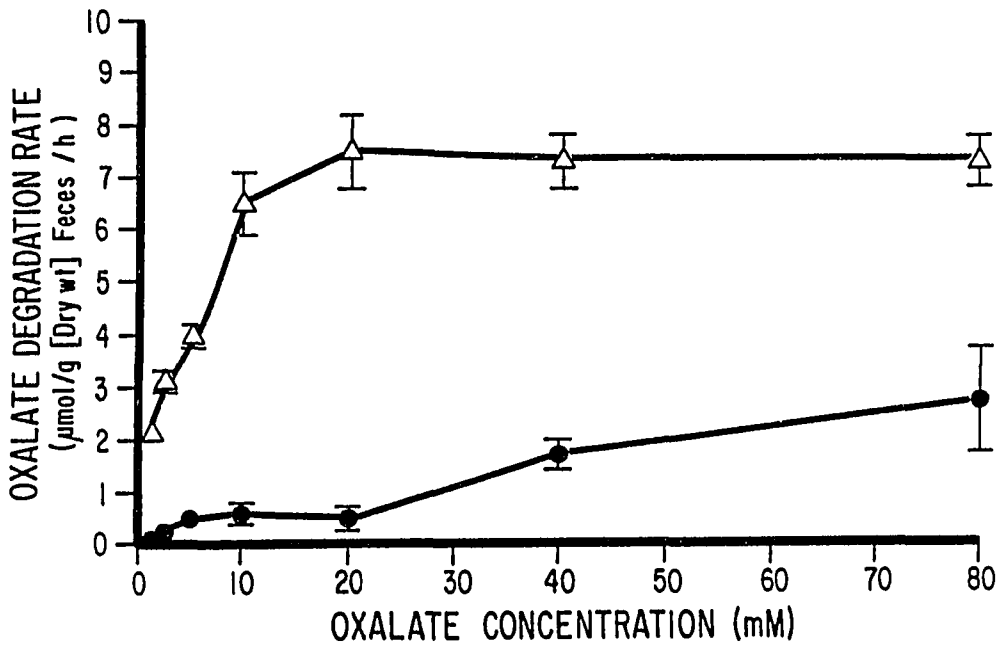

Figure B2. Effects of oxalate concentration on oxalate degradation by feces from Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.) fed the control diet (Teklad LM-485 mouse/rat diet) (●) and from inoculated rats fed the control diet supplemented with 2% sodium oxalate (△). These rats were each inoculated with 2.5 x 10^{10} viable cells of strain OxCR6. Feces were collected 21 days after inoculation. Each point is the mean ± standard deviation of triplicate tubes

Table B4. Effects of various antibiotics, gas phases, and temperatures on oxalate degradation by feces from laboratory rats[a]

Expt	Treatment	Oxalate degradation rate[b]	
		Control[c]	Inoculated[d]
1	Antibiotic[e]		
	None	0.4	11.0
	Cepthalothin	0.4	9.6
	Neomycin	0.4	8.7
	Polymxin B	0.2	2.8
	Clindamycin	0.2	9.1
	Tetracycline	0.4	8.2
	Chloramphenicol	0.4	6.9
2	Gas phase (1 atm)		
	CO_2	2.4	ND[f]
	H_2	2.0	ND
	N_2	1.5	ND
	O_2	1.6	ND
3	Temperature (°C)		
	4	1.6	1.1
	39	1.3	7.8
	65	1.1	2.5
	Autoclaved[g]	0	ND

[a]Feces from Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.) were collected overnight, diluted 10-fold in anaerobic dilution solution, and then homogenized before analysis.

[b]Micromoles of oxalate degraded per gram (dry weight) of feces per hour. Each value is the mean of triplicate tubes.

[c]Rats fed the control diet (Teklad LM-485 mouse/rat diet).

[d]Rats were each inoculated with 2.5×10^{10} viable cells of strain OxCR6 and were fed the control diet supplemented with 2% sodium oxalate. Feces were collected 17 to 23 days after inoculation.

[e]Antibiotic concentration (0.15 mg per ml of reaction mixture).

[f]ND, Not determined.

[g]Homogenate was autoclaved (121°C for 15 min) before analysis.

Table B5. Effects of differential centrifugation and dilution on oxalate degradation by feces and large bowel contents from laboratory rats[a]

Expt	Treatment	Oxalate degradation rate[b]	
		Control[c]	Inoculated[d]
1	Centrifugation		
	Diluted feces	1.4	4.2
	Centrifuged (150 x g for 2 min)		
	Supernatant fluid	0.5	3.7
	Pellet resuspended in ADS	0.9	3.0
	Fecal supernatant at 150 x g		
	Centrifuged (12,000 x g for 10 min)		
	Supernatant fluid	0	0
	Pellet resuspended in ADS	0.4	3.3
2	Dilution[e]		
	1:2.5	28.8[f]	ND[g]
	1:5	31.9	ND
	1:10	27.8	ND

[a]Feces from Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.) were collected overnight, diluted 10-fold in anaerobic dilution solution (ADS), and then homogenized before analysis.

[b]Micromoles of oxalate degraded per gram (wet weight) of feces per hour. Each value is the mean of triplicate tubes.

[c]Rats fed the control diet (Teklad LM-485 mouse/rat diet).

[d]Rats were each inoculated with 2.5×10^{10} viable cells of strain OxCR6 and were fed the control diet supplemented with 2% sodium oxalate. Feces were collected 23 days after inoculation.

[e]Cecal and large intestinal contents from Sprague-Dawley rats (Biolab Corp.) fed the Teklad 4% fat mouse/rat diet were pooled and then diluted in ADS.

[f]Nanomoles of oxalate degraded per ml of dilution per hour. Each value is the mean of duplicate tubes.

[g]ND, Not determined.

APPENDIX C.

SUPPLEMENT TO SECTION III

Table C1. Oxalate content of halogeton, hay cubes, oats, and corn

Feed	Batch[a]	% Oxalate (wt/wt)[b]
Halogeton	1	16.4
	2	13.4
	3	17.0
	4	13.0
Hay cubes	1	0.5
	2	0.9
	3	0.7
	4	0.8
Oats	1	0.02
Corn	1	0.02

[a]Represents different batches of feed that were analyzed.

[b]Oxalate content determined as described by Allison et al. (1981). Each value is the mean of duplicate or triplicate samples.

Table C2. Rates of oxalate degradation by ruminal contents from a sheep fed increasing levels of halogeton in the diet[a]

% Halogeton in diet[b]	Oxalate degradation rate[c]
0	0.03
2.6	0.10
5.1	0.18
9.4	0.26
12.3	0.33
19.0	0.62
32.0	1.38

[a]A 55-kg sheep fed a 50% ground hay-50% corn diet or a 60% ground hay cubes-40% oats diet.

[b]Halogeton contained 13% oxalate. Values for halogeton diets were obtained after 2 or more days of halogeton feeding.

[c]Micromoles of oxalate degraded per milliliter of strained ruminal fluid per hour. Each value is the mean of duplicate tubes.

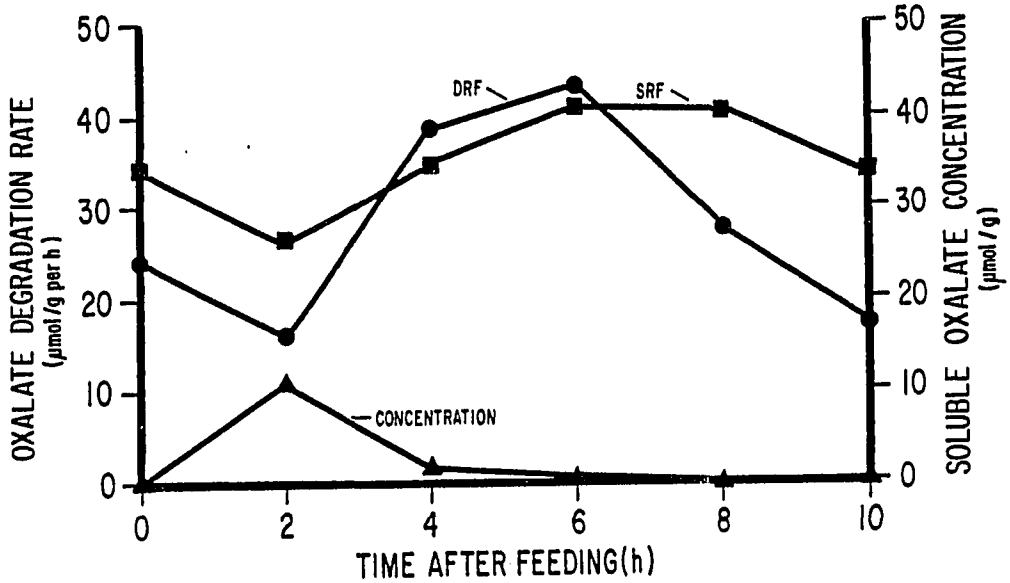

Figure C1. Postprandial changes in the rate of oxalate degradation by diluted (10-fold) ruminal fluid (DRF) and strained ruminal fluid (SRF). Ruminal contents were from a 55-kg sheep that had been fed 1 kg of a ground hay cubes-oats diet with 32% halogeton per day for 70 days before this experiment. Each point is the mean of duplicate tubes

Table C3. Clear zone production by O. formigenes strain OxB on a calcium oxalate medium in the presence of ruminal microorganisms

Inoculum[a]	Addition of OxB cells[b]	Colony count[c]
2×10^{-4}	-[d]	0
	+[e]	15
2×10^{-5}	-	0
	+	18
2×10^{-6}	-	0
	+	18
2×10^{-7}	-	0
	+	17
2×10^{-8}	-	0
	+	21

[a]Milliliters of ruminal fluid. Ruminal contents, collected 4 h after feeding, were strained and diluted (vol/vol) in anaerobic dilution solution (less the $CaCl_2$; Bryant and Burkey, 1953). Ruminal contents were from a 55-kg sheep that had been fed 1 kg of a ground hay cubes-oats diet with 32% halogeton per day for 34 days.

[b]OxB was grown for 5 h (OD_{600} = 0.21) in D broth with 100 mM oxalate.

[c]Number of colonies producing clear zones in D agar with 40 mM oxalate after 14 days of incubation. Each value is from a single tube.

[d]D agar inoculated only with ruminal fluid.

[e]D agar inoculated with ruminal fluid plus 2×10^{-7} ml of OxB culture.

Table C4. Postprandial changes in the pH of and the rate of oxalate degradation by ruminal contents from a sodium oxalate-fed sheep[a]

Time after feeding (h)[b]	pH	Oxalate degradation rate[c]
0	6.9	1.38
1	6.5	1.02
2	6.4	1.25
3	6.5	1.59
4	6.2	1.61
6	6.7	1.24

[a]A 55-kg sheep that had been fed 0.85 kg of a ground hay cubes-oats diet with 3% sodium oxalate per day for 86 days.

[b]After pH measurements, ruminal contents were diluted (10-fold) in anaerobic dilution solution (less the $CaCl_2$; Bryant and Burkey, 1953) before analysis.

[c]Micromoles of oxalate degraded per gram (wet weight) of ruminal contents per hour. Each value is the mean of duplicate tubes.

Table C5. The effects of dilution on the rate of oxalate degradation by a sample of ruminal contents from an oxalate-fed sheep[a]

Dilution[b]	Oxalate degradation rate[c]
Undiluted	1.63
1:2	1.46 (2.92)
1:4	0.81 (3.24)
1:8	0.44 (3.52)
1:16	0.15 (2.40)
1:32	0.07 (2.24)
1:64	0.02 (1.28)

[a]A 55-kg sheep that had been fed 0.85 kg of a ground hay cubes-oats diet with 3% sodium oxalate per day for 14 days.

[b]Ruminal contents, collected 5 h after feeding, were blended and then diluted (vol/vol) in anaerobic dilution solution (less the $CaCl_2$; Bryant and Burkey, 1953) before analysis.

[c]Micromoles of oxalate degraded per milliliter of diluted ruminal contents per hour. Each value is the mean of duplicate tubes. Values in parentheses are rates that have been corrected for dilution and are expressed on a per gram (wet weight) of ruminal contents basis.

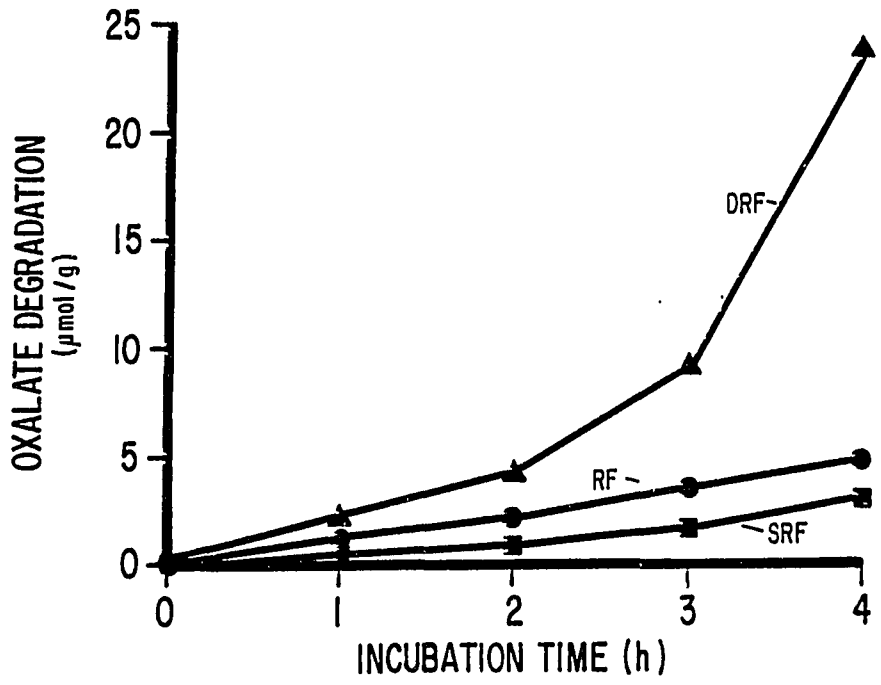

Figure C2. Time course of oxalate degradation by samples of ruminal fluid (RF); diluted (10-fold) RF (DRF); and strained ruminal fluid (SRF). Ruminal contents were from a 55-kg sheep that had been fed 0.85 kg of a ground hay cubes-oats diet with 3% sodium oxalate per day for 15 days before this experiment. Each point is the mean of triplicate tubes

Preparation of Ruminal Fluid

Clarified ruminal fluid (CRF)

The ruminal fluid was prepared from bovine ruminal contents collected 4 to 6 h after feeding. Ruminal contents were filtered through two layers of cheese cloth. The filtered ruminal contents were autoclaved (121°C for 5 min, slow exhaust). The particulate matter in the autoclaved ruminal contents was removed by centrifugation at 14,000 x g for10 min. The supernatant was further clarified by centrifugation at 35,000 xg for 10 min. The resulting clarified ruminal fluid (CRF) was then decanted into screw-cap bottles, autoclaved and then stored at 4°C.

Filter-sterilized ruminal fluid (FSRF) and heat-treated FSRF (HT-FSRF)

The ruminal fluid was prepared from bovine ruminal contents collected 4 h after feeding. The ruminal contents were filtered through two layers of cheesecloth. The particulate matter in the filtrate was removed by centrifugation at 14,000 x g for 15 min at 4°C in a model RC-2 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The supernatant was passed through a Millipore cassette filtration system (pore size, 0.5 μm; Millipore Corp., Bedford, Mass.). This filtrate was divided into two parts. The first part was filtered through Millex HA sterile disposable filter units (pore size, 0.22 μm; Millipore) into sterile screw-cap culture tubes (20 by 150 mm) and was referred to as filter-sterilized ruminal fluid (FSRF). The second part was autoclaved (121°C for 15 min, slow exhaust), cooled, and filtered through the

cassette filtration system to remove precipitated material. The filtrate was passed through disposable filter units into sterile tubes and was referred to as heat-treated FSRF (HT-FSRF). Both FSRF and HT-FSRF were stored at room temperature under CO_2 in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich). Before use, FSRF and HT-FSRF were first bubbled with CO_2 for 5 to 10 min and were then aseptically added to tubes of melted culture media just prior to inoculation. A large volume of FSRF and HT-FSRF was prepared so that comparisons would not be affected by differences between batches of ruminal fluid; however, experiments to determine whether there were such differences were not performed.

Table C6. The composition and preparation of medium 98-5[a]

Component[b]	Gram per liter
Glucose	0.250
Cellobiose	0.250
Soluble starch	0.500
Xylose	0.250
Resazurin	0.001
KH_2PO_4	0.225
K_2HPO_4	0.225
$(NH_4)_2SO_4$	0.450
NaCl	0.450
$MgSO_4 \cdot 7H_2O$	0.045
$CaCl_2$	0.023
Clarified ruminal fluid	400.000 ml
Distilled H_2O	600.000 ml
Agar	20.000
$NaCO_3$	4.000
Cysteine·HCl·H_2O	0.500

[a]Medium 98-5 with 0.025% xylose (Bryant and Robinson, 1961).

[b]Components other than the last two were mixed, and the pH was adjusted to 7.0. After boiling, the mixture was maintained under CO_2 while it was cooled to 50°C, while sodium carbonate and cysteine were added, and while 10-ml volumes were dispensed into culture tubes (18 by 150 mm). Tubes were held in a press while autoclaving (121°C for 15 min, fast exhaust).

Table C7. Colony counts of ruminal microorganisms in medium 98-5 supplemented with either clarified ruminal fluid (CRF), filter-sterilized ruminal fluid (FSRF), or heat-treated filter-sterilized ruminal fluid (HT-FSRF)

Medium	Ruminal fluid[a]	Colony count[b]
98-5[c]	CRF	9.67
98-5	FSRF	10.09
98-5	HT-FSRF	10.09

[a]Final concentration of ruminal fluids was 40%. FSRF and HT-FSRF were added to the melted medium just prior to inoculation.

[b]Log_{10}/g (wet weight) of ruminal contents. Each value is the mean of triplicate tubes after 20 days of incubation. Ruminal contents were from a 23-kg sheep that had been fed 0.66 kg of ground hay cubes with 1.5% sodium oxalate per day for 32 days.

[c]Medium 98-5 with 0.025% xylose (Bryant and Robinson, 1961).

Table C8. Oxalate degradation in D broth inoculated with ruminal contents from a sheep fed a halogeton diet[a]

Inoculum[b]	Oxalate medium[c]	
	20 mM	40 mM
1×10^{-2}	3[d] (2)[e]	3 (8)
1×10^{-3}	3 (8)	3 (48)
1×10^{-4}	3 (8)	3 (8)
1×10^{-5}	3 (48)	1 (48)
1×10^{-6}	1 (48)	-[f]
1×10^{-7}	-	-

[a]A 55-kg sheep fed 1 kg of a ground hay cubes-oats diet with 32% halogeton per day for 96 days.

[b]Gram (wet weight) of ruminal contents.

[c]D broth (Daniel et al., 1987) with either 20 or 40 mM sodium oxalate.

[d]Number of tubes out of three showing oxalate degradation after 48 days of incubation as determined by the calcium precipitation test (Dawson et al., 1980b).

[e]Days of incubation before oxalate degradation was observed.

[f]Oxalate degradation was not observed after 48 days of incubation.

Table C9. Initial attempts to enumerate viable anaerobic oxalate-degrading bacteria from the ruminal contents of a sheep[a]

Expt no.	% Halogeton in diet	Medium	Colony count[b]
1 (5/0.6)[c]	20	A[d]	0[e] (21)
2 (23/0.4)	20	A + 5% CRF	0 (31)
3 (3/1.4)	32	A	0 (30)
		A + 5% CRF	0 (30)
		D[f]	0 (30)
4 (34/ND[g])	32	D	0 (42)
5 (96/3.3)	32	D	0 (40)

[a]A 55-kg sheep fed a ground hay cubes-oats diet.

[b]Log_{10}/g (wet weight) of ruminal contents. Each value is the mean of duplicate tubes. Numbers in parentheses are total days of incubation.

[c]Days on diet/oxalate degradation rate (micromoles oxalate degraded per gram [wet weight] of ruminal contents per hour).

[d]Medium A with 40 mM oxalate, 0.05% yeast extract, and 7 mM $CaCl_2$ as described by Allison et al. (1985).

[e]0, No colonies with clear zones observed.

[f]D agar with 40 mM oxalate, 0.1% yeast extract, and 7 mM $CaCl_2$ as described by Daniel et al. (1987).

[g]ND, Not determined.

Table C10. The effects of different concentrations of oxalate, yeast extract, and calcium chloride in D agar on colony counts of anaerobic oxalate-degrading bacteria from the ruminal contents of a sheep[a]

Expt no.	Oxalate (mM)	Yeast extract (%)	$CaCl_2$ (mM)	Colony count[b]
1 (36/11.8)[c]	10	0.1	7	0[d] (41/93)
	20[e]	0.1	7	0 (00/93)
	40	0.1	7	0 (00/93)
	80	0.1	7	0 (00/93)
	100	0.1	7	0 (00/93)
2 (136/15.0)	20	0.1	14	6.35 (59/63)
	20	0.3	14	6.65 (36/63)
3 (178/9.2)	20	0.3	14	7.98 (21/49)
	20	0.5	14	7.86 (21/49)
	20	1.0	14	7.81 (21/49)

[a]A 55-kg sheep that had been fed 0.85 kg of a ground hay cubes-oats diet with 3% sodium oxalate per day.

[b]Log_{10}/g (dry weight) of ruminal contents. Each value is the mean of either duplicate or triplicate tubes. Numbers in parentheses are day of incubation that majority of colonies appeared/total days ofincubation.

[c]Days on diet/oxalate degradation rate (micromoles oxalate degraded per gram [dry weight] of ruminal contents per hour).

[d]30 to 300 colonies with clear zones per tube were not observed.

[e]D agar as described by Daniel et al. (1987).

Table C11. The effects of different concentrations of yeast extract, ruminal fluid, and calcium chloride in D agar on colony counts of anaerobic oxalate-degrading bacteria from the ruminal contents of a sheep[a]

Expt no.	Yeast extract (%)	Ruminal fluid[b]			$CaCl_2$ (mM)	Colony count[c]
		CRF (%)	FSRF (%)	HT-FSRF (%)		
1 (91/19.7)[d]	0.1[e]	–[f]	–	–	7	0[g] (56/66)
	0.1	20	–	–	7	0 (56/66)
2 (150/11.4)	0.1	–	–	–	7	0 (53/60)
	0.1	–	10	–	14	6.82 (35/60)
3 (206/25.7)	0.1	–	–	–	7	6.83 (42/66)
	0.3	–	–	–	7	7.14 (42/66)
	0.1	–	–	–	14	7.67 (42/66)
	0.3	–	–	–	14	7.28 (21/66)
	–	–	10	–	14	7.97 (42/66)
	–	–	20	–	14	8.10 (42/66)
	–	–	–	10	14	7.87 (42/66)
	–	–	–	20	14	0 (42/66)

[a]A 55-kg sheep that had been fed per day 0.85 kg of a ground hay cubes-oats diet with 3% sodium oxalate.

[b]CRF, Clarified ruminal fluid; FSRF, filter-sterilized ruminal fluid; HT-FSRF, heat-treated FSRF.

[c]Log_{10}/g (dry weight) of ruminal contents. Each value is the mean of duplicate tubes. Numbers in parentheses are day of incubation that majority of colonies appeared/total days of incubation.

[d]Days on diet/oxalate degradation rate (micromoles oxalate degraded per gram [dry weight] of ruminal contents per hour).

[e]D agar as described by Daniel et al. (1987).

[f]–, Not added to the culture medium.

[g]30 to 300 colonies with clear zones per tube were not observed.

Table C12. The effects of ruminal fluid and yeast extract concentrations in D agar on colony counts of anaerobic oxalate-degrading bacteria from the ruminal contents of a sheep[a]

Ruminal fluid type[b]	Ruminal fluid (%)	Yeast extract (0.1%)	Colony count[c]
None[d]	-[e]	+[f]	8.22 (49)
FSRF	10	-	8.48 (42)
	10	+	8.42 (28)
	20	-	8.54 (42)
	20	+	8.51 (28)
HT-FSRF	10	-	0[g] (00)
	10	+	0 (00)
	20	-	0 (42)
	20	+	8.45 (28)

[a]A 23-kg sheep that had been fed 0.7 kg of ground hay cubes with 2% sodium oxalate per day for 5 days.

[b]FSRF, Filter-sterilized ruminal fluid; HT-FSRF, heat-treated FSRF.

[c]Log_{10}/g (dry weight) of ruminal contents. Each value is the mean of triplicate tubes after 49 days of incubation. Numbers in parentheses are day of incubation that majority of colonies appeared.

[d]D agar (Daniel et al., 1987) with 14 mM $CaCl_2$.

[e]-, Not added to the culture medium.

[f]+, Added to culture medium.

[g]30 to 300 colonies with clear zones per tube were not observed.