BIOCHEMICAL EFFECT OF DICARBOXYLIC ACIDS ON OXALATE METABOLISM IN EXPERIMENTAL RATS AND STUDIES ON OXALATE DEGRADING BACTERIA

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ABBREVIATIONS

ANSA	-	1-Amino-2-naphthol-4-sulphonic acid
ATP	-	Adenosine triphosphate
BBMV	-	Brush border membrane vesicle
BSA	-	Bovine serum albumin
$CaCl_2$	-	Calcium chloride
CaOx	-	Calcium Oxalate
CPD	-	Calculi producing diet
$CuSO_4$	-	Copper sulphate
D NA	-	Deoxy ribonucl eic acid
DDH_2O	-	Double distilled water
DER	-	Diglycidyl, ether of polypropylene glycol
DMAE	-	Dimethyl amino ethanol
EDTA	-	Ethylene diamine tetra acetic acid
ESWL	-	Extracorporial shock wave lithotripsy
FDH	-	Formate dehydrogenase
GAGS	-	Glycosaminoglycans
GAO	-	Glycollic acid oxidase
GAD	-	Glycollic acid dehydrogenase
H_2SO_4	-	Sulphuric acid
HCl	-	Hydrchloric acid
HNO_3	-	Nitric acid
K_2HPO_4	-	Dipotassium hydrogen phosphate
$K_3Fe(CN)_6$	-	Potassium ferri cyanide
KH_2PO_4	-	Potassium dihydrogen phosphate
LB media	-	Luria Britani media
LDH	-	Lactate dehydrogenase
MDCK	-	Madine Darby Canine Kidney

$MgCl_2$	-	Magnesium chloride
Na_2CO_3	-	Sodium carbonate
Na_2HPO_4	-	Disodium hydrogen phosphate
$Na_2SO_4\ 7H_2O$	-	Sodium sulphite
NaCl	-	Sodium chloride
NAD	-	Nicotinamide adenine dinucleotide
NADPH	-	Nicotinamide adenine dinucleotide phosphate reduced
NaH_2PO_4	-	Sodium dihydrogen phosphate
NaOAc	-	Sodium acetate
$(NH_4)_2SO_4$	-	Ammonium sulphate
NSA	-	Non enyl succinic anhydride
OD	-	Optical density
OsO_4	-	Osmium tetroxide
RNA	-	Ribonucleic acid
RNase	-	Ribonuclease
SDS	-	Sodium dodecyl sulphate
TAE	-	Tris-Acetic acid-EDTA
TCA	-	Trichloro acetic acid
TE buffer	-	Tris-EDTA-Buffer
TEG	-	Tris-EDTA-Glacial acetic acid
TEM	-	Transmission electron microscopy
THP	-	Tamm - Horsfall glycoprotein
TPP	-	Thiamine pyro phosphate
VCD	-	Vinyl cyclohexane

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CHAPTER-I

GENERAL INTRODUCTION

1.1 Preface

The formation of renal stone composed of calcium oxalate is a complex process that remains poorly understood and treatment of idiopathic recurrent stone formers is quite difficult and this area has attracted lots of research workers.

Oxalic acid is one of the most highly oxidized organic compound widely distributed in the diets of man and animals, and ingestion of plants that contain high concentration of oxalate may lead to intoxication. Excessive ingestion of dietary oxalate may lead to hyperoxaluria and calcium oxalate stone disease.

The formation of calcium oxalate stone in the urine is dependent on the saturation level of both calcium and oxalate. Thus the management of one or both of these ions in individuals susceptible to urolithiasis appears to be important. The control of endogenous oxalate synthesis from its precursors in hyperoxaluric situation is likely to yield beneficial results and can be a useful approach in the medical management of urinary stones. A variety of compounds have been investigated to curtain endogenous oxalate synthesis which is a crucial factor, most of these compounds have not proved to be effective in the *in vivo* situation and some of them are not free from the toxic effect. The non-operative management of stone disease has been practiced in ancient India in the three famous indigenous systems of medicine, Ayurveda, Unani and Siddha, and proved to be effective. However the efficiency of most of these substances is still questionable and demands further study.

Man as well as other mammals cannot metabolize oxalic acid. Excessive ingestion of oxalic acid can arise from oxalate rich food and from its major metabolic precursors, glycollate, glyoxylate and ascorbic acid can lead to an acute oxalate toxicity. Increased levels of circulating oxalate, which can result in a variety of diseases including renal failure and oxalate lithiasis. The ability to enzymatically degrade oxalate to less noxious

substances, formate and CO_2, could benefit a great number of individuals including those afflicted with hyperoxaluria and calcium oxalate stone disease.

1.2 Literature Review

1.2.1 History

Oxalic acid is present either as the free acid or as the anion in many plants ingested by humans and animals (Libert and Franceschi, 1987). Urolithiasis, a disease characterized by the formation of urinary tract stones, affected 5 - 10% of the human populations (Daniel *et al.*, 1993). Calcium oxalate crystals were first recognized in urine in 1838 and in renal tissues at approximately the same time (Hockaday *et al.*, 1964; Hodgkinson and Zarembski, 1968). The effects of oxalic acid poisoning attracted attention and led to the identification of oxalate crystals in kidney tissues. In the 19th century, interest was generated in the field of oxalate and terms like oxaluria and oxalosis were introduced to describe the presence of oxalate crystals in urine, renal and extrahepatic tissues.

1.2.2 Oxalic acid

Oxalic acid (HOOC - COOH) is a white, crystalline strong dicarboxylic acid, the salts of which are widely distributed in plant tissues, and are claimed to be formed from the respiratory breakdown of carbohydrate or from protein metabolism. Oxalic acid is relatively a strong acid that is moderately soluble in water (8.7 g/100 g of water). It has a pKa 1 of 1.27 and a pKa 2 of 3.8 (Williams and Wandizilak, 1989; Smith, 1992).

Oxalic acid occurs in soluble and insoluble forms. At physiological pH, it forms soluble salts with sodium and potassium, whereas insoluble salt with calcium. A small amount of insoluble oxalate occurs as the magnesium salt and free oxalic acid occurs rarely and only in trace amounts (Oke, 1969).

Oxalic acid crystallizes from aqueous solutions as white dihydrate. Mono and dihydrate are the other two forms, have been described in biological materials. The commonest forms are mono and dihydrate which are known as whewellite and weddellite (Hagler and Herman, 1973). Oxalic acid is oxidized to CO_2 and H_2O by ferric

compounds and potassium permanganate. In the presence of zinc and hydrochloride it is reduced first to glyoxylic acid and then to glycollic acid (Hodgkinson and Zarembiski, 1968; Oke, 1969).

Oxalic acid is one of the most highly oxidized organic compound found in nature (Hodgkinson, 1977). In high concentration oxalic acid causes death in human and animals because of its corrosive effect, while smaller amounts can cause various pathological disorders, including hyperoxaluria, calcium oxalate stones, pyridoxine deficiency and even renal failure (Williams and Smith, 1968).

1.2.3 Urolithiasis

Aggregation of calcium salts of oxalate and phosphate may cause formation of mineral deposits in the kidney and urinary tract, and the process of stone formation is called urolithiasis. Stone disease of urinary tract has been recognized since ancient times and urinary calculi have been found in the tombs of Egyptian mummies dating back to 4800 B.C. (Shattock, 1905; Shattock and Whikhan, 1979) and in the graves of North American Indians from 1500 - 100 B.C. (Beck and Mulvanery, 1966). A bladder stone was found in an Egyptian skeleton more than 7000 years old (Riches, 1968).

1.2.4 Incidence of kidney stone in the world

Urinary calculi are one of man's oldest problems. Calculi in the urinary tract had appeared in an epidemic fashion in England during 1772 - 1816. Urolithiasis is a common disorder affecting 1% to 5% of the population in industrialized countries with a life time risk of 20% in white man and 5 to 10% in women (Sierakowski *et al.*, 1978). In United States, urolithiasis accounted for 0.9% of hospital discharges with a mean stay of 3 days, costing 1.83 billion dollars in 1993. In Thailand 19% of the surgical admissions were for urinary stones (Chuticorn *et al.*, 1967; Halstead and Valyasevi, 1967), of which 90% had bladder or urithral stones and over half the cases were boys under 6 years. In America, about 0.1% of the population had reported to the hospitals every year for treatment of renal stones (Boyce *et al.*, 1950). The Norwich area of England had a particularly high incidence and the Norwich hospital registered 1498 operations from 1772 to 1901 (Battyshaw, 1970). In Saudi Arabia, urolithiasis is the commonest problem. One in five of all patients attending urology clinics had urinary stones (Kassimi *et al.*,

1986). Urinary stones are generally considered to be uncommon amongst Africans (Blacklock, 1976). This is usually attributed to dietary rather than genetic causes. Both renal and vesical stones are common in North Sudan. Stones are more in South Sudan, which is inhabited by pure Africans. Baker *et al.* (1993) reported that trends in renal stone formation in the South Australian population, between 1977 and 1991 (3634 stones), with respect to age, sex and seasonal variation. Later by second half of the 19th century more attention had been laid in this field. Sir Henry Thompson became famous for his interest in medical therapy of bladder stones and he suggested the possibility of treatment of bladder stones by dissolution (Thorwald, 1956). It was only in 19th century that chemical analysis of stones were undertaken with a view to study the underlying causes of stone pathogenesis and prevents its recurrence.

In India, the incidence of calculus disease is generally accepted to be low in the south and high in the Northwest (Thind and Nath, 1969; Pendse and Singh, 1986). Rajasthan has been labelled as "stone area" (Pendse and Singh, 1986). A high and progressively increasing incidence of urolithiasis has been reported in Udaipur and some other parts of Rajasthan, in the western part of India (Pendse *et al.*, 1984; Pendse and Singh, 1986). McCarrison (1931) has reported high incidence of stone in the Northern part of India and low incidence in the south. But it is not likely to be true because of the wide spread use of indigenous remedies with good efficiency among the rural populations in this region. The type of stones encountered in the south area, mostly calcarious in nature containing predominantly calcium oxalate (Varalakshmi *et al.*, 1976).

1.2.5 Various types of urinary stones

Various types of urinary stones are listed in Table 1.2.1. Many patients having calculi in the bladder or kidneys have sediments in their urine and it has been suggested that the ingestion of muddy river water or water containing lime caused stone formation in the urinary tract (Anderson, 1966). There are four major types of stones. Urinary tract stones are usually classified according to the composition with the most frequently encountered (70%) being the calcium stone composed of calcium oxalate alone or calcium oxalate mixed with calcium phosphate (Ryall, 1993; Khan *et al.*, 1995; Buno Soto *et al.*, 1996; Balla *et al.*, 1998), uric acid (4.5%), cystein and struvite (2.2% and

Table - 1.2.1, The urinary stone constituents identified crystallographic techniques

Constituent	Formula	Mineralogical Name
Calcium oxalate monohydrate	$CaC_2O_4.H_2O$	Whewellite
Calcium oxalate dihydrate		
Uric acid	$CaC_2O4.2H_2O$	Weddellite
Uric acid dihydrate		
Ammonium acid urate	$C_5H_4N_4O_3$	----------
Sodium acid urate	$C_5H_4N{=}O3.2H_2O$	----------
Magnesium ammonium	----------	----------
phosphate	----------	----------
Magnesium dibasic phosphate	$MgNH_4PO_4.6H_2O$	Struvite
trihydrate (a degradation		
product of the above)	$MgHPO_4.3H_2O$	Newberyite
Carbonate apatite		
Hydroxyl apatite		
Calcium hydrogen phosphate		
dihydrate	$Ca_{10}(PO_4{\cdot}CO_3OH)_6$	Carbonate apatite
Tricalcium phosphate	$(OH)_2$	Hydroxyl apatite
Octacalcium phosphate	$Ca_{10}(PO_4)_6\ (OH)_2$	
Cystine		Brushite
Xanthine	$CaHPO_4.2H_2O$	Whitlockite
	$Ca_3(PO_4)_2$	---------
	$Ca_8H_2(PO_4)_6.5H_2O$	---------
	$C_6H_{12}N_2O_4S_2$	---------
	$C_5H_4N_4O_2$	

Ref. Prien and Frondel (1947)., Prien (1963)., Lonsdale and Sutor (1966)., Londale *et al* (1968) etc.

2.33% respectively) (Nordin and Hodgkinson, 1967). Stones associated with urinary infection are mainly phosphates (struvite or magnesium ammonium phosphate and calcium apatite) and they may also contain ammonium hydrogen urate. Most of the renal calculi are composed of calcium oxalate and/or calcium phosphate (Varalakshmi *et al.*, 1976). Phosphatic stones occur mainly in women and usually secondary to urinary tract infection (Richardson, 1967). Types of urinary calculi and its percentage vary at different regions, are summarized in Fig. 1.2.1.

The process of calcium oxalate crystallization in urine is a very complex, multifactorial process and the treatment of idiopathic recurrent stone formers is quite difficult and this area has attracted lots of research workers. Recurrent stones composed predominantly of calcium oxalate are more prevalent among males (Hodgkinson *et al.*, 1969) and it seems to be influenced by sex hormones (Richardson, 1967; Varalakshmi and Richardson, 1983a).

The formation of kidney stone is a consequence of increased urinary supersaturation with subsequent formation of crystalline particle. Since most of the solid particles crystallizing within the urinary tract will be excreted freely, particle formation by no means is equivalent to symptomatic stone disease (Finlaysan *et al.*, 1984). However, when solid particles are retaining within the kidney, they can grow to become full sized stones. Stone formation is a biological process that involves a physico-chemical aspect, called crystallization (Kok *et al.*, 1988).

Renal stones are composed of two important parts, namely the crystalline part (98% of the stone) which forms the bulk, and a mixture of high molecular weight substances like glycosaminoglycans and glycoproteins that forms the framework of matrix (2%) on which the low molecular weight components deposit and crystallize (Boyce, 1968; Marshall and Robertson, 1976; Koutsoukos and Nancollas, 1981). Based on the crystalline composition, about 50 types of stones have been recognized. Matrix has been described as a heterogenous material composed of about 64% protein, 9% nonaminosugars, 5% glucosamine, 10% bound water and 12% organic ash (Boyce, 1968). Several compounds have been isolated from the soluble part of the organic matrix

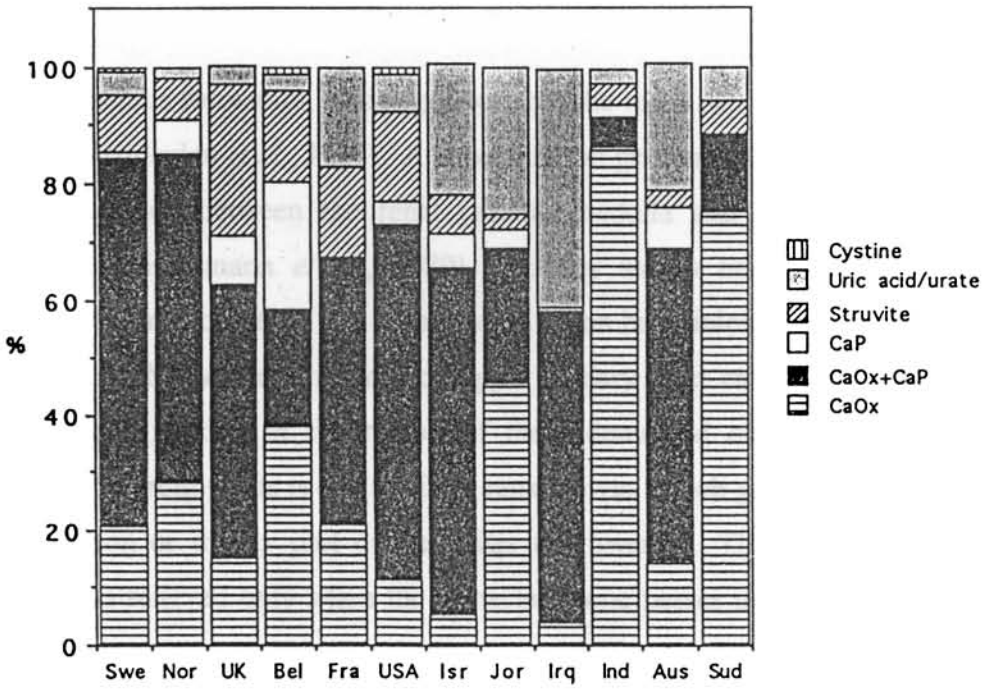


Fig.1.2.1 Stone composition in different parts of the world (Sweden, Norway, U.K., Belgium, Jordan, Iraq, India, Australia and Sudan)

of kidney stones, namely a mucoprotein called matrix substance A (Boyce *et al.*, 1962), a protein containing large amount of gamma carboxyglutamicacid (Gla) (Lian *et al.*, 1977), nephrocalcin, another Gla rich protein (Nakagara *et al.*, 1987), Tamm-Horsfall glycoprotein (Hess *et al.*, 1989; Grant *et al.*, 1973), renal lithostatine, a protein very similar to pancreatic stone protein (Sarles *et al.*, 1990), albumin (Boyce, 1968), glycosaminoglycans (mainly heparan sulfate) and free carbohydrate (Nishio *et al.*, 1985; Roberts and Resnick, 1986; Yamaguchi *et al.*, 1993). The formation of crystalline particle in tubular fluid as well as in urine comprises two major physico-chemical aspects - a thermodynamic one including supersaturation, results in nucleation of microcrystal and a kinetic one comprising rate of crystal nucleation, growth, aggregation and phase transformation (Kok *et al.*, 1988).

During calculus formation, it is suggested that the renal papilla has a central role (Randall, 1936; Vermculen *et al.*, 1967). A concentration gradient of calcium and oxalate has been demonstrated between the renal papilla, medulla and cortex (Wright and Hodgkinson, 1972; Hautmann *et al.*, 1980). Kidney stones may not produce any symptoms or signs for a long time but in rare cases it can be painless with haematuria or vague lain pains. The typical symptom of upper urinary tract calculus is the sudden severe colicky pain starting in the region of the kidney and occasionally radiating downwards into the groin. This is due to either renal pelvis, which gets impacted into the pelvi-uteral junction or in any part of the uriter downwards. The pain is sharp and excruciating and is relieved only when the impacted stone passes down the urethra into the bladder.

1.2.6 Factors influencing calcium oxalate stone formation

Increased excretion of oxalate derived from endogenous metabolic process, primarily from one of its major precursors or it can be secondary to the intake of oxalate or oxalate rich food. The history of stone disease implies that many diverse factors might be involved in its causation. Age and sex of an individual are the two important factors which govern urolithiasis (Pak, 1987). Several investigators have pointed out the maximum incidence of urinary lithiasis appears to occur in the 30 to 50 years old age group (Baily *et al.*, 1974). Hyperoxaluria and calcium oxalate stone diseases are more

prevalent in the age group 20 - 39 years (Vitale *et al.*, 1999). Richardson (1965) has shown a sex linked difference in the occurrence of oxalate urinary calculi. Testosterone administration is known to induce the action of major oxalate synthesizing enzyme glycollic acid oxidase in the liver, while oestradiol is reported to lower the enzyme activity in male rats (Varalakshmi and Richardson, 1983b). Calcium oxalate stones are more prevalent among males and it seems to be influenced by sex hormones (Richardson, 1967; Lee *et al.*, 1992; Iguchi *et al.*, 1999).

Stress, hot sunny climate, adult male exposed to affluent living conditions are suggested as additional risk factors for calcium oxalate stone formation (Anderson, 1973; Robertson *et al.*, 1978). The volume of water intake, subsequent urinary output, and the mineral content of water play a part in causing urolithiasis (Candarella *et al.*, 1998). High fluid intake and mineral water containing calcium and magnesium deserves to be considered as a possible therapeutic or prophylatic agent in calcium oxalate stone disease (Pak, 1999; Rodgers, 1997).

Diet has long been considered to be an important risk factor for stone disease. Dietary and metabolic factors, which influence the concentration of calcium and oxalate in urine are shown in Table 1.2.2. Oxalate poisoning may be encountered in grazing animals due to ingestion of oxalate rich plants or food contaminated with oxalate producing fungi (Seawright *et al.*, 1970; Andrews, 1971; James *et al.*, 1971).

Oxalate content of foods vary significantly, e.g. spinach, rhubarb, beets, buts, chocolate, tea, wheat bran and strawberries, while the oxalate in fruits is generally low (Hodgkinson, 1977; Brinkley *et al.*, 1981; Gregory, 1981) (Table 1.2.3). Among the food items tested spinach was capable of causing hyperoxaluria in normal subjects.

The other sources of oxalate are endogenous in nature and derived from the metabolism of glycine, ethanolamine, glycoaldehyde, glyoxylate, glycollate, serine, tryptophan, hydroxyproline, purines and ascorbic acid which leads to increased excretion of oxalic acid (Williams and Smith, 1968). Hyperoxalemia and hyperoxaluria may result from ethyleneglycol (anti-freeze) poisoning (Parry and Wallach, 1974)

Table 1.2.2. Mechanisms leading to increased risks of stones formation are described in the following foods

Food Type	Stone forming condition
Fat	Hypercalciuria
Carbohydrates	Hypercalciuria Hyperoxaluria Acidification of the urine
Protein, purines	Hypercalciuria Hyperoxaluria Hyperuricosuria Acidification of the urine
Vitamin A deficiency	Hypercalciuria Reduction in GAG excretion
Vitamin B_6 deficiency	Hyperoxaluria
Milk and dairy produce	Hyperoxaluria Hyperuricosuria
Food containing oxalic acid	Hyperoxaluria
Excessive common salt	Hypercalciuria
Alcohol (beer, wine, spirits)	Hyperuricaemia Acidification of urine
Lack of bulk food	Hypercalciuria Slower passage through the intestinal tract

Ref : (Hesse, 1986)

Liquids

MODERATE AMOUNTS

Apple juice

Beer

Coffee

Cola

Cranberry juice

Grapefruit juice

Instant tea

HIGH AMOUNTS

Cocoa

Fresh tea

Foods

Almonds

Asparagus

Beets

Cactus fruits

Cashew nuts

Concord grapes

Cranberries

Currants

Greens

Plums

Raspberries

Rhubarb

Spinach

Ref : Thomas, W.C., 1974.

Elevated urinary oxalate levels have been reported in diabetes, cirrhosis, Klinefelter's syndrome (associated with renal calculi), congestive heart failure, renal tubular acidosis, sarcodiosis and a variety of parasitic diseases including schistosomiasis, giarchiasis, amoebiasis and ascariasis (Dempsey *et al.*, 1960).

The urinary oxalate excretion increased in severe experimental pyridoxine deficient animals (Gershoff *et al.*, 1959; Daudon ***et al.***, 1987). Pyridoxine deficiency in experimental animals leads to hyperoxaluria presumably by reduced transamination of glyoxylate to glycine, a reaction in which pyridoxine act as a cofactor (Menon and Mahle, 1982). Deficiency of vitamin B6 leads to glyoxylate accumulation and its increased conversion to oxalate (Varalakshmi and Richardson, 1983b).

Patients with ileal disease have increased absorption of dietary oxalate, hyperoxaluria and an increased incidence of nephrolithiasis (Dowing *et al.*, 1971; Smith *et al.*, 1980). Malabsorption of fatty acids and bile salts is an important pathogenic factor in hyperoxaluria (Anderson and Basacus, 1981; Marangella *et al.*, 1982). The main cause of diarrhoea in hyperoxaluric patients is malabsorption of bile salts (Hofmann, 1967; Smith *et al.*, 1972; Stauffer *et al.*, 1973). Patients with steatorrhea, have increased frequency of renal stone formation (Kiertisin *et al.*, 1982). Cowley *et al.* (1987) reported that malabsorption of citrate, ascorbate and possibly other hydroxy carboxylic acids leads to hyperoxaluria.

1.2.7 Crystallization risk factors

The mechanism of stone formation is not clear. The four factors which encourage stone formation are

1. Increased concentration of stone forming constituents
2. Urine pH
3. Deficiency of inhibitors
4. Urinary tract obstructions

A prerequisite for urinary stone formation is urinary crystal formation. For this urine must be supersaturated with the offending salt. This occurs when excretion of the chemicals that constitute the crystals increases. The degree of supersaturation is usually high in patients with urinary stones (Robertson *et al.*, 1976). This high degree of supersaturation may be due to excretion of more calcium (Marshall *et al.*, 1972) and more

oxalate in urine (Robertson *et al.*, 1975). Oxalate is considered to be the most likely promoting factor since all the experimental data stress its importance rather than that of calcium (Robertson and Nordin, 1969; Robertson and Peacock, 1980). An overview of calcium oxalate kidney stone formation is shown in figure 1.2.2.

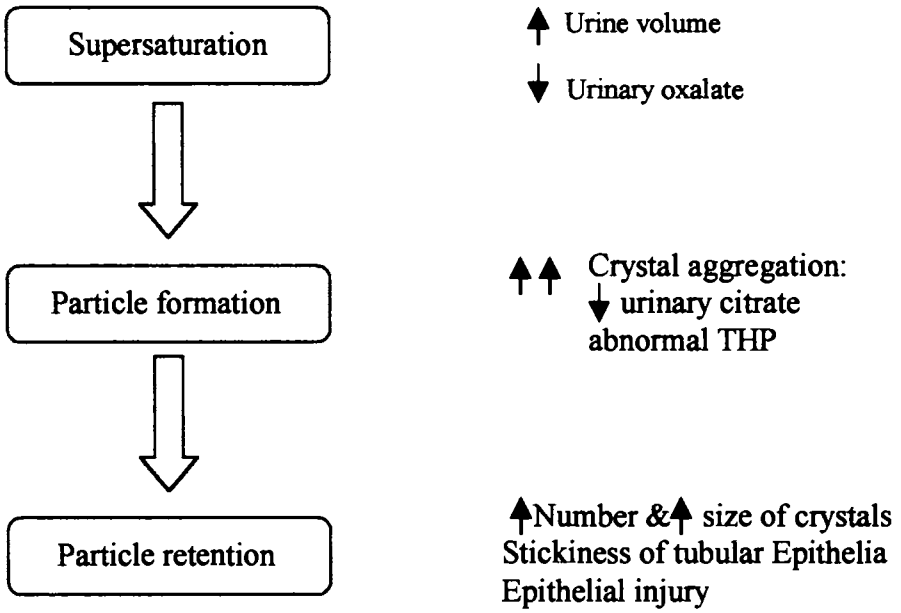


Fig.1.2.2. Overview on calcium oxalate kidney stone formation. Low urine volume and increased urinary oxalate are essential risk factors for abnormal urinary supersaturation.

Urinary pH plays an important role in determining the nature of the crystalline constituents in stone. Calcium oxalate stone usually occurs in acid urine (Gershoff, 1964). Calcium oxalate crystallization occurs more frequently around pH - 5. The risk of infective stone formation is especially high in alkaline urine and is induced by urease forming gram negative bacteria like *Proteus*, *S. aureus* and *Klebsiella.*

1.2.8 Urinary inhibitors

In normal urine, the concentration of calcium oxalate is four times more than its solubility, and infact precipitation occurs only when the supersaturation is 7 to 11 times its solubility (Coe and Parks, 1988). This is possible because many modifiers of calcium

oxalate crystallization are present in the urine. Inhibitors in the urine affect the formation, growth and aggregation of crystals. Two types of inhibitors are present in the urine. One group of inhibitors such as citrate and magnesium forms soluble complexes with calcium and oxalate. Pyrophosphate and glycosaminoglycans form the other group of inhibitors, which affect the formation, growth and aggregation of crystals (Bowyer *et al.*, 1979). Calcium oxalate crystal formation is inhibited by citrate, pyrophosphate, glycosaminoglycans, RNA fragments and nephrocalcin, with much of inhibition with large molecular weight compounds (Garside, 1982; Pak, 1987; Khan *et al.*, 1988). These inhibitors inhibit crystal formation at very low concentration. The low molecular weight inhibitors have account about 10-15% of total inhibitory activity of urine (Mayer and Smith, 1975) while the remaining activity is attributed to high molecular compounds.

Inorganic pyrophosphate was the first inhibitor isolated from urine by Fleisch and Bisaz (1962). This compound was found to inhibit the precipitation and aggregation of both calcium oxalate and calcium phosphate (Schwille *et al.*, 1988). Pyrophosphate and diphosphonates each inhibit precipitation of calcium phosphate from supersaturated solutions, whereas diphosphates also inhibit the growth of apatite crystals (Laminski *et al.*, 1990).

Urinary citrate appears to be an important factor in the crystallization process of calcium oxalate and calcium phosphate (Teselius *et al.*, 1993a). Citrate, by forming complex with free calcium reduce the calcium availability to form complexes with oxalate and phosphate and in this manner, could protect against stone formation (Grases *et al.*, 1989). Hallson *et al.* (1983) have shown that urinary citrate is highly effective in reducing calcium oxalate crystal formation. Further phosphocitric acid present in urine and also in the liver of rat is shown to be a powerful inhibitor of calcification process (Lehninger, 1977).

Magnesium form soluble complexes with oxalate and inhibit the calcium oxalate supersaturation (Fleisch, 1978; Teselius *et al.*, 1995). Very high magnesium intake certainly reduce the incidence of calcium oxalate lithiasis in rats (Lion *et al.*, 1966) and

there is clinical evidence which strongly suggest that magnesium therapy prevents recurrence of calcium oxalate urolithiasis (Johansson *et al.*, 1980; Su *et al.*, 1991).

Glycosaminoglycans are the main macromolecular inhibitors of growth and aggregation of crystals in the urine (Baggio *et al.*, 1982). The inhibitory activity of urinary chondroitin sulphates (Tiselius, 1981) and solubility of calcium oxalate are depended upon pH. Heparin is also a potent inhibitor of spontaneous precipitation of calcium oxalate (Koide *et al.*, 1990). Yamaguchi *et al.* (1993) reported that the main glycosaminoglycan in stone matrix was consistent with crystal surface binding substance and was heparine sulphate, with a strong inhibitory activity on calcium oxalate stone formation. A new urinary inhibitor of calcium oxalate formation was isolated from the urine of healthy subject, uronic acid rich protein (Atmani *et al.* 1996).

Nephrocalcin and Tamm-Horsfall protein are urinary glycoproteins that are potent inhibitors of calcium oxalate monohydrate crystal aggregation (Asplin *et al.* 1991). Nakagawa *et al.* (1983) isolated a glycoprotein nephrocalcin which accounted for 90% of the molecular inhibitory activity; THP is also reported as an inhibitor of calcium oxalate crystal aggregation (Hess *et al.*, 1989; Hess *et al.*, 1993; Miyake *et al.*, 1998). Shiraga *et al.* (1992) also found a fragment of osteopontin (uropontin) that was isolated from human urine, this protein inhibited calcium oxalate crystal growth. Uropontin is known to inhibit the growth and nucleation of calcium oxalate monohydrate crystal and it also impedes attachment of calcium oxalate crystal to cultured renal epithelial cells (Asplin *et al.*, 1998). RNA, synthetic polyadenylate and gastric pepsin, an acidic urine protein etc. can inhibit calcium oxalate crystal growth. RNA inhibits to the same extent as heparin (Scurr *et al.*, 1983).

1.2.9. Dietary sources, absorption, transport and excretion

Oxalate is the major constituent of many green leafy vegetables and plants and comprises about 15 - 20% of the total dry weight of the plant. Oxalate rich food enhanced excretion of urinary oxalate in normal volunteers, the increase was not proportional to the oxalate content of food. Milk, leaftea, powered coffee, cocoa, strawberry, raspberry, rhubarb, spinach and chocolate have high oxalate content.

Spinach, rhubarb and beet root have a high content of oxalate while the oxalate in fruit is generally low (Brinkley, *et al.*, 1981). The values are high for spinach (1236 mg) moderate for chocolate (126 mg) and tea (66 mg) and low for vegetable juice, canberry juice, pecans and orange juice (2-26 mg). Among the food items tested, spinach was capable of producing hyperoxaluria in normal subjects. The daily intake of dietary oxalate in man has been reported to vary from 70 mg - 980 mg in a typically western diet (Hodgkinson, 1977) and 80 mg - 2000 mg in Indian diets (Singh *et al.*, 1972).

Oxalate absorption from the gastrointestinal tract is normally quite low. In normal person about 5 - 10% of given oral dose of oxalate is absorbed within the intestine. Absorption takes place throughout the gastro-intestinal tract including the colon (Binder, 1974). Prenen *et al.* (1984) reported continuous oxalate absorption between one and eight hours after oxalate ingestion. No significant urinary excretion of C^{14} labelled oxalate was observed after 10 hours, when most of the administered radioactivity was still within the intestinal tract (Lindsjo *et al.*, 1989). Gregary (1981) was also reported that maximum oxalate absorption occurs in the maximal tubule of cortical region. Pinto and Paternan (1978) showed that oxalate was absorbed primarily by passive diffusion. However, the diffusion mechanism appears to be facilitated by a specific oxalate binding protein localized in the cytosol and hypothesized that at low concentration of oxalate, active binding took place whereas at high concentration passive diffusion was operative. Bile salts and fatty acids may increase colonic absorption by non-specific alteration of mucosal permeability (Dobbins and Binder, 1976). Prenen *et al.* (1984) showed that the peroxisomal part of the small bowel is a major absorption site of oxalate. Intestinal absorption of oxalate in castrated male rat was two fold that of normal male (Thind *et al.* 1985).

Oxalate transport was greatest in jejunum and least in colon and addition of calcium and magnesium did not cause significant changes in the rate or amount of oxalate transported (Madorsky and Finlayson, 1977). Oxalate can be transported across the epithelium by the paracellular (passive) and transcellular (active) pathway. Oxalate transport across cellular membranes is mediated by anion exchange transport proteins (Verkoelen and Romijm, 1996). Oxalate being the end product from various metabolisms

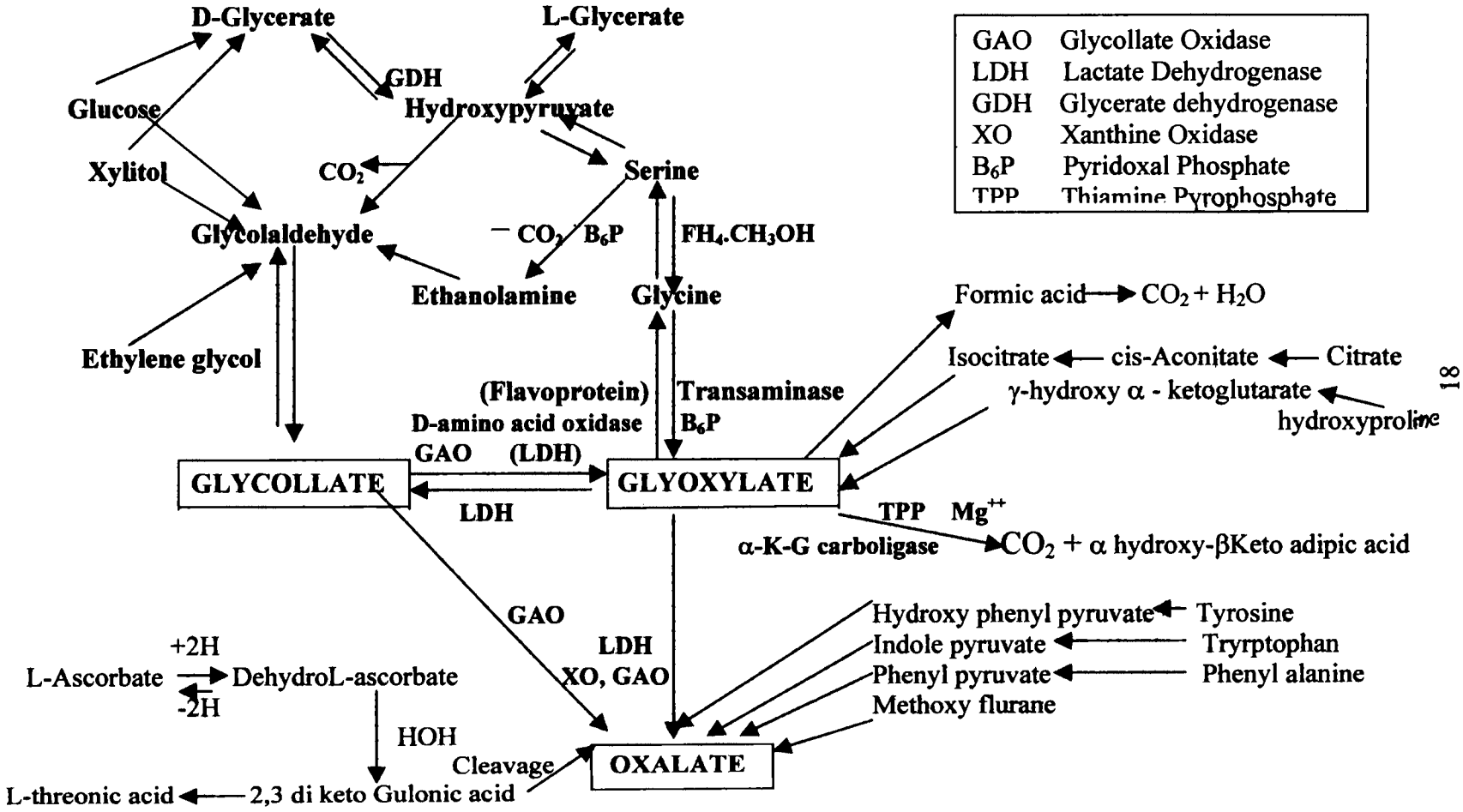
is transported as such across the microvillus membrane (Senckjian *et al.*, 1982; Menon and Mahle, 1982). Farooqui *et al.* (1981) has described that vitamin B_6 deficiency leads to induction of oxalate transport. Oxalate is transported by the small intestine in two steps; (i) oxalate uptake by brush border cells, and (ii) oxalate binding system. Oxalate transport by the intestinal brush border cell from rabbit and human by non-energy dependent diffusion mechanism (Pinto and Paternan, 1978). Transport of oxalate renal cortical brush border membrane vesicles (BBMV) is by passive diffusion. The rate of oxalate transport by the vesicles was decreased by the presence of both the Na^+ and K^+ gradient (Shigeo *et al.*, 1987).

In man and animals, oxalate is a non-essential end product of metabolism and is excreted unchanged in urine. In normal man the excretion of oxalate ranges from 20 - 60 mg per 1.73 liters per 24 hours (Archer *et al.*, 1957; Gibbs and Watts, 1969). When expressed as anhydrous oxalic acid it is from 10 - 15 g per 24 h (Hodgkinson, 1977). The amount of oxalate excreted in human feaces is normally approximately less than the amount that is ingested. Excretion is increased by the ingestion of a variety of substances that are normal constituents of the diet. These include glycine, glutamic acid and purines, gelatin a protein rich in glycine, hydroxyproline, tryptophan and ascorbic acid. Excretion has been reported to be reduced by the administration of ethinyl oestradiol (Zarembski and Hodgkinson, 1969).

1.2.10. Endogenous production of oxalate

In addition to the intestinal absorption of dietary oxalate, oxalate is synthesized from endogenous sources mainly from glycine, serine and ascorbic acid at a rate of about 1 mg/h (William and Smith, 1972; Watts, 1973; Auer *et al.*, 1998). Glyoxylate and glycollate are the immediate precursors of oxalate (Richardson and Tolbert, 1961; Holmes and Asimos, 1998) (Fig. 1.2.3).

Fig - 1.2.3. Pathways of oxalate biosynthesis



Glycine is the major source of endogenous oxalate and between 10 and 40% of oxalate is derived from it (Crowhall *et al.*, 1959; Dean *et al.*, 1968). Glycine is converted to glyoxylate either by oxidative deamination catalyzed by a flavoprotein, glycine oxalate or deaminoacid oxalate (Ratner *et al.*, 1944) or by transamination with 2-oxoglutarate brought about by a specific transaminase (Cammarta and Cohen, 1950). The transamination reaction is pyridoxal phosphate dependent. Some glycine may be metabolized to glyoxylate and therefore to oxalate more indirectly via serine → ethanolamine → glycolaldehyde → glycollate → glyoxylate, but this seems to be quantitatively less important (Liao and Richardson, 1972).

Serine conversion to glycine can lead to the formation of glyoxylate. Glyoxylate can also be formed from serine by initial decarboxylation forming glycolaldehyde and then glycollicacid (Kun *et al.*, 1954) which can be subsequently converted to oxalic acid (Richardson and Tolbert, 1961). This reaction is catalyzed by glycollic acid oxidase (GAO), a flavoprotein (glycollate: oxygen oxido reductase, EC 1.1.3.1). Once glyoxylic acid is found in the normal course of events, it is rapidly oxidized to formic acid and carbondioxide. The further oxidation of pyruvic acid, carbondioxide and water is also quite fast (Weinhouse, 1955). However, when the concentration of glyoxylate is very high, it is converted to oxalic acid.

Serine can also be converted to glycollate by multistep enzymatic reaction in which serine is first converted into ethanolamine or hydroxy pyruvate by serine decarboxylase or serine transaminase, respectively. These intermediates are irreversibly converted to gycollate via glycolaldehyde. This two-step reaction requires the enzymes ethanolamine oxidase, hydroxy pyruvate decarboxylase and aldehyde dehydrogenase. Ethylene glycol is converted to glycolaldehyde by the action of alcohol dehydrogenase. Glycolaldehyde is subsequently converted to glycollate. Carbohydrates can also be converted into glycolaldehyde under certain conditions. It has been shown that glycollate can be converted to oxalate directly by
glycollic acid dehydrogenase (GAD) present in the liver of man and rat (Fry and Richardson, 1979).

L-ascorbate is converted to oxalate through dehydro-L-ascorbic acid, 2.3 - diketogulonic acid. The first 2 carbon atoms of 2,3 - diketogulonic acid are converted to oxalic acid, other 4 carbon atoms are converted to L-threonic acid (Baker ***et al.***, 1966; Williams and Wandzilak, 1989; Auer *et al.* 1998). Ascorbic acid has been reported to provide 10-40% of the total urinary oxalate, glycine provided another 40% (Crowhall *et al.*, 1959 and Atkins *et al.*, 1964). Only a smaller portion of ingested vitamin C (L-ascorbic acid) is excreted as oxalic acid in man and a major portion is excreted unchanged (Hellmann and Burns, 1958).

Citric acid also appears to be a minor source of oxalate in the rat since it is readily available and 1 to 5% of the labelled acid was recovered as labelled oxalate (Hodgkinson, 1978). The most direct route from citrate appears to be citrate→ isocitrate → glyoxylate → oxalate. The key step in this sequence (isocitrate → glyoxylate) was thought to be contained to plants but the reaction has also been demonstrated in animal tissues, including rat skin (Brown and Box, 1968). An alternate explanation is that citric acid is converted to oxalate by intestinal flora, many of which are known to contain the enzymes necessary for the glyoxylate pathway (Hodgkinson, 1977).

Glyoxylate is oxidized to oxalate in the cytosol by lactate dehydrogenase (LDH) (Sawaki *et al.*, 1966) and by glycollic acid oxidase in the peroxisomes (de Duve and Daudhuin, 1966; Masters and Holmes, 1977).

Hydroxypyruvate is a minor precursor of oxalate (Gambardella and Richardson, 1978; Raghavan and Richardson, 1983). Hydroxypyruvate has been shown to increase endogenous oxalate via glycolaldehyde → glycollate → glyoxylate → oxalate. The amino acid phenylalanine, tyrosine and tryptophan have shown to be converted to oxalate (Gambardella and Richardson, 1977).

Hydroxyproline is one of the minor precursors of oxalate (Tawashi *et al.*, 1980). Hydroxyproline increases urinary oxalate level via 2-oxy, 4-hydroxy glutarate and glyoxylate (Adams, 1970).

1.2.11 Hyperoxaluria

Hyperoxaluria is a condition which is associated with increased excretion of oxalic acid (43 mg/day for men and 32 mg/day for women). Urinary oxalate in normal man varies between 10 and 50 mg/24 h. Hyperoxaluria is considered to play a crucial role in calcium oxalate renal stone disease (Verkoelen and Romijm, 1996; Buno soto *et al.*, 1996). Hyperoxaluria can result from increased endogenous production and increased intestinal absorption or a renal transport defect (Table1.2.4). Hyperoxaluria in man may be classified in to primary (genetic) and secondary types.

Table 1.2.4. Hyperoxaluric/hyperoxalemic states: classification

Increased oxalate production
Primary hyperoxaluria
Type I
Type II
Oxalate precursor load
Ethylene glycol intoxication
Methoxyflurane anesthesia
Xylitol hyperlimentation
Pyridoxine deficiency
Increased oxalate absorption
Enteric hyperoxaluria
Renal oxalate absorption
Renal oxalate leak
Hyperoxalemic oxalosis of renal failure

Primary hyperoxaluria is a rare inherited disease induced by an enzymatic deficiency responsible for high endogenous production of oxalate (Daudon *et al.*, 1998), which has an autosomal recessive mode of inheritance. The true incidence of primary hyperoxaluria is difficult to establish but it is considerably less than 1% of patients with calcium oxalate urinary stone. In patients with primary hyperoxaluria, urinary oxalate

excretion has averaged 240 mg/24 h and has exceeded 400 mg/24 h. Primary hyperoxaluria was first diagnosed during life in 1953 (Newns and Black, 1953). Two types have been identified, namely type I and type II.

Type I primary hyperoxaluria is also known as glycolicaciduria and results from a block in glyoxylate metabolism. Urinary excretion of oxalic acid, glycollic acid and glyoxylic acid are increased considerably (Williams and Smith, 1968) and also increased endogenous synthesis of oxalate and glycollate. Excretion of glycollic acid in normal subject varies from 15-60 mg/ 24 h (Hockaday *et al.*, 1965). In primary hyperoxaluria type I, it usually exceeds 100 mg/24 h. It is due to the deficiency of cytoplasmic enzyme alpha ketoglutarate - glyoxylate carboligase (Williams and Smith, 1978). Recent investigations have shown that this disorder is caused by deficiency or functional abnormality of peroxisomal alanine glyoxylate amino transferase in the liver (Fig.1.2.4) (Danpure and Purdue, 1995). This enzyme acts on glyoxylate and alanine in peroxisome to produce pyruvate and glycine. Glutamate - glyoxylate aminotransferase is a cytoplasmic enzyme that acts on cytoplasmic glyoxylate and glutamate to form glycine and 2-oxoglutarate. Pyridoxine is a co-factor in this latter reaction.

Danpure and Jenning (1986) have reported alanine glyoxylate aminotransferase enzyme activity to be low in the liver of hyperoxaluric patients than control human subjects.

Type II primary hyperoxaluria, or L-glycericaciduria is caused by the deficiency of the enzyme glyoxylate reductase/D-glycerate dehydrogenase (D-glycerate: NAD^+ oxireductase) (Williams and Smith, 1968), the enzyme which catalyses the reduction of hydroxypyruvate to glycerate and the reduction of glyoxylate to glycollate (Fig.1.2.4). Both NADH and NADPH can be used as co-factors. In the absence of this enzyme hydroxypyruvate accumulated and is reduced to L-glycerate in the presence of lactate dehyrogenease (Fig1.2.5). Urinary excretion of oxalate and L-glyceric acid is elevated, but the glycollate and glyoxylate excretions were normal. The excretion of L-glyceric acid is 200-600 mg/24 h.

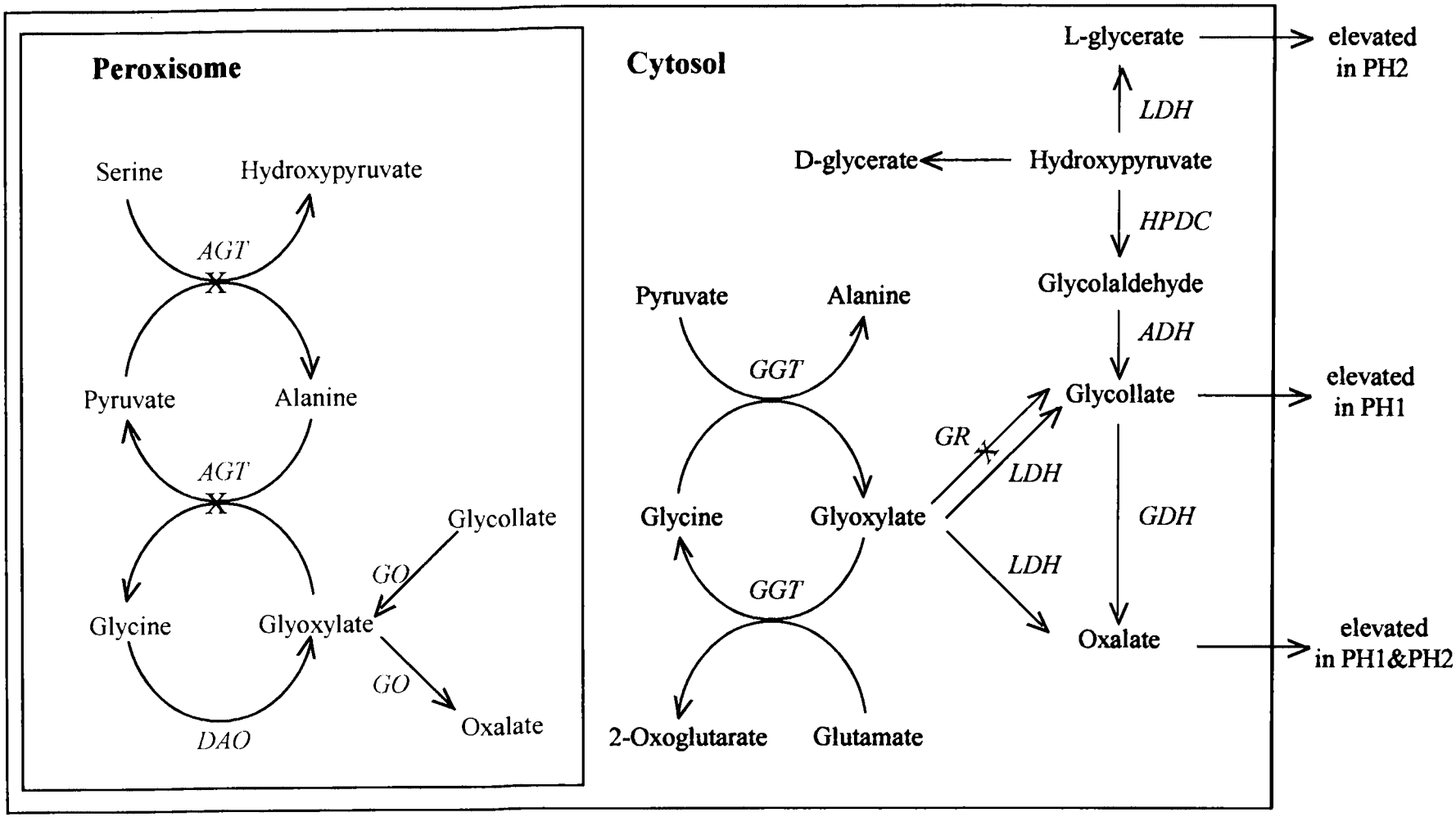
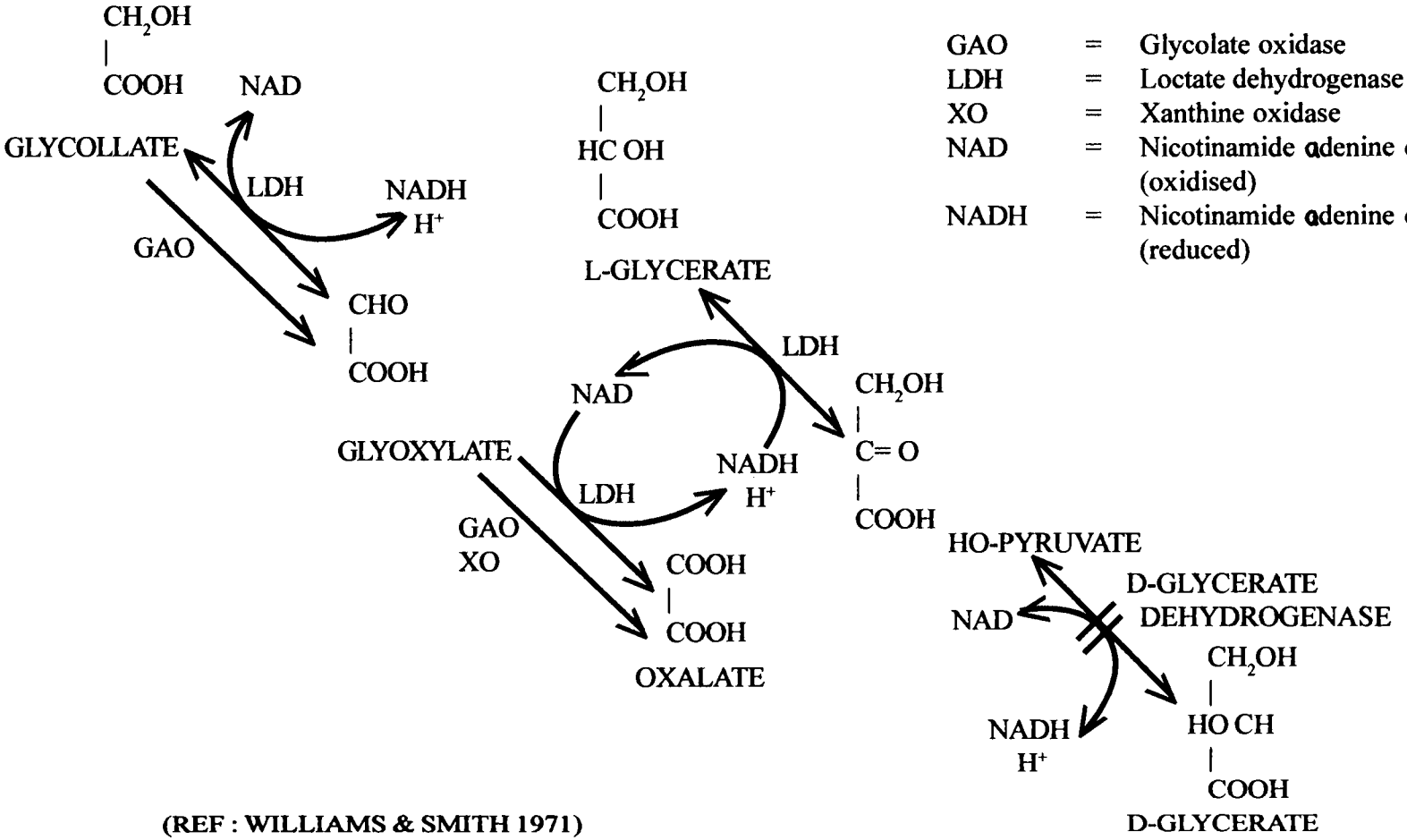


Figure 1.2.4. Some important pathways involved in oxalate, glyoxylate, and glycolate metabolism. The metabolic roles of HPDC, ADH and GDH are not well established. Solid crosses the metabolic blocks in PH1 that lead to elevated oxalate and glycollate synthesis; open crosses show the metabolic blocks in PH2 that lead to elevated oxalate and L-Glycerate synthesis. The peroxisomal membrane is presumed to be freely permeable to all of the metabolites shown. AGT, alenine: glyoxylate amino transferase; GO, glycollate oxidase; DAO, D-amino acid oxydase; GGT, glutamate; glyoxylate amino transferrase; LDH, lactate dehydrogenase; GR, glyoxylate reductase/D-glycerate dehydrogenase; HPDC, hydoxy pyruvate decarboxylase; ADH, aldehyde dehydrogenase; GDH, glycollate dehydrogenase. (Modified from Danpur, C.J and Purdue, PE, 1995)

FIGURE 1.2.5. THE MECHANISM OF OXALATE SYNTHESIS IN PRIMARY HYPEROXALURIA, TYPE - II



ABBREVIATIONS

GAO	=	Glycolate oxidase
LDH	=	Loctate dehydrogenase
XO	=	Xanthine oxidase
NAD	=	Nicotinamide adenine dinucleotide (oxidised)
NADH	=	Nicotinamide adenine dinucleotide (reduced)

(REF : WILLIAMS & SMITH 1971)

Secondary hyperoxaluria is an important factor in calcium oxalate stone formation and it can be also occur from increased endogenous production of oxalate, primarily from the intake of oxalate or oxalate rich food and secondarily from one of its major precursors. The oxalate content of the food varies significantly, e.g. spinach,

The other sources of oxalate are endogenous in nature and are derived from the metabolism of glycine, ethanolamine, glycolaldehyde and ascorbic acid which leads to increased excretion of oxalic acid (Crawhall, 1959; Auer ***et al.***, 1998; Holmes and Assimos, 1998) Hyperoxalemia and hyperoxaluria may result from ethylene glycol poisoning (de Water *et al.*, 1996; Tamilselvan *et al.*, 1997).

Methoxiflurane (2,2-dichloro-2, 1-fluro methyl ether) is a general anaesthetic that has been implicated in the production of postoperative renal dysfunction (Mazze *et al.*, 1971; Silverberg *et al.*, 1971). The renal dysfunction following methoxyflurane may be due to the additive effects of both oxalate and fluoride. Xylitol was also considered as ideal agents for endogenous oxalate overproduction. Hyperoxaluria and oxalate deposits in the renal parenchyma have been reported with the use of methoxyflurane.

Pyridoxine is an essential cofactor in the conversion of glyoxylate to glycine and its deficiency can lead to glyoxylate accumulation, shunting it down the oxalate pathway (Daudon, *et al.*, 1987). The urinary oxalate excretion increased in severe experimental pyridoxine deficient animals (Andrews *et al.*, 1960; Gershoff *et al.*, 1959). Pyridoxine deficiency in experimental animals leads to hyperoxaluria presumably by reduced transamination of glyoxylate to glycine, a reaction in which pyridoxine acts as a cofactor. Deficiency of vitamin B_6 leads to glyoxylate accumulation and its increased conversion to oxalate (Varalakshmi and Richardson, 1983b, Sharma *et al.*, 1990).

Another type of hyperoxaluric syndrome has been recognized in patients with a variety of malabsorptive states in which the gastro-intestinal absorption of oxalate is increased and they came under the category of enteric hyperoxaluria (Admirand *et al.*, 1971; Smith *et al.*, 1972, Seftal and Resnick, 1990). In these patients the oxalate excretion usually varied between 100 and 300 mg/24 h. The urinary glycollate and

glyoxylate concentrations were within the normal range. Patients with ileal diseases have increased absorption of dietary oxalate hyperoxaluria and an increased incidence of nephrolithiasis (Dowing *et al.*, 1971, Smith *et al.*, 1980). Malabsorption of fatty acids and bile salts is an important pathogenic factor in hyperoxaluria (Anderson and Bosacus, 1981; Marangella *et al.*, 1982). The main cause of diarrhoea in hyperoxaluric patients is malabsorption of bile salts (Smith *et al.*, 1972; Stauffer *et al.*, 1973; Hofmann, 1987).

Gregory (1981) has reported that small bowel bypass surgery in patients leads to hyperoxaluria. Individuals with an intact bowel absorbs less than 10 percent of the dietary oxalate whereas individuals with a shortest bowel can absorb upto 50 per cent of the same, predominantly from the colon. Hyperoxaluria may be also due to malabsorption of citrate, ascorbate and possibly other hydroxycarboxylic acids which act as crystal inhibitors (Cowley *et al.*, 1987).

1.2.12 Preventive measures

The medical management of stone disease has resulted in a significant reduction in the rate of stone formation. The causes of hyperoxaluria largely determine the type of treatment to be given. However, early detection, treatment of urinary tract infection if present, surgical removal of kidney stone to relieve urinary obstruction etc., have proved beneficial. Most patients with kidney stones have at least one identifiable physiologic derangement that results in abnormal level of one or more stone forming constituents, promoters, or inhibitors; often, these derangements are correctable. Consequently, not only can stone formation be reduced (Pak, 1973) but also the post surgical stone free rate can be improved (Suzuki *et al.*, 1994). Measures have already been taken to lower the degree of over saturation of urine with calcium oxalate by increased water therapy and use of diuretics etc. Recently Pak (1999) reported that a high fluid intake alone can inhibit the recurrence of stone formation in single stone formers. In patients with no identifiable metabolic abnormality, increased fluid intake alone may be sufficient to prevent future recurrence. High water intake leading to increased urinary out put decreased the incidence of urinary calculi in those patients who are predisposed to the disease. A high fluid intake is the only nutritional modification that may be applied in all forms and causes of urolithiasis except infective stones.

Increased oxalate in urine is mainly of endogenous origin. Techniques to inhibit the endogenous synthesis of oxalate from its precursors seem to be an ideal therapeutic solution. Vitamin B_6 deficiency leads to hyperoxaluria accompanied by hyperglycollaturia. Increased urinary excretion of both these acids could be restricted by the administration of vitamin B_6 (Kasidas and Rose, 1984; Sharma *et al.*, 1990). Pyridoxine supplementation with dietary oxalate restriction may be more effective than dietary restriction alone and is a simple maneuver that may be warrant a short trial in hyperoxaluric patients.

Dietary restriction has been one of the commonly advocated aspects of stone therapy as it has been assumed that the constituents of renal stones must come from the ingested food. Dietary oxalate potentially plays an important role in the pathogenesis of calcium oxalate urolithiasis. Robertson *et al.* (1978) have found urinary oxalate to be a significant risk factor for stone formation. Most urinary oxalate is of dietary origin, except in rare situation of increased endogenous synthesis of oxalate or substrate availability (Williams, 1978). As dietary oxalate plays an important role in the pathogenesis of oxalate, dietary restriction of oxalate intake has been used as therapy to reduce the risk of recurrence of oxalate kidney stones (Massey *et al.* 1993).Dietary measures are to decrease the consumption of sugars, ascorbic acid (Auer *et al.*, 1998), purines, fat and proteins like gelatin and collagen (Dussol and Berland, 1996; Giamini *et al.*, 1999). Dietary restriction of calcium is also an efficient practice in reducing urinary calcium excretion (Messa *et al.*, 1997).

Allopurinol treatment can lower urinary oxalate excretion (Felstrome *et al.*, 1985, Pak, 1999). Allopurinol has also been used primarily in patients with calcium oxalate urolithiasis (Coe and Raison, 1973).

The administration of magnesium salt was first advocated on the ground that it reduced the urinary excretion of oxalate (Gershoff and Rien, 1967; Su *et al.*, 1991). Magnesium oxide therapy has been reported to be therapeutic benefit in a small number of patients with both types of primary hyperoxaluria (Silver and Brendler, 1971).

Cellulose phosphate is a substance, which has been most useful in attempts to reduce the hyper-absorption of calcium (Hallson *et al.*, 1976) and in treatment of patients with calcium containing renal stones (Pietreak and Kokot, 1973).Alanine is used effectively to prevent phosphate calculi formation in rats (Chow *et al.*, 1974). Isocarboxazide is reported to reduce oxalate excretion by 40% (Smith *et al.*, 1972).

Thiazide therapy has a good effect in preventing renal calculi (Yendt *et al.*, 1970; Pak, 1999). It has a good effect in renal and absorptive hyperoxaluria (Yendt and Cohanim 1973 & 1978; Hallson *et al.*, 1976). Several others have reported the beneficial effect of thiazide treatment (Rose and Harrison, 1974; Coe, 1977; Pak, 1979). It has also been described that the excretion of various inhibitors, such as zinc and pyrophosphate are increased during treatment with thiazides (Bridgeman and Finlayson, 1978).

Orthophosphate salts have been used as a prophylactic treatment for recurring calcium containing kidney stones (Smith *et al.*, 1973). Orthophosphate and pyridoxine have been used in combination in patients with primary hyperoxaluria with some success. Phosphate administration lowers urinary calcium, whereas pyridoxine lowers urinary oxalate, resulting in a decreased saturation of calcium oxalate (Smith, 1992). Pyrophosphate had a less marked effect on calcium oxalate urolithiasis (Grases *et al.*, 1989). Sodium pentosan polysulphate treatment lowered oxalate and calcium levels in the sodium glycollate induced calcium oxalate stone forming rats (Subha and Varalakshmi, 1993).

Colestipol and aluminium hydroxide administration might reduce dietary oxalate absorption in patients with enteric hyperoxaluria (Laker and Hoffman, 1981) Cholestryamine, a bile salt binding resin, has been successfully used in the treatment of hyperoxaluria due to malabsorption (Smith *et al.*, 1972; Stauffer *et al.*, 1973). Urinary excretion of oxalate, liver protein content, GAO and LDH levels are normalised in taurine fed hyperoxaluric rats (Thalwar *et al.*, 1985).

Structural analogues of oxalate, glycollate and glyoxylate were considered in the *in vitro* experiments (Smith *et al.*, 1972). Of the oxalate analogues, oxalate hyrazide was

found to be the most potent inhibitor of LDH catalyzed oxalate synthesis. Hydroxy methane sulphonate was found to be a potent inhibitor of erythrocyte LDH and also GAO (Solomons *et al.*, 1967). Another analogue of oxalate hydrazide, oxamate hydrazide partially inhibits oxalate synthesis when administered intravenously with [^{14}C] glyoxylate (Goldberg *et al.*, 1965; Coe and Strunk, 1970). Some of the inhibitors, which are useful include p-chloromercuri benzoate, n-heptonoate and DL-phenyl lactate. These inhibit GAO and prevent oxalate synthesis in the perfused rat liver (Liao and Richardson, 1973). However, most of these inhibitors have not proved effective in the *in vivo* situation and some of them are not free from toxic effect.

The effects of sodium acetate, a monocarboxylic acid salt and sodium succinate, a dicarboxylic acid salt, were studied on partially purified liver GAO, which produced non-competitive and competitive types of inhibitions respectively (Senthil and Varalakshmi, 1995). Fry and Richardson (1979) have evaluated the inhibitory effects of some mono and dicarboxylic acids on purified liver GAO preparations and they obtained good inhibitions with oxalate, malonate and succinate. Recently Saso *et al.* (1998) found out that certain mono and dicarboxylic acids have the capacity of inhibiting liver GAO and LDH and thereby reducing the endogenous production of oxalate. Yagiswa *et al.* (1998) studied the inhibitory effects of succinate on calcium oxalate lithiasis.

Pyruvate and bicarbonate salts inhibit urinary calculi formation in rats, not by decreasing oxalate synthesis but by increasing urinary citrate concentration (Yoshihide *et al.*, 1986). Pyruvate administration regulates kidney and liver glycollate dehydrogenase as well as liver glycollate oxidase activity (Murthy *et al.*, 1985). Hesse *et al.*, (1986) have shown the possible prevention of urolithiasis with potassium-sodium-citrate mixture. The administration of 9 or 12 g of potassium-sodium- citrate mixture induced a significant reduction in calcium excretion in the urine.

Melon and Thomas (1971) claimed that succinic acid could prevent oxalate lithiasis in rats. Reports by Thomas *et al.* (1977) and Thind *et al.* (1978) showed a decrease in urinary oxalate excretion by stone formers treated with succinic acid. The possible mechanism by which succinic acid exhibits the effect of urinary oxalate may be

due to the fact that this dicarboxylic acids may compete with oxalate for the specific cell surface sites for intestinal absorption, thereby reducing oxalate concentration (Thind *et al.* 1978) in both fluids. The possibility of controlling calcium oxalate crystal growth in urine and consequent stone formation with tartarate looks attractive (Sur *et al.*, 1981, Hallson and Rose, 1984; Rose and Hallson, 1984). The inhibitory effect of L(+) tartaric acid on calcium oxalate crystal formation, both *in vitro* and *in vivo*, was studied by Selvam *et al.*, (1990 and 1992).

The non operative management of stone disease has been practiced in ancient India in three famous indigenous systems of medicine, Ayurveda, Unani, and Siddha and proved to be effective (Dymock *et al.*, 1976). Some traditional medicines also are there, including *Tribulus terrestris*, *Coleus aromaticus*, *Berberies vulgaris*, *Crataeva religiosa*, *Spinacia oleracea*, *Dolichos biflorus* etc (Kritikar *et al.*, 1918; Nadkarni, 1976).

In the southern part of India, banana stem kernel juice (Pith) (*Musa paradisiaca*) is widely used to treat patients with urinary stones. However, the efficiency of most of the substances is still questionable and demands further study.

Tartaric acid also known as *Acidum tartaricum* is an odourless, white crystalline substance having an acidic taste. It is soluble in water and alcohol, sparingly soluble in ether and insoluble in chloroform. Since it has two asymmetric carbon atoms, it shows optical isomerism. Two asymmetric carbon atoms in tartaric acid are attached to the groups H, OH and COOH. L(+) tartarate is the naturally occurring isomer and is commonly found as a constituent of berberry, papaya, coconut, rozelle hemp, potato, grapes, wines and tamarind. Tamarind is the fruit of a tree, *Tamarindus indica* which grows in the tropical areas. In the Indian subcontinent it is consumed in large amounts in the south and reported low incidence of stones in the south has attributed to the use of tamarind pulp (Colabawalla, 1971). Tartaric acid is known to be safe for oral administration. Tartarate is used as laxative. The usual dose for an adult is 0.3 to 2.0 g/day and for children, one to three quarters of the adult dose. The dicarboxylic acid is a constituent of effervescent powder and granules, which are used in bakery products, soft drink industry and confectionary products.

Maleic acid [(Z) - Butene dioic acid] is a dicarboxylic acid with repulsive astringent taste and acidulus odour. It is soluble in water, ethyl alcohol, acetone and glacial acetic acid. It has a melting point of 138 - 139^{0}C, Pka 1 - 1.03, Pka 2 - 6.27. On heating above the melting point it is converted partly to tannic acid and partly to anhydride. Maleic acid is found as a constituent of many traditional plants, eg. *Tribulus terestris*, commonly found in South India, which acts as diuretic and can be used for the treatment of kidney stone disease.

Malic acid (Hydroxy succinic acid or Hydroxy butane dioic acid), dicarboxylic acid which is naturally occurring as L-isomer. It is soluble in methanol, diethyl ether, ethanol, acetone, dioxane and water, insoluble in benzene. It is a constituent of apple and many other fruits and plants like Berberis vulgaris, a medicinal plant commonly found in the Indian subcontinent, which is an astringent, diuretic and antibilious, mainly used in biliary renal calculi.

1.2.13. Microbial degradation of oxalate

Since oxalic acid is produced as an end product by a wide diversity of plants, animals and microbes, it should not be surprising that microbes able to attack oxalate are widely distributed in natural ecosystem (Allison *et al.*, 1995). The chemical nature of oxalate would appear to limit microbial appetite for it. Further more, the high oxidation state of oxalate dictates that only small amount of energy can be made available through further oxidations of the molecules. Oxalate coupled with its ability to act as powerful chelating agents not only results in limited possibilities for its catabolism and use in energy production but also makes oxalate toxic to most forms of life, especially mammals. Oxalic acid is produced in large quantities as a product of metabolism by virtually all life forms. This high rate of synthesis coupled with a general ability to catabolize oxalic acid can lead to rapid accumulation. Salts of oxalic acids are widely distributed in the diets of man and animals and ingestion of plants that contain high concentration of oxalate may lead to intoxication (Lung *et al.*, 1994).

In mammals, dietary oxalate that is absorbed from the intestinal tract is not metabolized and is excreted unchanged in urine (Hodgkinson, 1977). Increased urinary oxalate excretion may arise when dietary oxalate intake is increased or oxalate absorption is increased due to intestinal disease (Menon and Mehle, 1982; Laker, 1983). Urolithiasis, a disease characterised by the formation of urinary tracts stones, affects 5-10 percent of the human population. Approximately 70% of these stones contain calcium oxalate as a major component (Ryall, 1993). Though the effects of diet and intestinal absorption on oxalate excretion have been extensively studied (Hodgkinson, 1977), relatively little is known about bacterial degradation of oxalate in the mammalian intestinal tract and its influence on the absorption and excretion of dietary oxalate.

Unfortunately there are no known, naturally occuring oxalate degrading or metabolizing enzymes in vertebrates (Lung *et al.*, 1994). It is catabolized by a limited number of bacterial species by an activation -decarboxylation reaction, which yields formate and CO_2. The first process can be accomplished either aerobically or anaerobically while the second is strictly aerobic (Chandra and Shelina, 1975).

Bacteria that use oxalate as a carbon and energy source have been isolated from many environments including gastrointestinal tract of many animals (Allison and Cook, 1981). Oxalate is degraded by microbial population in the gastrointestinal tract of human (Allison *et al.*, 1986) ruminants (Morris and Gaercia Rivera, 1955), and certain non-ruminant microbes (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 and Cook, 1981; Allison *et al.*, 1977).

Most of the known bacterial oxalate degraders are aerobes or facultative anaerobes, which use O_2 as final electron acceptor during the growth on oxalate. Only two strictly anaerobic oxalate degraders have been reported: a Clostridial strain, isolated from donkey dung (Bhat, 1966) and a strain of *Desulfovibrio*, isolated from mud as an oxamate utilizer (Postgate, 1963). A number of oxalate degrading bacteria has been isolated from soil (Knutson *et al.*, 1980). Dawson *et al.* (1980) reported the isolation of

oxalate degrading bacteria from ruminal content of sheep. Similar bacteria have been isolated from human faeces (Allison *et al.*, 1986), the faecal content of guinea pig, swine and lake sediments which, frequently contain significant amount of oxalate (Smith *et al.*, 1985). Zaitsev *et al.* (1993) isolated *Bacillus oxalophilus*, a mesophilic oxalate degrading bacterium from the rhizosphere of sorrel. Both aerobic and anaerobic oxalate degrading bacteria isolated from various environments were reported to have the ability of decreasing the increased absorption of oxalate, which is a major cause of calcium oxalate stones (Lung *et al.*, 1991; Daniel *et al.*, 1993; Lung *et al.*, 1994).

1.3 Scope and objectives of the present work

Aggregation of calcium salts of oxalate and phosphate may cause formation of mineral deposits in the kidney and urinary tract, and are known as calculi or stones. The urinary stones encountered to southern part of India are composed of calcium oxalate either purely or mixed with phosphate. The process of urolithiasis is a very complex and multifactorial and the treatment of calcium oxalate recurrent stone formers are difficult. Increased urinary excretion of oxalate has been encountered in oxalate stone patients and controlling hyperoxaluria may be advantageous in preventing the calcium oxalate crystallization process. Although precipitation of calcium oxalate depends on urine saturated with both calcium oxalate ions in a metastable state, it has been argued that oxalate concentration is more significant in the formation of urinary calcium oxalate stone. Thus the management of oxalate in individual susceptible to urolithiasis would seem especially important. A variety of chemical compounds and famous indigenous medicinal system have a preventive role on calcium oxalate crystallization process. However, the efficiency of most of the substances was not proved to be effective in the *in vivo* situation and demand further study.

Unfortunately, there are no known naturally occuring oxalate metabolizing enzyme in vertebrates. Bacteria that use oxalate as a carbon source have been isolated from many environments including gastrointestinal tract of many animals. Oxalate is catabolized by a limited number of bacterial species by an activation decarboxylation

reaction, which yields formate, and CO_2 could benefit a great number of individuals in the biomedical field. The current study is an attempt to find a means of lowering oxalate concentration in individuals susceptible to recurrent calcium oxalate stone disease.

The specific objectives of the present work are as follows

1. To study the effect of certain mono and dicarboxylic acids on calcium oxalate crystal growth *in vitro*.
2. To investigate the effect of dicarboxylic acids on oxalate metabolism in experimental hyperoxaluric rats.
3. To study the biochemical effect of sodium glycollate and dicarboxylic acids on oxalate metabolism in experimental stone forming rats.
4. Isolation and characterization of oxalate degrading bacteria.

Chapter 2

IN VITRO STUDIES ON THE EFFECT OF MONO AND DICARBOXYLIC ACIDS ON CALCIUM OXALATE CRYSTAL GROWTH

CHAPTER-II

IN VITRO STUDIES ON THE EFFECT OF MONO AND DICARBOXYLIC ACIDS ON CALCIUM OXALATE CRYSTAL GROWTH

2.1 Introduction

Calcium oxalate is one of the most common constituents of urinary stones (Ryall, 1993; Baker *et al.*, 1993; Khan, 1995; Buno Soto, 1996; Balla, 1998). Crystal growth and aggregation are essential steps in stone formation (Fleisch, 1978). Crystal growth is one of the prerequisites for particle formation in stone disease (Finllayson *et al.*, 1984). The process whereby crystals in solution stick together in order to form a large particle is called aggregation or agglomeration (Drach 1986; Coe *et al*, 1991; Coe *et al.*, 1992; Ryall 1993). Aggregation is a very fast process, allowing formation of larger particles within seconds (Blomen, 1982). Aggregation of particles in solution is governed by an interplay of several basic forces with either aggregating or disaggregating effects (Roberson *et al.*, 1981; Hess, 1991). Calcium oxalate crystal can be easily produced *in vitro* and a number of investigators have designed experiments to study the formation of crystals and the effects of various substances on their growth rates.

Lyon and Vermeulon (1965) grew calcium oxalate crystals on wire loops suspended in simple salt solutions by the slow diffusion of calcium and oxalate ions through filter paper wicks in to the media. They examined growth of crystals in the presence of potential inhibitors. Using a similar method, Sutor (1969) examined calcium oxalate crystal growth in the presence of a wide variety of substances in acetate buffer. Later Sutor and Wooley (1970) examined the action of several compounds using human urine. Welshman and McGeown (1972) used an analogous experiment and have described a method for the estimation of crystal growth by measuring the calcium content of crystals. The effect of compounds on the formation of crystal over a 24h period was studied.

If the increasing amount of substance capable of crystallization are added to pure water at a given pH and temperature, eventually a high enough concentration reached for

crystals to form. When crystals begin to form, the solution has become saturated with the substance. When two or more substances are combined to form the crystal (eg. Calcium oxalate), the level of saturation is governed by the product of the concentration of the substances. The point at which saturation is reached and crystallization begins is referred to as the solubility product (SP), which is defined as the product of molar concentrations of the two substances at the point of saturation. pH and temperature are always specified for any crystallization process. Alteration in either factor may greatly change the amount of substance or solute that may be held in solution. Most important factors that contribute to supersaturation of calcium oxalate are the concentrations of calcium, oxalate, citrate and magnesium (Tiselius, 1982). It was shown by Robertson *et al.* (1981) that the crystallization of calcium oxalate for a certain supersaturation was highly dependent on the oxalate/calcium ratio. Asplin *et al.* (1998) and Jiang *et al.* (1998) studied the effect of human uropontin on calcium oxalate crystallization, which is known to inhibit the growth and nucleation of calcium oxalate monohydrates. Many investigators have described that urinary macromolecules such as glycosaminoglycans (GAGS), Tamm-Horsfall glycoprotein (THP) and also other urinary macromolecules act as inhibitors of calcium oxalate stone formation (Nakagava *et al.*, 1983; Hess *et al.*, 1989). Yoshimura *et al.* (1997) reported that heparan sulphate and dermetan sulphate may inhibit calcium oxalate crystal formation. Recent experimental studies have indicated that small urinary ions such as citrate, magnesium and phosphocitrate retarded the mineralisation rate of calcium oxalate (Nancollas *et al.*, 1991; Rodgers *et al.*, 1996).

Most of the inhibitors exert their effects by one of the two possible mechanisms. Firstly they may act by complexing with either Ca^{2+} or $C_2O_4^{2-}$ ions and thereby reduce the level of calcium oxalate supersaturation in the crystallizing solution. This can lead to a reduction in the thermodynamic driving force necessary for crystal nucleation and growth. Citrate and magnesium come under this category. The remaining inhibitors act at relatively low concentration by absorbing on to the surface of the crystal and subsequently slow down the kinetics of crystal growth and agglomeration. Most of the studies were performed using either diluted urine or low concentrations of additives so as to modify the rates of crystal growth and / or aggregation of calcium oxalate crystal suspended in metastable solution of the salt.

The above works have shown that a number of substances can alter the rate of growth or agglomeration of seed crystals of calcium oxalate. Hence it was brought worthwhile to investigate the effect of some mono and dicarboxylic acids on calcium oxalate crystal growth in the *in vitro* situation. The *in vitro* studies also permit rigorous control of experimental conditions and at the same time greater freedom can be exercised in varying the conditions of crystallization, which are impossible in the living system.

2.2 Materials and Methods

2.2.1 Biochemicals and their sources

Biochemicals used in the present study were mostly of analytical grade purchased locally from SRL, BDH, Qualigens and MERCK, India.

2.2.2 *In vitro* calcium oxalate crystal formation studies

Calcium oxalate crystals were produced *in vitro* according to the method of Boumann and Wacker (1980).

2.2.2.1 Reagents

1. Basic solution (pH - 6.0)

Sodium chloride	-	0.15 M
Sodium cacodylate	-	5 mM
Sodium azide	-	0.2 %

2. Crystal inducer

Calcium oxalate	-	2.04 mg/ml

3. Calcium chloride - 0.1 M
4. Sodium oxalate - 0.2 M
5. Test solutions

Different concentrations of L(+) tartaric acid, maleic acid, malic acid, succinic acid, malonic acid and pyruvic acid (0.1 mM, 0.2 mM and 0.4 mM).

All the solutions were made using the basic solution.

2.2.2.2 Procedure

To 1 ml of 0.1 M $CaCl_2$ contained in 10 ml centrifuge tubes, were added 0.1 - 1 ml of 0.2 M sodium oxalate. Calcium oxalate crystal growth was induced by the addition of 0.02 ml of a well mixed 2.04 mg/ml calcium oxalate crystal suspension. The final volume was made up to 4.1 ml with the basic solution. The samples were incubated on a mechanical shaker at 100 - 120 oscillations/minute for 90 minutes. The crystals were removed by centrifuging the samples at 3000 rpm for 30 minutes. The calcium concentrations in the supernatant was determined and compared with the control, containing only 0.1 ml of 0.1 M $CaCl_2$.

In another set of experiments L(+) tartaric acid, maleic acid, malic acid, succinic acid, malonic acid and pyruvic acid, at concentrations of 0.1 mM, 0.2 mM and 0.4 mM were included to study their effects on crystal growth and compared with their suitable controls (without the above acids).

2.2.3 Calcium estimation

The method adopted was that of Gindler and King (1972).

2.2.3.1 Principle

Calcium forms a blue complex with methylthymol blue in alkaline solution and the intensity of the colour was measured at 612 nm.

2.2.3.2 Reagents

1. Methyl thymol blue reagent - 1000 ml

Methyl thymol blue (sodium salt)	-	180 mg
Polyvinyl pyrrolidone	-	6.0 g
8- hydroxy quinoline	-	7.2 g
Conc. Hydrochloric acid	-	10 ml

Dissolved and made upto 1000 ml with D.D.H_2O

2. Alkaline sulphite solution - 1000 ml

Sodium sulphite ($Na_2SO_3.7.H_2O$)	-	24 g
Monoethanolamine	-	220 ml

Dissolved and made upto 1000 ml with D.D.H_2O

3. Working colour reagent - 1000 ml

Methyl thymol blue reagent	-	500 ml
Alkaline sulphite solution	-	500 ml

This reagent is stable at room temperature for 2 days. (Avoid refrigeration).

4. Standard calcium solution (10 mg/100 ml)

Pure dry calcium carbonate	-	125 mg
1 N HCl	-	3 ml

Mixed, warmed on a water bath to remove excess acid, transferred to a 500 ml measuring flask and made to the mark with double distilled water.

5. Sodium chloride solution - 100 ml

NaCl	-	9 g
D. D. H_2O	-	100 ml

2.2.3.3 Procedure

Made an initial 10 fold dilution of the standard calcium chloride solution and the test sample with saline. Pipetted out 0.5 ml of diluted sample, diluted standard or saline (as blank) in to a test tube and added 3 ml working colour reagent. Mixed well and read the extinction of the solution at once against the blank at 612 nm using a spectrophotometer.

Concentration of calcium was calculated by

$$\text{Calcium content (mg / 100 ml)} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 10$$

2.3 Results

In vitro experiment measures the capacities of certain mono and dicarboxylic acids to inhibit a given amount of calcium oxalate from crystal growth. Fig. 2.1 and Table 2.1 shows the increasing trend of calcium oxalate growth in percent, with

increasing concentration of sodium oxalate. Sodium oxalate concentration ranged from 0.01 - 0.1 mM, with constant amount inducer (calcium oxalate) added. The 0.1 M calcium chloride content was also kept constant. The growth was negligible at zero concentration and gradually increased up to 87% at 0.1 mM sodium oxalate concentration.

Table 2.2A, 2.2B, 2.2C, 2.2D, 2.2E and 2.2F represent the inhibitory capacities of mono and dicarboxylic acids, L(+) tartaric acid, maleic acid, malic acid, succinic acid, pyruvic acid and malonic acid respectively. Mono and dicarboxylic acids were added at concentrations of 0.1 mM, 0.2 mM and 0.4 mM respectively. The calcium content in the supernatents was estimated, which increased with increasing concentration of inhibitors. The inhibition was maximal at 0.4 mM, submaximal at 0.2 mM and minimal at 0.1 mM.

The percentage growth and inhibition relative to control were calculated. 0.1 mM concentration of L(+) tartaric acid, maleic acid, malic acid, succinic acid, pyruvic acid and malonic acid brought about 44.38 %, 58.38 %, 61.83 %, 66.19 %, 68.72 % and 79.67% crystal growth when 0.01 mM sodium oxalate concentration was used. It was further reduced at 0.2 mM concentration of inhibitors (37.29 %, 47.19 %, 55.02 %, 53.98 %, 61.86 % and 70.82 % growths with the above acids respectively). The inhibition of crystal growth was almost complete at 0.4 mM concentration of inhibitors being 79.08 %, 60.02 %, 58.06 %, 51.82 %, 40.53 % and 30.03 % with these acids respectively, when 0.01 mM sodium oxalate was used. A similar trend was seen with increasing amounts of sodium oxalate and crystal growth increased up to 95.07 %, 94.77 %, 93.99 %, 93.68 %, 90.19 % and 91.84 %, at 0.1 mM concentration of L(+) tartaric acid, maleic acid, malic acid, succinic acid, pyruvic acid and malonic acids, with 0.2 mM concentration of the inhibitors, it drops to 75.02 %, 70.23 %, 82.57 %, 84.20 %, 83.02 % and 88.07 % respectively. 0.4 mM concentration of L(+) tartaric acid, maleic acid, malic acid, succinic acid, pyruvic acid and malonic acids produced 27.13 %, 31.08 %, 21.68 %, 17.23 %, 19.05 % and 12.76 %, inhibitions of crystal growth respectively.

Best fit line drawn by linear regression is also shown in Fig. 2.2A, 2.2B, 2.2C, 2.2D, 2.2E and 2.2F.

Table 2.1. Effect of Sodium oxalate concentration on calcium oxalate crystal growth *in vitro.*

Conc. of sodium oxalate. (mM)	% of crystal growth.
0.01	10.3
0.02	19.5
0.03	25.4
0.04	35.0
0.05	43.0
0.06	51.4
0.07	57.1
0.08	63.1
0.09	75.5
0.10	81.0

Figure 2.1 Calcium oxalate crystal growth with increase in concentration of sodium oxalate

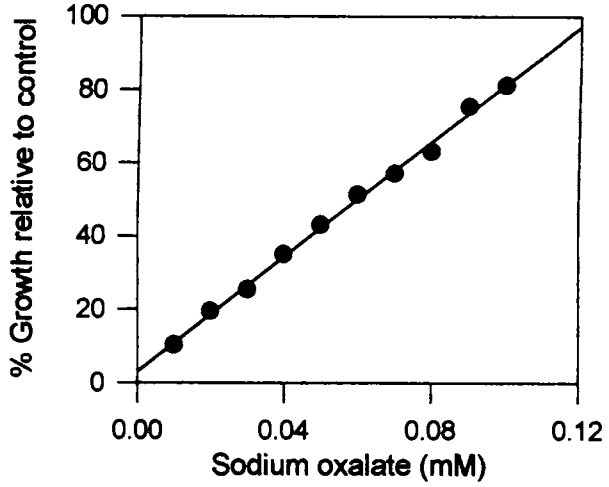


Table 2.2A Calcium oxalate crystal growth and inhibition relative to control with L(+) Tartaric acid

Conc.of Sodium oxalate (mM)	Without tartarate		0.1mM tartarate		0.2mM tartarate		0.4mM tartarate	
	% growth	% inhibition	% growth	% inhibition	% growth	% inhibition	% growth	% inhibition
0.01	100	0	44.38	55.62	37.29	62.71	20.92	79.08
0.02	100	0	56.29	43.71	39.88	60.12	24.19	75.81
0.03	100	0	67.91	32.09	41.57	58.43	32.17	67.83
0.04	100	0	70.44	29.56	46.09	53.91	38.59	61.42
0.05	100	0	78.62	21.38	50.38	49.62	40.15	59.85
0.06	100	0	82.44	17.56	54.87	45.13	48.67	51.33
0.07	100	0	86.85	13.15	61.04	38.96	51.38	48.62
0.08	100	0	90.20	9.80	64.88	35.12	63.28	36.72
0.09	100	0	93.88	6.12	73.39	26.61	71.82	28.18
0.10	100	0	95.07	4.93	75.02	24.98	72.87	27.13

Figure 2.2A Effect of L(+)tartaric acid on calcium oxalate crystal growth

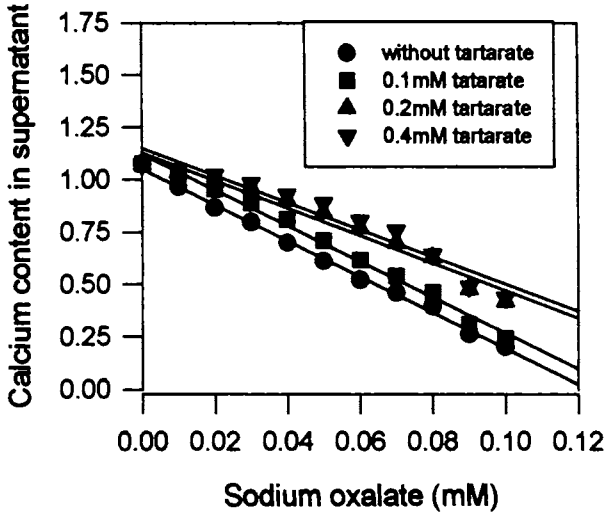


Table 2.2B Calcium oxalate crystal growth and inhibition relative to control with maleic acid

Conc.of Sodium oxalate (mM)	Without Maleic acid.		0.1mM maleic acid.		0.2mM maleic acid		0.4mM maleic acid	
	% growth	% inhibition	% growth	% inhibition	% growth	% inhibition	% growth	% inhibition
0.01	100	0	58.38	41.62	47.19	52.81	39.98	60.02
0.02	100	0	61.88	38.12	50.11	49.89	41.84	58.16
0.03	100	0	68.17	31.83	55.42	44.58	44.77	55.23
0.04	100	0	71.86	28.14	59.24	40.76	48.47	51.53
0.05	100	0	76.11	23.89	61.01	38.99	52.02	47.98
0.06	100	0	80.64	19.36	65.65	34.35	55.16	44.84
0.07	100	0	82.11	17.89	67.91	32.09	58.11	41.11
0.08	100	0	88.97	11.03	68.19	31.81	60.09	39.91
0.09	100	0	92.92	7.08	69.08	30.92	65.94	34.06
0.10	100	0	94.77	5.23	70.23	29.77	68.92	31.08

Figure 2.2B Effect of maleic acid on calcium oxalate crystal growth

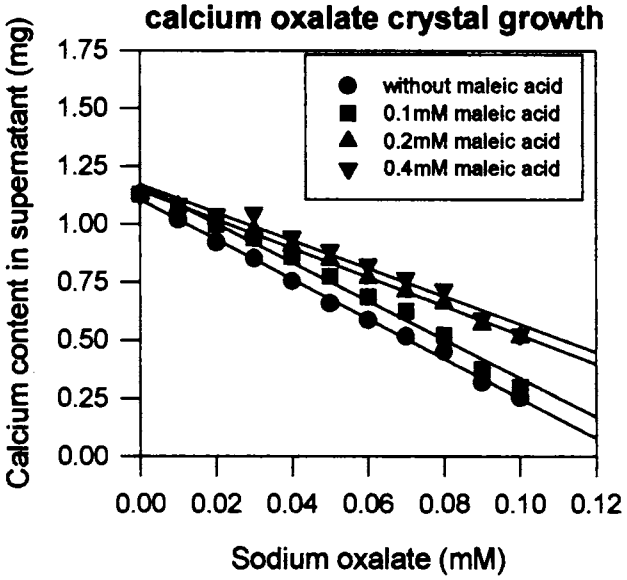


Table 2.2C Calcium oxalate crystal growth and inhibition relative to control with malic acid.

Conc.of Sodium oxalate (mM)	Without Malic acid.		0.1mM malic acid.		0.2mM malic acid		0.4mM malic acid	
	% growth	% inhibition	% growth	% inhibition	% growth	% inhibition	% growth	% inhibition
0.01	100	0	61.83	38.17	55.02	44.98	41.94	58.06
0.02	100	0	66.58	33.42	58.11	41.89	47.95	52.02
0.03	100	0	70.03	29.97	61.97	38.03	50.65	49.35
0.04	100	0	72.12	27.88	65.82	34.18	54.99	45.01
0.05	100	0	78.97	21.03	69.02	30.98	58.17	41.83
0.06	100	0	80.88	19.12	71.97	28.03	62.66	37.34
0.07	100	0	82.37	17.63	74.39	25.61	66.83	33.17
0.08	100	0	85.66	14.34	78.96	21.04	70.04	29.96
0.09	100	0	90.82	9.18	80.78	19.22	72.32	27.68
0.10	100	0	93.99	6.01	82.57	17.43	78.32	21.68

Figure 2.2C Effect of malic acid on calcium oxalate crystal growth in vitro.

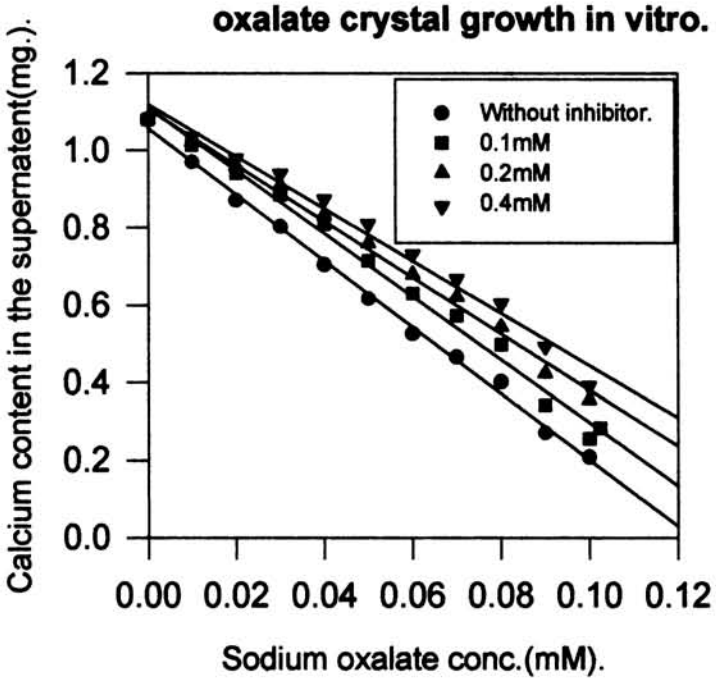


Table 2.2D Calcium oxalate crystal growth and inhibition relative to control with succinic acid.

Conc.of Sodium oxalate (mM)	Without Succinic acid.		0.1mM Succinic acid.		0.2mM Succinic acid.		0.4mM Succinic acid.	
	% growth	% inhibition	% growth	% inhibition	% growth	% inhibition	% growth	% inhibition
0.01	100	0	66.19	33.81	53.98	46.02	48.18	51.82
0.02	100	0	68.48	31.52	58.24	41.76	51.47	48.53
0.03	100	0	71.33	28.67	61.56	38.44	58.91	41.09
0.04	100	0	76.02	23.98	66.01	30.99	61.29	38.71
0.05	100	0	80.93	19.07	71.84	28.16	68.01	31.99
0.06	100	0	84.19	15.81	74.99	25.01	70.22	29.78
0.07	100	0	88.92	11.08	79.14	20.86	75.13	24.87
0.08	100	0	90.01	9.99	82.74	17.26	80.19	19.81
0.09	100	0	92.02	7.98	83.88	16.12	81.99	18.01
0.10	100	0	93.68	6.32	84.2	15.8	82.77	17.23

Figure 2.2D Effect of succinic acid on calcium oxalate crystal growth *in vitro*

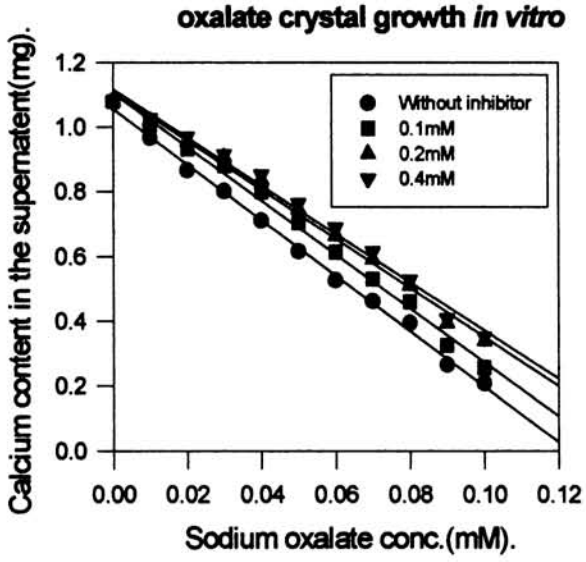
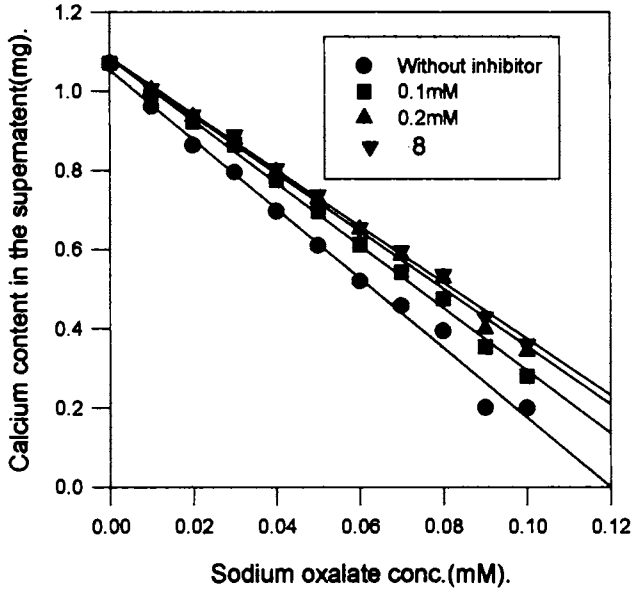


Table 2.2E Calcium oxalate crystal growth and inhibition relative to control with pyruvic acid

Conc.of Sodium oxalate (mM)	Without pyruvic acid.		0.1mM pyruvic acid.		0.2mM pyruvic acid.		0.4mM pyruvic acid.	
	% growth	% inhibition	% growth	% inhibition	% growth	% inhibition	% growth	% inhibition
0.01	100	0	68.72	31.28	61.86	38.14	59.47	40.53
0.02	100	0	70.88	29.12	65.02	34.98	62.99	37.01
0.03	100	0	74.40	25.60	69.67	30.33	66.02	33.98
0.04	100	0	79.01	20.99	72.99	27.01	70.89	29.11
0.05	100	0	81.29	18.71	74.59	25.41	72.25	27.75
0.06	100	0	83.13	16.87	76.12	23.88	75.37	24.63
0.07	100	0	85.96	14.04	78.99	21.01	77.12	22.88
0.08	100	0	87.88	12.12	80.32	19.68	78.84	21.16
0.09	100	0	88.32	11.68	82.14	17.86	79.13	20.87
0.10	100	0	90.19	9.81	83.02	16.98	80.95	19.05

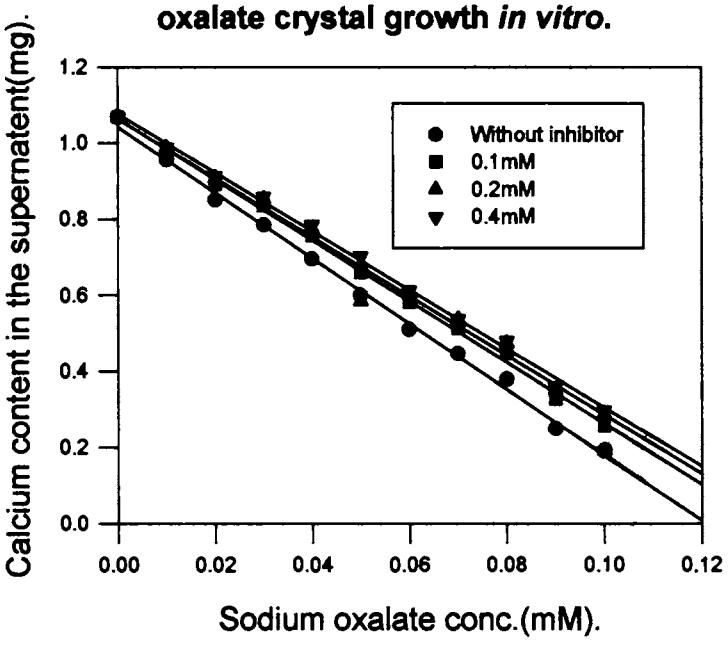
Figure 2.2E Effect of pyruvic acid on calcium oxalate crystal growth *in vitro.*



Tables 2.2F Calcium oxalate crystal growth and inhibition relative to control with malonic acid.

Conc.of Sodium oxalate (mM)	Without malonic acid.		0.1mM malonic acid.		0.2mM malonic acid		0.4mM malonic acid	
	% growth	% inhibition	% growth	% inhibition	% growth	% inhibition	% growth	% inhibition
0.01	100	0	79.67	20.33	70.82	29.18	69.97	30.03
0.02	100	0	80.82	19.18	73.01	26.99	71.34	28.66
0.03	100	0	81.97	18.08	75.87	24.13	73.82	26.18
0.04	100	0	83.22	16.78	78.92	21.08	75.46	24.54
0.05	100	0	85.81	14.19	81.86	18.14	78.02	21.98
0.06	100	0	87.01	12.99	83.25	16.75	81.22	18.78
0.07	100	0	88.97	11.03	85.02	14.98	84.97	15.03
0.08	100	0	89.89	10.11	86.09	13.91	85.41	14.59
0.09	100	0	90.11	9.89	87.14	12.86	86.16	13.84
0.10	100	0	91.84	8.16	88.07	11.93	87.24	12.76

Figure2.2F Effect of malonic acid on calcium oxalate crystal growth *in vitro*.



2.4 Discussion

The primary mechanism in stone formation is crystallization, dependent inturn upon circumstances of supersaturation and nucleation. Studies have been carried out to measure the effect of various modifiers on calcium oxalate crystallization by different techniques (Robertson *et al.*, 1973; Rose and Sulaiman, 1984). Robertson and Scurr (1986) used a continuous crystallizer and artificial urine and showed, that of the main modifiers of the calcium oxalate crystallization, which were active within the normal range of urinary concentrations. Ebisuno *et al.* (1993) studied the inhibitory effect of urinary macromolecules on the aggregation of calcium oxalate crystals using an aggregometer. The rate of crystal sedimentation in a suspension of calcium oxalate monohydrate crystal was determined spectrophotometrically in the presence and absence of dialysed urine, citrate urine and citrate (Teselius *et al.*, 1993). The inhibitory effect of urine pyrophosphate, citrate and magnesium are well established (Ryall *et al.*, 1981; Mayer and Thomas, 1982).

In this chapter, efforts have been made to study the effects of various mono and dicarboxylic acids on calcium oxalate crystal growth by direct precipitation of calcium oxalate in centrifuge tubes, in the presence of varying concentrations of mono and dicarboxylic acids. The above acids exhibit significant differences in the crystal inhibitory potential. Of the six inhibitors tested, L(+) tartaric acid was the most potent inhibitor of calcium oxalate crystal growth (79.08 %) followed by maleic acid (60.02 %), malic acid (58.06 %), succinic acid (51.80 %), pyruvic acid (40.53 %) and malonic acid (30.03 %). The above observations go to confirm that L(+) tartaric acid, maleic acid, malic acid, succinic acid, pyruvic acid and malonic acid have anticrystallizing effect on the growth of calcium oxalate crystals.

Rose and Hallson (1984) and Hallson and Rose (1984) have studied the effect of tartarate upon calcium oxalate precipitation from whole urine and Saso *et al.* (1998) studied the inhibitory effect of some of the dicarboxylic acids on calcium oxalate crystallization *in vitro*, lends support to our observations.

Chapter 3

EFFECT OF MONO AND DICARBOXYLIC ACIDS ON OXALATE METABOLISM IN EXPERIMENTAL HYPEROXALURIC RATS -SHORT TERM STUDIES

CHAPTER- III

EFFECT OF MONO AND DICARBOXYLIC ACIDS ON OXALATE METABOLISM IN EXPERIMENTAL HYPEROXALURIC RATS - SHORT TERM STUDIES

3.1 Introduction

Hyperoxaluria is considered to play a crucial role in calcium oxalate renal stone disease (Hatch, 1993). Hyperoxaluria has been encountered in idiopathic recurrent stone formers (Varalakshmi and Anandam, 1979a; Robertson and Peacock, 1980; Baggio *et al.*, 1983). It is likely to be of endogenous origin since such patients were also identified to manifest a subclinical state of vitamin B_6 deficiency (Varalakshmi *et al.*,1979b). The mechanism responsible for the increment in urinary excretion of oxalate could involve oxalic acid synthesis. Buno Soto *et al.* (1996) reported that excessive ingestion of oxalate rich food leads to hyperoxaluria. The control of endogenous oxalate synthesis and exogenous supply in hyperoxaluric situation is likely to yield beneficial effects. Obviously this type of control has to be exercised on oxalate biosynthesis pathway from its precursors. The rational approach here will be to prevent the conversion of the immediate precursors, glycollate and glyoxylate to oxalate. It has been emphasized that changes in oxalate concentration are more important than changes in calcium concentration in determining the oversaturation of urine with calcium oxalate (Hodgkinson, 1978; Baggio *et al.*, 1983).

Glycollate has been successfully used to experimentally induce hyperoxaluria in rats, which is a major precursor of endogenous oxalate synthesis (Chow *et al.*, 1978; Varalakshmi and Richardson, 1983b; Murthy *et al.*, 1983; Selvam and Varalakshmi, 1989). Massive deposition of oxalate crystals is known to occur in the renal tissue under this condition mimicking nephrocalcinosis, nephrolithiasis and associated hyperoxaluria. This method of induction has been used in the present work.

Liver is well established to be the primary site of oxalate synthesis (Liao and Richardson, 1972; Varalakshmi and Richardson, 1983a). GAO and LDH, the two liver

enzymes play an important role in this aspect. An abnormal induction of these enzymes results in hyperoxaluria. Several reports indicate that the use of various compounds to inhibit the endogenous oxalate synthesis through these two liver enzymes (Liao and Richardson, 1972; Fry and Richardson, 1979). Reports by Sur *et al.* (1981), Anasuya (1988) and Selvam and Varalakshmi (1990 & 1992) have indicated usefulness of tartaric acid as inhibitor of calcium oxalate crystallization. Rose and Hallson (1984) and Selvam and Varalakshmi (1989) have shown that isomers of tartarate reduced calcium oxalate precipitation in the *in vitro* system. Further Hallson and Rose (1984) have reported that L(+) tartarate reduced calcium phosphate precipitation in urine.

Hautman *et al.* (1978) and Yagisawa *et al.* (1998) have shown the effect of succinate treatment in hyperoxaluric patients. Sodium malate and sodium succinate administration inhibited urinary calculi formation in experimental stone forming rats . Saso *et al.* (1998) demonstrated the inhibitory action of succinic acid, malic acid and gluconic acid on calcium oxalate crystallization.

The above studies have provoked us to carry out a comparative study of some dicarboxylic acids and to study their biochemical effects on oxalate metabolism in experimental hyperoxaluric rats.

3.2 Materials and Methods

3.2.1 Biochemicals and their sources

Biochemicals used in the present study were mostly of analytical grade purchased locally from SRL, HI MEDIA and MERCK, India. Sodium glycollate, glyoxylic acid and tartaric acid were purchased from Sigma Chemical Co., U.S.A.

3.2.2 Experimental animals

Adult male albino rats of Wistar strains, weighing between 150-250 g were obtained from Small Animal Breeding Center, Kerala Agricultural University, Mannuthy for the experimental studies. Animals were acclimatized to the animal house condition

for ten days. Water was given *ad libitum*. Animals were fed with commercial rat feed. No special arrangements were made for heating, cooling or lighting in the animal house.

3.2.3 Experimental induction of hyperoxaluria in rats

Hyperoxaluria was induced by the oral administration of sodium glycollate using stomach tube (Murthy *et al.*, 1981; Selvam and Varalakshmi, 1989), 100 mg/ml in 0.9 % saline/day/rat.

3.2.4 Experimental set up

The animals were divided into eight groups of six animals each.

Group I - Rats were received commercial diet, served as control.

Group II - Rats were administered with sodium glycollate for seven days (to induce hyperoxaluria).

Group III - Rats were administered with sodium glycollate for seven days followed by L(+) tartarate (100 + 50 mg/ml in 0.9 % saline/day/rat) for seven days.

Group IV - Rats were administered with sodium glycollate for seven days followed by maleic acid (100 + 50 mg/ml in 0.9 % saline/day/rat) for seven days.

Group V - Rats were administered with sodium glycollate for Seven days followed by malic acid (100 + 50 mg/ml in 0.9 % saline/day/rat) for seven days.

Group VI - Rats were administered with sodium glycollate for seven days followed by succinic acid (100 + 50 mg/ml in 0.9 % saline/day/rat) for seven days.

Group VII - Rats were administered with sodium glycollate for seven days followed by pyruvic acid (100 + 50 mg/ml in 0.9 % saline/day/rat) for seven days.

Group VIII - Rats were administered with sodium glycollate for seven days followed by gallic acid (100 + 50 mg/ml in 0.9 % saline/day/rat) for seven days.

After the experimental period the animals were weighed and placed in metabolic cages for 24 h and the urine samples were collected in clean, dry containers with a drop of concentrated hydrochloric acid as preservative. During urine collection, food was withdrawn in order to avoid contamination of urine samples. The urine was filtered, measured and stored at 4^0C. The animals were sacrificed by decapitation. Liver and kidneys were excised from the body, washed with cold 0.15 M KCl and their weights were recorded.

The following biochemical investigations were carried out.

3.2.5 Estimations in liver

Assay of enzymes

3.2.5.1 Preparation of tissue homogenate

A 10 % homogenate of the washed tissue were prepared (liver & kidney) using 0.01 M sodium phosphate buffer, pH 7.0. The homogenate was subjected to centrifugation at 12,000 rpm for 30 minutes at 4^0C. The supernatent fraction was used for the assays of glycollate oxidase (GAO) and lactate dehydrogenase (LDH).

3.2.5.2 Glycollate oxidase (E.C.1.1.3.1)

The method adopted was that of Lui and Roels (1970). The principle of the method is based on converting the substrate, glycollate to glyoxylate by the liver enzyme GAO (Richardson and Tolbert, 1961). The glyoxylate is then made to react with phenyl hydrazine to form the hydrazone which on oxidation with potassium ferricyanide in the presence of HCl forms a formazan, which is a pink coloured compound, the wavelength of which can be measured at 517 nm in a spectrophotometer.

Glycollate → glyoxylate

glyoxylate + phenylhydrazine → glyoxylate phenyl hydrazine.

Glyoxylate phenyl hydrazine + $K_3Fe(CN)_6$ → Formazan (Pink colour)

3.2.5.2.1 Reagents

1. 0.2 M Potassium phosphate buffer - pH 8.3 (100ml)

   Potassium dihydrogen phosphate - 2.72 g

   pH adjusted with 5 M sodium hydroxideand made upto 100 ml with D.D.H_2O

   It was stored in the cold room.

2. Sodium glycollate (10 μmoles/0.1 ml) - 100 ml

   Sodium glycollate - 98 mg

   Dissolved and made upto 100 ml with D.D.H_2O

   Stored in cold room.

3. 8 % TCA - 100 ml

   TCA - 8 g

   D.H_2O - 100 ml

4. 2 % phenyl hydrazine - 100 ml

   Phenyl hydrazine - 2 g

   D.H_2O - 100 ml

   Filtered the solution through a Whatman No.1 filter paper to get a clear solution, which was prepared just before use and kept in ice.

5. 10 % potassium ferricyanide - 100 ml

   Potassium ferricyanide - 10 g

   D.H_2O - 100 ml

   Prepared just before use and kept in ice.

6. Chilled concentrated hydrochloric acid (Analar)

7. 0.05 M sodium phosphate buffer - pH 7.0.

   Solution (a) - 50 ml

   Na_2HPO_4 - 445 mg

   D. H_2O - 50 ml

   Solution (b) - 50 ml

   NaH_2PO_4 - 390 mg

   D. H_2O - 50 ml

   To prepare 100 ml 0.05 M sodium phosphate buffer, mixed 30.5 ml solution (a) and 19.5 ml solution (b), made up to 100 ml with D. H_2O

8. Stock glyoxylic acid

Glyoxylic acid - 10 mg

$D.H_2O$ - 10 ml

Kept in a freezer

The standard solution was prepared by mixing 0.1 ml of stock solution in 4.9 ml of distilled water. This solution contained 20 μg/ml. It was prepared on the day of use and kept it in ice.

3.2.5.2.2 Procedure

The reaction mixture of GAO containing 1.0 ml of 0.2 M potassium phosphate buffer, pH 8.3 and 0.2 ml of liver homogenate (supernatent) was pre-incubated for 10 minutes at 37^0C. Then 0.1 ml of sodium glycollate was added and the incubation continued for 30 minutes with constant shaking at 37^0C. The reaction was stopped by adding 0.7 ml of 8 % TCA. Enzyme blanks were setup in which the homogenate was added after arresting the reaction with TCA. All the tubes were then centrifuged at 4000 rpm for 10 minutes and the glyoxylate present in the supernatent was estimated as bellow.

0.2 ml of the TCA supernatent was made up to 1.0 ml with distilled water, followed by the addition of 1.0 ml of 0.05 M sodium phosphate buffer pH 7.0. All the tubes were kept at 25^0C after 10 minutes of temperature equillibriation, 0.1 ml of phenyl hydrazine was added. Exactly after 2 minutes 1.2 ml of chilled concentrated HCl was added slowly within 1 minute after the addition of acid, 0.1 ml of 10 % potassium ferricyanide was added. The tubes were kept at 25^0C exactly for 2 minutes and the colour developed was read at 517 nm in a spectrophotometer. Standards were also set up side by side with different concentrations of sodium glyoxylate. The same procedure was followed from the addition of 0.05 M sodium phosphate buffer.

One unit of GAO is defined as the enzyme required to produce 0.1nanomole of glyoxylate per minute at 37^0C.

3.2.5.3 Lactate dehydrogenase (L-lactate: NAD oxido reductase, E.C.1.1.1.27).

The method adopted for the estimation of liver and kidney LDH was that of King (1965).

3.2.5.3.1 Principle

The method is based on the ability of LDH to convert lactate to pyruvate with the help of the coenzyme nicotinamide adenine dinucleotide (NAD+). The pyruvate formed is made to react with 2,4-dinitrophenyl hydrazine in hydrochloric acid. The hydrazone formed turned into an orange coloured complex in alkaline medium, which is measured at 420 nm in a spectrophotometer.

3.2.5.3.2 Reagents

1. 0.1 M glycine buffer - 1000 ml

Glycine	-	7.505 g
Sodium chloride	-	5.85 g

   Dissolved and made upto 1000 ml with $D.H_2O$

2. Buffered substrate - 200 ml

Glycine buffer	-	125 ml
0.1 N sodium hydroxide	-	75 ml
Lithium lactate	-	4 g

   Mixed well and kept in the cold room.

3. NAD - 10 ml

NAD	-	50 mg
$D.H_2O$	-	10 ml

   Kept at 0 - 4^0C.

4. 2,4-dinitrophenyl hydrazine - 100 ml

2,4-dinitrophenyl hydrazine	-	20 mg
Hot normal HCl (1 N)	-	100 ml

5. 0.4 N sodium hydroxide - 1000 ml

Sodium hydroxide	-	16 g

   Dissolved and made upto 1000 ml with $D.H_2O$

6. Standard sodium pyruvate

Sodium pyruvate	-	11 mg
Buffered substrate	-	100 ml

This solution contained 1 μmole of pyruvate/ml and was kept at 4^0C.

3.2.5.3.3 Procedure

Pipetted out 1.0 ml of buffered substrate and 0.1 ml of tissue homogenate (supernatent) in to each of the two tubes and placed both tubes in a water bath at 37^0C. The tubes were allowed to reach the temperature of the bath (for 10 minutes). Then to one tube (the test) 0.2 ml of NAD solution was added and shaken well. The tubes were once again incubated at 37^0C for another 20 minutes. Exactly after that time the reaction was stopped by adding 1.0 ml of the dinitrophenyl hydrazine reagent to each tube, and shaken well to mix, and the other tube (blank) 0.2 ml NAD was added, mixed. It was once again left in a water bath, for further 20 minutes. After removal from the bath 9.9 ml of 0.4 N sodium hydroxide was added and read at 420 nm in a spectrophotometer within 1 to 5 minutes of adding the alkali.

One LDH unit is defined as the enzyme required to produce 0.1 micromole of pyruvate per minute at 37^0C.

3.2.5.4 Estimation of protein

The protein content of the tissue was estimated by the method of Lowry *et al.* (1951).

3.2.5.4.1 Reagents

1. Alkaline copper reagent (Protein reagent)
   (a) 2 % Na_2CO_3 in 0.1 M NaOH.
   (b) 1 % $CuSO_4$
   (c) 2 % Sodium potassium tartarate

   Protein reagent was prepared at the time of use by mixing the above solution in the ratio 100 (a): 1 (b): 1(c).
2. Folin's reagent - 1:1 ratio

Folin's reagent - 1 ml

$D.H_2O$ - 1 ml

Mixed and stored at 4^0C.

3. Standard Bovine Serum Albumin (BSA) (1 mg / ml)

BSA - 100 mg

$D.H_2O$ - 100 ml

3.2.5.4.2 Procedure

10 µl of the tissue homogenate was taken and made up to 1 ml with water. To this 4 ml of protein reagent was added, mixed and allowed to stand at room temperature for 10 minutes. Later 0.5 ml of Folin's phenol reagent was added and shaken well. The water blank and standards were treated in a similar manner. The sample mixture was kept for 30 minutes at room temperature. The blue colour formed was measured at 700 nm in a spectrophotometer.

3.2.6 Estimations in Kidney

Assay of enzymes

3.2.6.1 Preparation of tissue homogenate - (As in section 3.2.5.1)

3.2.6.2 Lactate dehydrogenase - (As in section 3.2.5.3)

3.2.6.3 Protein estimation - (As in section 3.2.5.4)

3.2.6.4 Estimation of oxalate

3.2.6.4.1 Preparation of tissue homogenate

0.3 g of kidney tissue was homogenized in 3 ml 0.9 % KCl, acidified with concentrated HCl (1ml/100 ml). The aliquots were used for the estimation of oxalic acid. Kidney oxalate was measured using a modified procedure of Hodgkinson and Williams (1972).

3.2.6.4.2 Reagents

1. Electrolytic zinc

Metalic zinc pieces of about 250 mg were cleaned just before used by immersing briefly in freshly prepared 10 N HNO_3. After washing thoroughly in distilled water, the zinc was ready for use.

2. Chromotropic acid solution - 1 %

Chromotropic acid - 1 g

Mixed, made upto 100 ml with D.H_2O, filtered and stored at 4^0C.

3. Oxalic acid standard

Potassium oxalate monohydrate - 1.023 g

Mixed, made upto 100 ml with D.H_2O

This solution contained 5 mg of anhydrous oxalic acid/ml.

3.2.6.4.3 Procedure

0.1 ml of the above aliquot was taken in a boiling tube and made up to 1 ml with distilled water. 1 ml of 4 N H_2SO_4 was added. Then added a piece of freshly cleaned zinc, heated in a boiling water bath for 30 minutes. Removed zinc, washed with 0.5 ml of 1 % chromotropic acid solution, added the washings of the tube. Standards in the range of 50 µg into 250 µg were taken and treated as above. Added 0.5 ml of concentrated sulphuric acid to both standards and test solutions. Heated for 30 minutes in boiling water bath. Added 15 ml of 10 N sulphuric acid to all the tubes through their sides slowly with constant stirring. Cooled and read at 570 nm in a spectrophotometer. The colour was stable for several hours.

The values are expressed as mg/g wet tissue.

3.2.6.5 Estimation of stone forming constituents

Kidney tissue was digested according to the method of Ballentine and Barford (1957).

3.2.6.5.1 Wet ashing

A known weight of kidney tissue was taken in a pyrex Kjeldahl Flask. To this 5 ml of concentrated nitric acid was added, followed by 1.0 ml of perchloric acid. The

same was then digested over a sand bath until the solution become pale yellow in colour. If the colour of the digest was brown, more of nitric acid and perchloric acid were added and the oxidation was repeated. The final solution was made up to a known volume. Aliquots of this were used to estimate the inorganic constituents, calcium and phosphorus.

3.2.6.5.2 Estimation of calcium - (As in section 2.2.2)

3.2.6.5.3 Estimation of Phosphorous

The phosphorous content was estimated by the method of Fiske and Subbarow (1925).

3.2.6.5.3.1 Reagents

1. Ammonium molybdate I solution

   2.5 % ammonium molybdate in 3 N sulphuric acid.

2. Ammonium molybdate II solution

   2.5 % ammonium molybdate in 5 N sulphuric acid.

3. ANSA

ANSA	-	50 mg
15 % sodium bisulphite	-	195 ml
20 % sodium sulfate	-	5 ml

   Stored in a brown bottle at 0^0C.

4. Stock standard solution - 100 ml (80 µg of phosphorus / ml)

Potassium dihydrogen phosphate	-	35.1 mg

   Dissolved and made upto 100 ml with D.H_2O

3.2.6.5.3.2 Procedure

0.5ml of the wet ashing solution was made up to 2.0 ml with distilled water. To this added 1ml of ammonium molybdate II solution. Standards in the range of 4 µg to 20 µg were taken and made up to 2 ml with water and to each of these was added 1 ml of ammonium molybdate I reagent. Along with the standard a blank was also run. To all the tubes 0.4 ml ANSA reagent was added and mixed gently after each addition. Kept at

room temperature for 10 minutes and the absorbance was measured at 600 nm in a spectrophotometer.

The values are expressed as mg/g wet tissues.

3.2.7 Analysis in urine

Preserved 24 h urine samples were analyzed for the following constituents after making 10 ml with distilled water.

3.2.7.1 Estimation of calcium

The method adopted was that of Gindler and King (1972) as in section -2.2.3

3.2.7.2 Estimation of phosphorous

(Fiske and Subbarow (1925) - (As in section 3.2.6.5.3)

3.2.7.3 Estimation of oxalate

Pretreatment of the urine samples were performed using a modified method of Hodgkinson and Williams (1972).

3.2.7.3.1 Procedure

Acidified the urine samples by adding concentrated HCl (1 ml/100 ml urine) to ensure the solubilisation of any crystal of calcium oxalate which may present. Measured 0.5 ml of urine in to a 25 ml graduated stoppered centrifuge tube followed by 1.5 ml of water and 1 drop of 0.04 % bromothymol blue indicator solution. Adjusted the solution to pH 7.0 (green) by the addition of dilute sodium hydroxide or dilute acetic acid solution. Added 2.0 ml of saturated aqueous solution of calcium sulfate followed by 14 ml of ethanol, mixed gently and allowed the solution to stand at room temperature for atleast 3 h (preferably overnight). Centrifuged at 2000 rpm for 10 minutes, carefully decanted the supernatent fluid and allowed the tube to drain for a few minutes on a filter paper. Wiped the mouth of the tube and dissolved the precipitate in 2.0 ml of 2 N H_2SO_4 solution. The remaining part of the procedure for urinary oxalate estimation was the same as described in earlier section 3.2.6.4

3.3 Results

The effect of administering glycollate and mono and dicarboxylic acids on the body, liver and kidney weight along with the tissue protein levels are presented in table 3.1. The treatment did not produce any significant change in any of them, when compared with the pair fed control rats.

3.3.1 Changes in liver and kidney enzymes

The effect of treatment on the levels of oxalate synthesizing enzymes, liver GAO and LDH and kidney LDH are shown in table 3.2. Since GAO is present only in the liver, its activity was not determined in the kidneys. A significant increase in GAO activity ($p<0.001$) was observed in glycollate administered rats (group II) (2.12 ± 0.11 units/mg protein) from that of group I controls (1.21 ± 0.60 units/mg protein). A slight reduction in GAO activity was observed in glycollate fed rats treated with L (+) tataric acid, maleic acid, malic acid, succinic acid, pyruvic acid and galic acids (group III to group VIII) (1.59 ± 0.90, 1.61 ± 0.11, 1.67 ± 0.12, 1.69 ± 0.13, 1.71 ± 0.12, 1.75 ± 0.18, compared with group II (2.12 ± 0.11 units/mg protein respectively). Interestingly L(+) tartaric acid , maleic acid and malic acid found to be more effective in reducing GAO level.

LDH, that regulates many biochemical reactions in the body was found to increase slightly in the liver of glycollate administered group II rats (0.67 ± 0.05 unit/mg protein), when compared with group I controls (0.52 ± 0.03 units/mg protein). No drastic change was observed in the liver LDH, in mono and dicarboxylic acid administered hyperoxaluric rats (group III to group VIII) from that of group II (hyperoxaluric rats). Kidney LDH exhibited a pattern similar to the above. It was significantly ($p<0.05$) elevated in the glycollate fed group II rats as compared to the control (0.72 ± 0.05 and 0.54 ± 0.05 units/mg protein respectively). Dicarboxylic acid treatment in hypeoxaluric rats (group III to VIII) lowered the kidney LDH when compared with group II (0.58 ± 0.04, 0.60 ± 0.05, 0.61 ± 0.05, 0.64 ± 0.04, 0.64 ± 0.04 and 0.66 ± 0.05 units/mg protein respectively and 0.72 ± 0.05 in group II hyperoxaluric rats).

3.3.2 Calcium, oxalate and phosphorous in kidney tissue

The extent of deposition of the stone components in the kidney tissue as affected by various treatments was looked in to and suitable comparisons were made (table 3.3, fig. 3.1). The calcium deposits in the renal tissue were high in glycollate administered group II rats (0.46 ± 0.04 mg/g wet tissue and 0.38 ± 0.02 in group I control, respectively). L(+) tartaric acid , maleic acid, malic acid, succinic acid, pyruvic acid and and galic acid administration slightly reduced the kidney calcium in glycollate treated rats (group III to group VIII; 0.39 ± 0.03, 0.40 ± 0.03, 0.41 ± 0.04, 0.40 ± 0.03, 0.41 ± 0.04 and 0.41 ± 0.04 respectively)

Glycollate administration enhanced the renal deposition of oxalate (0.69 ± 0.06 and 0.57 ± 0.06 in group II and group I respectively). Kidney oxalate content was lowered in mono and dicarboxylic acid treated hyperoxaluric rats (group III to VIII), when compared with group II.

Analysis of phosphorus in the kidney tissue revealed an enhancement in the glycollate treated group II rats (2.26 ± 0.18 and 1.86 ± 0.09 mg/g wet tissue in group II and group I respectively.

3.3.3 Changes in the urinary excretion pattern of calcium, oxalate and phosphorous

The 24 h urinary excretion of calcium, oxalate and phosphorous as assessed after the administration of glycollate and mono and dicarboxylic acids are given in table 3.4 and fig 3.2)

The urinary excretion of calcium was high in glycollate administered group II rats (1.91 ± 0.18 mg/24h) as compared with group I control (1.43 ± 0.11 mg/24h). Administration of L(+) tartaric acid , maleic acid and malic acid in hyperoxaluric rats brought about a slight reduction in the urinary calcium level (1.48 ± 0.09, 1.51 ± 0.13, 1.54 ± 0.14 mg/24h respectively) whereas succinic acid pyruvic acid and galic acid

reduced the calcium excretion level (1.60 ± 0.15, 1.62 ± 0.16 and 1.65 ± 0.18 mg/24h respectively) as compared with group II rats.

As per the expectation, the excretion of oxalate was significantly ($p<0.001$) increased in the glycollate fed group II rats, resulting in hyperoxaluria (2.7 ± 0.17 and 0.43 ± 0.03 mg/24h urine, group II and group I rats respectively). L(+) tartaric acid maleic acid, malic acid, succinic acid, pyruvic acid and galic acid significantly ($p<0.001$) reduced the oxalate levels (0.91 ± 0.09, 0.99 ± 0.07, 1.03 ± 0.09, 1.35 ± 0.11, 1.38 ± 0.12 and 1.55 ± 0.14 respectively)

Phosphorous level was found to be slightly elevated in the glycollate administered rats (group II; 3.57 ± 0.31) when compared with group I controls (3.11 = 0.21 mg/24h). Mono and dicarboxylic acid treatment in hyperoxaluric rats slightly reduces the phosphorous level.

Table- 3.1 Effect of glycollate, mono and dicarboxylic acids on the body weight, tissue weight (liver and kidney) and protein content of experimental rats (values are ± SEM of 5-6 separate experiments)

Parameters	Group I Control	Group II Glycollate	Group III Glycollate and L(+) tartarate	Group IV Glycollate and Maleic acid	Group V Glycollate and Malic acid	Group VI Glycollate and Succinic acid	Group VII Glycollate and Pyruvic acid	Group VIII Glycollate and Galic acid
Increase in body wt. after 14 days	2.55± 0.31	2.16 ± 0.12	2.46± 0.23	2.40± 0.19	2.38± 0.17	2.25± 0.17	2.22± 0.16	2.30± 0.18
Liver wt. (g/100g body wt.)	3.54 ± 0.30	3.48± 0.30	3.52± 0.30	3.51± 0.29	3.53± 0.21	3.50 ± 0.30	3.53± 0.31	3.52± 0.29
Kidney wt. (g/100g body wt.)	0.89± 0.08	0.97± 0.08	0.91± 0.08	0.93 ± 0.09	0.92± 0.09	0.95± 0.08	0.96± 0.09	0.97± 0.08
Liver protein (mg/g wet tissue)	168.31± 7.33	158.14± 6.80	162.89± 7.10	161.13± 6.98	164.38± 7.13	161.33± 7.05	162.41± 7.10	159.99 ± 6.98
Kidney protein (mg/g body wt.)	183.91± 9.08	177.18 ±7.80	181.08± 7.38	179.79± 7.33	182.77± 7.10	179.79 ± 7.10	178.48 ± 7.23	177.08± 6.99

Table-3.2.The effect of glycollate and carboxylic acid administration on the level of oxalate synthesising enzymes,GAO & LDH
[Values are mean ± SEM of 5-6 separate experiments]

Enzyme (units/ mg. of protein)	Group I Control	GroupII Glycollate	Group III Glycollate and L(+)tartarate	Group IV Glycollate and Maleic acid	Group V Glycollate and Malic acid	Group VI Glycollate and Succinic acid	Group VII Glycollate and Pyruvic acid	Group VIII Glycollate and Galic acid
Liver GAO	1.21± 0.06	2.12± 0.11***	1.59± 0.09	1.61± 0.11	1.67± 0.12	1.69± 0.13	1.71± 0.12	1.75± 0.18*
Liver LDH	0.52± 0.03	0.67± 0.05	0.55± 0.04	0.56± 0.04	0.57± 0.05	0.57± 0.03	0.59± 0.03	0.60± 0.01
Kidney LDH	0.54± 0.05	0.72±* 0.05	0.58± 0.04	0.60± 0.05	0.61± 0.05	0.64± 0.04	0.64± 0.04	0.66± 0.05

GAO- glycolic acid oxidase, LDH- Lactate dehydrogenase
One unit of GAO- enzyme required to produce 0.1nanomole of glycoxilate/min at 37^0C.
One unit of LDH- Enzyme required to produce 0.1µmole of pyruvate/min at 37^0C.

***P<0.001 and *P<0.05 when compared to control.

Table-3.3. Kidney tissue deposition of oxalate, calcium and phosphorus
(Values are mean ± SEM of 5-6 separate experiments.)

Parameters (mg/g wet tissue).	Group I Control	Group II Glycollate	Group III Glycollate and L(+) tartarate	Group IV Glycollate and Maleic acid	Group V Glycollate and Malic acid	Group VI Glycollate and Succinic acid	Group VII Glycollate and Pyruvic acid	Group VIII Glycollate and Galic acid
Oxalate	0.57± 0.06	0.69± 0.06	0.59± 0. 05	0.61± 0.04	0.61± 0.04	0.62± 0.05	0.63± 0.06	0.64± 0.06
Calcium	0.38± 0.02	0.46± 0.04	0.39± 0.03	0.40± 0.03	0.41± 0.04	0.40± 0.03	0.41± 0.04	0.41± 0.04
Phosphorus	1.86± 0.09	2.26± 0.18	1.91± 0.11	1.98± 0.17	2.08± 0.12	2.11± 0.15	2.15± 0.12	2.18± 0.14

Figure 3.1 Stone forming constituents in the kidney

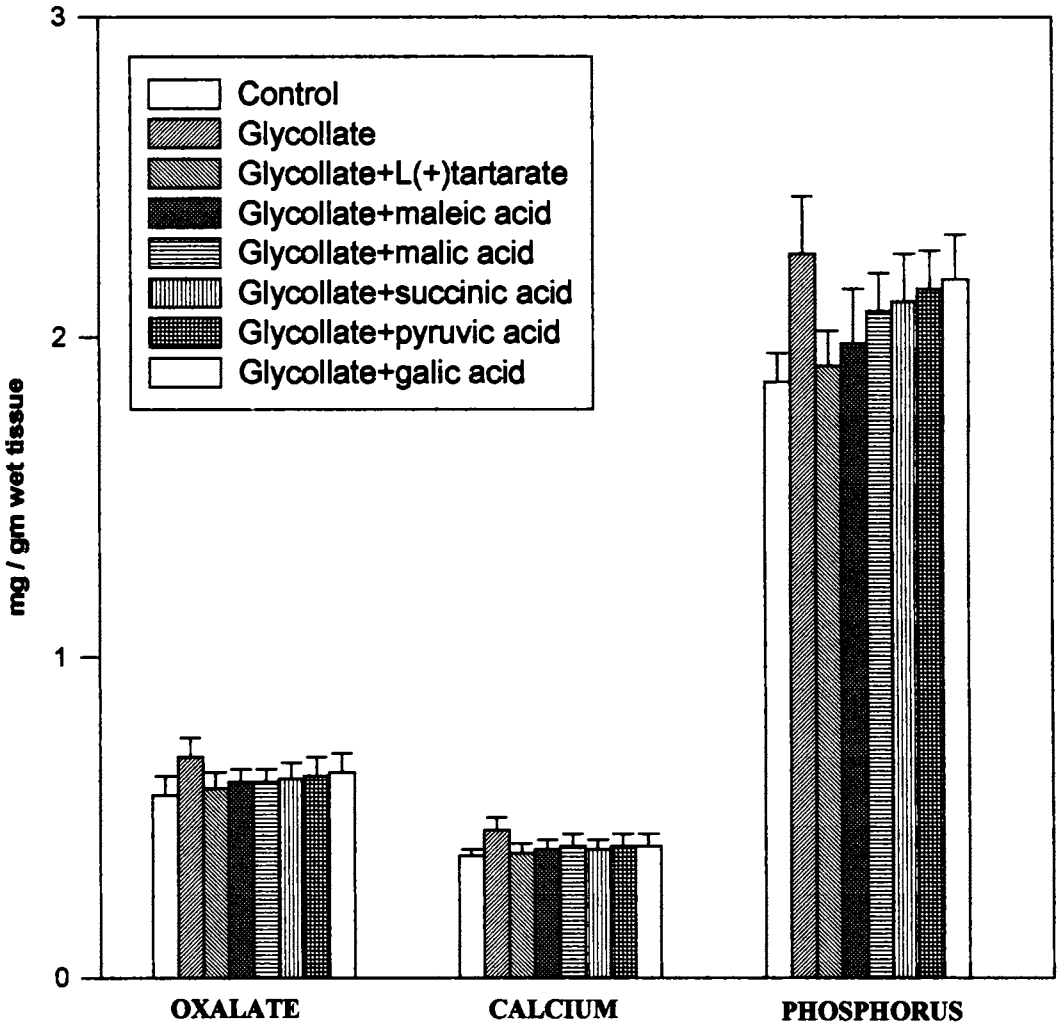
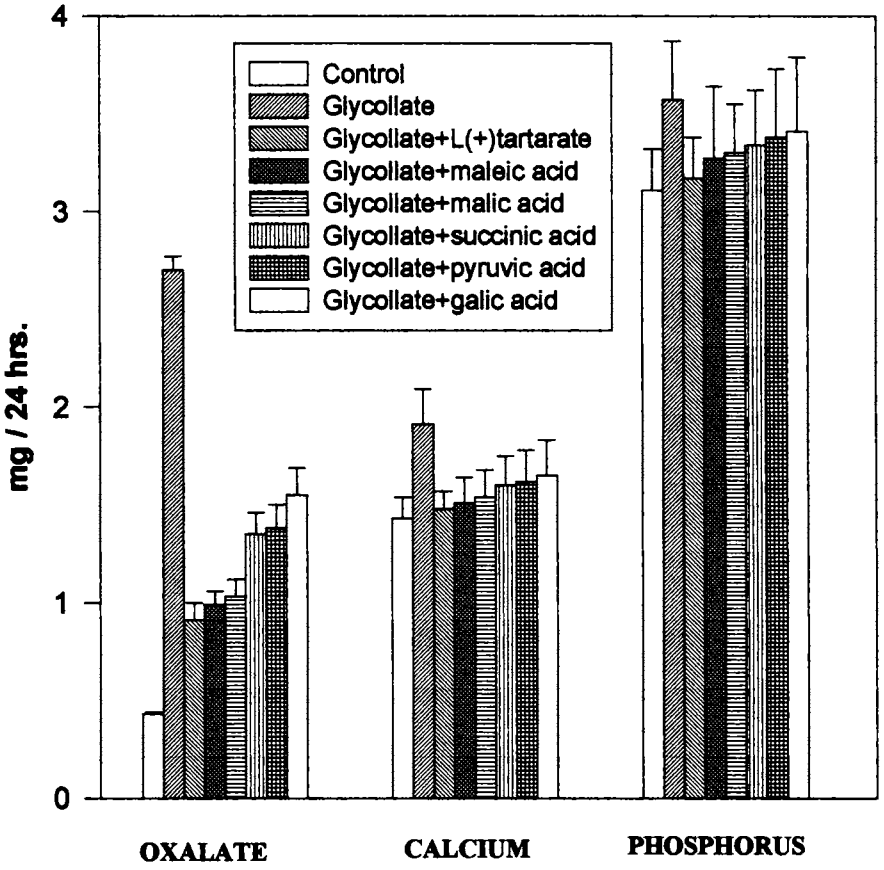


Table- 3.4. 24h. urinary excretion of oxalate, calcium, and phosphate.
(Values are mean ± SEM of 5-6 separate experiments.)

Parameters (mg/24h. urine.	Group I Control	Group II Glycollate	Group III Glycollate and L(+)tartarate	Group IV Glycollate and Maleic acid	Group V Glycollate and Malic acid	Group VI Glycollate and Succinic acid	Group VII Glycollate and Pyruvic acid	Group VIII Glycollate and Galic acid
Oxalate	0.43± 0.01	2.70± 0.07***	0.91±* 0.09♦♦♦	0.99±** 0.07♦♦♦	1.03±** 0.09♦♦♦	1.35±*** 0.11♦♦♦	1.38±*** 0.12♦♦♦	1.55±*** 0.14♦♦♦
Calcium	1.43± 0.11	1.91± 0.18	1.48± 0.09	1.51± 0.13	1.54± 0.14	1.60± 0.15	1.62± 0.16	1.65± 0.18
Phosphorous	3.11± 0.21	3.57± 0.3	3.17± 0.21	3.27± 0.37	3.30± 0.25	3.34± 0.28	3.38± 0.35	3.41± 0.38

♦♦♦P<0.001 when compared to group II.
***P<0.001, **P< 0.01 and *P<0.05 when compared to control.

Figure 3.2 24 hrs. urinary excretion of oxalate, calcium and phosphorus



3.4. Discussion

Hyperoxaluria was produced in rats with the administration of sodium glycollate, for a week, which is one of the major precursor and it has been used successfully by many others (Chow *et al.*, 1975; Moorthy *et al.*, 1983 and Selvam and Varalakshmi, 1989).

Glycollate is converted to glyoxylate by oxidation, catalyzed by the liver enzyme GAO, which is a flavin linked peroxisomal enzyme (Masters and Holmes, 1977). It has been found to have a greater affinity for glycollate than glyoxylate (Ushijima, 1973).

Reduction in oxalate synthesis would be the most direct therapeutic approach for which, glyoxylate, the immediate precursor of oxalate has to be reduced to glycollate, which has then to be transaminated to glycine (Dean *et al.*, 1968). Alternatively glyoxylate has to be decarboxylated by TPP-dependent reaction catalyzed by 2-oxoglutarate: glyoxylate carboligase (Williams and Smith, 1972). Since with the possible exceptions of ascorbic acid (Baker, *et al.*, 1966) and the aromatic amino acids (Cook and Henderson, 1969) all known oxalate precursors and metabolized via glyoxylate, the inhibition of the oxidation of glyoxylate to oxalate is the most reasonable site for regulation. (Liao and Richardson, 1972).

Liver is well established to be the major site of endogenous oxalate synthesis and two major enzymes involved in oxalate synthesis, GAO and LDH. GAO is predominantly localized in the liver, which has a great affinity for glycollate than glyoxylate. LDH is a cytosolic enzyme present in most of the body tissues and fluids, which regulate many biochemical reactions. According to certain reports, LDH seemed to be the major enzyme in oxalate synthesis (Gibbs and Watts, 1973). It is able to catalyse the simultaneous oxidation and reduction of glyoxylate (Warren, 1970). Studies with hepatectomised rats have provided evidence that GAO is the major enzyme in oxalate synthesis in the liver, the primary site of oxalate formation (Farinelli and Richardson, 1983; Varalakshmi and Richardson, 1983a). GAO was lowered significantly by castration whereas LDH activity was not affected. The possible correlation between

GAO play an important role in the control of endogenous oxalate formation in rats. Thus attempts were directed towards inhibition of GAO which may allow a more selective reduction of oxalate synthesis without disruption of metabolic process.

As per the expectation, glycollate administration significantly increased the oxalate excretion in group II rats (table 3.3). The levels were lowered in the dicarboxylic acid treated hyperoxaluric rats, but were not brought back to normal level. A reduction of oxalate excretion reflects lowered endogenous synthesis of oxalate from glycollate by inhibiting liver GAO.

Fry and Richardson (1979) have described the inhibitory effects of some mono and dicarboxylic acids on liver GAO preparation. Later Sur *et al.* (1981) and Rose and Hallson (1984) had demonstrated the inhibitory action of tartarate on calcium oxalate crystallization in urine. Saso *et al.* (1998) demonstrated the inhibitory action of certain dicarboxylic acids on calcium oxalate crystallization lending support to our findings. Our findings have obvious implications for the development of mono and dicarboxylic acids as therapeutic agents.

Chapter 4

BIOCHEMICAL EFFECT OF DICARBOXYLIC ACIDS ON OXALATE METABOLISM IN EXPERIMENTAL STONE FORMING RATS -LONG TERM STUDIES

CHAPTER- IV

BIOCHEMICAL EFFECT OF DICARBOXYLIC ACIDS ON OXALATE METABOLISM IN EXPERIMENTAL STONE FORMING RATS - LONG TERM STUDIES

4.1 Introduction

Urinary stone disease has afflicted humankind since antiquity. A bladder stone was found in an Egyptian skeleton more than 7000 years old (Riches, 1968). The problem of urolithiasis dates back to the days of Hippocrates and even in India it has been an ancient one, as mentioned in the old Samhitas like Charaka (210 B.C. - 170 A.D.) and Sushruta (176 A.D. - 340 A.D.), where the non-operative management of stone disease was practiced by the indigenous medical practitioners, with several herbo-mineral preparations. Many advances have been made in the evolution and management of patients who form stones. The historical panorama reveals several fundamental changes in the understanding and treatment of this disorder.

The risk factors and the mechanisms favouring calcium oxalate stone formation are still awaiting solution in the biomedical field. The occurrence of urinary tract stones and their location has a characteristic geographical distribution. The major emphasis of current research is in the area of oxalate stones because of the wide spread occurrence (Prien and Frondel, 1947; Varalakshmi *et al.*, 1976) and difficult treatment. Other types of stones have well established diagnostic and treatment criteria.

Calcium oxalate stones are the predominant variety. In India close to 90% of the urinary stones are pure calcium oxalate. Whewellite and weddelite are the monohydrate and dihydrate forms of calcium oxalate, existing in the crystalline form in urinary calculi and urine. The monohydrate form is more common in stone than the dihydrate, because of the greater stability of whewellite.

The formation of kidney stone is a consequence of increased urinary supersaturation with subsequent formation of crystalline particle. Calcium oxalate is

insoluble over the urinary pH range of 4.5 to 8.0. So growth can produce in both acid and alkaline urine. The stones are dense, hard and often difficult to be cut. Their structure usually consists of concentric bands of fine grained material.

Stone formation is a biological process that involves a physicochemical aspect which leads to crystallization (Kok *et al.* 1988). Urinary stones or concretions are essentially crystalline in nature, but in union with an amorphous proteinaceous material referred to as the organic matrix, the frame work of the stone, constitutes about 2.5 to 5% of the dry weight (Boyce and Gravey, 1956). Several compounds have been isolated from the soluble part of the organic matrix of kidney stone, namely glyco and mucoproteins including matrix substance A and Tamm - Horsefall mucoprotein both of which are antigenic (Boyce *et al.*, 1962; Hess *et al.*, 1989). The organic matrix is structurally related to the gross morphology of concretion and has a definite architectonic role in the morphology of stones (Boyce *et al.*, 1968) Vermeulen and his co-workers (1968) were put forth the crystallization concepts, a stone is essentially a crystalline material and the matrix is an adventitious inclusions during growth. However, the interrelationship between the matrix and the crystals and their relative importance in calculogenesis are not clear.

Significant advances in the surgical and medical management of calcium oxalate nephrolithiasis during the last two decades have improved the outlook for many recurrent stone formers (Lisa, 1997). Calcium oxalate stone patients usually have recurrence but do not exhibit any metabolic, hormonal or pathological abnormalities (Randall, 1936). Patients with recurrent stones, no etiologic factor can be deductant hence they are referred to as idiopathic. Most of them however exhibit mild hyperoxaluria (Varalakshmi and Anandam, 1979, Marangella *et al.*, 1982). Idiopathic calcium oxalate lithiasis has a male preponderance and it seems to be influenced by sex hormones (Richardson, 1967; Lee *et al.*, 1992, and Iguchi *et al.*, 1999).

Urine is a highly complex solution, containing under normal circumstances, a favourable balance of crystalloids and colloids. Several factors are involved in the crystallization process, the major factors are supersaturation, matrix initiation, deficiency

of inhibitors, presence of promoters, epitaxy and combinations of these. The sequence of events leading to urinary stone formation is as follows.

Saturation → Supersaturation → Nucleation → Crystal growth or aggregation → crystal retention → stone formation (Balaji and Menon, 1997).

The presence of urinary inhibitors has attracted lot of research and several interesting and conflicting observations have been made (Dent and Sutor, 1971; Fleisch, 1978; Tiselius, 1987; Edyvane, 1987). Deficiency of urinary inhibitors in stone formers seems to be the answer to the problem of stone pathogenesis. Inhibitors in urine affect formation, growth and aggregation of crystals (Robertson *et al.*, 1973;; Fellstrom *et al.*, 1982; Miyake *et al.*, 1998).

During calculus formation it is suggested that the renal papilla has a central role (Randall, 1936; Vermculan *et al.*, 1967). A concentration gradient of calcium and oxalate has been demonstrated between the renal papilla, medulla and cortex (Wright and Hodgkinson, 1972; Hautman *et al.*, 1980). Kidney stones may not produce any symptoms as signs for a long time while in others, it can be painless with haematuria or vague lain pains. The typical symptom of upper urinary tract calculi is the sudden severe colicky pain starting in the region of the kidney and occassionally radiating downwards into the groin. This is due to either a renal pelvis, which gets impacted into the pelvi-ureteral junction or in any part of the ureter downwards. The pain is sharp and excruciating and is relieved only when the impacted stone passed down the ureter in to the bladder.

Urinary stone disorder is a multifactorial one arising from an abnormal combination of a number of risk factors. The method of prevention is still not clear. Surgical removal of stone is the method of choice but is found to cause recurrence and ultimately the kidneys gets diseased. Extracorporial shock wave lithotripsy (ESWL) is useful in disintegration of the stones. However in order to prevent the recurrence selective therapy / prophylaxis is necessary.

Since the major source of oxalate is from the endogenous synthesis in the liver, the more rational approach to control hyperoxaluria will be to curtail the synthesis. This is possible by affecting the enzyme systems - GAO and LDH so as to prevent the conversion of the immediate precursor of oxalate. Based on this reasoning several structurally resembling compounds of oxalate were tried (Smith *et al.*, 1972). Oxamate hydrazide was found to be a potent inhibitor of LDH - catalyzed oxalate synthesis. n-Heptonoate and DL- phenyl lactate were found to inhibit GAO (Liao and Richardson, 1973). However, these compounds have failed in the *in vivo* situation and some of them are not free from toxic effects. Certain mono and dicarboxylic acids were found to inhibit liver GAO (Fry and Richardson, 1979). Succinate and succimide therapy have proved effective in hyperoxaluric conditions (Yagisawa *et al.*, 1998). It was of interest to study whether L(+) tartarate, maleic acid and malic acid, might affect the endogenous oxalate synthesis. Reports by Sur *et al.* (1981) Hallson and Rose (1984), Rose and Hallson (1984) and Selvam and Varalakshmi (1990 and 1992) have suggested the use of tartarate to prevent urolithiasis. Our preliminary works (Chapter III - short term studies), with mono and dicarboxylic acids such as tartaric acid, maleic acid, malic acid, succinic acid, pyruvic acid and galic acid, in hyperoxaluric rats, yielded results favouring regulation of oxalate metabolism. Hence the three major dicarboxylic acids, L(+) tartaric acid , maleic acid and malic acids were selected with the primary aim of investigating its effect on oxalate metabolizing enzymes in the liver and kidneys in experimental calculogenic rats. The secondary objective was to investigate the effect of these dicarboxylic acids on renal deposition of calcium, oxalate and phosphorous and lastly, urinary excretion pattern of calcium, oxalate, phosphorous and magnesium were studied.

The observations made on the above lines have been discussed in the light of the current literature.

4.2 Materials and Methods

4.2.1 Biochemicals and their sources

Biochemicals used in the present study were mostly of analytical grade purchased locally from SRL, HI MEDIA and MERCK, India., Sodium glycollate, glyoxylic acid,

tartaric acid and all the chemicals used for Electron microscopic experiments were purchased from Sigma Chemical Co., U.S.A.

4.2.2 Experimental animals

Adult male albino rats of Wistar strains, weighing between 150 - 250 g, obtained from Small Animal Breeding Center, KAU, Mannuthi, were used for the experimental studies. The animals were acclimatized to the animal house condition for ten days. The animals were fed with commercial rat field and water was given *ad libitum.*

4.2.3 Experimental induction of calcium oxalate lithiasis in rats

The method adopted was that of Chow *et al.* (1975).

4.2.4 Preparation of calculi producing diet (CPD)

The method adopted was that of Chow *et al.* (1975). Commercial rat feed was finely powdered, supplimented with sodium glycollate (3 % concentration, w/w) and pelleted with water. The pellets were dried in an oven at 30°C for 2 days and used as the calculi producing diet.

4.2.5 Experimental setup

The animals were divided into eleven groups comprising of six animals in each.

Group I	-	Received commercial diet and served as control.
Group II	-	Rats fed with CPD for 30 days to induce stone formation.
Group III	-	Received CPD for 30 days followed by L(+) tartaric acid (50 mg/ml of 0.9% saline/day/rat) for 15 days.
Group IV	-	Received CPD for 30 days followed by maleic acid (50 mg/ml of 0.9% saline/day/rat) for 15 days.
Group V	-	Received CPD for 30 days followed by malic acid (50 mg/ml of 0.9% saline/day/rat) for 15 days.
Group VI	-	Received CPD with L(+) tartaric acid (50 mg/ml of 0.9% saline/day/rat) for 30 days.
Group VII	-	Received CPD with maleic acid (50 mg/ml of 0.9%

saline/day/rat) for 30 days.

Group VIII - Received CPD with malic acid (50 mg/ml of 0.9% saline/day/rat) for 30 days.

Group IX - Received L(+) tartaric acid (50 mg/ml of 0.9% saline/day/rat) for 30 days.

Group X - Received maleic acid (50 mg/ml of 0.9% saline/day/rat) for 30 days.

Group XI - Received malic acid (50 mg/ml of 0.9% saline/day/rat) for 30 days.

After the experimental period, 24 h urine samples were collected using hydrochloric acid as preservative. The animals were sacrificed by decapitation, liver and kidneys were excised from the body and their weights were recorded, a small portion of both kidney and liver tissues were preserved for histopathological and electron microscopic studies.

Investigations carried out

The following estimations were carried out in the liver and kidney.

4.2.6 Preparation of tissue homogenate.

A 10% of homogenate of the washed tissue were prepared in 0.01M sodium phosphate buffer - pH 7. The homogenate was subjected to centrifugation at 12000 rpm for 30 minutes at 4^0C. The supernatant fraction was used for the assays of the following

Oxalate synthesising enzymes

4.2.6.1 Glycollate oxidase (GAO)

The activity of the enzyme, GAO was assayed by the method of Lui and Roels (1970) as in section 3.2.5.2.

4.2.6.2 Lactate dehydrogenase

The activity of the enzyme in the liver and kidney tissues was estimated according to the method of King (1965) as in section 3.2.5.3.

4.2.6.3 Protein estimation

Protein content of the tissues was estimated by the method of Lowry et al (1951) as in section 3.2.5.4.

4.2.7 The following investigations were carried out in kidney tissue.

4.2.7.1 Estimation of oxalate - As in section 3.2.6.4.

4.2.7.2 Estimation of phosphorous

Phosphorous estimation was carried out according to the method of Fiske and Subbarow (1925) as in section 3.2.6.5.3.

4.2.7.3 Estimation of calcium

Calcium estimation was carried out according to the method of Gindler and King (1972) as in section 2.2.3.

4.2.8 Analysis in urine

Preserved urine samples were analyzed for oxalate, calcium, phosphorous and magnesium.

4.2.8.1 Urinary calcium, phosphorous and oxalate, were estimated as in sections 2.2.3, 3.2.6.5.3, 3.2.7.3 respectively.

4.2.8.2 Estimation of magnesium

Magnesium estimation was carried out by the method of Neill and Neely (1956).

4.2.8.2.1 Reagents

1. 0.05% titan yellow - 200 ml

   Powdered titan yellow - 0.1 g

   $D.H_2O$ - 200 ml

2. 2 N NaOH
3. 0.1% gum ghatti

4. Stock standard (1 mg/ml) - 100 ml

$MgCl_2$ $6H_2O$ - 8.458 g

Dissolved and made upto 100 ml with $D.H_2O$

5. Working standard (5 µg/ml)

Stock standard - 1 ml

$D.H_2O$ - 199 ml

6. Calcium chloride solution

$CaCl_2$ - 13.88 mg

Dissolved and made upto 100 ml with $D.H_2O$

4.2.8.2.2 Procedure

1 ml of the urine sample was made up to 2 ml with water. To this 1 ml of titan yellow, 1 ml of gum ghatti, 2 ml of 2 N NaOH and 1 ml of calcium chloride were added. Standards in the range of 10-50 µg and a blank with 1ml distilled water were also treated as above. The purple colour formed was read at 620 nm using a spectrophotometer.

Values are expressed as mg/24 h urine.

4.2.9 Histopathological studies

Histopathological studies were carried out in liver and kidney tissues.

4.2.9.1 Reagents

1. Bauin's fluid

Picric acid (aqueous saturated) - 75 ml

37-40% Formaldehyde - 25 ml

Acetic acid - 5 ml

2. Aqueous eosin - 1 %

Aqueous eosin - 3 g

Dissolved and made upto 300 ml with $D.H_2O$

3. Alcoholic eosin - 1%

Alcoholic eosin - 3 g

70% ethanol - 300 ml

4. Harris Hematoxylin solution

Hematoxylin crystals	-	5 g
Absolute alcohol	-	50 ml
Ammonium/potassium aluminium sulphate	-	100 g
D.H_2O	-	1000 ml
Mercuric oxide (red)	-	2.5 g

Hematoxylin was dissolved in alcohol and then dissolved the alum in hot water. The two solutions were mixed and then boiled as rapidly as possible. Then mercuric oxide was added slowly and the solution was reheated (for one minute) until it became dark purple. Then the solution was cooled, 40 ml acetic acid was added. Filtered the solution before use.

5. Acid: Alcohol mixture	-	100 ml
Concentrated HCl	-	1 ml
70% Alcohol	-	99 ml
6. Ammonia water		
Liquid ammonia	-	1 ml
D.H_2O	-	99 ml

4.2.9.2 Procedure

4.2.9.2.1 Fixation

Immersion fixation procedure was carried out. Kidney and liver tissues were cut into small bits and fixed by keeping in Bauin's fluid for 48 h.

4.2.9.2.2 Washing

The tissues were washed using running tap water for 48 h to remove the Bauin's fluid.

4.2.9.2.3 Dehydration

The washed tissues were then dehydrated using different grades of alcohol.

70% alcohol	-	overnight
80% alcohol	-	1h
90% alcohol	-	1h
100% alcohol	-	1h

4.2.9.2.4 Clearing

Clearing was carried out using xylene. The steps are:

(i)	Alcohol + Xylene (1:1)	-	1h
(ii)	Xylene	-	30 minutes
(iii)	Xylene + Wax mixture	-	over night

4.2.9.2.5 Embedding

Embedding was carried out using the procedure introduced by Spurr (1969). The tissues were made into blocks using paraffin wax having a melting point ranging from 50^0C to 62^0C. The tissues from the xylene - wax mixture were transferred to melted wax and kept for 30 minutes. After 3 changes (30 minutes each), the tissues were embedded in the paraffin wax. L-shaped metal blocks were used for the block preparation. Using two L-shaped metal blocks, a rectangular paraffin block was prepared with tissues embedded in it. After cooling, the paraffin blocks were detached from the metal blocks and used for sectioning.

4.2.9.2.6 Sectioning

Sections were made using a microtome. The sections were obtained in long paraffin ribbons. These ribbons were cut into small pieces and transferred to glass slides, which were smeared with egg albumin. These ribbons were straightened using hot water and fixed on the slides by heating. These slides were used for staining.

4.2.9.2.7 Procedure

The following steps were carried out. Eosin - Hematoxylin staining was carried out in coplin jars.

1.	Xylene	-	3 minutes
2.	Xylene	-	4 minutes
3.	Absolute alcohol	-	4 minutes

4. Absolute alcohol	-	4 minutes
5. 95% alcohol	-	4 minutes
6. 95% alcohol	-	4 minutes
7. 70% alcohol	-	5 minutes
8. Distilled water	-	5 minutes
9. Harris Hematoxylin	-	5 minutes
10. Washing in tap water		
11. Tap water	-	5 minutes
12. D.H_2O	-	rinse
13. Acid : alcohol	-	just a dip
14. D.H_2O	-	rinse
15. D.H_2O	-	rinse
16. Ammonia water	-	2 to 4 dips
17. Tap water	-	10 minutes
18. D.H_2O	-	5 minutes
19. Aqueous eosin	-	1 minutes
20. 70% alcohol	-	2 to 3 dips
21. Alcoholic eosin	-	3 to 4 dips
22. 95% alcohol	-	2 to 3 dips
23. 95% alcohol	-	2 to 3 dips
24. Absolute alcohol	-	2 dips
25. Absolute alcohol	-	2 to 3 dips
26. Air dry		
27. Xylene	-	5 minutes

The slides were mount with DPX mountant using cover slips and were observed under light microscope.

4.2.10 Electron microscopic studies

The ultrastructural changes occurring in liver and kidney tissues were studied using Transmission Electron Microscope (TEM) (ZESS - Germany).

The steps used are as follows:

4.2.10.1 Fixation

Fixation is the first step in the biological specimen preparation for TEM. The tissues are cut into small bits of 3mm length and were put to death almost instantaneously by fixatives. Commonly used fixatives are glutaraldehyde and Osmium tetroxide (OsO_4).

4.2.10.1.1 Primary Fixation

1. The tissues were preserved in 3% cacodylate buffered glutaraldehyde (0.1 M) for 3 h
2. The specimens were washed with cacodylate buffer (0.1 M) - 3 washes of 15 minutes duration.

4.2.10.1.2 Post fixation/Secondary fixation

3. The tissues were fixed again in 1% osmium tetroxide (OsO_4) for 2 h.
4. OsO_4 was drained and the tissues were washed with fresh buffer - 3 washes of 15 minutes duration.
5. The tissues were washed again with double distilled water (3 washes).

4.2.10.2 Dehydration

The vials with specimen bits were filled with 30% ethanol or acetone (dehydrants) and allowed for 10 minutes at 4^0C. The dehydration was continued as follows at 4^0C.

59% acetone	-	15 minutes, 2 changes
70% "	-	15 minutes, 2 changes
90% "	-	15 minutes, 2 changes
100% "	-	15 minutes, 2 changes

4.2.10.3 Plastic infiltration

Infiltration was carried out at room temperature with the liquid resin with which embedding of tissues were carried out. Plastic formula of Spurr embedding media was given below.

a) Vinyl cyclohexane (VCD)

b) Diglycidyl, ether of polypropylene glycol (DER)

c) Non enyl succinic anhydride (NSA)

d) Dimethyl amino ethanol (DMAE)

DMAE is the accelerator and first three ingredients are epoxy resin, flexibilizer and hardner respectively. They are mixed and stirred vigorously.

Fresh plastic mixture for infiltration was prepared in the ratio as shown below:

	Embedding media		Acetone	
6.	a)	1	3	- 1 h
7.	b)	1	1	- 1 h
8.	c)	3	1	- 1 h

4.2.10.4 Embedding

Embedding medium was prepared by taking the resin and adding DMAE in the appropriate proportion. Plastic capsules, beam capsules, silicon moulds, gelatin capsules were used for embedding.

9. Embedding medium was poured in the moulds and the tissues were immersed carefully at the bottom of the mould.

4.2.10.5 Polymerization

10. The embedded tissues in the mould were kept in an incubator at 70^0C for 12-24 h, till it completely polymerized.

4.2.10.6 Ultrathin Sectioning

11. The specimen blocks were taken out from the moulds and ultrathin sections were prepared with the help of an ultramicrotome by standard techniques.

4.2.10.7 Staining

The sections were stained in lead nitrate and uranyl acetate with the help of an ultrastainer. These stained sections were collected on the microgrids before observation.

4.2.10.8 Photography

The sections on the microgrids were observed in the screen of the electron microscope and the electron micrographs were taken.

4.3 Results

Experimental induction of urolithiasis in rat is possible by employing several methods (Table 4.1). Here the method adopted was that of Chow *et al.* (1974 and 1975). Calculi producing diet (CPD), consists of 3% glycollate mixed with normal feed. Glycollate is the major precursor of oxalate in the endogenous biosynthetic pathway and the CPD administration for a month can produce calcium oxalate lithiasis.

The results of administration of stone forming diet and dicarboxylic acids on body, liver and kidney weight and protein concentrations are presented in Table 4.2. Body weight differences from the initial period to that at final sacrifice (after 30 days) and tissue weights and protein content were not significant between the affected groups.

4.3.1 Liver and kidney enzymes

The activities of the tissue enzymes are shown in Table 4.3. Liver GAO was significantly increased in stone forming rats, group II (3.97 + 0.21 units/mg protein) compared with pair fed controls (1.68 + 0.13 unit/mg protein). L(+) tartaric acid , maleic acid and malic acid treatment considerably reduced the enzyme level as seen in group III to VIII rats (2.62 ± 0.23, 2.73 ± 0.21, 2.75 ± 0.23, 1.95 ± 0.11, 2.11 ± 0.14, 2.18 ± 0.12 units/mg protein respectively). Dicarboxylic acid treated normal rats (groups IX, X and XI) lowering the GAO activity when compared with control (1.51 ± 0.13, 1.58 ± 0.09 and 1.61 ± 0.11).

Liver and kidney LDH was slightly increased in stone formers (0.65 ± 0.05 and 0.68 + 0.05 unit/mg protein respectively) when compared with group I (0.45 ± 0.02 and 0.50 ± 0.04 unit/mg protein respectively). No drastic change was observed in the other groups from that of normals.

4.3.2 Renal tissue deposition of calcium oxalate and phosphorous.

The kidney tissue deposition of calcium oxalate and phosphorous levels are shown in Table 4.4 and Fig. 4.1. CPD feeding increased the renal deposition of calcium and phosphorous with a marked increase in the oxalate content from their pair fed controls. It

was interesting to note that calcium deposition was reduced when compared to that of group II in stone formers with dicarboxylic acid treatment, Group III to VIII rats. Similarly oxalate was reduced significantly ($p<0.001$) in calculogenic rats but did not equal to that of control. A reduction was also seen in these groups with respect of phosphorus level. The calcium/oxalate ratio was lowered to 0.57 from that of 0.67 observed in normal (Fig.4.2).

4.3.3 Urinary excretion of stone forming constituents

Table 4.5 and fig. 4.3 and 4.4. depicts the results of urinary excretion of stone forming constituents in the experimental animals. The calcium level was increased significantly ($p<0.001$) in stone forming rats (2.54 ± 0.25 mg/24 h) from that of control (1.50 ± 0.13 mg/24 h). Dicarboxylic acids lowered the calcium level in stone forming group III to VIII, but the values did not equal to that of the control group.

CPD feeding significantly ($p<0.001$) increased the urinary oxalate level when compared with their pair fed controls (4.12 ± 0.39 and 0.46 ± 0.04 mg/24 h). A significant decrease ($p<0.001$) in urinary oxalate content was observed in dicarboxylic acid along with CPD fed rats (group III to V), while dicarboxylic acid on CPD fed stone forming rats (group VI to VIII) decreased the oxalate levels when compared with group II calculogenic rats. The seven stone forming groups (group II to VIII) exhibited a decrease in Ca/oxalate ratio from that of the control rats (Fig. 4.5).

Urinary phosphorus level was increased in CPD fed calculogenic rats (3.07 ± 0.28 mg/24 h) compared with control rats (2.67 ± 0.22 mg/24h). Dicarboxylic acid treatment along with CPD reduced the phosphorous level from that of group II calculogenic rats.

Magnesium levels were found to be reduced significantly ($p<0.001$) in stone forming rats when compared with control (1.39 ± 0.12 and 2.99 ± 0.26 respectively). Dicarboxylic acid treatments elevated the Mg levels in groups III to VIII. It was shown that in group II stone forming rats, Mg/oxalate and Mg/Ca ratios decreased significantly when compared with the that of group I control rats (Fig.4.5).

4.3.4 Histopathological and Electronmicroscopic observations in liver and kidney tissues

Light microscopic examination of Hemotaxylin - Eosin (H-E) stained liver sections of 30 days CPD-fed animals showed focal necrosis (Fig. 4.18) which was not observed in dicarboxylic acid treated liver cells (Fig. 4.25 - 4.27). In dicarboxylic acid treated caliculogenic rats the degree of cell necrosis were considerably minimum (Fig. 4.19 - 4.24).

Cystic dialation and extensive necrosis of renal tubules were noticed in calculogenic rats (Fig.4.7). The structural changes at cellular levels were reversed by dicarboxylic acid treatments (Fig. 4.8 - 4.13).

The ultra structural examination of CPD-fed calculogenic rat liver cells showed nuclear damage and enlargement and increase in the number of cells exhibiting cytoplasmic vacuolisation (Fig.4.38). The damage caused was reversed by dicarboxylic acid treatment (Fig.4.39- 4.41).

Ultra structural examination of kidney tissues of CPD-fed stone forming rats showed calcium oxalate crystal aggregation in the renal intestitial cells (Fig. 4.30) and a marked dialation in the distal tubules were also observed (Fig. 4.31). Dicarboxylic acid treatments helped to regain the structural alterations in the kidney cells caused by calculogenic CPD feeding (Fig. 4.33 - 4.36).

Table 4.1 Experimental production of calcium oxalate renal stones in animals

Method	Animals	Time required to produce renal calculi (days)	Reference
Low phosphorous diet	Weanling male rats (Wistar)	63	Coburn and Packett (1962)
Pyridoxine-deficient diet containing 3% glycene	Adult male rats (Charles River)	42	Andrus *et al.* (1960)
Pyridoxine -deficient diet containing 3% glycene	Adult male rats (Harlan) and Adult female rats (Holtzman)	56	Lyon *et al.* (1966a)
Pyridoxine -deficient diet containing 3% glycene	Adult male rats (Sprague-Dawley)	56	Borden and Lyon (1969)
Pyridoxine -deficient diet containing 3% glycollic acid	Adult male rats (Wistar)	21	Chow *et al.* (1974)
Ethylene glycol (0.25% in drinking water)	Adult male rats (Charles River)	28	Gershoff and Andrus (1962)
Ethylene glycol (1% in drinking water)	Adult male rats (Sprague-Dawley)	28	Lyon *et al.* (1966b)
Ethylene glycol (1% in drinking water)	Adult male rats (Wistar)	35	Vaille *et al.* (1971)
Glycollic acid (3% in normal diet)	Adult male rats (Wistar)	21	Chow *et al.* (1975)
Hydroxyproline (2.5g/Kg daily, intraperitoneally)	Adult male and female rats (Wistar)	1-4	Thomas *et al.* (1971)
Hydroxyproline (3g/Kg daily, intraperitoneally)	Gunea pig	1-4	Thomas *et al.* (1971)

Table 4.2. Body weight, tissue weight and tissue protein concentration of control and experimental rats
(Values are mean ± SEM of 5-6 separate experiments)

Particulars	Group I (Control)	Group II (CPD)	Group III CPD and L(+) tartarate	Group IV CPD and Maleic acid	Group V CPD and Malic acid	Group VI CPD + L(+) tartarate	Group VII CPD + Maleic acid	Group VIII CPD + Malic acid	Group IX L(+) tartaric acid only	Group X Maleic acid only	GroupXI Malic acid only
Change in body weight after 30 days	12.83± 1.91	10.14± 1.23	11.03± 1.51	11.14± 1.18	11.28± 1.08	12.01± 1.91	11.91± 1.45	11.39± 1.18	12.54± 2.13	12.11± 2.11	12.76± 2.03
Liver weight (g/100gbody weight)	3.63± 0.31	3.34± 0.30	3.38± 0.31	3.41± 0.33	3.39± 0.29	3.48± 0.33	3.52± 0.31	3.56± 0.31	3.53± 0.31	3.59± 0.33	3.55± 0.31
Kidney weight(g/100g body weight)	0.93± 0.08	0.89± 0.07	0.91± .07	0.89± 0.08	0.92± 0.08	0.92± 0.08	0.91± 0.09	0.91± 0.08	0.93± .09	0.92± 0.08	0.91± 0.08
Liver protein (mg/g wet tissue)	171.81± 7.5	166.93 ± 7.1	168.81± 7.8	167.11± 7.6	169.34± 7.9	169.46± 8.3	168.28± 8.1	170.83± 8.2	171.11± 7.9	169.9± 8.1	170.63± 7.9
Kidney protein (mg/g wet tissue	186.43± 9.1	181.54 ± 8.9	183.18± 8.6	183.98± 8.8	182.72± 8.1	185.11± 8.3	184.96± 8.1	185.91± 8.9	185.60± 8.1	186.1± 8.2	185.99± 8.10

Table 4.3. The effect of glycollate and dicarboxylic acid administration on the level of oxalate synthesising enzymes-GAO and LDH
(Values are mean ± SEM of 5-6 separate experiments)

Enzyme (Units/ mg protein)	Group I (Control)	Group II (CPD)	Group III CPD and L(+) tartarate	Group IV CPD and Maleic acid	Group V CPD and Malic acid	Group VI CPD + L(+) tartarate	Group VII CPD + Maleic acid	Group VIII CPD + Malic acid	Group IX L(+) tartaric acid only	Group X Maleic acid only	Group XI Malic acid only
Liver LDH	0.45± 0.02	0.65± 0.05	0.54± 0.04	0.56± 0.05	0.59± 0.06	0.51± 0.03	0.55± 0.04	0.55± 0.05	0.46± 0.03	0.45± 0.05	0.46± 0.04
Liver GAO	1.68± 0.13	3.97± 0.21***	2.62± 0.23** ♦♦♦	2.73± 0.21** ♦♦♦	2.75± 0.23 ** ♦♦♦	1.95± 0.11 ♦♦♦	2.11± 0.14 ♦♦♦	2.18± 0.12 ♦♦♦	1.51± 0.13 ♦♦♦	1.58± 0.09 ♦♦♦	1.61± 0.11
Kidney LDH	0.50± 0.04	0.68± 0.05	0.56± 0.07	0.59± 0.04	0.59± 0.06	0.54± 0.04	0.56± 0.06	0.58± 0.05	0.49± 0.04	0.50± 0.04	0.52± 0.04

♦♦♦P<0.001 when compared to group II.
***P<0.001, **P< 0.01 when compared to control.

One unit of GAO (Glycollic Acid Oxidase) –Enzyme required to produce 0.1nanomole of glyoxylate/min. at 37°C
One unit of LDH (Lactate Dehydrogenase) –Enzyme required to produce 0.1μ mole of pyruvate/min. at 37°C

Table 4.4 -Kidney tissue deposition of oxalate, calcium and phosphorus
(Values are mean ± SEM of 5-6 separate experiments)

Parameters (mg/g Wet tissue)	Group I (Control)	Group II (CPD)	Group III CPD and L(+)tartarate	Group IV CPDand Maleic acid	GroupV CPD and Malic acid	Group VI CPD + L(+) tartarate	GroupVII CPD + Maleic acid	Group VIII CPD + Malic acid	Group IX L(+) tartaric acid only	Group X Maleic acid only	Group XI Malic acid only
Oxalate	0.53± 0.02	1.19± 0.08 ***	0.81± 0.04*♦♦♦	0.85± 0.07** ♦♦	0.91± 0.08***♦	0.60± 0.05♦♦♦	0.71± 0.06♦♦♦	0.79± 0.08♦♦♦	0.53± 0.03♦♦♦	0.52± 0.04 ♦♦♦	0.54± 0.04♦♦♦
Calcium	0.36± 0.04	0.68± 0.05 ***	0.55± 0.05	0.58±* 0.06	0.60±* 0.06	0.41±♦ 0.03	0.47±♦ 0.04	0.49± 0.04	0.36± 0.02♦♦♦	0.38± 0.04 ♦♦♦	0.35± 0.03♦♦♦
Phosphorus	1.83± 0.12	2.89± 0.18 ***	2.15± 0.18	2.28± 0.19	2.35± 0.20	1.94± 0.12♦♦	2.06± 0.13♦	2.16± 0.18	1.84± 0.11♦♦♦	1.83± 0.12 ♦♦♦	1.82± 0.16♦♦♦
Ca/Ox	0.67	0.57	0.67	0.68	0.66	0.68	0.66	0.62	0.69	0.73	0.64

♦♦♦P<0.001, ♦♦P<0.01 and ♦P< 0.05 when compared to group II.
***P<0.001, **P< 0.01 and *P<0.05 when compared to control.

Figure 4.1. Stone forming constituents in the kidney

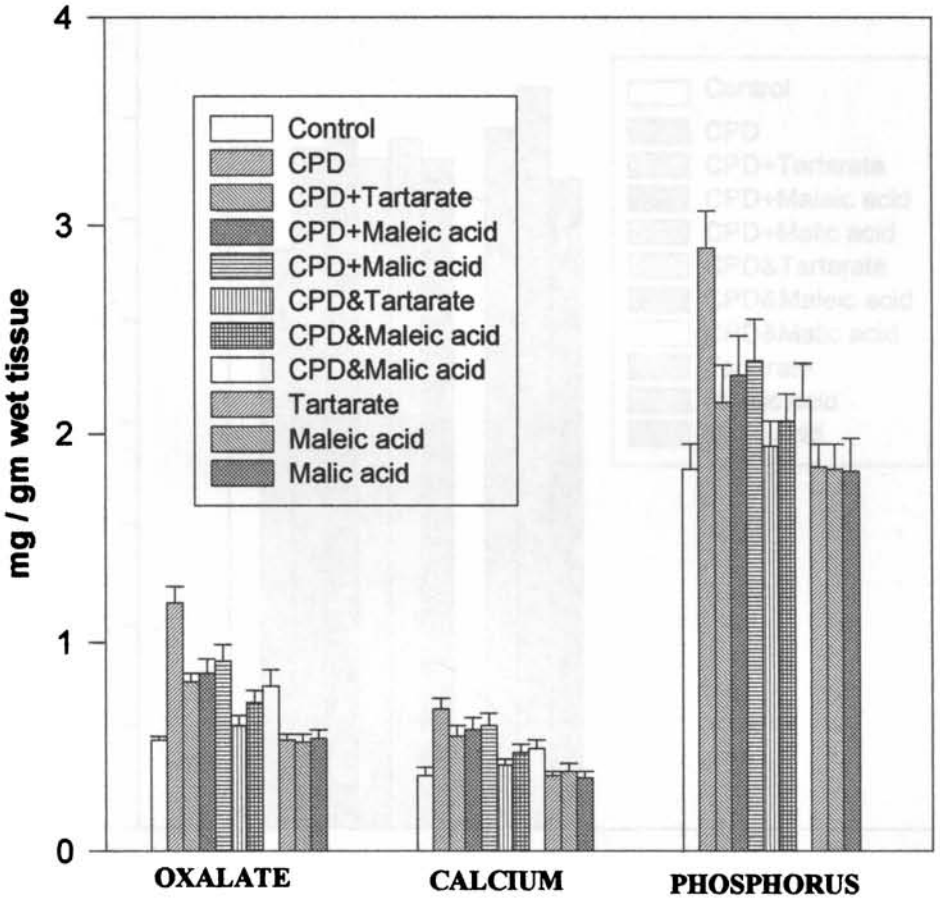


Figure 4.2.Stone forming constituents in the kidney (Ca/Oxalate)

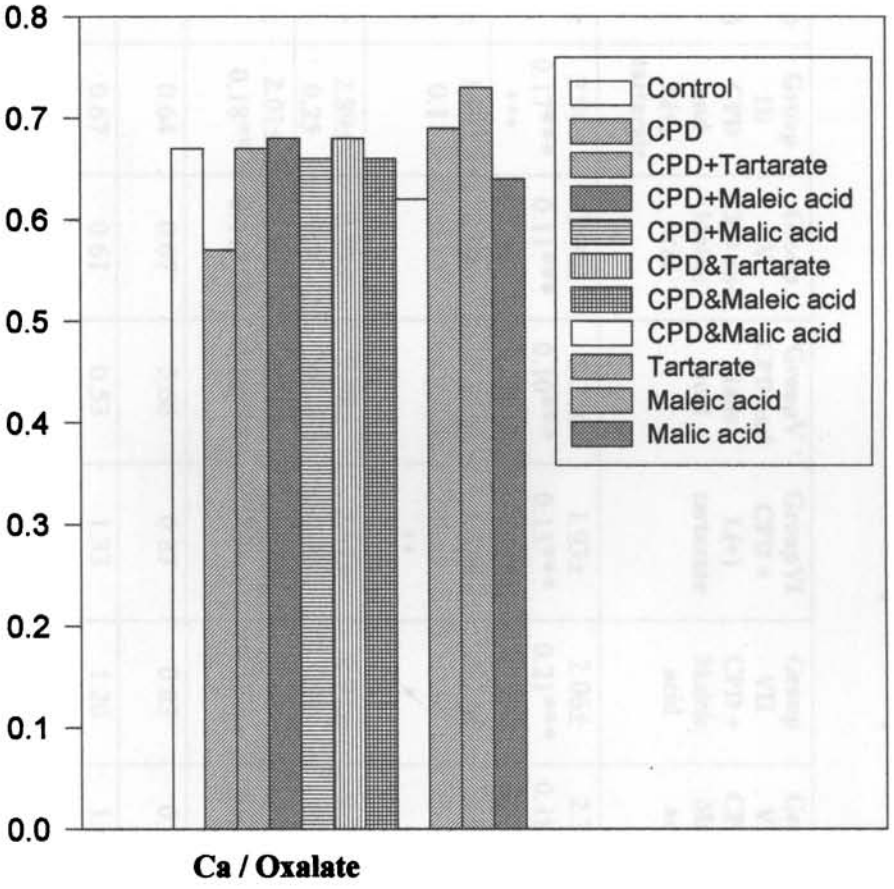


Table 4.5. 24 h. Urinary excretion of oxalate, calcium, phosphorus and magnesium (Values are mean ± SEM of 5-6 separate experiments)

Parameters (mg/24h urine)	Group I (Control)	Group II (CPD)	Group III CPD and L(+) tartarate	Group IV CPD and Maleic acid	GroupV CPD and Malic acid	GroupVI CPD + L(+) tartarate	Group VII CPD + Maleic acid	Group VIII CPD + Malic acid	GroupIX L(+) tartaric acid only	GroupX Maleic acid only	GroupXI Malic acid only
Oxalate	0.46± 0.04	4.12± 0.39 ***	2.98± 0.17*** ♦♦♦	3.08± 0.11*** ♦♦	3.22± 0.10*** ♦	1.93± 0.13*** ♦♦♦	2.06± 0.21*** ♦♦♦	2.36± 0.18*** ♦♦♦	0.45± 0.03 ♦♦♦	0.48± 0.04 ♦♦♦	0.47± 0.04 ♦♦♦
Calcium	1.50± 0.13	2.54± 0.25 ***	1.91± 0.11	2.08± 0.15	2.13± 0.11	1.68± 0.15 ♦♦	1.73± 0.14 ♦	1.78± 0.16 ♦	1.49± 0.13 ♦♦♦	1.51± 0.18 ♦♦♦	1.50± 0.13 ♦♦♦
Phosphorus	2.67± 0.22	3.07± 0.28	2.89± 0.25	2.93± 0.23	2.98± 0.28	2.67± 0.22	2.71± 0.25	2.74± 0.22	2.68± 0.21	2.67± 0.22	2.65± 0.25
Magnesium	2.99± 0.26	1.39± 0.12* **	2.01± 0.18**	1.89± 0.11***	1.71± 0.12***	2.58± 0.19♦♦♦	2.48± 0.11♦♦♦	2.38± 0.12♦♦	2.91± 0.18♦♦♦	3.01± 0.19♦♦♦	2.90± 0.11♦♦♦
Calcium/ Oxalate	3.26	0.61	0.64	0.67	0.66	0.87	0.83	0.75	3.31	3.14	3.19
Mg/ Oxalate	6.50	0.33	0.67	0.61	0.53	1.33	1.20	1.00	6.4	6.27	6.17
Mg/Ca	1.99	0.53	1.05	0.90	0.80	1.53	1.43	1.33	1.95	1.99	1.93

♦♦♦P<0.001, ♦♦P<0.01 and ♦P< 0.05 when compared to group II.
***P<0.001, **P< 0.01 when compared to control.

Figure 4.3. 24h urinary excretion of oxalate and calcium

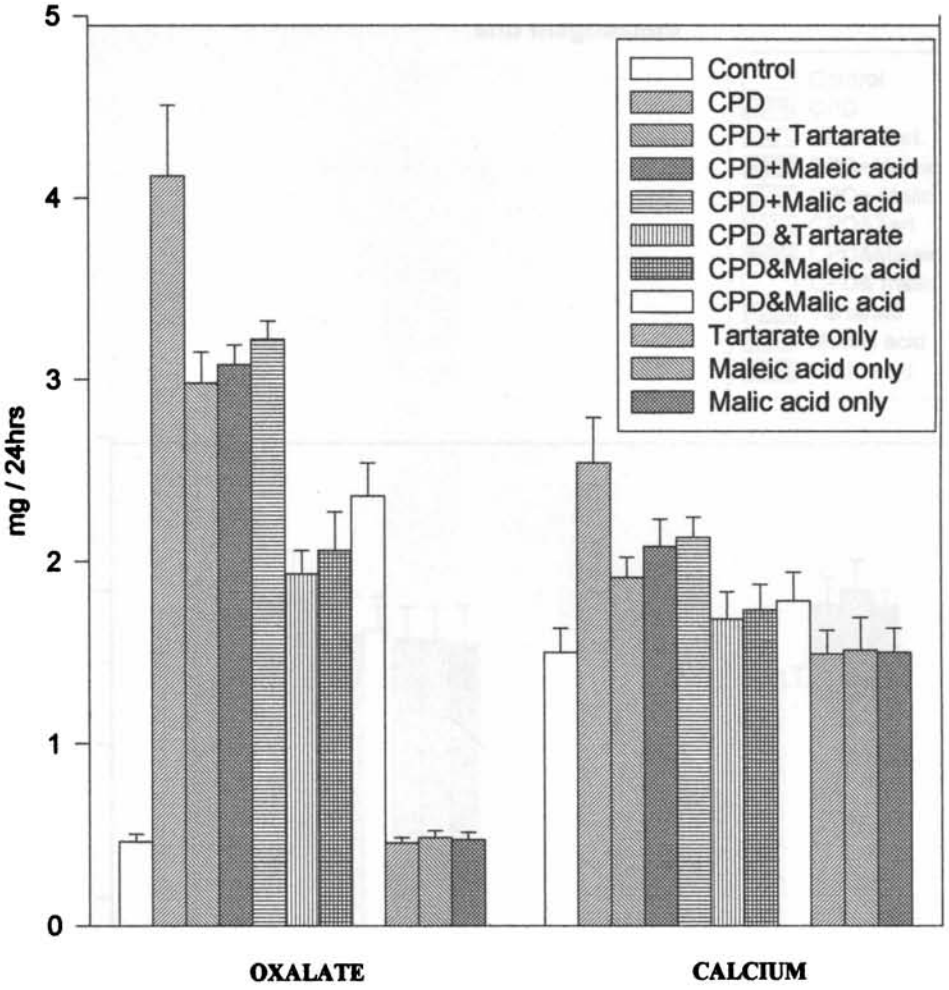


Figure 4.4. 24h urinary excretion of phosphorus and magnesium

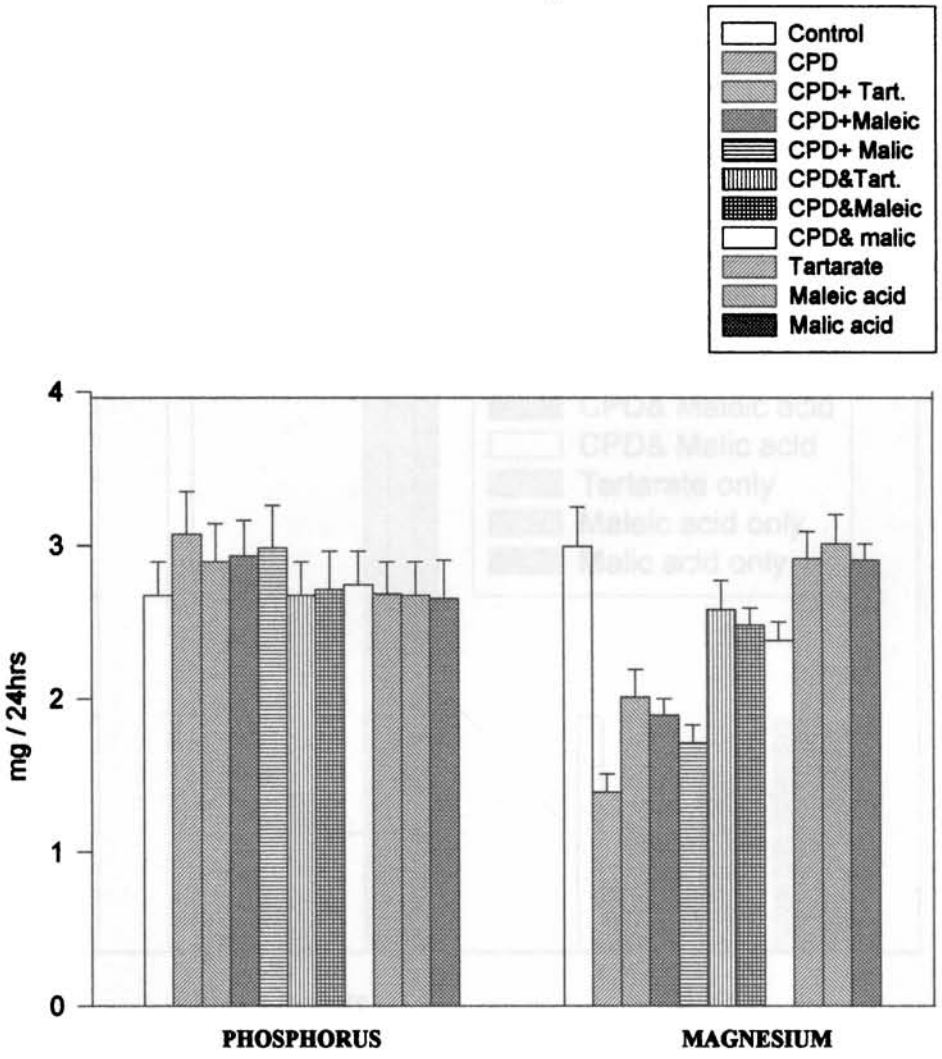
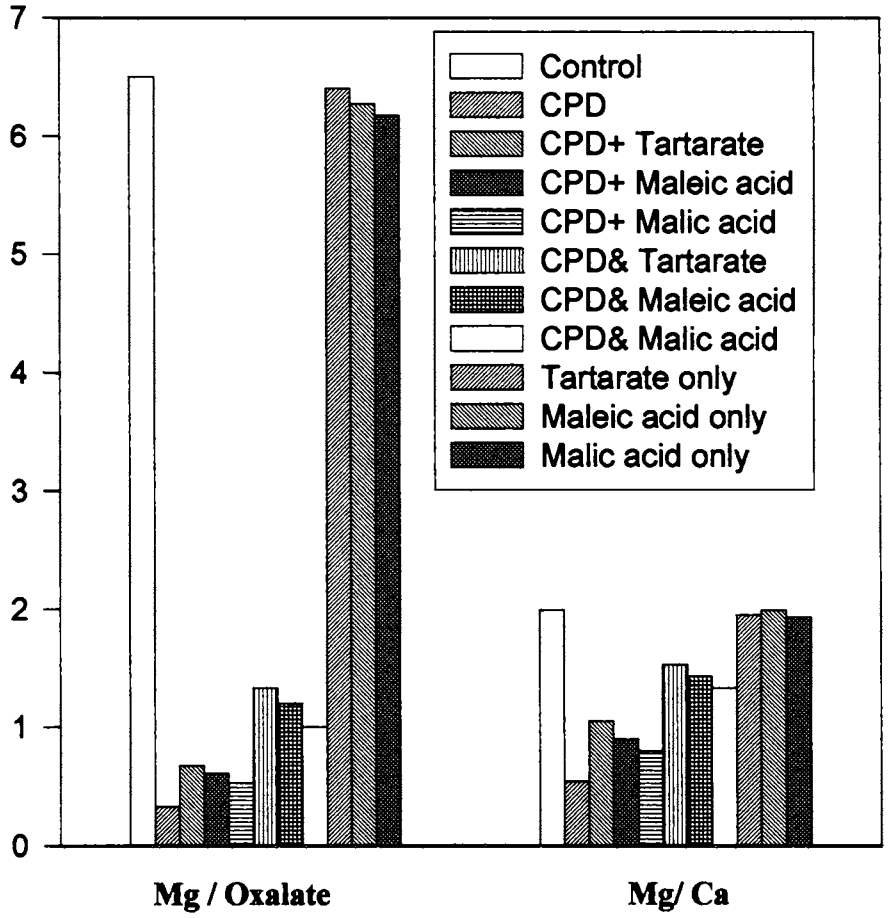


Figure 4.5. Urinary excretion of stone forming constituents



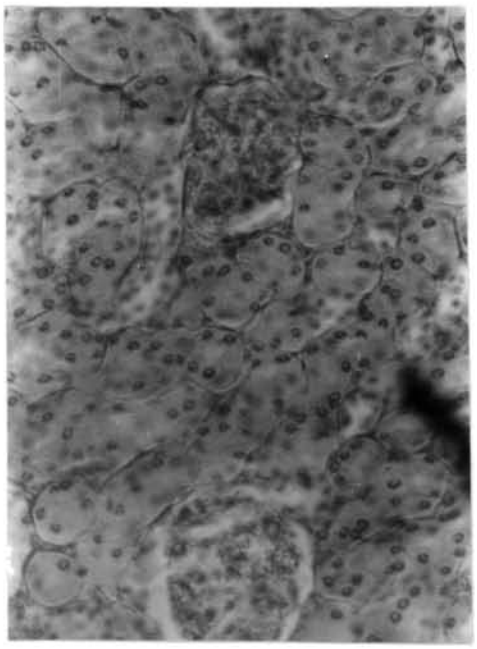


Figure 4.6. Normal rat kidney cells with Bowman's capsule and renal tubules (H-E x 200)

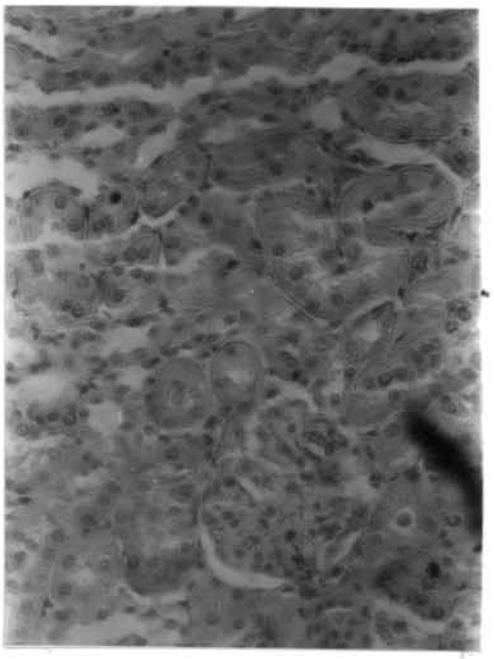


Figure 4.7. Normal kidney cells after 30days of CPD feeding (H-E x 200)

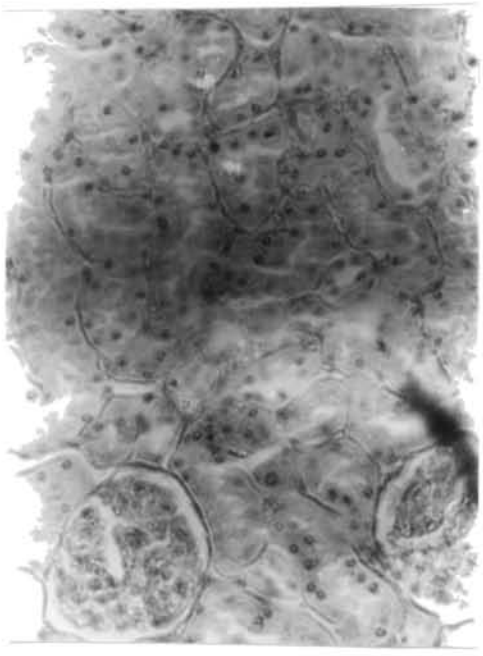


Figure 4.8. CPD fed rat kidney after 15days of L(+) tartarate treatment (H-E x 200)

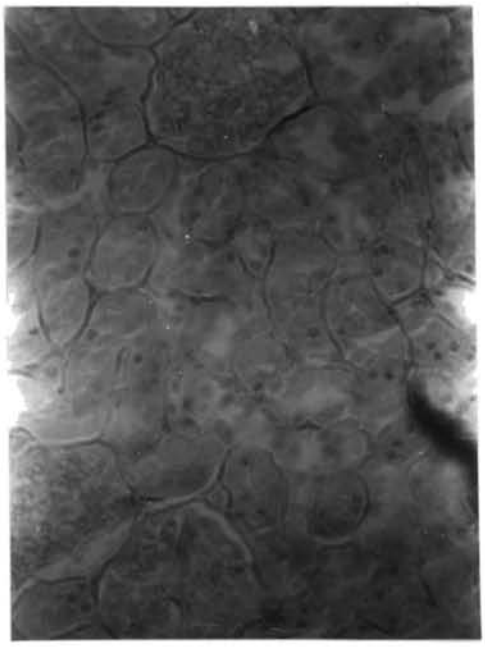


Figure 4.9. CPD fed rat kidney after 15days of maleic acid treatment (H-E x 200)

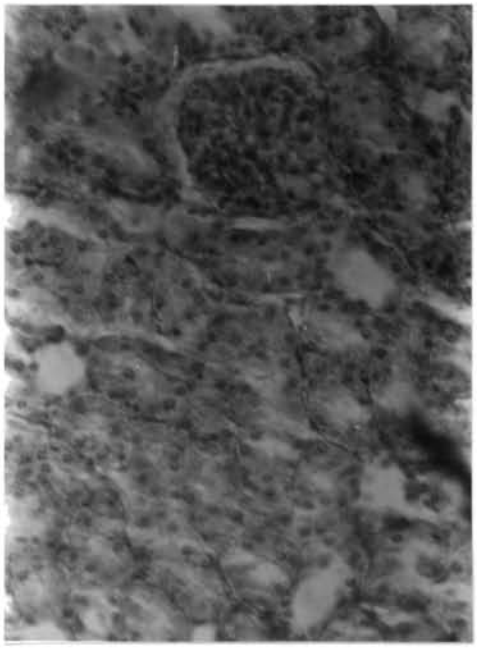


Figure 4.10. CPD fed rat kidney after 15days of malic acid treatment (H-E x 200)

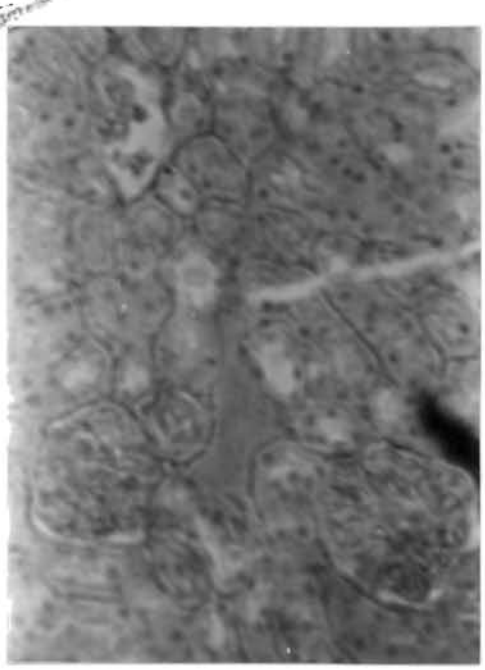


Figure 4.11. Normal kidney cells after 30days of CPD and tartarate treatment (H-E x 200)

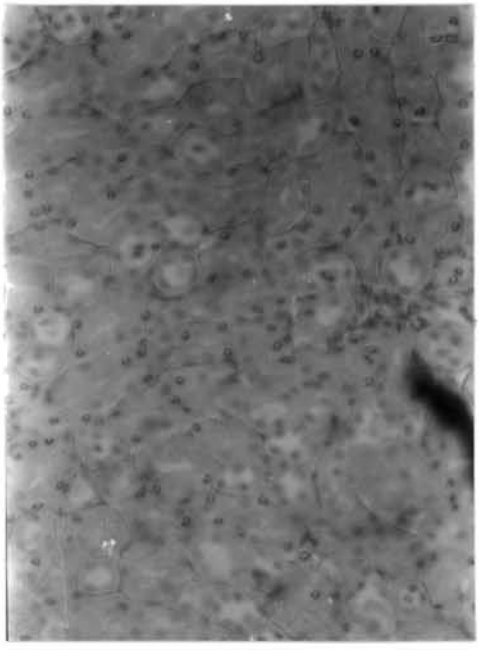


Figure 4.12. Normal kidney cells after 30days of CPD and maleic acid treatment (H-E x 200)

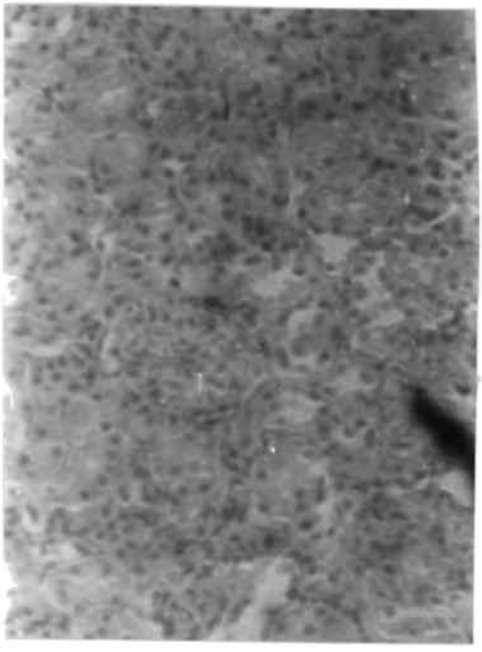


Figure 4.13. Normal kidney cells after 30days of CPD and malic acid treatment (H-E x 200)

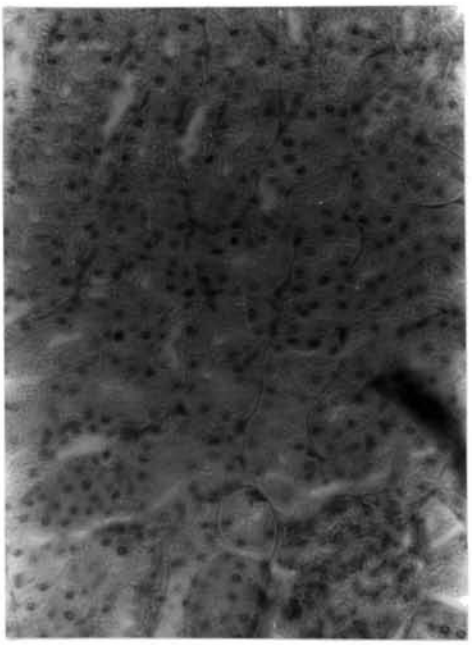


Figure 4.14 L(+) tartarate treated rat kidney (H-E x 200)

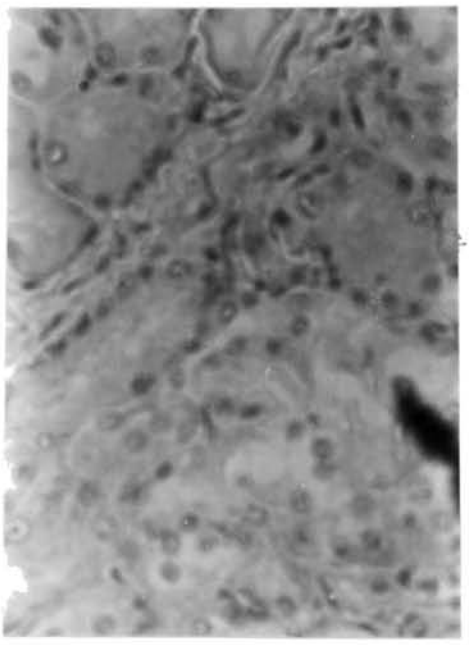


Figure 4.15 Rat kidney after 30 days of maleic acid treatment (H-E x 200)

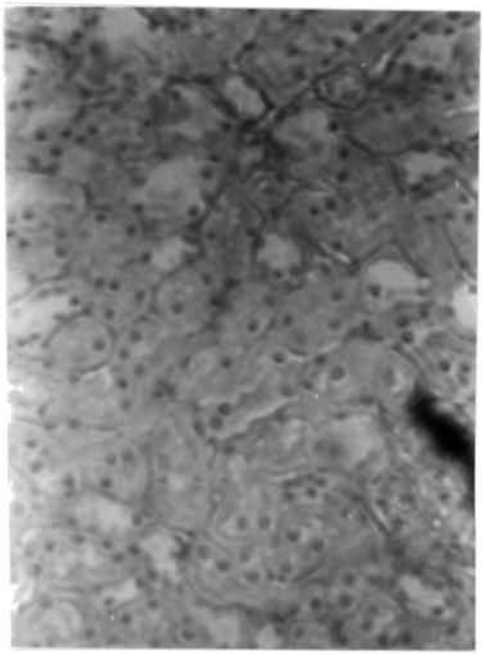


Figure 4.16. Rat kidney after 30 days of malic acid treatment (H-E x 200)

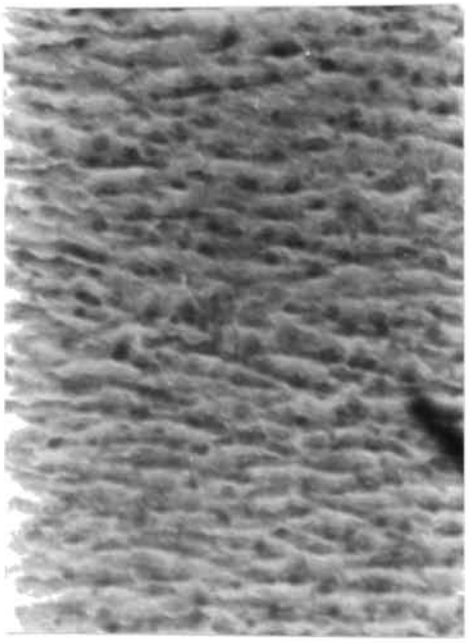


Figure 4.17. Liver tissue of normal rat (H-E x 200)

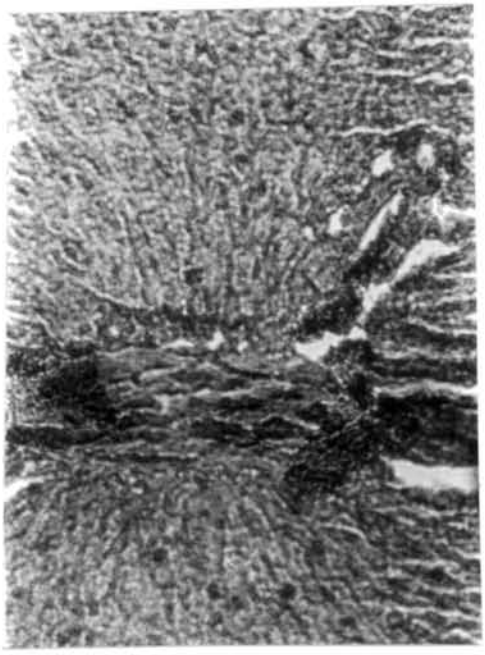


Figure 4.18. Liver tissue of CPD fed rat (H-E x 200)

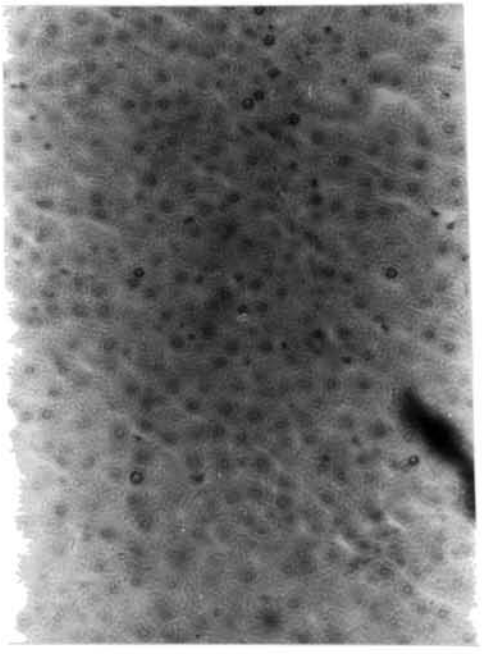


Figure 4.19. CPD fed rat liver after 15days of L(+) tartarate treatment (H-E x 200)

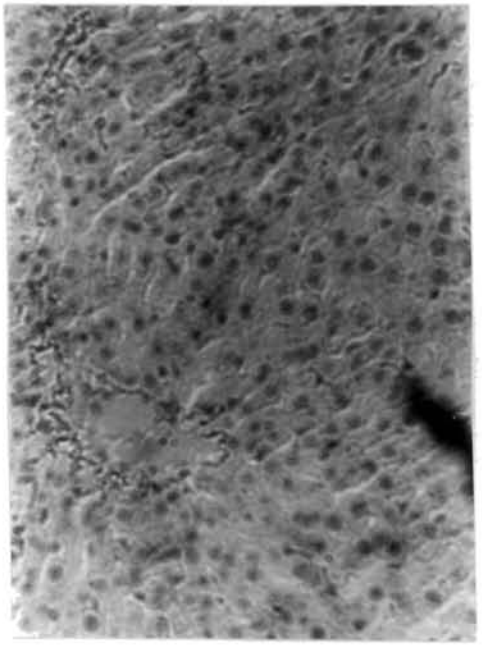


Figure 4.20. CPD fed rat liver after 15days of maleic acid treatment (H-E x 200)

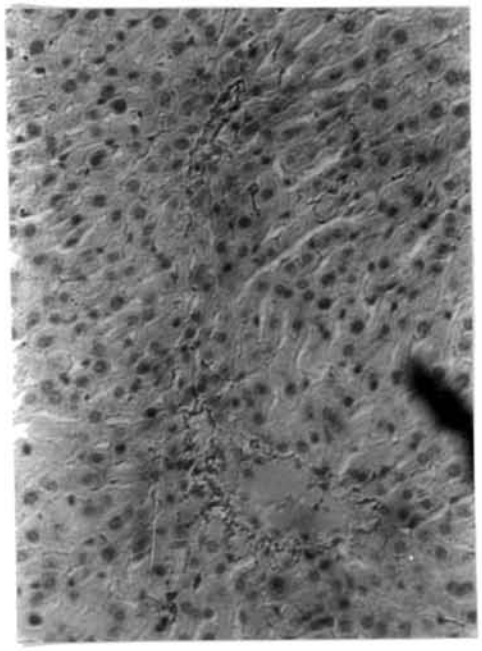


Figure 4.21. CPD fed rat liver after 15days of malic acid treatment (H-E x 200)

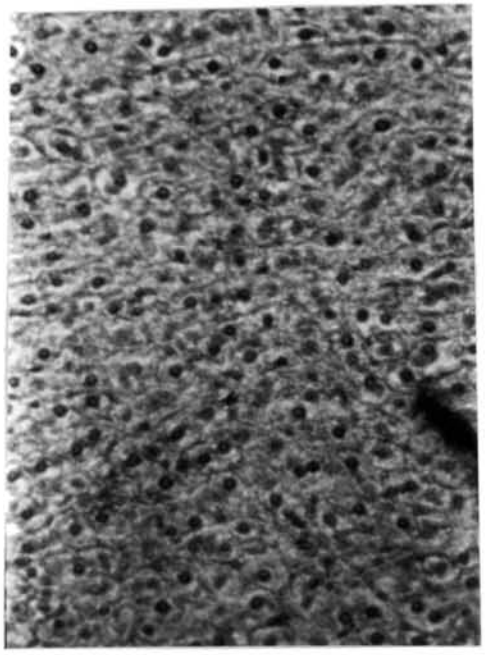


Figure 4.22. Normal rat liver after 30days of CPD and L(+) tartaric acic treatment (H-E x 200)

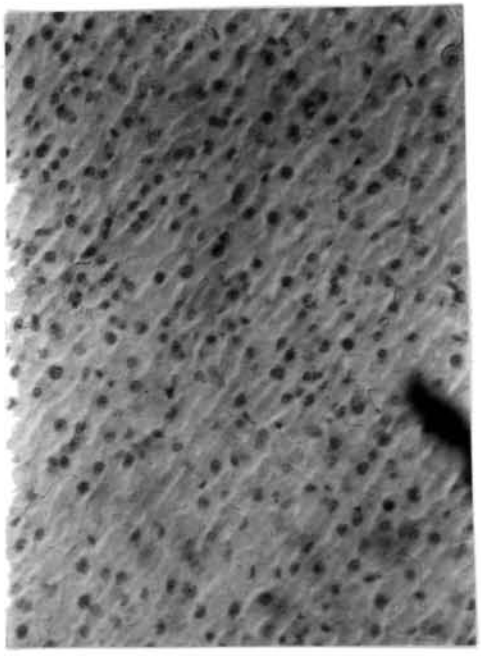


Figure 4.23 Normal rat liver after 30days of CPD and maleic acid treatment (H-E x 200)

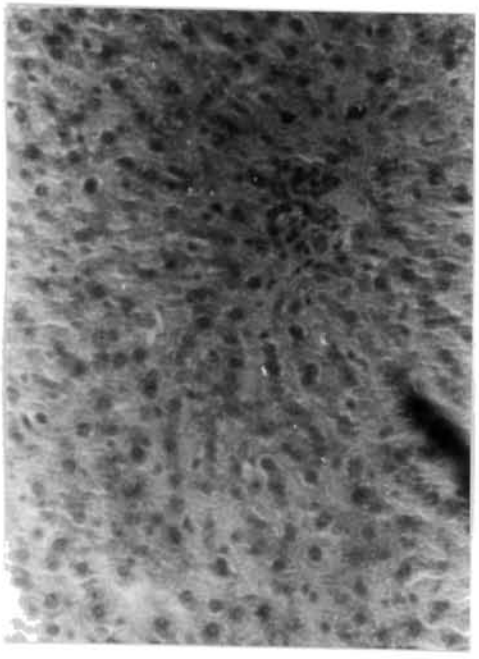


Figure 4.24. Normal rat liver after 30days of CPD and malic acid treatment (H-E x 200))

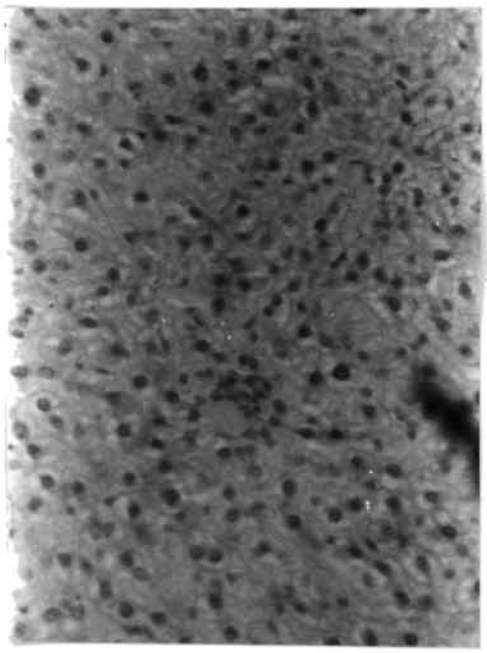


Figure 4.25. Normal rat liver after L(+) tartaric acic treatment for 30 days (H-E x 200)

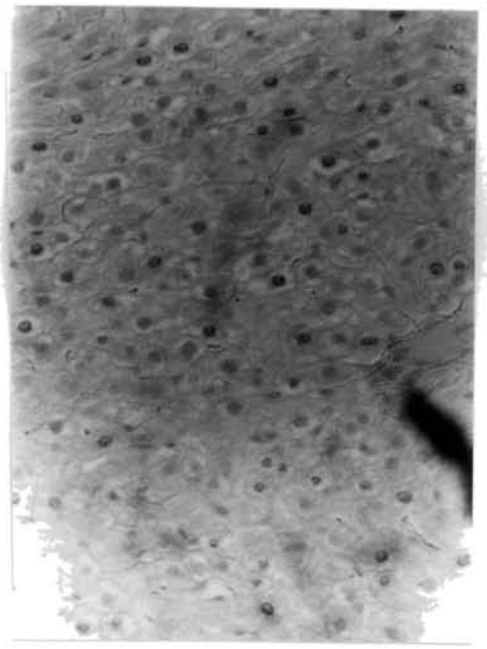


Figure 4.26. Normal rat liver after maleic acic treatment for 30 days (H-E x 200)

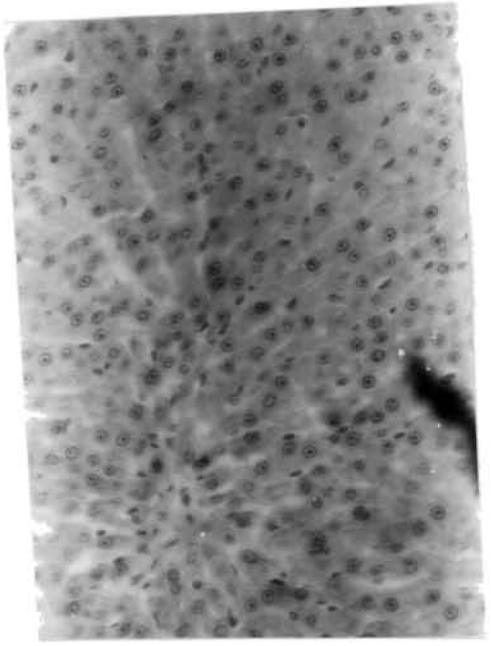


Figure 4.27. Malic acid treated rat liver (H-E x 200)

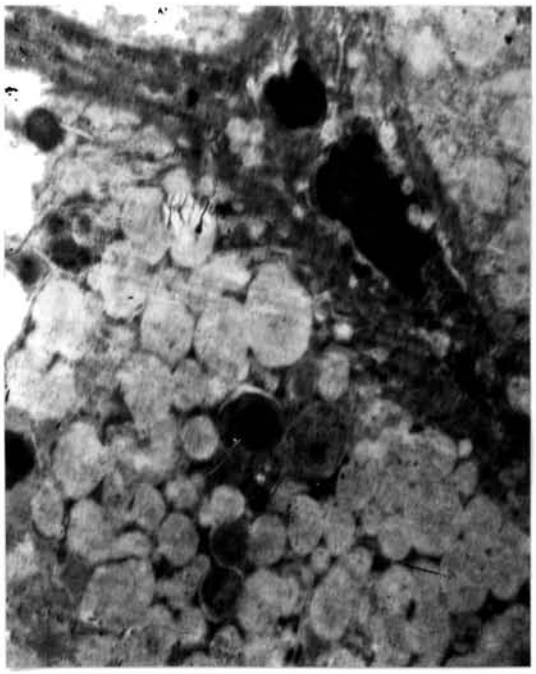


Figure 4.28. Normal rat kidney. TEM x 3500

Figure 4.29. Normal rat kidney showing foot process. TEM x 3500

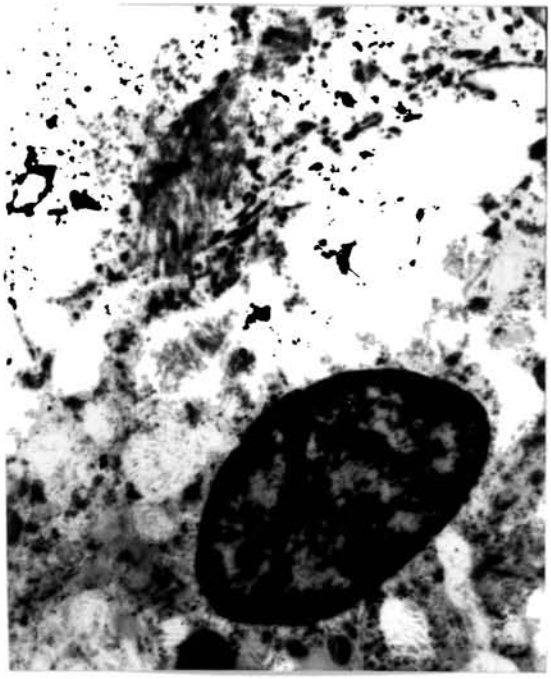


Figure 4.30. CPD fed rat kidney showing calcium oxalate aggregation in the intercalated cells. TEM 5000

Figure 4.31. Electron micrograph of CPD fed rat kidney showing dialated distal tubules. TEM 5000

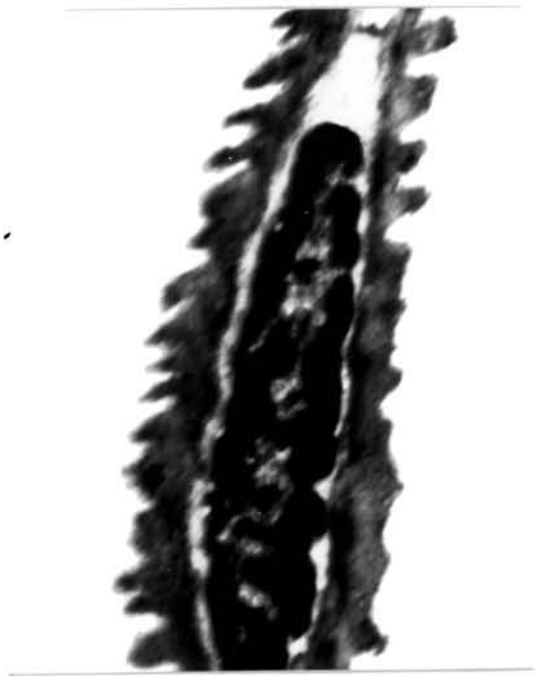


Figure 4.32. Electron micrograph of CPD fed rat kidney showing an enlarged striated foot process. TEM 5000

Figure 4.33. Electron micrograph of CPD fed rat kidney after 15 days tartarate treatment showing distal convoluted tubules. TEM 12000

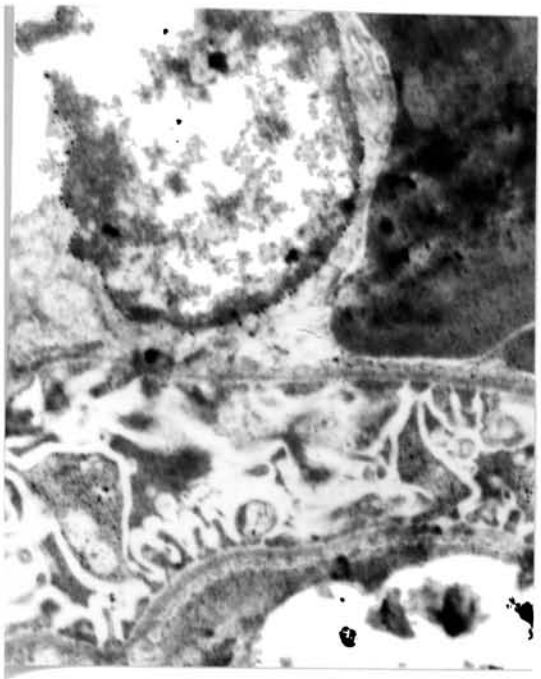


Figure 4.34 Electron micrograph of kidney of CPD fed rats after 15 days L(+) tartarate treatment showing luminar aspect of the tubuleTEM x 15000

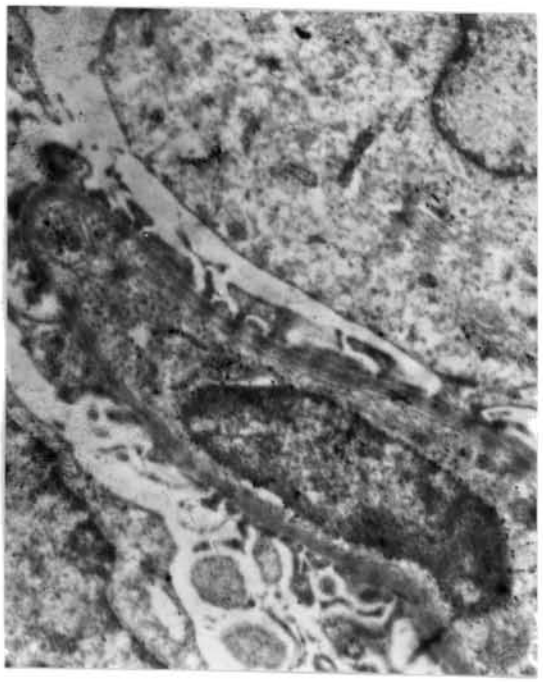


Figure 4.35. Electron micrograph of CPD fed rat kidney after 15 days of maleic acid treatment TEM x 10000

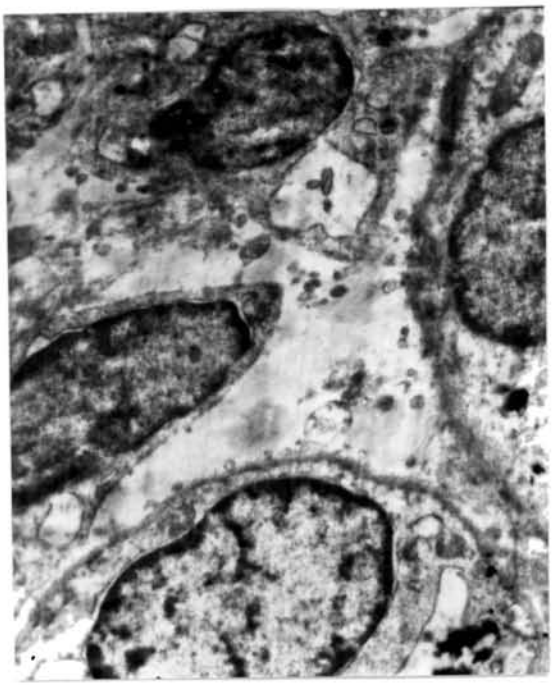


Figure 4.36. Electron micrograph of CPD fed rat kidney after 15days of malic acid treatment TEM x 8000

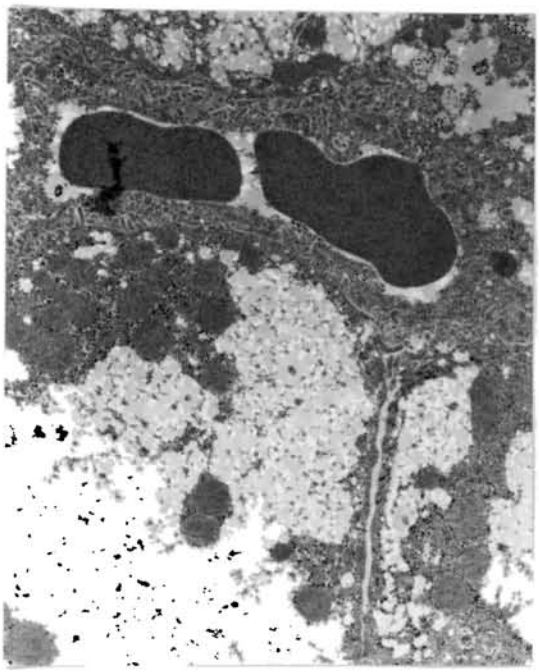


Figure 4.37. Electron micrograph of normal rat liver showing RBC in the sinusoidal space TEM x 6000

Figure 4.38. Electron micrograph of CPD fed rat liver TEM x 6000

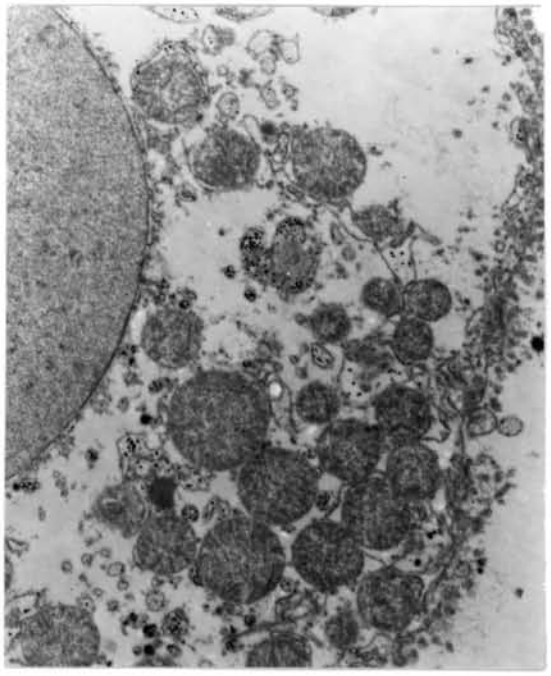


Figure 4.39. Electron micrograph of CPD fed rat liverr after 15days of L(+) tartarate treatment.TEM x 8000

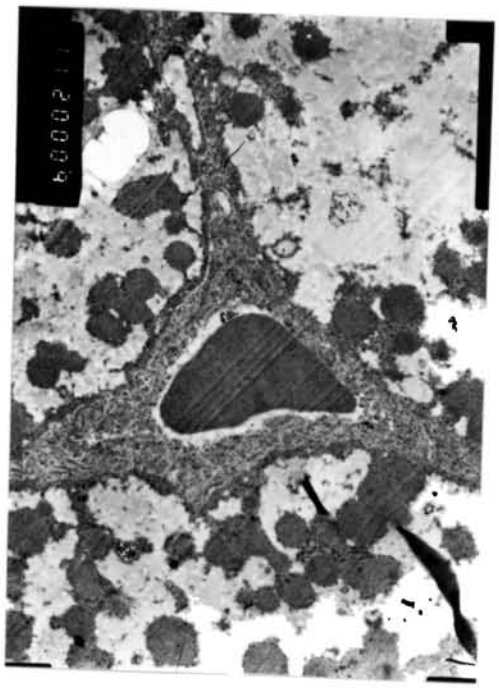


Figure 4.40. Electron micrograph of CPD fed rat liverr after 15days of maleic acid treatment. TEM x 6000

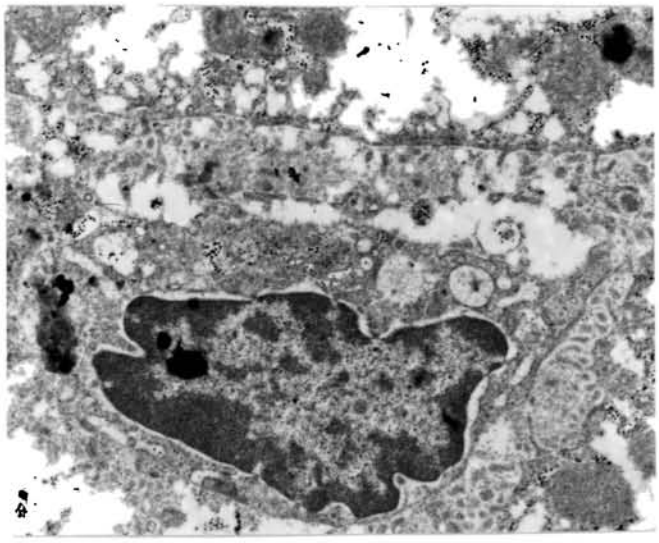


Figure 4.41. Electron micrograph of CPD fed rat liverr after 15days of malic acid treatment. TEM x 6000

4.4 Discussion

Calcium oxalate urolithiasis is a common occurrence among the population of India and controlling endogenous synthesis of oxalate is likely to be a useful approach to medical management. Our short term studies (chapter III) for 7 days with mono and dicarboxylic acids such as L(+) tartaric acid , maleic acid, malic acid, succinic acid, pyruvic acid and malonic acid yielded beneficial results in regulating oxalate metabolism in experimental hyperoxaluric rats. Here we selectively studied the influence of L(+) tartaric acid , maleic acid and malic acid on oxalate metabolism in calcium oxalate stone forming rats.

Liver is known to be the primary site of endogenous oxalate synthesis, and the role of the two enzymes GAO and LDH has been well recognised (Varalakshmi *et al.*, 1989). Results of our long term experiments show that the GAO activity was significantly increased in CPD fed calculogenic rats. Increased GAO activity has been recognised in rats fed with pyridoxine deficient diet (Varalakshmi and Richardson 1983), in glycollate fed rats (Moorthy *et al.*, 1983; Selvam and Varalakshmi 1989), and in CPD fed rats (Selvam and Varalakshmi 1990; Jayanthi *et al.*, 1994). The enzyme catalyzes both the oxidation of glycollate to glyoxylate and glyoxylate to oxalate. An abnormal induction of this enzyme results in hyperoxaluria. During this condition increased deposition of calcium oxalate in kidney usually ends with death from renal failure. Treatment of these pathological conditions includes the regulation of the endogenous synthesis of oxalate to prevent saturation of the urine with calcium oxalate.

Administration of L(+) tartaric acid , maleic acid and malic acid brought about a marked reduction in the enzyme levels thereby assigning an important role to the dicarboxylic acids in regulating oxalate synthesis via the liver. GAO activity was not detected in the kidney and reports to be devoid of the enzyme in this tissue (Fry and Richardson 1979). Kidney LDH was slightly increased in the stone forming rats. But its contribution to oxalate synthesis from glyoxylate is minimal (Leo and Richardson, 1972). Thind and Nath (1977) did not observe any change in the total LDH activity in the rat kidneys in the experimental urolithiasis. However, they did observe an increase in the

activity of LDH isoenzyme, which indicates an alteration in the cortex. No significant change in the LDH activity was seen with dicarboxylic acid treatments. This has an advantage since it can be used without affecting the normal metabolic reaction of LDH.

CPD feeding increased the kidney deposition of calcium and phosphorous with a very marked increase in the oxalate contents from their pair fed controls. Calcium and oxalate content was reduced in group III to group V rats when compared with group II calculogenic rats. Houtman *et al.*, (1980) reported that the calcium and oxalate concentration in the renal tissue play key roles in the pathogenesis of papillary calcification and eventual stone formation. Supersaturation of these constituents in the kidney has seen in the CPD fed calculogenic rats was controlled to a greater extent with dicarboxylic acids which reflects its beneficial action in this connection.

The resultant increases in calcium and oxalate in CPD fed rats (Table 4.4) are likely to be associated with marked increase in calcium oxalate super saturation and crystalluria. Elevated level of oxalate in urine found in stone patients (Pendse, 1984), pyridoxine deficient rats (Moorthy *et al.*, 1981) and sodium glycollate fed rats (Rangaraju and Selvam, 1987; Varalakshmi and Selvam, 1990).

Phosphorous level was slightly elevated in CPD fed calculogenic rats. High levels of phosphorous excretion are significantly important in the formation of phosphate stones. However, in the pathogenesis of oxalate stones, its action is rather controversial.

Magnesium level was significantly lowered in stone forming group II rats. Low magnesium excretion has been reported earlier in stone formers (Hodginson, 1974; Varalakshmi and Anandam, 1979a). There was a considerable increase in magnesium levels in dicarboxylic acid treated rats. Goren *et al.*, (1978) also have made a similar observation.

Histopathological studies of kidney tissues of CPD fed rats showed significant alteration in the cellular level. Extensive necrosis and cystic dialation of renal tubules were observed. Poonkuzhali *et al.* (1994) reported extensive necrosis and degenerative

changes in the tubular epithelial cells with occasional cell casts in kidney sections of sodium oxalate injected rats. Oxalate exposure might have deleterious effects of renal mitochondria (Strzeleki and Menon, 1996). Oxalate can also produce additional changes in renal tubular cell function. de Water *et al.* (1996) reported that the occurrence of calcium oxalate crystals coincided with morphological changes, glomerular damage, and tubular dialation and necrosis, and an enlargement of the intestitium, lending support to our findings.

We observed some sort of cellular damage in the liver cells of CPD fed calculogenic rats. Interestingly dicarboxylic acid administration were found to normalise the altered cellular structure in the kidney and liver cells to some extent. Similar changes were noticed by Chow *et al.* (1978). Calcium oxalate is found to be a potent urolithic agent and histological observation shows a good evidence for this.

Ultrastructural studies with calculogenic kidney tissue showed calcium oxalate crystal aggregation and distal tubular dialation as evidenced by figures. Kohijimoto *et al.* (1996). and Ebisuno *et al.* (1997) showed the endocytosis of calcium oxalate crystals to the three kinds of tubular cells (Madin-Darby canine kidney (MDCK) cells, rats and human kidneys) by scanning electronmicroscopy (SEM) and also reported crystal addition and endocytosis might be a vital process and microvilli of cells play an important role in this process. CPD fed liver cells also showed cellular damage and deformalities. Dicarboxylic acid treatments helped a lot to regain the deformalities caused in the cellular level.

The exact mechanism of oxalate induced oxalate cellular damage and the repair mechanism by dicarboxylic acid were not known. Further investigations are needed to substantiate the beneficial effects of long term treatment with dicarboxylic acid and the mechanism CPD induced urolithiasis.

Chapter 5

ISOLATION AND CHARACTERISATION OF OXALATE DEGRADING BACTERIA

CHAPTER-V

ISOLATION AND CHARACTERIZATION OF OXALATE DEGRADING BACTERIA

5.1 Introduction

The formation of calcium oxalate stone in the urine is dependent on the saturation level of both calcium and oxalate, thus the management of oxalate in individuals susceptible to urolithiasis would seem especially important. In man and animals oxalate is a nonessential toxic end product of metabolism and is excreted unchanged in urine. The majority of oxalate in plasma and urine is derived from endogenous metabolism of glycollate, glyoxylate, and ascorbic acid and to a lesser degree, tryptophan. In addition, between 10 and 20% of the urinary oxalate is absorbed from the diet, especially through ingestion of leafy vegetables and plant materials (Hodgkinson, 1977).

Either abnormal synthesis or hyperabsorption of oxalate can lead to a serious condition referred to as hyperoxaluria. There is a strong association between increased levels of urinary oxalate and calcium oxalate stone disease in man. Since there are no known naturally occurring enzymes in vertebrates capable of degrading or metabolising oxalate, it is catabolised by a limited number of bacterial species by an activation-decarboxylation reaction to yield formate and CO_2. The first process can be accomplished either aerobically or anaerobically, while the second is strictly aerobic.

Soil bacteria, examples *Pseudomonas oxalis* and *Thiobacillus* are capable of converting oxalic acid to formic acid and CO_2 in an aerobic reaction that requires ATP, coenzyme A, Mg^{2+}, Thiamine pyrophosphate (TPP) and acetate (Kornberg and Elsden, 1961). Anaerobic oxalate degradation was found to occur in sediments taken from a diversity of aquatic environments (Smith and Oremland, 1983). Oxalate is degraded by ruminal microbes and by mixed bacterial population in the large intestine of other herbivores (Hagmaier *et al.*, 1981), while there is evidence for oxalate degradation by bacteria in human faeces (Chandra and Shethna, 1977), information concerning the nature of oxalate degradaters in the human bowel or of their significance is lacking. Rates of

oxalate degradation by mixed bacterial population in faecal content from wild rats ranged from 2.5 to 20.6 μmole/g (dry wt.)/h (Daniel *et al.*, 1987). Rieu-Lesme, (1995) also reported that the oxalate could serve as a substrate for growth of spore forming, gram negative acetogenic bacteria from the rumen of a mature deer. *Oxalobacter formigenes*, an anaerobic bacterium that utilises oxalate as a major source of carbon and energy is responsible for oxalate degradation in mammalian intestinal tract (Dowson *et al.*, 1980;Allison *et al.*, 1985; Weaver *et al.*, 1992;Han *et al.*, 1995). The large intestine of the laboratory rats were experimentally colonised with a strain of *Oxalobacter formigenes* to examine their effect of the fate of dietary oxalate and found that there was consistent trend towards less oxalate excretion in faeces of rats after colonisation with *Oxalobacter formigenes* (Danial *et al.*, 1993).

Recently Sidhu *et al.* (1998) reported that absence of *Oxalobacter formigenes*, the intestinal aerobic oxalate degrading bacteria, increased the absorption of oxalate and there by causing hyperoxaluria.

The ability to enzymatically degrade oxalate to less noxious substances, formate and CO_2 could benefit a great number of individuals in the biomedical field. In this chapter, we initiate the study to test the feasibility of isolation of oxalate degrading bacteria from various environments such as soil, water and decayed colocasia stem and to identify the potential strains capable of maximum oxalate degradation.

5.2 Materials and Methods

5.2.1 Biochemicals and their sources

Media chemicals were purchased from HI MEDIA. λ Hind III molecular marker and restriction enzymes were purchased from Genei, Bangalore. RNase from Sigma and FDH from E-MERCK. All other chemicals were purchased locally from SRL, CDH, BDH.

5.2.2 Collection of samples

Soil and water samples were collected from terrestrial and marine environment of Cochin and decayed colocasia samples were collected from vegetable waste, in sterilised polythene bags and were stored at 4^0C.

5.2.3 Isolation of bacterial colonies

5.2.3.1 Preparation of the sample

1ml of each sample was diluted to 10 ml with sterile distilled water. Serial dilution blanks were prepared up to 10^{-5} dilution.

5.2.3.2 Media

The readymade nutrient agar medium was used to enumerate the bacteria from the collected samples.

5.2.3.3 Plating procedure

Conventional pour plate technique was employed. 1ml each of serially diluted blanks was used as inoculum for inoculating nutrient agar media. After plating the plates were incubated at 30^0C for 2-3 days. The bacterial colonies were observed for their colony morphology, shape, size and colour. Later single celled colonies were picked and subcultured on nutrient agar slants and maintained at 4^0C.

5.2.4 Isolation of oxalate degrading bacterial isolates.

5.2.4.1 Medium

Nutrient agar oxalate (Nutrient agar (Himedia) + 0.5 - 1% oxalate) and mineral salt oxalate medium was used for isolating oxalate degrading bacterial isolate.

5.2.4.2 Medium composition

K_2HPO_4	-	0.1%
KH_2PO_4	-	0.1%
$MgSO_4\ 7H_2O$	-	0.05%
NaCl	-	0.05%
$(NH_4)_2SO_4$	-	0.1%

pH - 7± 0.2

0.5 - 1% of sodium oxalate was added to the above medium.

5.2.4.3 Inoculum and inoculation procedure

Preculture of the isolate was prepared by inoculating 5 ml of nutrient broth and incubating at 30^{0}C in mechanical shaker for 12 h The overnight grown cultures were serially diluted (10 - 5) with 0.9% NaCl and 100µl of the bacterial samples were transferred in to nutrient agar oxalate medium by spread plate technique. The isolate with, comparatively faster growth rate were selected as potential isolates for further studies.

For confirmation of oxalate degradation the above bacterial isolates were grown on mineral salt oxalate broth for 24h and the growth was measured in terms of turbidity.

5.2.5 Identification of bacterial isolates

The potential isolates were identified based on biochemical and morphological characteristics as outlined by Bergey's Manual of Systematic Bacteriology, after purification by repeated streaking on nutrient agar plates.

The cultures were maintained on nutrient agar and subcultured periodically at regular intervals of 15 days. Stock cultures were maintained in the same medium. Culture purity was checked once in a month by repeated streaking on nutrient agar plates.

5.2.6 Growth Studies

5.2.6.1 Media

Growth studies were carried out using nutrient broth oxalate media, prepared by additionally supplying oxalate in the range 0.5 - 1% to the readymade nutrient broth. The medium was autoclaved at 121^{0}C, 15 lb pressure for 15 minutes and used.

5.2.6.2 Preparation of inoculum

1. A loop full of 24 h old culture of the bacterial strain was first grown in 5ml nutrient broth oxalate medium for 12h at room temperature (28 ± 2^{0}C).

2. 1ml of the culture broth was then aseptically transferred into 50 ml of nutrient broth and incubated in a rotary shaker at 200 rpm for 12 h at room temperature.
3. Cells were harvested by centrifugation at 10,000 rpm for 10 minute.
4. The harvested cells were washed repeatedly with sterile physiological saline (0.95% NaCl) and resuspended in 10 ml of the same saline.
5. The prepared cell suspension (0.5 O.D at 600nm) was used as inoculum, and stock culture was kept at 4^0C until used.

5.2.6.3 Measurement of growth

The growth of bacteria in the medium was determined in terms of turbidity in the culture broth, by measuring absorbance at 600 nm in UV visible spectrophotometer. Growth was expressed as optical density (O.D.)

5.2.6.4 Optimisation of growth parameters

Various environmental parameters that influence the growth of the selected strains namely - *Acinetobacter* sp., *Alcaligenes* sp. and *Xanthobacter* sp. were studied to optimise the growth. The different parameters optimised for growth include incubation temperature, substrate concentration and pH.

5.2.6.4.1 Temperature

Optimum temperature for maximum growth was determined by growing the bacterial cultures at various incubation temperatures (25^0C, 30 ± 2^0C, 37^0C and 40^0C) for a period of 33 h on a rotary shaker. Growth was determined as in the section -5.2.6.3

5.2.6.4.2 pH

Optimum pH required for maximum growth was determined by subjecting the bacteria to various pH conditions (5,6,7,8 & 9). After 33 h of incubation on a rotary shaker at room temperature growth was determined as described in section 5.2.6.3

5.2.6.4.3 Substrate concentration

Optimum substrate concentration was determined by growing the bacteria at various substrate (oxalate) concentrations (0.05%, 0.1%, 0.5% and 1.5%) for a period of

33 h on a rotary shaker at room temperature. Growth was measured as mentioned under section -5.2.6.3

5.2.7 Antibiotic resistance profile

Selected strains were tested for resistance to antibiotics, namely Ampicillin, Streptomycine, Kanamycine, Tetracycline and Chloramphenicol, incorporated in LB media at a concentration of 100 μg/ml.

5.2.8 Estimation of oxalate degradation by the selected bacterial strains

Oxalate degrading capacity of the selected bacterial strains was examined by growing the bacterial isolates in nutrient broth oxalate medium. The inoculum was incubated for 33 h on a rotary shaker. Oxalate and formate was estimated in culture broth at regular intervals of time (6,12,18,24 & 30 h) by withdrawing the media at corresponding time intervals.

5.2.8.1 Oxalate estimation

Estimation of oxalate was carried out according to the method of Hodgkinson and Williams (1972) as described earlier (section -3.2.7.3)

5.2.8.2 Formate estimation

Formate estimation was carried out by using the method of Triebig and Schaller (1980).

5.2.8.2.1 Principle

The principle of the method is the oxidation of formic acid to carbondioxide with nicotinamide adenine dinucleotide (NAD) in the presence of enzyme formate dehydrogenase (FDH).

$$HCOOH + NAD \xrightarrow{FDH} CO_2 + NADH + H^+$$

The amount of NADH formed is equivalent to the quantity of formic acid, which was measured at 340 nm. in a spectrophotometer

5.2.8.2.2 Reagents

1. Potassium phosphate buffer - 0.15 M, pH 7.5

Solution (a) - 100ml

KH_2PO_4	-	2.04 g
$D.H_2O$	-	100 ml

Solution (b) - 100ml

K_2HPO_4	-	14 g
$D.H_2O$	-	100 ml

The phosphate buffer contains 14 ml of solution (a) and 100 ml of solution (b)

2. NAD solution (42 mmol/lit)

NAD	-	330 mg
$D.H_2O$	-	11 ml

3. FDH solution (20U/ml)

FDH - lyophilisate	-	45.5mg
$D.H_2O$	-	1 ml

Stored at 0^0C

4. Formic acid solution (1g/lit)

Formic acid	-	1 g
$D.H_2O$	-	1000ml

5.2.8.2.3 Procedure

0.1ml of the sample was mixed with 0.5 ml NAD solution and 1ml potassium phosphate buffer. Then the reaction mixture was made up to 3 ml with distilled water. Standards in the range of 50 - 250 μg. and a blank were also treated in the same way. Mixed and kept at room temperature for 2 minutes. Then the optical density was measured at 340 nm against blank in a spectrophotometer. Then to the same reaction mixture added 0.05 ml of FDH solution, mixed kept for 2 min. at room temperature, again taken the O.D. at 340 nm against the same blank containing 0.05 ml FDH solution. Difference in optical density (δ E) will be equivalent to the amount of NADH which inturn represents the amount of formate present.

(δ E) = [(Final reading of sample - initial reading of sample) - (Final

reading of blank - initial reading of blank)]

The amount of formate was expressed as mg/ml.

5.2.9 Isolation of plasmid DNA.

Large scale isolation of plasmid DNA was carried out by a modified method, based on alkaline lysis procedure, of Susan (1995).

5.2.9.1 Bacterial strains and growth conditions.

Oxalate degrading bacteria, namely *Acinetobacter* sp., *Alcaligenes* sp. and *Xanthobacter* sp. were used. All the cultures were grown in LB broth at 37^0C under agitated condition. Strains were maintained in LB agar plates containing ampicillin (100 µg/ml) and 0.5% oxalate.

5.2.9.2 Reagents

1. LB broth
2. LB agar
3. Agarose
4. Ampicillin stock (1 mg/ml)
5. Solution 1

   50 mM glucose

   25 mM Tris buffer

   10 mM EDTA

   Adjusted to pH - 8.0 with NaOH or HCl.

   Sterilised
6. Solution II - 100ml

   0.2 M NaOH

   1% SDS

   mixed, 2 M NaOH - 10 ml, 20% SDS - 5ml, and Sterile D.H_2O - 85 ml
7. Solution III

   3 M Potassium acetate - pH 5.5

   pH adjusted with glacial acetic acid
8. Lysozyme - 10 mg/ml of solution I
9. Phenol : Chloroform (1:1)

Phenol (melted) - 440 ml

Chloroform - 440 ml

Mixed and added

NaCl - 26.4 gm

Mixed and added

2 M Tris (pH-7) - 440 ml

Removed the aqueous (top phase) and added 440 ml of 50 mM Tris (pH-8).

Removed the aqueous phase

10. 3 M sodium acetate
11. RNase A - 10 mg/ml (preboiled)
12. Isopropanol (saturated with 20xSSC)

(20xSSC -. 3 M NaCl + 0.3 M sodium citrate

13. TE buffer - pH 8 (100 ml)

1 M Tris buffer - 1 ml

0.5 M EDTA - 2 ml

Sterile D.H_2O - 97 ml

pH - adjusted with NaOH

14. Ethanol

5.2.9.3 Procedure

1. 2.0 ml of LB broth containing 0.5% of oxalate with ampicillin (50 µg/ml) was inoculated with the strains and incubated at 37^{0}C overnight, in an environmental shaker.
2. 2.0 ml of overnight grown culture was subcultured into a 100 ml LB broth containing 0.5% oxalate with ampicillin (50 µg/ml) and incubated for 8 - 16 h at 37^{0}C with vigorous shaking.
3. Harvested the cells by centrifugation at 8,000 rpm for 5 minutes. Decanted the medium and the cell pellet stored at –20^{0}C for 5 minutes.
4. Resuspended the cells in 7 of ml solution-I, pipetted the solution up and down to resuspend the cell completely.
5. Transferred the resuspended cells to a new sterile screw capped centrifuge tube.

6. Added 1.0 ml of lysozyme (10 mg/ml) in solution-I to the cell suspension, mixed well by inverting the capped centrifuge tube and incubated at room temperature for 10 minutes.
7. Added 16 ml of solution-II to the cell suspension and mixed gently by inverting the capped centrifuge tube.
8. Incubated on ice for 5 minutes.
9. Added 12 ml ice cold solution-III into the cell suspension and mixed gently by inverting the capped centrifuge tube.
10. Incubated the tubes on ice for 15 minutes.
11. Centrifuged at 12,000 rpm for 5 minutes at 4^0C.
12. Transferred the supernatant solution into 50 ml centrifuge tube.
13. Added 0.6 volume of ice cold isopropanol to precipitate the nucleic acids. Inverted the capped centrifuge tubes several times to ensure thorough mixing.
14. Centrifuged at 8,000 rpm for 5 minutes.
15. Decanted and drained off the supernatant under vacuum desiccator chamber for 10 minutes. Resuspended the pellet in 5 ml of cold sterile distilled water and transferred the solution to a 15 ml corex centrifuge tube.
16. Added 0.5 ml of 3 M NaOAc (sodium acetate) solution, 50 µl RNase (10 mg/ml pre-boiled), mixed well and incubated at 37^0C for 30 minutes.
17. Added 5 ml of phenol:chloroform solution, mixed well and centrifuged at 5,000 rpm for 5 minutes to separate the phases completely. Carefully transferred the aqueous phase into a clean 15 ml corex tube and repeated the phenol:chloroform extraction procedure two times, until the interfaces between the two phases were clear.
18. Added 10 ml of ice cold ethanol and incubated at $^-20^0$C for 30 minutes.
19. Centrifuged at 10,000 rpm for 10 minutes at 4^0C to pellet out the nucleic acid and decanted the ethanol.
20. Washed the pellet with 500 µl of cold 70% ethanol, centrifuged at 10,000 rpm for 10 minutes at 4^0C and pellet was dried under vacuum.
21. Resuspended the dried DNA in 100 µl TE buffer (pH 8.0).
22. Transferred the suspended DNA into sterile microfuge tubes and stored at 4^0C

5.2.9.4 Agarose Gel Electrophoresis

5.2.9.4.1 Reagents

1. 50x TAE) -100 ml

Tris buffer	-	12.1 g
Glacial acetic acid	-	2.9 ml
0.5 M EDTA	-	1 ml

2. Loading dye (Bromophenol blue) - 10ml

Sucrose	-	6 g
0.5 M EDTA	-	0.5ml
Bromophenol blue	-	25 m
Sterile D.H_2O	-	10 ml

5.2.9.4.2 Procedure

Agarose gels can be used to separate DNA molecules of different size and shape. Agarose gel electrophoresis was carried out in a submerged gel apparatus, 200 mg agarose was dissolved in 20 ml of sterile distilled water, boiled and cooled to 50^0C. To this 400 µl of 50 × TAE buffer was added and mixed. Later agarose was poured on to a miniplatform with the inserted comb and the gel was allowed to set for 30 minutes at room temperature. Removed the comb and the gel was placed on a horizontal tank with electrophoresis buffer (1×TAE). 10 µl of DNA sample, added with10 µl loading dye, was loaded into the slot. λ Hind III digest DNA was used as marker. The gel was run at 50 volts.

5.2.9.5 Staining of Agarose Gel

The agarose gel was stained with ethidium bromide (0.5 µg/ml) for 30 minutes and destained with distilled water for 15 minutes at room temperature. The image of the DNA bands was captured using an imagemaster gel documentation system.

5.2.9.6 Spectrophotometric Determination of Plasmid DNA

The concentration of plasmid DNA suspended in TE buffer was estimated by the optical density at 260 nm and 280 nm in an UV-spectrophotometer and the 260/280 ratio was calculated.

5.2.10 Bacterial Transformation Studies

5.2.10.1 Bacterial Culture, Plasmids and Growth Conditions

An ampicillin sensitive phenotype, E.coli ($Krgl^-$) and *Serratia* sp., (bacterial strain, isolated from rat's small intestine) were used as the hosts for transformation studies. Plasmid isolated from *Xanthobacter* sp. was used. The host bacterial strains were maintained in LB broth.

5.2.10.2 Preparation of Competent Cells

Competent cells were prepared and transformed as described by Susan (1995).

1. 2.0 ml of two sets of LB broth was inoculated with *E.coli* ($Krgl^-$) and *Serratia* sp, grown overnight at 37^0C in an environmental shaker.
2. Fresh overnight cultures (2%) were used to inoculate 50 ml L B and the cells were grown to A 600 =0.50 to 0.60
3. Cells were harvested by centrifugation at 7,000 rpm for 5 minutes, at 4^0C in sterile centrifuge tubes.
4. Harvested cell pellets were suspended in 20 ml of ice cold 10 mM NaCl. and resuspended the cells by gently pipetting the solution up and down onto the cell pellet.
5. Centrifuged the cells at 7,000 rpm for 5 minutes at 4^0C.
6. Supernatant was decanted and the cell pellet was gently resuspended in 5 ml of ice cold 30 mM $CaCl_2$ solution.
7. Incubated on ice for 20 minutes.
8. Centrifuged at 7,000 rpm for 5 minutes at 4^0C.
9. The supernatant solution was decanted and the cell pellet was gently resuspended in 1.0 ml of ice-cold 20 mM $CaCl_2$ and kept in ice until used for transformation experiment.

5.2.10.3 Protocol for Transformation Technique

1. Mixed 10 µl of TE suspended plasmid DNA with 90 µl of 30 mM $CaCl_2$ and chilled the tubes on ice for 5 minutes.
2. To this 200 µl of competent cells prepared earlier was added, tightly closed the cap of the centrifuge tube and inverted the tube to mix the DNA and cell gently.
3. Placed the tubes with cells and DNA on ice for 1 h.
4. After 1 hour the tubes with cells and DNA were given heat shock at 42^0C for exactly 2 minutes and cells were incubated again on ice for 5 minutes.
5. To the contents 2.7 ml of LB (pre-warmed to 37^0C) was added to the cell and aerated for 60 minutes to enable the expression of genes coding drug resistance.
6. 200 µl of aliquots of the transformed cell suspension was spread on the LB agar plates containing ampicillin (50 µg/ml) and incubated the plates at 37^0C for overnight.

Transformation efficiency was calculated by the following formula.

$$\text{Transformation efficiency} = \frac{\text{Number of transformed cells}}{\mu\text{g of DNA used}} \times \frac{\text{Final Vol. of cell suspension}}{\text{Vol. of cell suspension used}}$$

5.2.11 Isolation of plasmid from transformed *Serratia* sp. and *E.coli*

Plasmid DNA was isolated from the transformed bacterial cells using the procedure of Birnboin and Doly (1979).

5.2.11.1 Reagents

1. TEG (Tris buffer - EDTA Glucose) - 1 M, 50 ml, pH - 8

   Tris buffer - 25 mM

   EDTA - 25 mM

   Glucose - 50 mM

   Mixed and sterilised

2. 0.8 M sodium hydroxide
3. 4% SDS
4. 3 M potassium acetate - pH - 4.8
5. 70% ethanol

6. TAE (50x) - 50 ml - pH - 8 - as in section -5.2.9.4
7. Loading dye
8. Ethidium bromide (staining solution)

   0.5g ethidium bromide was dissolved in 5ml sterile D.H2O
9. TE buffer - 10ml - pH - 8

   1M Tris base - 100 µl

   0.5 M EDTA - 200 µl

   Sterile D.H_2O - 9.7 ml

   Mixed and sterilised
10. Ethanol
11. Ampicillin stock - 1 mg/ml
12. LB agar
13. LB broth
14. Agarose
15. RNA stock - 1 ml

Ribonuclease	-	10 mg
Tris buffer (1M) (pH - 7.4)	-	1 µl
NaCl (0.9%)	-	100 µl
Sterile D.H_2O	-	899 µl

5.2.11.2 Procedure

1. 1 ml of log phase cells containing the plasmid (grown in LB broth with ampicillin) was harvested by centrifugation at 10,000 rpm 10 minutes at 4^0C in sterile microfuge tubes.
2. The harvested cells were suspended in 100µl TEG buffer, mixed and incubated on ice for 5 minutes.
3. The cells were lysed by adding 100µl of NaOH (0.8 M) and 100µl of 4% SDS, mixed gently and incubated on ice for 10 minutes.
4. The chromosomal DNA was precipitated by adding 150µl of 3 M potassium acetate, mixed gently and incubated on ice for 5 minutes.
5. Centrifuged the mixture at 10,000 rpm for 10 minutes at 4^0C.

6. Transferred the supernatant to a fresh sterilised microfuge tube.
7. The plasmid DNA was precipitated by adding 900 µl of absolute alcohol.
8. The plasmid DNA was recovered by centrifuging at 10,000 rpm for 10minutes at 4^0C.
9. Washed the pellet twice in 400µl of 70% ethanol.
10. Suspended the washed pellets in 10µl TE buffer and stored 4^0C.

5.2.12 Agarose gel electrophoresis and staining

The isolated plasmid DNA was detected in an agarose gel as described in sections 5.2.9.4 and 5.2.9.5.

5.2.13 Curing of plasmid DNA

Plasmid mediated oxalate degradation property was confirmed by curing the plasmid DNA from both *Xanthobacter* sp. and transformant. By treating the strains with ethidium bromide 100 µg/ml and Nalidixic acid (150 µg/ml) (in Nutrient Agar oxalate medium with 100 µg/ml ampicillin) The strains were grown at 42^0C in LB medium with these reagents to the mid log phase and then spread on Nutrient agar plates containing oxalate (0.5%) and ampicillin (100 µg/ml) and without oxalate and ampicillin (negative control). Incubated the plates at 37^0Cfor 24 h. Scored the viable count of bacteria grown over the control and test plate (Inga *et al*, 1998).

5.2.14 Oxalate degradation by the wild and transformed *Serratia* sp.

The percentage of oxalate degradation by both wild and transformed *Serratia* sp. were carried out using the same method described as in section -3.2.7.3

5.3 Results

5.3.1 Isolation of oxalate degrading bacteria

Oxalate degrading bacteria were qualitatively screened from terrestrial and marine environments and vegetable waste (Colocasia stem). Bacterial colonies were selected based on colony morphology, colour etc. from nutrient agar plates. Fourteen isolates from

soil, twenty three from water and five isolates from plant sources were obtained. They were undergone potential secondary screening in oxalate medium.

Three potential isolates, one from each source were selected after secondary screening and were found to have faster growth rate in mineral salt oxalate medium. The potential isolates were identified based on the morphological and biochemical characteristics as outlined by Bergy's Manual of Systematic Bacteriology. The isolates were tentatively identified as *Acinetobacter* sp., *Alkaligenes* sp. and *Xanthobacter* sp. from terrestrial, marine and plant sources respectively.

5.3.2 Optimization of growth parameter

Various growth parameters of the above bacterial strains were optimized.

5.3.2.1 Temperature

The incubation temperature of each bacterial strains were optimized by growing them under different temperatures and it was found that optimum temperature for the growth of both *Alcaligenes* sp. and *Xanthobacter* sp. was to be 30^0C and that of *Acinetobacter* sp. was 37^0C. (Figure 5.1, 5.2, 5.3 respectively).

5.3.2.2 pH

The bacterial strains were grown in media with 5 different pH. *Acinetobacter* sp. and *Alcaligenes* sp. showed faster growth in media with pH 7 and *Xanthobacter* sp. with pH 8. The optimum pH for the growth of the above strains are shown in figure 5.4, 5.5, and 5.6 respectively.

5.3.2.3 Substrate concentration

To optimize substrate concentration the bacterial cultures were grown in media with different substrate (oxalate) concentrations. *Acinetobacter* sp. and *Alcaligenes* sp. can grow at a faster rate when the substrate concentration was 0.5% whereas *Xanthobacter* sp. have an optimum substrate concentration of 1% figure 5.7, 5.8, and 5.9 respectively.

5.3.3 Antibiotic resistance profile

The selected bacterial strains were tested for their ability to grow in different antibiotic media (Table -5.1). From the results it was observed that both *Acinetobacter* sp. and *Alcaligenes* sp. were resistant to antibiotics such as ampicillin, streptomycin and kanamycin (100 μg/ml medium), while it was sensitive to tretacycline and chloramphenicol. *Xanthobacter* sp. was resistant to ampicillin, streptomycin and chloramphenicol and sensitive to kanamycin and tretacycline

5.3.4 Oxalate degrading capacity

The percentage of oxalate degradation of the selected bacterial strains was observed mainly by the substrate (oxalate) utilization rate. From the results we noticed that *Xanthabacter* sp. posses maximum utilization of oxalate (86.26%) (Table 5.2 - Figure 5.10) whereas *Acinetobacter* sp.(79.4%) and *Alcaligenes* sp. (73.18%) respectively and also we observed the oxalate degrading capacity of the above strains through the formation of the product (formate) in the culture medium (Table-5.3).

5.3.5 Plasmid isolation and Transformation

Plasmid DNA was extracted from the above bacterial isolates and examined by agarose gel electrophoresis. One of the strains, *Xanthobacter* sp., found to harbor a plasmid DNA of about 4 kb (Figure 5.11). The concentration of plasmid DNA was estimated (Table 5.4). To determine whether the bacterial culture, *Xanthobacter* sp. harbor plasmid capable of utilizing oxalate, we attempted to cure the strain with ethidium bromide (100 μg/ml) and nalidixic acid (150 μg/ml). We observed that *Xanthobacter* sp. had lost its ability to grow in the oxalate medium with antibiotic marker. We tried to introduce the plasmid (about 4 kb) in to the intestinal bacteria *Serratia* sp., isolated from rat intestine, by transformation. The plasmid transformed *Serratia* sp. was able to grow in the oxalate rich medium and its ability to degrade oxalate is shown in Figure 5.12. The transformant of *Serratia* sp. contained a plasmid corresponding to that seen in *Xanthobacter* sp. (Figure 5.11)

Table 5.1 Antibiotic Resistance.

Antibiotics / Bacterial strains	Ampi-Cillin	Strepto-Mycin	Kanam-ycin	Tetra-cyclin	Chloram-Phenicol
Acinetobacter sp.	+	+	+	-	-
Alcaligenes sp	+	+	+	-	-
Xanthobacter sp	+	+	-	-	+

+ Resistant
- Sensitive

Table 5.2 Substrate (oxalate) utilisation

Time(h)	% of oxalate remaining in the culture media		
	Acinetobacter sp.	***Alcaligenes sp.***	***Xanthobacter sp.***
0	100%	100%	100%
6	84.4%	86.3%	85.2%
12	68.91%	75.83%	63.8%
18	67.31%	71.46%	58.92%
24	37.5%	39.31%	20.16%
30	20.6%	26.02%	13.74%

Table 5.3 Formate production (μg/ml) by *Xanthobacter* sp., *Acinetobacter* sp. and *Alcaligenes* sp. at various time intervals

Bacterial → strains / **Time in h ↓**	***Xanthobacter* sp.**	***Acinetobacter* sp.**	***Alcaligenes* sp.**
0	0	0	0
6	120	68.07	61.25
12	189.20	111.79	132.85
18	240.32	123.01	134.89
24	241.01	181.28	151.67
30	255	205.11	168.47

Table 5.4 Spectrophotometric determination of plasmid DNA

Plasmid	O.D. at 260 nm	O.D. at 280 nm	Ratio of O.D. 260/280	DNA content (μg/ml)
Xanthobacter sp.	1.01	0.45	2.24	11.20

Figure 5.1 Growth curve of *Alcaligenes sp.* at different temperatures.

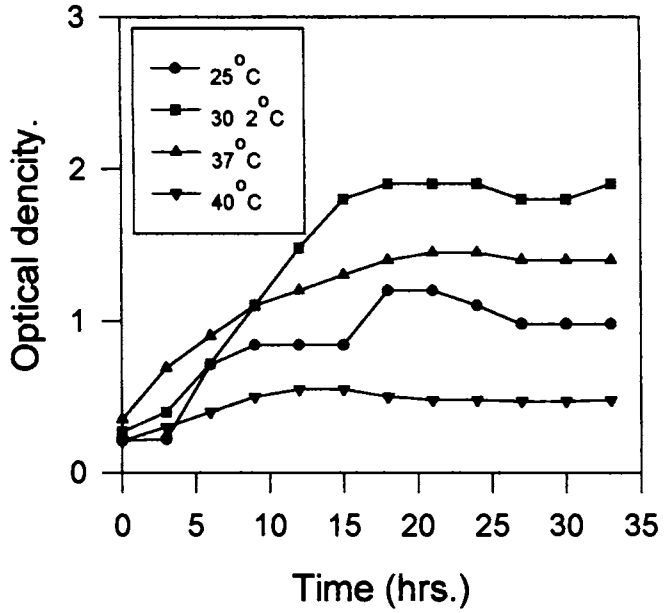


Figure 5.2 Growth curve of *Xanthobacter sp.* at different temperatures.

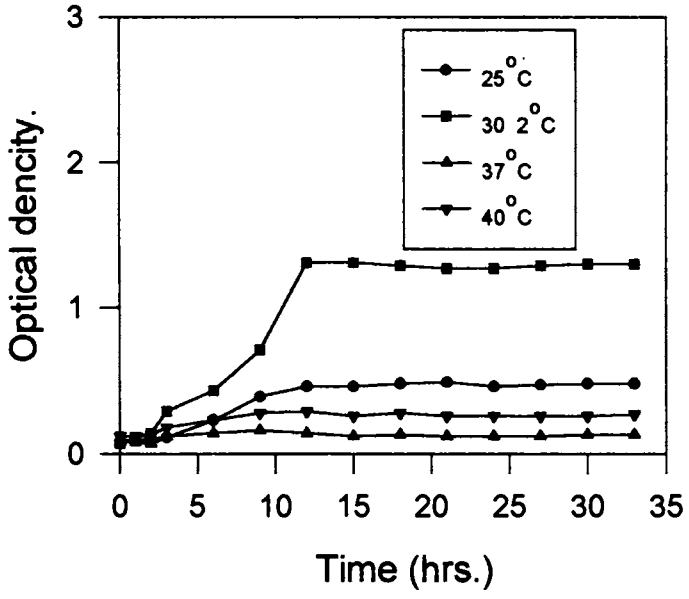


Figure 5.3 Growth curve of *Acinetobacter sp.* at different temperatures.

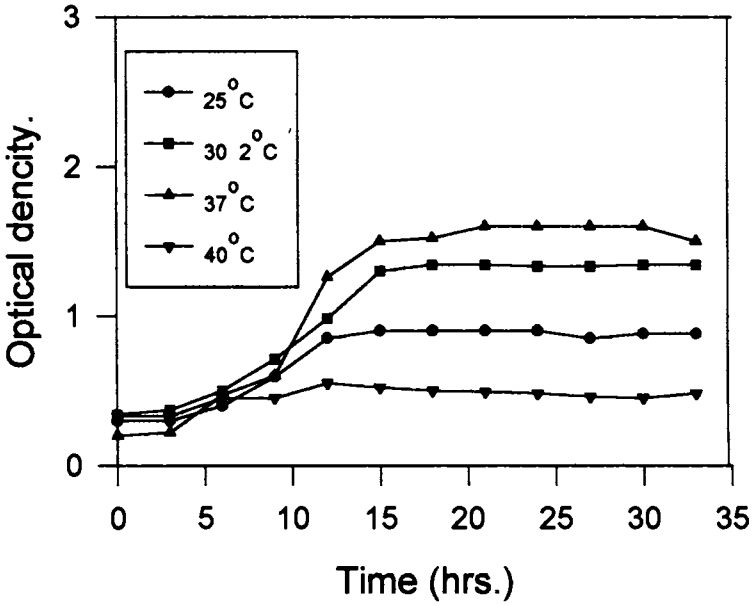


Figure 5.4 Growth curve of *Acinetobacter sp.* at different pH.

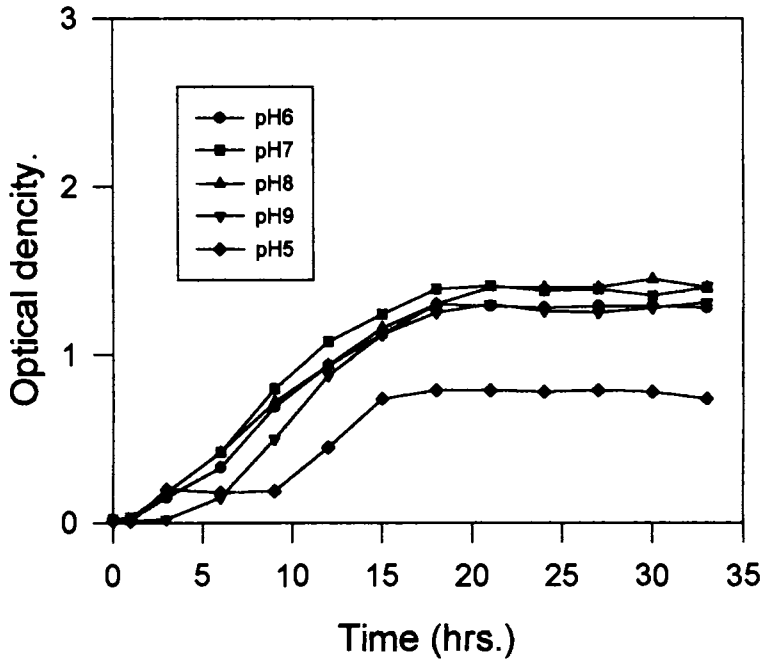


Figure 5.5 Growth curve of *Alcaligenes sp.* at different pH.

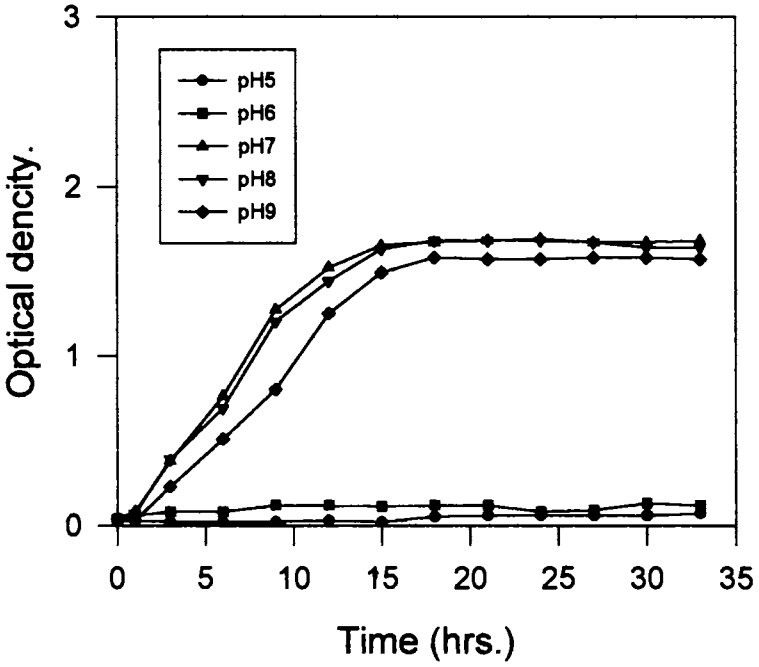


Figure 5.6 Growth curve of *Xanthobacter sp.* at different pH.

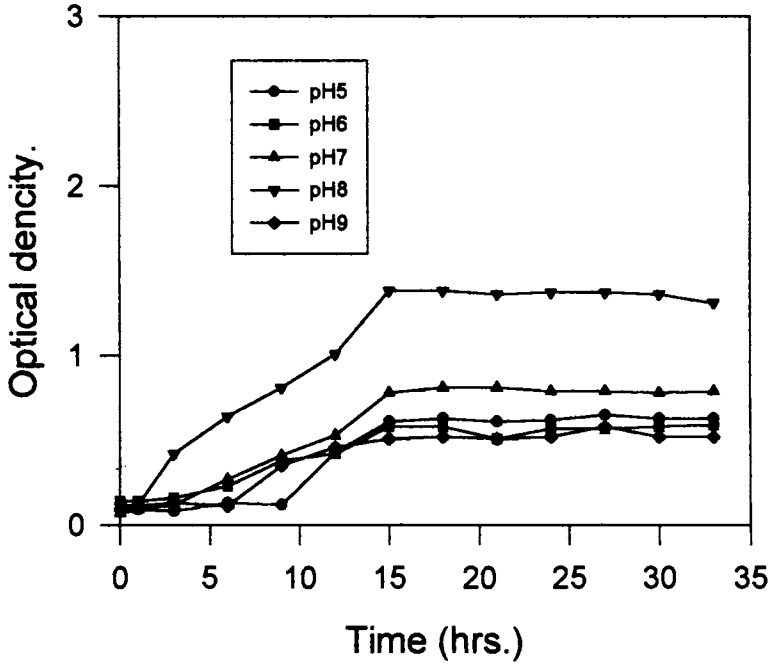


Figure 5.7 Growth curve of *Acinetobacter* sp. at different substrate concentrations.

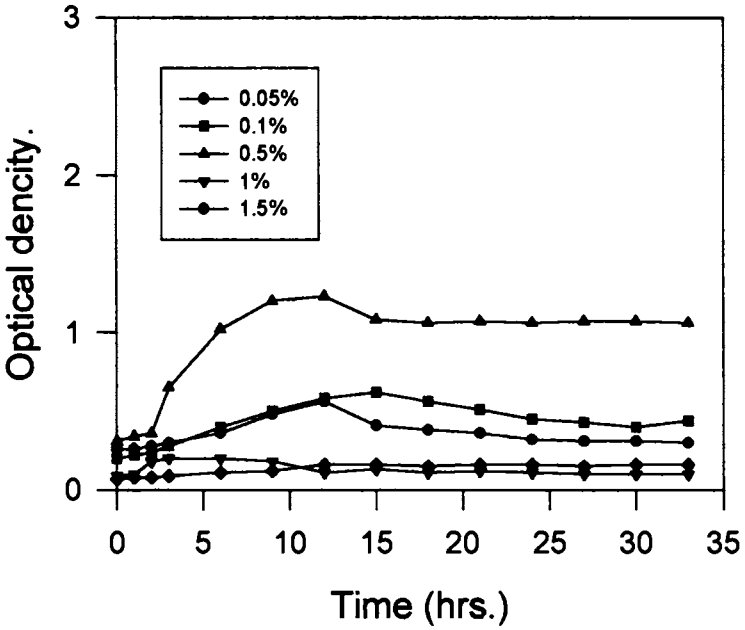


Figure 5.8 Growth curve of *Alcaligenes sp.* at different substrate concentrations.

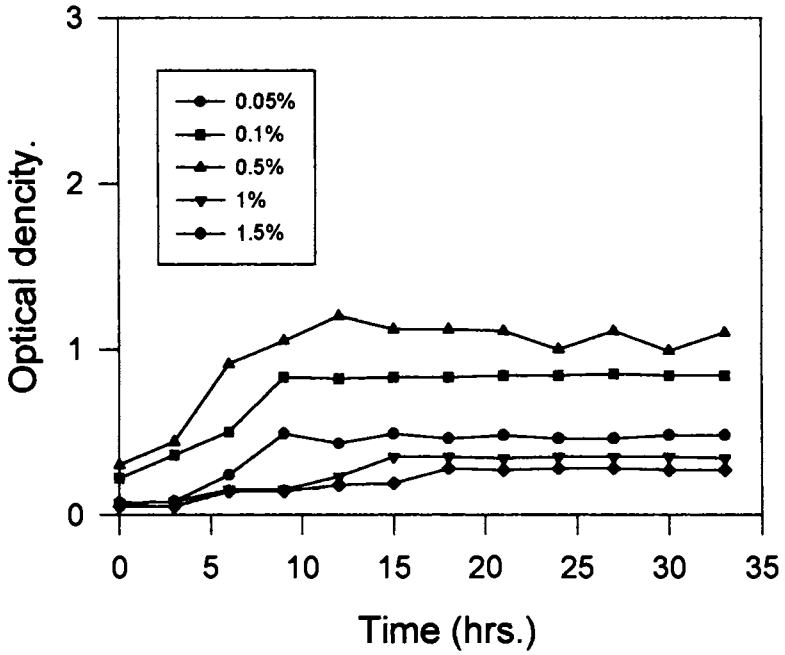


Figure 5.9 Growth curve of *Xanthobacter sp.* at different substrate concentrations.

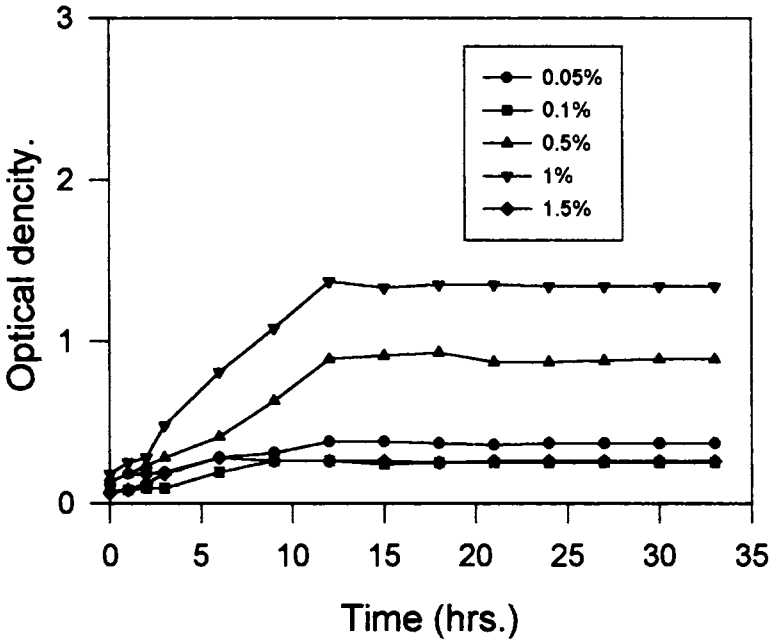
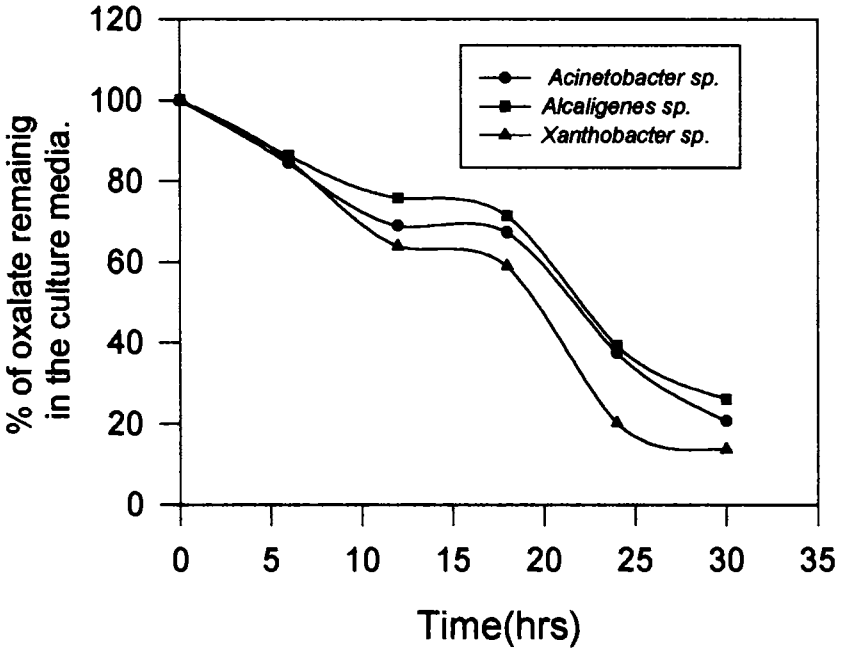


Figure 5.10 Oxalate utilization curve



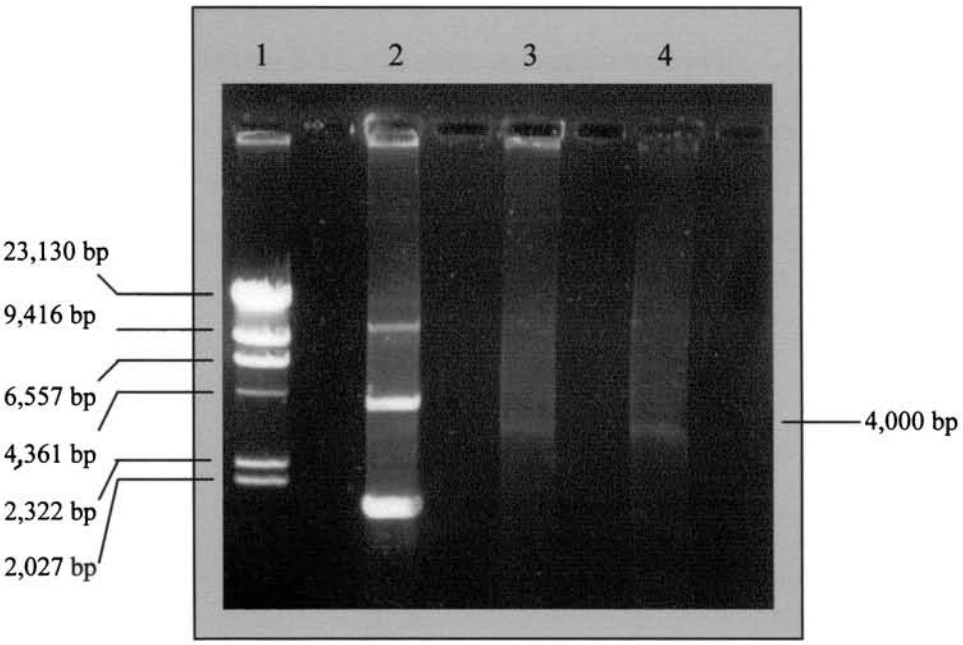
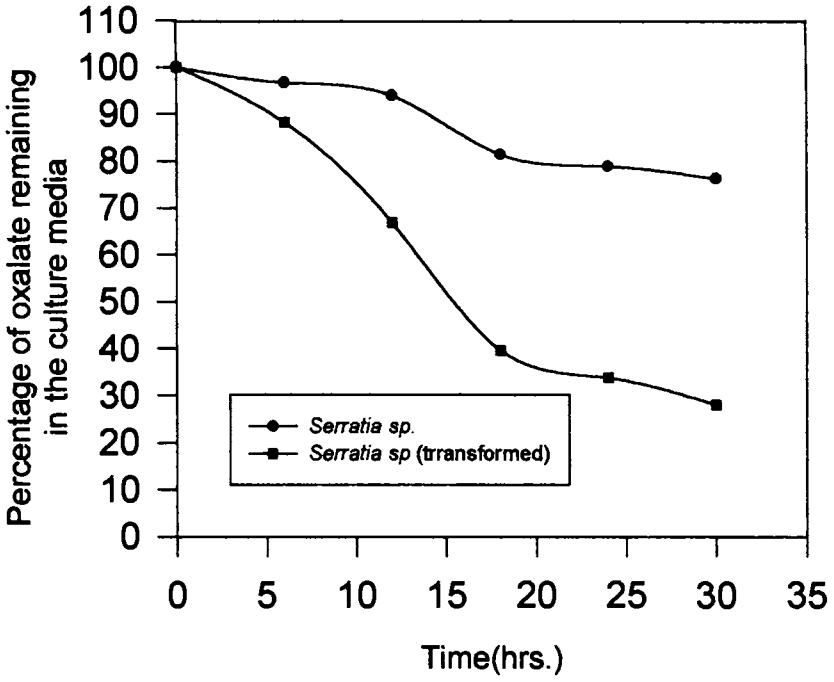


Figure 5.11 Isolation of recombinant plasmid DNA

Lane- 1, λ Hind III digest DNA molecular weight marker
Lane- 2, Plasmid DNA from *Xanthobacter* sp.
Lane- 3, Plasmid DNA from *Xanthobacter* sp., digested with pstI
Lane- 4, Plasmid DNA from transformed *Serratia* sp.

Figure 5.12 Oxalate utilization curve



5.4 Discussion

Oxalic acid is present in small amounts in many plants eaten by humans and animals. High intakes of dietary oxalate may lead to hyperoxaluria with enhanced risk for formation of renal stones. Thus the management of oxalate in individuals susceptible to urolithiasis could seem especially important. There are no known naturally occurring oxalate degrading or metabolizing enzymes in vertebrates, we initiated the study to test the feasibility of isolation of oxalate degrading bacteria from, vegetables waste (Colocasia stem) marine and terrestrial environment to identify the potential strains capable of maximum oxalate degradation.

The three isolated bacterial strains, *Acinetobacter* sp., *Alkaligenes* sp. and *Xanthobacter* sp, from terrestrial, marine and vegetable sources respectively are capable of utilizing oxalate at a comparatively factor rates. The percentage degradation of oxalate by *Xanthobacter* sp. isolated from Colocacia stem was found to be maximum when compared to other bacterial strains isolated from marine and terrestrial environment. Aerobic soil bacteria such as *Pseudomonas oxalaticus* and *Thiobacillus novellus*, are capable of converting oxalic acid to formic acid and CO_2 (Komberg and Elsden, 1961; Quayle et al, 1961, Daniel et al, 1993 and Lug et al 1994 reported that O.*formigenes*, an anaerobic bacterium that degrades oxalate to CO_2 formate, colonises the intestinal tract of man and other animals.

The plasmid DNA detected in *Xanthobacter* sp. was purified and analysed by electrophoresis in agarose gel. A single covalently closed circular DNA was detected, the size of the plasmid DNA was estimated to about 4kb by its mobility in agarose gel with λ Hind III digested marker. There has been no report on the mechanism of plasmid determined oxalate degraders.

Despite of the relative importance of oxalate metabolism in the environment and despite the fact that the existence of oxalate metabolic mechanism has been known for approximately 30 years, virtually nothing is known of the genetic element that controls the expression of oxalate degrading enzymes. In an attempt to improve the oxalate

degrading power of intestinal bacteria, *Serratia* sp., isolated from rat intestine by conventional techniques having minimal oxalate degrading capacity, we introduced the plasmid DNA from the oxalate degrading bacteria, *Xanthobacter* sp. The transformant was shown to harbour a plasmid of the same size as that in *Xanthobacter* sp. The transformant has the ability to degrade oxalate at a higher rate (72%). This results indicate that the plasmid can replicate in the intestinal bacteria, *Serratia* sp. and that it posses genetic information necessary for degradation of oxalate.

Thus our study points out the possibility to reintroduce the transformed *Serratia* sp. into the indigenous intestinal flora of adult laboratory rats, may provide an animal model in which the influence of a transformed microorganism on the fate of dietary oxalate in mammals. Studies with these rats may be useful for gaining a better understanding of factors influencing the absorption of dietary oxalate and its relation to urinary stone formation in human.

SUMMARY

SUMMARY

The role of dicarboxylic acid on oxalate metabolism in experimental hyperoxaluric and Calcium oxalate stone forming rats and isolation and characterisation of oxalate degrading bacteria has been studied under four major sections in the thesis and the results obtained are summarised as follows:

1. *In vitro* calcium oxalate crystal growth studies.
2. Short term studies in hyperoxaluric rats.
3. Long term studies in CPD fed calculogenic rats.
4. Isolation and characterisation of oxalate degrading bacteria.

Section I *In vitro* calcium oxalate crystal growth studies

The method of Bauman and Walker had been used for measuring the inhibitory effect of mono and dicarboxylic acids on the growth and aggregation of calcium oxalate crystals. L(+) tartaric acid, maleic acid, malic acid, succinic acid, pyruvic acid and malonic acid are able to protect a given mass of calcium oxalate from growth. The percentage of crystal growth inhibition increases with increasing concentration of the above acids. L(+) tartaric acid brought about significant reduction in the calcium oxalate crystal growth (20.92%) followed by maleic acid (39.98%), malic acid (41.94%), succinic acid (48.18%), pyruvic acid (59.47%) and malonic acid (69.97%). The results of the above study show the inhibitory action of mono and dicarboxylic acid.

Section II short term studies in hyperoxalic rats

The role of mono and dicarboxylic acid administration on oxalate metabolism in glycollalte induced hyperoxaluric rats were investigated.

Liver GAO activity in the glycollalte induced hyperoxaluric rat was significantly increased. GAO, the major liver enzyme involved in the endogenous synthesis oxalate, which led to increased renal and urinary excretion pattern of oxalate along with the associated calcium. Mono and dycarboxylic acid treatment lowering the level of oxalate synthesising enzyme GAO in the liver, along with kidney tissue and urinary excretion stone forming constituents. LDH activity was found to increase slightly in the liver and kidneys of hyperoxaluric rats and decrease with mono and dicarboxylic acid treatment. The lack of effect of LDH is of advantage, since dicarboxylic acid can be used without affecting the normal metabolic reaction of LDH.

The possibility of regulating oxalate metabolism in hyperoxaluric condition by way of inhibiting liver GAO and super saturation of calcium and oxalate were controlled to greater extent with mono and dicarboxylic acid which indicate its beneficial action in this respect.

Section III Longterm studies in CPD fed calculogenic rats

Experimental calcium oxalate lithiasis was induced in male rats by feeding calculate producing diet for 30 days. The effect of L(+) tartaric acid, maleic acid and malic acid were investigated in kidney and liver tissue and in urine.

Liver is the major tissue to synthesis oxalate endogenously. The key enzyme in oxalate synthesis, GAO activity was elevated in stone formers. Dicarboxylic acid administration decreased GAO activity significantly compared to CPD fed rats but the activity was still higher than the control activity in calculogenic rats. The influence of dicarboxylic acid play and important role on the liver GAO activity which in turn affect the oxalate content in the renal tissue and urine. Liver and kidney LDH activity was increased in CPD fed rats compared to control rats. The enhanced LDH activity was restored to nearly normal in both liver and kidney tissue of dicarboxylic treated rats.

The renal deposition of stone forming constituents elevated in CPD fed rats. Calcium deposition was reduced considerably with dicarboxylic acid treatment when

compared to stone formers. Similarly oxalate content was reduced in calculogenic rats administered with dicarboxylic acid, but not equal to that of control rats. Urinary supersaturation with respect stone forming constituents is generally considered to be one of the causative factors in calculogenesis. CPD rats exhibited increased urinary exhibition of calcium, oxalate and phosphorous and lowered the magnesium level. Dicarboxylic treatment lowered the calcium and oxalate levels considerably increased the magnesium concentration but the values did not equal to that of control. The above investigation indicate that dicarboxylic acids, along with other inhibitors of calcium oxalate crystallization in urine, may have additive effect and thereby hinders stone formation.

Light microscopic examination of Hematoxilin-Eosin (H.E) stained liver and kidney section showed various morphological deformalities. CPD feeding increased endogenous oxalate production which inturn produced focal necrosis in liver tissues. At the same time cystic dialation and extensive necrosis observed in renal tissues. These structural changes at cellular level caused by CPD feeding was reversed considerably by dicarboxylic acid treatment.

Electron microscopic (TEM) examination in stoneforming rats showed the ultrastructural changes occurring in liver and kidney tissues of CPD fed calculogenic rats. We observed nuclear damage and enlargement and cytoplasmic vacuolization in liver tissue of CPD fed rats and calcium oxalate crystal aggregation in the renal intestitial cells and marked dialation in the distal tubules in kidney tissues which was quite evident from the photograph. Dicarboxylic acid treatment helped to regain the structural alteration caused by CPD feeding, to a certain extent.

Section IV Isolation and Characterisation of oxalate degrading bacteria

The formation of calcium oxalate in the urine is dependent on the saturation level of both calcium and oxalate. The management of these ions in individuals susceptible to calcium oxalate stone appears important. Oxalic acid, a highly toxic end product of metabolism, which is catabolised by limited number of bacterial species to yield formate and CO_2. In order to lower the plasma and urinary oxalate concentration in recurrent

calcium oxalate stone formers we have initiated a study of isolation and characterisation of oxalate degraders from vegetable waste (Colocasia stem) and water and soil sources. These organism requires oxalate as a sole source of carbon for growth and utilised oxalate at a rate of 86%, 79% and 73% respectively within 32 h of growth. The strain isolated from colocasia stem, *Xanthobacter* sp. capable of degrading oxalate at a higher rate which harboured a plasmid DNA of about 4kb, as potential strain for further studies.

In an attempt to improve the oxalate degrading ability of the intestinal bacteria, *Serratia* sp., isolated from rat intestine we introduced the plasmid DNA from *Xanthobacter* sp. by transformation. The resulting transformant carry a plasmid DNA of same molecular size as that of *Xanthobacter* sp. and capable of maximum oxalate degradation compared with the parental strain.

These results indicate the scope for utilising these bacterial strains as powerful weapons to eradicate kidney stone disease.

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LIST OF PUBLICATIONS

1. **A. Naseema**, Sheji Mary and G. S. Selvam. (1998), Isolation and characterization of oxalate degrading bacteria from marine and terrestrial environment of Cochin. *Journal of Scientific and Industrial Research* 57(10 & 11): 795-799.

2. **A. Naseema**, Pius S. Padayatti and C. S. Paulose. (1995), Mechanism of wound healing induced by chitosan in streptozotocin diabetic rats. *Current Science* 69(5): 461-464.

SCIENTIFIC MEETINGS ATTENDED AND PAPERS PRESENTED

1. National seminar on "Prospects of Biotechnology in the Next Millenium" sponsored by UGC, Cochin, November 26-27, 1999.

2. "Effect of certain dicarboxylic acids on oxalate metabolism in experimental hyperoxaluric rats". Paper presented in the National Conference on Molecular Diagnostics, June-1998,Trivandrum, Kerala, India.

3. "Isolation and characterization of oxalate degrading bacteria from marine and terrestrial environment of Cochin". Paper presented in the International Conference on Frontiers in Biotechnology (ICFB'97), November-1997, Trivandrum, Kerala, India.

4. "Mechanism of wound healing induced by chitosan in streptozotocin diabetic rats". Paper presented in the National symposium on Relevance of Biotechnology in Industry, March-1995, Cochin, Kerala, India.