Chapter 4

BIOCHEMICAL EFFECT OF DICARBOXYLIC ACIDS ON OXALATE METABOLISM IN EXPERIMENTAL STONE FORMING RATS -LONG TERM STUDIES
CHAPTER- IV
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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-Horsfall 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 inclusion 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; Vermuculan 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 pain. The typical symptom of upper urinary tract calculi 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 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, supplemented 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.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Received commercial diet and served as control.</td>
</tr>
<tr>
<td>II</td>
<td>Rats fed with CPD for 30 days to induce stone formation.</td>
</tr>
<tr>
<td>III</td>
<td>Received CPD for 30 days followed by L(+) tartaric acid (50 mg/ml of 0.9% saline/day/rat) for 15 days.</td>
</tr>
<tr>
<td>IV</td>
<td>Received CPD for 30 days followed by maleic acid (50 mg/ml of 0.9% saline/day/rat) for 15 days.</td>
</tr>
<tr>
<td>V</td>
<td>Received CPD for 30 days followed by malic acid (50 mg/ml of 0.9% saline/day/rat) for 15 days.</td>
</tr>
<tr>
<td>VI</td>
<td>Received CPD with L(+) tartaric acid (50 mg/ml of 0.9% saline/day/rat) for 30 days.</td>
</tr>
<tr>
<td>VII</td>
<td>Received CPD with maleic acid (50 mg/ml of 0.9% saline/day/rat) for 30 days.</td>
</tr>
</tbody>
</table>
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°C. 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.H₂O - 200 ml
2. 2 N NaOH
3. 0.1% gum ghatti
4. Stock standard (1 mg/ml) - 100 ml
   \[ \text{MgCl}_2 \cdot 6\text{H}_2\text{O} \] - 8.458 g
   Dissolved and made up to 100 ml with D.H\textsubscript{2}O

5. Working standard (5 \(\mu\)g/ml)
   Stock standard - 1 ml
   D.H\textsubscript{2}O - 199 ml

6. Calcium chloride solution
   \[ \text{CaCl}_2 \] - 13.88 mg
   Dissolved and made up to 100 ml with D.H\textsubscript{2}O

4.2.8.2 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 \(\mu\)g and a blank with 1 ml 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 up to 300 ml with D.H\textsubscript{2}O

3. Alcoholic eosin - 1%
   Alcoholic eosin - 3 g
   70\% ethanol - 300 ml
4. Harris Hematoxylin solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoxylin crystals</td>
<td>- 5 g</td>
</tr>
<tr>
<td>Absolute alcohol</td>
<td>- 50 ml</td>
</tr>
<tr>
<td>Ammonium/potassium</td>
<td></td>
</tr>
<tr>
<td>aluminium sulphate</td>
<td>- 100 g</td>
</tr>
<tr>
<td>D.H₂O</td>
<td>- 1000 ml</td>
</tr>
<tr>
<td>Mercuric oxide (red)</td>
<td>- 2.5 g</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrated HCl</td>
<td>- 1 ml</td>
</tr>
<tr>
<td>70% Alcohol</td>
<td>- 99 ml</td>
</tr>
</tbody>
</table>

6. Ammonia water

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid ammonia</td>
<td>- 1 ml</td>
</tr>
<tr>
<td>D.H₂O</td>
<td>- 99 ml</td>
</tr>
</tbody>
</table>

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 - overnight

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°C to 62°C. 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 (OsO₄).

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 (OsO₄) for 2 h.
4. OsO₄ 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°C. The dehydration was continued as follows at 4°C.

- 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:

<table>
<thead>
<tr>
<th>Embedding media</th>
<th>Acetone</th>
</tr>
</thead>
<tbody>
<tr>
<td>6. a)</td>
<td>1</td>
</tr>
<tr>
<td>7. b)</td>
<td>1</td>
</tr>
<tr>
<td>8. c)</td>
<td>3</td>
</tr>
</tbody>
</table>

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°C 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% glycocolate mixed with normal feed. Glycocolate 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 Hematoxylin - Eosin (H-E) stained liver sections of 30 days CPD-fed animals showed focal necrosis (Fig. 4.10) 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. 4.19). 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. 4.39). 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 intestinal cells (Fig. 4.39) 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>
<thead>
<tr>
<th>Method</th>
<th>Animals</th>
<th>Time Required to Produce (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low phosphorous diet</td>
<td>Adult male and female rats (Wistar)</td>
<td>63</td>
</tr>
<tr>
<td>Ethylene glycol (0.25% in drinking water)</td>
<td>Adult male rats (Charles River)</td>
<td>28</td>
</tr>
<tr>
<td>Ethylene glycol (1% in drinking water)</td>
<td>Adult male rats (Sprague-Dawley)</td>
<td>35</td>
</tr>
<tr>
<td>Ethylene glycol (1% in drinking water)</td>
<td>Adult male rats (Wistar)</td>
<td>35</td>
</tr>
<tr>
<td>Glyceric acid (3% in normal diet)</td>
<td>Adult male rats (Wistar)</td>
<td>21</td>
</tr>
<tr>
<td>Hydroxyproline (2.5g/kg daily)</td>
<td>Adult male and female rats (Wistar)</td>
<td>4</td>
</tr>
<tr>
<td>Chlorite acid (3%) in normal diet</td>
<td>Adult male rats (Wistar)</td>
<td>21</td>
</tr>
<tr>
<td>Pyridoxine-deficient diet</td>
<td>Adult male rats (Sprague-Dawley)</td>
<td>21</td>
</tr>
<tr>
<td>Pyridoxine-deficient diet</td>
<td>Adult male rats (Hartley) and adult female rats (Wistar)</td>
<td>21</td>
</tr>
<tr>
<td>Pyridoxine-deficient diet</td>
<td>Adult male rats (Wistar)</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 4.1 Experimental production of calcium oxalate renal stones in animals

References:
- Cobum and Packett (1962)
- Andrus et al. (1960)
- Lyon et al. (1966a)
- Lyon et al. (1966b)
- Chow et al. (1974)
- Chow et al. (1975)
- Thomas et al. (1971)
- Borden and Lyon (1969)
- Vail et al. (1971)
### Table 4.2. Body weight, tissue weight and tissue protein concentration of control and experimental rats

<table>
<thead>
<tr>
<th>Group</th>
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<tr>
<td></td>
<td>V</td>
<td>VD</td>
<td>VM</td>
<td>IX</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>IV</td>
</tr>
<tr>
<td>(CPD)</td>
<td>CPD</td>
<td>CPD</td>
<td>CPD</td>
<td>CPD+</td>
<td>L(+)</td>
<td>Maleic tartaric</td>
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<td>8.3</td>
<td>8.1</td>
<td>8.9</td>
<td>8.1</td>
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Values are mean ± SEM of 5-6 separate experiments and experimental rats.

(Change in body weight after 30 days)
Table 4.3. The effect of glycollate and dicarboxylic acid administration on the level of oxalate synthesising enzymes-

<table>
<thead>
<tr>
<th>Group</th>
<th>Enzyme Group I (Units/l (Control) mg protein)</th>
<th>Enzyme Group II (CPD)</th>
<th>Enzyme Group III (L(+)) Maleic acid</th>
<th>Enzyme Group IV (Malic acid)</th>
<th>Enzyme Group V (L(+)) Tartaric acid + CPD</th>
<th>Enzyme Group VI (CPD)</th>
<th>Enzyme Group VII (L(+)) Maleic acid + CPD</th>
<th>Enzyme Group VIII (L(+)) Tartaric acid + CPD</th>
<th>Enzyme Group IX (L(+)) Tartaric acid only</th>
<th>Enzyme Group X (Maleic acid only)</th>
<th>Enzyme Group XI (Malic acid only)</th>
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<tbody>
<tr>
<td>I</td>
<td>Liver 0.45± 0.52±</td>
<td>LDH 0.02± 0.04±</td>
<td>GAO 0.13± 0.15±</td>
<td>Kidney 0.50± 0.65±</td>
<td>CPD 0.4± 0.5±</td>
<td>L(+) Maleic acid 0.4± 0.04±</td>
<td>Malic acid 0.5± 0.03±</td>
<td>Tartaric acid + CPD 0.6± 0.03±</td>
<td>Tartaric acid + CPD 0.6± 0.03±</td>
<td>Maleic acid 0.4± 0.04±</td>
<td>Maleic acid 0.4± 0.04±</td>
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<tr>
<td>II</td>
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<td>0.0± 0.0±</td>
<td>0.0± 0.0±</td>
<td>0.0± 0.0±</td>
<td>0.0± 0.0±</td>
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<td>0.0± 0.0±</td>
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<tr>
<td>III</td>
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<td>0.0± 0.0±</td>
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<tr>
<td>IV</td>
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<td>0.0± 0.0±</td>
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<tr>
<td>V</td>
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<td>VI</td>
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<tr>
<td>VII</td>
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<td>VIII</td>
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<td>0.0± 0.0±</td>
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<tr>
<td>XI</td>
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<td>0.0± 0.0±</td>
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</table>

Values are mean ± SEM of >6 separate experiments.

One unit of LDH (Lactate Dehydrogenase) - Enzyme required to produce 0.1 ml of pyruvate/min at 37°C.

One unit of GAO (Glycollic Acid Oxidase) - Enzyme required to produce 0.1 mmole of glyoxylate/min at 37°C.

When compared to control. 100>p>0.01, **p>0.001, ***p>0.0001.
Table 4.4 - Kidney tissue deposition of oxalate, calcium and phosphorus

<table>
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<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
</tr>
</thead>
<tbody>
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<td>Oxalate (mg/g)</td>
<td>Calcium (mg/g)</td>
<td>Phosphorus (mg/g)</td>
<td>Ca/Ox</td>
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<tr>
<td></td>
<td>Wet tissue</td>
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<td>0.69</td>
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<td>0.47</td>
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<td>0.06±0.07</td>
<td>0.64±0.30</td>
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</table>

Values are mean ± SEM of 5-6 separate experiments.

*p < 0.05 when compared to control.

**p < 0.01 when compared to control.

***p < 0.001 when compared to control.
Figure 4.1. Stone forming constituents in the kidney

- Oxalate
- Calcium
- Phosphorus

Control
CPD
CPD + Tartarate
CPD + Maleic acid
CPD + Malic acid
CPD & Tartarate
CPD & Maleic acid
CPD & Malic acid
Tartarate
Maleic acid
Malic acid

mg / gm wet tissue
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)

<table>
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<td>Calcium</td>
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<td>2.08±</td>
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<td>0.12±</td>
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<td>0.23±</td>
<td>0.28±</td>
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<td>0.25±</td>
<td>0.22±</td>
<td>0.25±</td>
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<tr>
<td>Magnesium</td>
<td>2.99±</td>
<td>1.39±</td>
<td>2.01±</td>
<td>1.89±</td>
<td>1.71±</td>
<td>0.26±</td>
<td>0.12±</td>
<td>0.18±</td>
<td>0.11±</td>
<td>0.12±</td>
<td>0.18±</td>
<td>0.11±</td>
<td>0.18±</td>
<td>0.11±</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 5-6 separate experiments. When compared to control, 10'<p<0.05 and *p<0.01. **p<0.001.
Figure 4.3. 24h urinary excretion of oxalate and calcium
Figure 4.4. 24h urinary excretion of phosphorus and magnesium

Control
CPD
CPD+ Tart.
CPD+ Maleic
CPD+ Malic
CPD&Tart.
CPD&Maleic
CPD& malic
Tartarate
Maleic acid
Malic acid

mg / 24hrs

PHOSPHORUS
MAGNESIUM
Figure 4.5. Urinary excretion of stone forming constituents

![Bar chart showing the 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 30 days of CPD feeding (H-E x 200)

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

Figure 4.9. CPD fed rat kidney after 15 days of maleic acid treatment (H-E x 200)
Figure 4.10. CPD fed rat kidney after 15 days of malic acid treatment (H-E x 200)

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

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

Figure 4.13. Normal kidney cells after 30 days 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 15 days of L(+) tartarate treatment (H-E x 200)

Figure 4.20. CPD fed rat liver after 15 days of maleic acid treatment (H-E x 200)
Figure 4.21. CPD fed rat liver after 15 days of malic acid treatment (H-E x 200)

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

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

Figure 4.24. Normal rat liver after 30 days of CPD and malic acid treatment (H-E x 200)
Figure 4.25. Normal rat liver after L(+) tartaric acid treatment for 30 days (H-E x 200)

Figure 4.26. Normal rat liver after maleic acid 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 dilated 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 tubule TEM 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 15 days 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 liver after 15 days of L(+) tartarate treatment TEM x 8000
Figure 4.40. Electron micrograph of CPD fed rat liver after 15 days of maleic acid treatment. TEM x 6000

Figure 4.41. Electron micrograph of CPD fed rat liver after 15 days 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.