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2.1 SCOPE OF REVIEW:

The scope of the present review is to investigate the excretion patterns of lithogenic substances (calcium, oxalate, uric acid and phosphate), inhibitors (citrate, pyrophosphate, polyphosphates), inhibitory activity and metals (magnesium, iron, copper and zinc) amongst idiopathic calcium oxalate stone formers. Further, the contribution by high molecular weight inhibitors and promoters is reviewed to understand the nucleation, epitaxial growth and aggregation of these crystals, resulting in the formation of urinary calculi. The role played by biological time fluctuations (circadian and circannual) is emphasized to get a better insight into the metabolic role played by these moieties in the final formation of urinary stones.

2.2 HISTORICAL ASPECTS:

The ancient malady of urinary stones continues to pose a universal health problem even today. The earliest stone was discovered in the mummy of a sixteen year old Egyptian boy, buried in about 4800 B.C. (Shattock, 1905; Ellis, 1969). Ironically, after more than 6500 years of preservation, the stone was pulverized during the 1941 bombings. Early accounts of urinary tract stone disease have been made by Susruta (from India), Hippocrates (from Greece) and Celsus (from Rome). Galenus (131-201AD), who was interested in the multi-factorial aspects of this disease, related stone formation to nutrition, heredity and
climate. From the start considerable medical efforts have been spent on treatment and research. Surgical treatments were already performed in ancient Greece and India (Schneider and Doberentz, 1979).

Oxalic acid was discovered by Angelus Sala in the sixteenth century and was found to be associated with urinary stones. Wollaston (1810) and Donne (1839), in the early part of the nineteenth century, first recognized calcium oxalate crystals in the urine and renal stones. Earlier workers (Gaglio, 1887; Pohl, 1896) had correctly postulated that "oxalic acid is not destroyed in the animal body", a statement which still holds true.

Bladder stones occur rather frequently in some countries (Turkey, India, Thailand etc.) probably due to a general deficiency of animal protein and/or fats (Anderson, 1962; 1973) and to relatively low hygienic standards. Hippocrates (460-370 BC), who had earlier differentiated between vesical (bladder) and renal (kidney) stones, studied the problem which threatens the modern world even today. Well-developed western countries like England, the Scandinavian countries, Germany, Holland and the U.S.A. show a rising incidence and prevalence of stones formed in the kidney and in the higher parts of the ureter (Anderson, 1973; Robertson et al, 1979; Iguchi et al, 1984). These are mostly rather small stones that leave the body spontaneously or after simple dietary measures. The majority of these stones are made either of pure calcium oxalate or a mixture of calcium oxalate and calcium
phosphate, thus emphasizing the need for studying oxalic acid metabolism.

It has been observed that with the changing pattern of life in the present day world, the incidence of vesical stones has decreased considerably while those formed in the kidney and upper urinary tract have increased significantly. Increased stress situations in life today have been suggested to enhance the risk of stone formation (Brundig, 1981). A high intake of animal protein (Anderson, 1973; Robertson, 1978, 1979, 1980) and a high economic standard in the developed countries could be correlated to an increasing incidence of renal calculi. Anderson (1973) pointed out the paradoxical situation that both a shortage and a surplus of dietary animal protein may have a promoting effect on stone formation. These facts substantiate the steadily increasing importance of this multifactorial affliction (Robertson, 1980, 1981) that causes so much pain and agony.

Since last two decades several aspects of urolithiasis and oxalate metabolism have been reviewed (Hagler and Herman, 1973; Hodgkinson, 1978; Drach, 1978; Nath et al, 1984). Literature on urolithiasis is also being updated every four years at international meets. The sixth of this series was held recently (July, 1988) at Vancouver, Canada.

2.3 ROLE OF OXALATE IN UROLITHIASIS:

In the biological systems oxalate occurs in the form
of calcium salts with its three forms i.e. calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD) and calcium oxalate trihydrate (COT). Calcium oxalate trihydrate is a very unstable form whereas, the other two types are abundantly found in the human system. Recently, it has been reported that the conversion of COD to COM in biological system triggers crystalluria and aggregation of this salt; as COM is more membranolytic as compared to COD (Weissner et al, 1986).

2.3.1 Oxalate metabolism:

Calcium oxalate has been identified as a major component of two-thirds of all the urinary stones (Thind and Nath, 1969; Williams, 1974). The human body gets its oxalic acid from exogenous sources i.e. dietary oxalate absorbed through the intestine, as well as by endogenous synthesis from its precursors viz. ascorbic acid, glycine, serine, glycolate, hydroxyproline etc.

Most of the oxalate in the diet exists in the form of highly insoluble calcium salts which is unabsorbable. However, nearly 5 percent of the ingested dietary oxalate is absorbed and excreted in the urine (Marshall et al, 1972; Chadwick et al, 1973). The average daily intake of oxalate varies from 100-400 mg/day and the foods like leafy vegetables, sorrel, rhubarb and spinach have been found to be rich in oxalate. Among the beverages tea, coffee and cocoa contain a high oxalate content. Approximately, 10 to 20 percent of the excreted oxalate arises from absorbed
dietary oxalate and the remainder of the oxalate in the urine arises as a result of endogenous production from its two major precursors viz. glyoxylate and ascorbic acid (Ney et al, 1981). Conversion of ascorbic acid to oxalic acid apparently occurs in the liver by a process involving diketogluconic acid with the first two carbons of ascorbic acid being converted to oxalate. Apparently the enzymes involved in the conversion of ascorbic acid to oxalate are saturable at low concentrations of ascorbic acid, since ingestion of fairly large doses of ascorbic acid does not appear to increase urinary oxalate. On the other hand, some studies have demonstrated that with high doses of ascorbic acid (greater than 5 gm per day) some elevation of urinary oxalic acid does occur (Kallnor, 1979; Schmidt et al, 1981), and under certain conditions ascorbic acid can undergo apparent nonenzymatic oxidation to oxalate in urine (Chalmers et al, 1985). An increased urinary oxalate and decreased ascorbate excretion in recurrent stone formers as compared to controls have been demonstrated after oral (2 gm) administration of ascorbic acid, suggesting an increased conversion of ascorbate to oxalate in the gastrointestinal tract of recurrent stone formers (Chalmers et al, 1986). However, little is known about the factors controlling this metabolite conversion but it is estimated that approximately 35 to 50 percent of urinary oxalate comes from ascorbic acid (Rivers, 1987).

The other major precursor of oxalate is glyoxylate,
which accounts for the synthesis of approximately 50-70 percent of urinary oxalate. The major sources of glyoxylate in man include glycine, glycolic acid and serine. Glycine is converted to glyoxylate by D-amino acid oxidase. Glycolate is oxidized to glyoxylate by glycolic acid oxidase and serine can be converted to glycine or to glycolic acid via ethanolamine and glycoaldehyde (Williams and Wandzilak, 1989). The detailed reactions and conversions of various precursors to glycolate-glyoxylate-oxalate have been shown in Fig. 1.

2.3.2 Absorption and Excretion of oxalate:
Most ingested oxalate appears to be bound by intraluminal calcium in the small intestine and it is excreted as insoluble calcium oxalate complexes (Earnest et al, 1974). It is this phenomenon that probably accounts for the fact that only 10-20 percent of ingested oxalate is absorbed in normal individuals. The concentration of intraluminal calcium appears to have a major role in determining the amount of dietary oxalate absorbed and an inverse relationship exists between the amount of calcium ingested and the amount of oxalate absorbed by the gastrointestinal tract. The mechanisms of oxalate absorption by the gastrointestinal tract as studied by various authors (Caspary, 1977; Schwartz et al, 1980) suggested that this transport was non-energy dependent, non-saturable, passive process. However, these experiments were performed with calcium as a buffer system, and it was
FIG. 1

METABOLIC PATHWAYS OF OXALATE BIOSYNTHESIS IN ANIMALS
Various enzymes involved in the metabolic pathway of oxalate

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Enzyme</th>
<th>Coenzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glycolic Acid Oxidase</td>
<td>FMN</td>
</tr>
<tr>
<td>2</td>
<td>Lactate dehydrogenase</td>
<td>NAD⁺</td>
</tr>
<tr>
<td>3</td>
<td>D-amino acid oxidase</td>
<td>FMN</td>
</tr>
<tr>
<td>4</td>
<td>Glyoxylate transaminase</td>
<td>B₆</td>
</tr>
<tr>
<td>5</td>
<td>Aldehyde dehydrogenase</td>
<td>NAD⁺</td>
</tr>
<tr>
<td>6</td>
<td>Ethanolamine oxidase</td>
<td>FAD/FMN</td>
</tr>
<tr>
<td>7</td>
<td>Serine decarboxylase</td>
<td>PALP</td>
</tr>
<tr>
<td>8</td>
<td>Serine transaminase</td>
<td>PALP</td>
</tr>
<tr>
<td>9</td>
<td>Hydroxypyruvate decarboxylase</td>
<td>TPP</td>
</tr>
<tr>
<td>10</td>
<td>Serine: Glycine-hydroxymethyl transferase</td>
<td>Folate</td>
</tr>
<tr>
<td>11</td>
<td>Glycolate dehydrogenase</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>4-Hydroxy-2-Ketoglutarate aldolase</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>Glyoxylate carboligase</td>
<td>TPP</td>
</tr>
<tr>
<td>14</td>
<td>Hexose monophosphate shunt</td>
<td>–</td>
</tr>
<tr>
<td>15</td>
<td>Non-enzymatic conversion</td>
<td>–</td>
</tr>
<tr>
<td>16</td>
<td>Alcohol dehydrogenase</td>
<td>NAD⁺</td>
</tr>
</tbody>
</table>
later demonstrated that calcium was required to maintain the integrity of the conductive pathway across gastrointestinal epithelium. Isolated segments of rat and rabbit colon demonstrated a net transport of oxalate and chloride (Freel et al, 1980). The studies of Prenen et al (1984) demonstrated a peak absorption of oxalate between 2-4 hours after ingestion. More recently, Knickelbein et al (1986) demonstrated the presence of oxalate transport in brush border membrane vesicles of rabbit ileum. Oxalate could be transported in exchange for either hydroxyl or chloride ions and it could be inhibited by the anion exchange inhibitor, 4,4'-di-isothiocynatostilbene-2,2'-disulfonic acid (DIDS). Among other organic acids only formate and oxaloacetate could stimulate oxalate and chloride uptake. These studies confirmed the cellular absorption of oxalate as an active process of transport.

Increased absorption of dietary oxalate can explain the modest hyperoxaluria seen in some patients with idiopathic calcium oxalate nephrolithiasis. Schwille et al (1984) showed that with fasting, the amount of urinary oxalate in patients in idiopathic stone disease did not differ from that in controls, but patients exhibited a greater degree of postprandial hyperoxaluria. However, Allison et al (1986) demonstrated the degradation of oxalate by gastrointestinal bacteria from human, and subsequently releasing carbon dioxide, thus preventing the absorption of oxalate in the intestine. Recently, Lindsjo et al (1989) indicated the increased uptake of both calcium
and oxalate in patients with recurrent formation of calcium oxalate stones.

Oxalate is freely filtered at the glomerulus. Micropuncture studies indicate that there may be two secretory systems in the proximal tubule and oxalate is secreted in the early part of the proximal convoluted tubules and is probably not transported further down the tubule (Weinman et al., 1978). Later, Osswald and Hautmann (1979) studied the renal handling of oxalate in six subjects by the rapid injection of $^{14}$C-oxalate into the renal artery and the subsequent collection of urine via a catheter inverted into the renal pelvis. A 2-3 fold higher excretion of oxalate was demonstrated as compared to inulin or creatinine, confirming that tubular secretion has a major role in oxalate excretion in man. The above observation was further confirmed in various animals like rat (Weinman et al., 1978) and chicken (Tremaine et al., 1985). Tremaine and associates (1985) demonstrated saturable excretory transport of oxalate through the chicken renal tubular cells in vivo. The transport of oxalate was not affected by probenecid, suggesting a separate system from that of uric acid, but it was reduced by α-ket-glutaric acid, indicating a possible involvement of dicarboxylate transporter. In a recent study using the immobilized oxalate-oxidase method for determining plasma and urine oxalate, the fractional excretion (FE) of oxalate in normals and idiopathic calcium oxalate stone formers was
less than 1.0, suggesting that oxalate is reabsorbed within
the nephron (Wilson et al., 1988). Further investigation is
needed to confirm this observation and define the site of
oxalated reabsorption (Williams and Wilson, 1990).

2.4 MINERAL METABOLISM:

Mineral composition of urinary calculi invariably
shows that more than 90% of the stones are composed of
calcium, magnesium and phosphate besides oxalate. Thus the
pathogenesis of renal calculi is strongly linked with
disorders of mineral metabolism. The earliest evidence of
histological change in the kidney of magnesium deficient
rats was the appearance of spherical monoliths in the thin
lumen of the nephron, which were thought to be the origin
of renal stones (Modlin, 1967). Subsequently Oreopaulos et
al. (1969) showed the low magnesium/calcium ratio as one of
the factors of initiation of human urinary calculi.
Characteristic sites of calcium deposition were found to be
located in the lumen of the loop of Henle and in the
collecting tubule (Cooke et al., 1971), and idiopathic
hypercalciuria has been found to be one of the disorders of
stone formation (Insogna et al., 1985). The intrarenal
environment of calcium, magnesium and phosphate vary
considerably within the various parts of the nephron and in
the tissue of the papilla, according to the food intake and
metabolic state of the person. Such variations in minerals
play a key role in both stone formation and crystal
aggregation (Berg et al., 1986).

2.4.1 Homeostatic mechanisms controlling calcium metabolism

Calcium is the most abundant cation in the body, and as almost all calcium is in the bones, the total body calcium can be assessed by the bone mass, which averages about 1 kg in man. Only 1% of the calcium occurs in the extracellular fluid and various other soft tissues, and has been recognized as the key ion in regulating cellular functions (Lyles et al., 1981). Although concentration of ionic Ca in extra-cellular fluid is about 1.3 mM/l (Gupta, 1968), its concentration in the cell compartments is very low ($10^{-7}$-$10^{-8}$M), because higher concentrations of free calcium is known to inhibit many important physiological processes.

Besides cellular calcium, the serum calcium levels are also precisely regulated within a narrow range and its association with proteins and other ligands are pH and temperature dependent (Gupta, 1967, 1967a, 1967b, 1968). For plasma levels to remain constant, the sum of all the inputs of calcium into the extracellular fluid must balance the sum of all the outputs from the extracellular fluid. Schematic representation of homeostatic calcium balance in man occurs in the gastrointestinal tract, bone surface and in the kidney (Fig. 2). Regulation and homeostasis of calcium thus depends on the movement of calcium from these sites. Vitamin 'D' and parathyroid hormone determine the equilibrium of these processes.
FIG. 2 SCHEMATIC REPRESENTATION OF HOMEOSTATIC CALCIUM BALANCE IN MAN
Schematic representation of the action of Vitamin D on the intestinal epithelial cell is shown in Fig. 3.

The homeostatic process mobilizes 2000-4000mg calcium per day and involves hormonal regulation. Any increase in the calcium concentration above the one predicted from bone mineral solubility is due to the effect of PTH on cellular metabolic functions and that the mobilization of calcium from the bone requires both PTH and 1,25-(OH)$_2$-D$_3$. PTH has at least two effects on calcium mobilization, firstly it rapidly stimulates the translocation of Ca$^{++}$ from bone to the extracellular fluids as measured by the $^{45}$Ca and secondly it also promotes the development, on arrival, of new multinucleate cells in the later stages (Carafoli, 1987).

2.4.2 Physiological basis for idiopathic hypercalciuria:

An increased urinary calcium concentration is a factor favouring the nucleation and precipitation of calcium oxalate or apatite from urine and subsequent crystal growth. Hypercalciuria is defined as urinary excretion of more than 300 mg per kg. BW per day. Hypercalciuria was first observed by Flocks et al, 1939, while Henneman et al (1958) exhibited hypercalciuria without hypercalcemia and hypophosphaturia. Coe et al (1979) classified such patients with idiopathic hypercalciuria and proposed following pathophysiological mechanisms:
FIG. 3 SCHEMATIC REPRESENTATION OF THE ACTION OF VITAMIN D ON THE INTESTINAL CELL
2.4.2.1 Intestinal calcium hyperabsorption:

Patients with idiopathic hypercalciuria have been shown to exhibit increased rates of calcium absorption in comparison to healthy subjects (Pak et al., 1972; Lemann, 1980). Moreover, in idiopathic hypercalciuria patients exhibit an exaggerated calcium excretion following ingestion of standard calcium load (Pak et al., 1975; Broadus et al., 1978) reflecting an intestinal calcium absorption resulting from increased production of 1,25-(OH)$_2$ vitamin D$_3$ (Kaplan et al., 1977; Gray et al., 1977; Broadus et al., 1984). Moreover, 1,25-(OH)$_2$ vitamin D$_3$ measured by the tritium labeled vitamin D$_3$ also showed an increased production rate amongst hypercalciuric patients.

However, Favus et al. (1988) showed that in idiopathic hypercalciuria, hyperabsorption of intestinal calcium may be independent of circulating vitamin D$_3$. He further justified his observation that vitamin D$_3$ mediated transport could increase without any change in circulating 1,25-(OH)$_2$-D$_3$ levels through an increase in enterocyte vitamin D receptors which may amplify the target tissue response to 1,25-(OH)$_2$ vitamin D$_3$.

Studies performed with duodenal brush border membrane vesicles from chick and rat intestine and erythrocyte ghosts revealed that uptake of calcium in the membrane is saturable and involves mostly binding (Sharma et al., 1979; Bikle et al., 1983; Bronner et al., 1986). It was shown that $^{45}$Ca binding with RBC ghost was dependent on cholesterol content per RBC. Further, Deluca (1988) showed
that the stimulatory effect of vitamin D₃ on calcium absorption is secondary to a variety of structural and biochemical changes viz. increase of the mucosa of the microvilli or increased synthesis of specific transport proteins and enzymes in the brush border membrane surface. Borle (1981) had initially demonstrated that calmodulin activates the calcium, magnesium - ATPase in the basolateral membrane and this augments the calcium transport from the cell to the blood. The total cellular calcium concentration determines the amount of calcium binding proteins (CaBP) produced in response to 1,25-(OH)₂ vitamin D₃. Pinto et al (1985) showed that calcium binding proteins of stone formers have higher capacity to transport calcium ions as compared to control subjects. This effect may occur by modulation of transcription or translation of mRNA for Ca binding proteins. The CaBP mediates the release of mitochondrial calcium and phosphate at the site of the basolateral calcium pump in exchange for sodium. Further, Deluca, (1988) and Borke et al, (1988) characterized the vitamin D dependent protein which has been located abundantly in the enterocyte membrane. However, controversy still exists regarding the mechanisms that account for the augmentation of intestinal calcium absorption among the patients with idiopathic hypercalciuria and whether this augmentation is a primary process or a compensatory leak, related to more generalized activities of calcium transport (Lemann and Gray, 1989).
2.4.2.2 Increased production of 1,25-(OH)_2 vitamin D_3 and parathyroid hormone

Increased production of 1,25-(OH)_2 vitamin D_3 is a well known hormonal stimulus for intestinal calcium absorption. Kaplan et al (1977) and Broadus et al (1984) exhibited higher serum 1,25-(OH)_2 vitamin D_3 concentrations amongst patients with idiopathic hypercalciuria as compared to control subjects. Studies on the effects of calcium metabolism during elevated 1,25-(OH)_2 vitamin D_3 in healthy volunteers have also demonstrated stimulation of net intestinal calcium absorption and increased daily urinary excretion of calcium (Adams et al, 1982; Mairhofer et al, 1984b).

Mechanisms accounting for elevated serum 1,25-(OH)_2 vitamin D_3 concentrations amongst patients with idiopathic hypercalciuria is still controversial. Serum immunoreactive parathyroid hormone concentrations have been reported to be normal amongst idiopathic hypercalciuric patients (Pak et al, 1974; Shen et al, 1977). Urinary cyclic adenosine monophosphate as a reflection of parathyroid hormone activity was also found to be normal (Broadus et al, 1978; Coe et al, 1982). However Korker (1987) confirmed the stimulation of 1,25-(OH)_2-D_3 production due to parathyroid hormone.

Insogna et al (1985) showed that idiopathic hypercalciuria patients produce more 1,25-(OH)_2 vitamin D_3 as determined by isotope clearance methodology. Since the major regulators of renal 1-hydroxylase (1-OHase) are not
present in most patients, the stimulus for increased 1,25-(OH)_{2} vitamin D_{3} biosynthesis is unknown (Coe and Favus, 1986). The 1-OHase is a complex of three proteins located on the inner mitochondrial membrane of renal proximal tubule (Deluca and Schnoes, 1984). The specificity of 1-OHase appears to reside in the cytochrome P-450 component, while the other two proteins renonenedoxin reductase and renonenedoxin are similar to those found in association with other cytochrome P-450 mixed function oxidases. This transport could also mediate through vitamin D dependent calcium binding protein (Bronner and Stein, 1988; Deluca, 1988).

Another theory to explain the intestinal hyperabsorption of calcium in idiopathic hypercalciuria suggests that the primary defect is a renal tubular phosphate leak (Gray et al., 1977) that produce mild hypophosphataemia. The low serum phosphate levels stimulate the synthesis of 1,25-(OH)_{2}-D_{3} which augments the intestinal absorption of calcium (Coe and Favus, 1986). Patients with fasting hypercalciuria and elevated parathyroid hormone are believed to have a secondary intestinal over absorption of calcium to compensate for a primary defect in renal tubular calcium conservation (Coe et al., 1986; Van, 1987). Since the molecular basis for renal tubular transport of calcium is not known, further insight into the nature of the defect in idiopathic hypercalciuria is possible after the isolation and characterization of the putative transporter (Borke et al., 1990).
2.4.3 Role of phosphorus in idiopathic urolithiasis:

The daily intake of phosphorus in man is 1100 ± 300 mg and most of the phosphorus absorbed is inorganic, either present in the diet or liberated from organic phosphorus before absorption. The role of phosphate compounds as inhibiting substances was discovered accidentally by Rosenstein (1936), who was in search of an active agent which can effectively deal with the scaling problem in the industry. Inorganic phosphorus is present in calcified tissues such as bone, teeth and in body fluids viz. blood, urine and saliva. There are many reports indicating that phosphate supplementation when given on a long term basis reduces the recurrence of stone formation in idiopathic calcium stone disease without any serious side effects (Smith et al, 1973; Peacock et al, 1981; Gray et al, 1983 and Churchill, 1987). Oral phosphate supplements affect the calcium lithiasis by reducing urinary supersaturation of calcium salts and secondly by increasing the excretion of crystallization inhibitors mainly pyrophosphate. A combined potassium phosphate and thiazide therapy was employed for the successful treatment of recurrent idiopathic urolithiasis (Klein and Griffith, 1982; Insogna et al, 1989; Roberts and Knox, 1990).

Harrison and Harrison (1961) using everted gut sacs of rat small intestine showed that the highest ability to transport phosphate against a concentration gradient was in the jejunum followed by duodenum and ileum. Similar findings were reported by Nordin (1976). Isolated brush
border membrane vesicles from chick intestine were used to demonstrate Na\(^+\) dependent phosphate uptake (Fontaine et al., 1979). Phosphate kinetics indicated two components, a saturable Na\(^+\) dependent uptake and a linear, non-saturable Na\(^+\) independent passive diffusion (Paterlik, 1978). Presence of multiple sodium dependent phosphate transport processes in proximal brush border membranes has recently been shown by Walker et al. (1987). Broadus et al. (1984) showed that oral phosphate therapy was capable of lowering plasma 1,25-(OH)\(_2\) vitamin D\(_3\) and reduced calcium absorption and subsequent excretion. Oral intake of phosphorus also determines the serum concentrations of 1,25 dihydroxy vitamin D\(_3\) by determining its production in humans (Portale et al., 1986). However, the antihypercalciuric effect of phosphorus, mediated through the plasma 1,25-(OH)\(_2\) vitamin D\(_3\) is still open to speculation (Kaplan et al., 1987).

2.4.4 Role of magnesium in idiopathic urolithiasis:

The source of magnesium in most of the human foods is from plant and animal sources with cereals and vegetables contributing more than 67% of the daily magnesium intake. Urinary excretion of magnesium in man is around 100 mg/day. Magnesium excretion, similar to that of calcium and phosphorus is dependent on filtration and partial reabsorption by the kidney.

In the gastrointestinal tract magnesium is mainly absorbed from the ileum (Danielson et al., 1979). Studies of Jacob and Forbes (1970) showed an increased absorption of
magnesium due to the action of vitamin D₃ or is secondary to changes in calcium and phosphorus absorption. Magnesium inhibits calcium absorption from the intestine which is concentration dependent (Smith et al, 1973) thereby confirming that calcium and magnesium absorption in the gut as well as in the renal tubule, occurs by way of a common transport carrier as shown by Berg et al. (1986).

The inhibitory action of magnesium on calcium oxalate crystal growth by rapid evaporation technique has been shown by Hallson et al (1982). The administration of 400mg magnesium oxide resulted in a significant increase in magnesium and calcium excretion. The mechanism of increased calcium excretion is not fully understood, but might be attributable to a shift between Ca⁺ and Mg²⁺ in the skeleton (Petner et al. 1978). Gulati et al. (1988) suggested that magnesium decreased the oxalate excretion, either by affecting its absorption or by regulating the endogenous synthesis after oral supplementation of magnesium in hyperoxaluric rats. Lindberg et al (1990) also showed the beneficial effect of magnesium oxide and magnesium citrate against calcium oxalate crystallization amongst stone formers.

Urinary excretion of magnesium has also been found to be slightly lower in stone formers than in control subjects (Takasaki, 1975; Beckman et al., 1979). Tiselius, (1980) showed that magnesium can increase the solubility of calcium oxalate presumably by forming ion pairs or
complexes with oxalate ions, which are thus unavailable for precipitation with calcium. The optimal concentration of magnesium required to inhibit the crystallization of calcium oxalate in human urine is yet to be worked out. Significant reduction in the growth rate and nucleation rate of calcium oxalate crystals has been observed by Li et al. (1985). The mechanism by which the magnesium ions prevent the calcium oxalate crystallization is still obscure. Toshitsugu et al. (1987) reported that the presence of magnesium ions acts as a stabilizer of thermodynamically unstable calcium oxalate dihydrate and prevents its conversion to calcium oxalate monohydrate, which is a major constituent of urinary stones.

2.5 PROCESSES OF STONE FORMATION:

The process of urinary stone formation is more complex than one would intuitively expect. The urinary tract is a flow system, where, in order to regulate body fluids, urine is produced by filtration and subsequent reabsorption processes. The composition of the freshly filtrated pre-urine changes during its passage through the nephrons, and the fluids of the more than million nephrons in a kidney are concentrated in the renal pelvis. The magnitude of the processes is reflected by the fact that two kidneys produce 180 litres of filtrate each day which is more than 4 times the total water content of the body, of which only an average amount of 1-2 litres is excreted as urine (Kessel and Kardon, 1979). Many diseases may
influence the composition of the formed urine, apart from dietary influences. The concentrated urine at any point of the urinary tract contains certain amounts of stone constituents which determine the tendency to form crystals (the driving force). On the other hand, renal anatomy can provide favourable milieu to form crystals (both intra-and extracellular) and constitutes the renal hydrodynamics (Finlayson, 1977; Hautmann et al., 1980). Some places in the urinary tract may have very poor flow characteristics i.e. the supply of fresh solution is disturbed. Crystals which are present in the supply solution have a bigger chance to stay in those dead volume sites (Schulz et al., 1980).

Urine has a transit time of a few minutes, approximately 3 minutes for the nephron (Hautmann et al., 1979) and about 8-12 minutes for the whole kidney (Finlayson, 1977). The transit time is one of the factors that determine the mean crystal residing time for any particle. Apart from this, crystals which grow or agglomerate to sizes 10-15 μm have a chance to stick in the narrow parts of the nephron. The nucleation sites and hydrodynamics, together with the driving force for crystallization determine the nucleation and growth process. Crystal nucleation is the establishment of smallest unit lattice of a crystal species and can be either homogenous (i.e. pure nucleus) or heterogenous, a foreign body or other crystal form which can lower the formation product, with resultant crystallization. Homogenous nucleation of a salt occurs in the unstable zone
of supersaturation.

In the metastable zone homogenous nucleation is less likely but preformed crystals may grow and aggregate. Nucleation, crystal growth or aggregation cannot occur in this zone. Most urines of stone formers as well as normal subjects are metastable and supersaturated with calcium oxalate (Robertson, 1976). In the supersaturated urine, a crystal will thus form either by a process of homogenous or heterogenous nucleation and this crystal will subsequently grow or aggregate to give rise to stone formation (Finlayson, 1977).

Moreover, in stone forming patients calcium oxalate dihydrate somehow gets converted to calcium oxalate monohydrate, which is more prone to precipitation in urine solution as compared to calcium oxalate dihydrate (Ligabue et al, 1979). Such precipitation could be developed either as free particles in the urine or as a result of secondary crystallization on another particle or substance. Thus ultimate formation of renal stone is thought to be the final outcome of at least three processes namely nucleation, crystal growth and aggregation (Robertson et al, 1981; Finlayson, 1982; Blomen & Bijovet, 1983; Thorne & Resnick, 1982). Moreover, all these processes are further controlled by various promoters and inhibitors of crystallization in the etiology of urinary stone formation.
2.6 INHIBITORS OF CALCIUM OXALATE CRYSTAL GROWTH:

Calcium oxalate is the most crystalline constituent of urinary stones and it exists in urine as two different phases viz. calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD). Calcium oxalate monohydrate is the predominant constituent of kidney stones and thus extensive attention has been devoted to the identification of inhibitors of calcium oxalate monohydrate crystal growth and aggregation, because they play an important role in urolithiasis. Several inhibitors of calcium oxalate crystal growth and aggregation have been so far identified in human urine but it has not been possible to assess the accurate percentage contribution of each of these to the total inhibitory activity. It has been demonstrated that the effects of various inhibitors are roughly additive and thus each contributes partly to the solubilization of calcium oxalate (Finlayson, 1974; Drach et al., 1982; Azoury et al., 1984). The calcium stone formers may have deficiency of not one but several of the inhibitors that are present in the urine. The nature of some of these inhibitors is discussed as follows:

2.6.1 Cationic Inhibitors:

Many cations (Mg$^{2+}$, Mn$^{2+}$, Cr$^{3+}$, Co$^{2+}$, Cd$^{2+}$, Sr$^{2+}$ and Fe$^{2+}$) have been found to inhibit in vitro crystallization (Bird and Thomas, 1963; Feagin et al., 1969; Barker et al., 1970; Meyer and Thomas, 1982). The concentration of these ions required to inhibit the rate of in vitro
crystallization by 50 percent ranges from $1 \times 10^{-4}$M to $1 \times 10^{-2}$M. When magnesium is excreted into urine in significant amounts, it tends to increase the solubility of calcium phosphate and calcium oxalate (Berg et al., 1976; Hallson et al., 1982). Effect of high magnesium concentration is due to a complex formation between magnesium and oxalate. Berg et al. (1976) showed that high magnesium concentration also prevents the conversion of Wedellite (COD) into Whewellite (COM). Boskey and Posner (1982, 1985) postulated that Mg$^{2+}$ acts by inhibiting the formation of Ca-acidic phospholipid phosphate complex by competing with Ca$^{2+}$ for sites on the phospholipid moiety. Once this complex was formed, magnesium ions were found to have no effect on calcium oxalate crystallization or precipitation (Nunziata et al., 1984).

Zinc is another trace metal which has been often implicated in the inhibition of urinary stone formation (Elliot & Eusebio, 1967; Elliot & Rebeiro, 1973). Meyer and Angino (1977) studied the trace metal content of urinary stones composed of calcium oxalate or a mixture of calcium oxalate and calcium phosphate by emission spectroscopy. The trace metals found in amounts 0.001% or more were iron, copper, zinc, tin, lead and aluminium. The inhibitory effects of each of these trace metals on crystal growth of calcium oxalate and calcium phosphate were also studied. The metal ions copper (II), zinc (II), tin (II) and aluminium (III) did affect the crystallization at the physiological concentrations prevalent in normal urine.
The role of trace metals in the metabolic processes which leads to the urinary stone disease has been speculated (Meyer & Angino, 1977). Thomas Jr. (1982) and Meyer and Thomas Jr. (1982) have studied the inhibitory effect of trace metals, citric acid complexes on calcium oxalate growth. The high molecular weight Fe (III) : citric acid complex formed at low ratios of citrate to Fe (III) in solution showed an effective inhibition of calcium oxalate crystal growth. The effect was very specific and could not be observed for Al (III) or Cr (III). Anasuya and Narasingha Rao (1983) reported that silicon and fluoride accelerated the calcium uptake while magnesium inhibited this process.

Tatsuro et al. (1980) demonstrated that the decrease of hydroxyproline in the bones of Cd²⁺ treated rats is due to an inhibition of collagen synthesis. Bonner et al. (1981) have observed that single s/c injection of CdCl₂ (1.5 mg Cd/kg) produced a decrease in plasma calcium concentration and a decrease in the concentration of both Cd²⁺ and Zn²⁺ in femur of the male rats. Repeated administration of Cd²⁺ caused marked hypocalciuria with increased excretion of alkaline phosphatase in the urine.

Pandey and Patel (1981) showed that cobalt administration not only inhibits hydroxyapatite formation, but also gets incorporated into the bone. Bachra and Fisher (1969) postulated that Mg²⁺, Sr²⁺ and Fe³⁺ are inhibitors of nucleation and not of crystal growth. Blumenthal and
Posner (1984) found that aluminium acted by delaying the formation of hydroxy apatite and also the transformation of amorphous calcium phosphate to hydroxy apatite.

2.6.2 Anionic inhibitors:

It has been reported in many studies (Fleisch and Bisaz, 1962; Bird and Thomas, 1963; Bachra, 1969; Pak, 1972) that several anions (\(\text{HCO}_3^-\), \(\text{P}_2\text{O}_7^{2-}\), \(\text{F}^-\), \(\text{SiO}_4^2\), \(\text{CrO}_4^{2-}\) and phosphonates) in addition to inhibiting the spontaneous precipitation of calcium oxalate also inhibit the crystallization of calcium oxalate.

2.6.2.1 Fluoride:

Taves and Neuman (1964) reported that \(\text{F}^-\) can cause either inhibition or stimulation of \textit{in vitro} mineralization depending upon the ion product \((\text{MCa}^{2+} \times \text{M} \text{HPO}_4^{2+})\) in the soluble phase. They showed that fluoride can stimulate the crystallization below the ion product of \(1.5 \times 10^{-6} \text{M}^2\) but inhibited it above \(2.5 \times 10^{-6} \text{M}^2\). Eriesson (1980) showed that combined supply of \(\text{F}^-\) and \(\text{Ca}^{2+}\) promotes the formation of new well mineralized bone tissue. On the contrary Smid and Kringer (1980) showed that enamel formed during an excessive intake of \(\text{F}^-\) was hypomineralized. This hypomineralization during fluorosis was caused by a pathological change in the protein of enamel matrix. \(\text{F}^-\) was shown to act by affecting the uptake of amino acids.

Yonese \textit{et al} (1981) have shown that when after demineralization, bovine enamel was remineralized in Ca \(\text{F}^-\)-containing remineralization solution, the stoichiometry of
mineralization and electron microscope studies indicated the formation of fluoroapatite rather than CaF$_2$. Lin et al (1981) demonstrated that at low F$^-\)$ concentrations, fluoroapatite appears to be formed at the surface hydroxyapatite through adsorption and at high F$^-\)$ concentration CaF$_2$ was formed on OHAP by surface precipitation. Sharma (1982) have shown that F$^-\)$ administration to rabbits for a period of 136 days caused defective collagen formation. Fluoride was shown to act by interfering with cross linkage formation and maturation of collagen. Margolis et al (1982) and Larsen and Thorsen (1984), however, demonstrated that F$^-\)$ administration to rats stimulated mineralization.

2.6.2.2 Pyrophosphate and polyphosphate ions:

Pyrophosphate is one of the earlier urinary constituents, identified as a potent inhibitor of calcium oxalate crystal growth and aggregation (Fleisch and Bisaz, 1962; Nancollas and Gardner, 1974; Meyer and Smith, 1975). Its concentration in the urine varies between 1 to 7x10$^{-5}$M which is high enough to inhibit calcium oxalate crystallization. It delays various processes involved in the formation of the solid phase viz. heterogeneous nucleation, crystal growth and crystal aggregation by getting tightly adsorbed on the crystal surface.

Fleisch et al, (1964) suggested that pyrophosphate blocked the active sites of mineralization by binding to the side chains of the basic amino acids in the collagen
and this binding might be the controlling factor in mineralization of the soft tissue. He further postulated that the process might be started by the hydrolysis of pyrophosphate. Inhibition of aggregation, is more likely due to a change in the zeta potential of the crystal surface which alters the attraction or repulsion between the crystals (Fleisch, 1978).

Studies by Termine and Conn (1976) and William & Sallis (1981) demonstrated that minimum structural requirements of an acidic inhibitor are the presence of two acidic groups of which one must be a phosphate and the other could either be a phosphate or carboxylate.

Liu et al (1982) studied the retardation of calcium oxalate monohydrate crystallization by polyphosphate ions. The polyphosphate ions get strongly adsorbed on the crystal surface and the inhibition increases with the chain length. It was observed that polyphosphate ions are much more effective in inducing the precipitation of calcium oxalate dihydrate. Recently, Martin (1984) showed that most of the inhibitors of calcium oxalate crystallization induce the precipitation of COD and strongly inhibit its phase transformation to the thermodynamically more stable COM.

The inhibitory nature of pyrophosphate has also been confirmed by Hosking et al (1983) and Smith (1985). Meyer (1984) suggested that if homeostasis of the various crystallization inhibitors is assumed to occur in the urinary system, then it would appear that strongly
adsorbed, highly specific inhibitor such as PPi would be most important in controlling urolithiasis.

2.6.2.3 Citrate and Phosphocitrate:

Citric acid plays an important role in the prevention of renal stones. Citrate can bind calcium ions in a soluble complex (Sutor, 1969) and in addition to ion pairing, the surface adsorption phenomenon also contributes to the inhibitory effect of citrate (Wagner and Finlayson, 1978; Curreri et al, 1981). Bercevic and Milhofer (1979) and Meyer and Selinger (1980) while studying the effect of citrate ions on crystalline apatite precipitation observed that in the presence of citrate ions, the crystal growth of calcium phosphate was slowed down. Schwille (1979) and Leskover (1982) demonstrated that in patients with recurrent calcium calculi of urinary tract, urinary concentration of both citrate and phosphate were below normal. They postulated that lower levels of urinary citrate might be responsible for the decreased inhibitory activity of the urine in case of kidney patients.

Thomas (1982) and Meyer and Thomas (1982) have demonstrated that citric acid forms unique complexes with small polyvalent metals like Fe$^{3+}$, Cr$^{3+}$ and Be$^{2+}$ and these complexes were found to be potent inhibitors of mineralization. Out of these Fe$^{3+}$ citrate complex was found to be the most potent.

Phosphocitrate, a low molecular weight highly acidic organic moiety in human urine and rat liver mitochondria
has been found to be a very potent inhibitor of hydroxyapatite crystal formation (Lehninger, 1977). Williams and Sallis (1981) synthesized this compound and studied its influence on the calcium oxalate crystal growth. They reported the synthesis of phosphocitric acid via the phosphorylation of triethyl citrate with phenylene phosphochloridate and hydrogenolysis of the product to yield triethyl phosphocitrate. Brown and Sallis (1984) synthesized N-sulpho-2-amino-tricarballylate (SAT) a new analogue of phosphocitrate, which is very well absorbed, is resistant to enzymatic degradation and is rapidly cleared into the urine. Their data suggested that SAT is more effective in preventing calcium oxalate nephrolithiasis than phosphocitrate and also lacks the bone anticalcifying effect.

Hallson and Rose (1985) have demonstrated that like citrate, L(+) tartarate also inhibited the precipitation of calcium and phosphate and this inhibitory effect was enhanced in the presence of Mg\(^{2+}\) (Yasukawa et al, 1987). Recently Lindberg (1990) has shown the beneficial effect of dietary magnesium citrate complex against COM crystallization, while other workers (Hauser et al, 1990) used potassium citrate to prevent the stone formation.

2.6.2.4 Organic dye-stuffs:

Dye-stuffs like methylene blue, hydroxyanthraquinone have been tested as prophylactics against stone formation (Terhorst and Melchior, 1972). Leskover and
Hartung (1979) tested the effect of certain organic dyes which are used as food and drug additives on calcium oxalate crystal growth. Almost all the dye-stuffs tested viz. Methylene blue, Phenol red, Evans blue, Indigo and Carmine, showed a strong inhibitory effect on calcium oxalate crystal growth and aggregation. The mechanism of inhibition is due to the adsorption of the dyes on the crystal surface. The inhibitory effect of these dyes could be seen only at very high concentrations, well above the biological levels and their normal contribution to the inhibitory activity of urine is quite insignificant.

2.6.2.5 Amino acids:

Urinary amino acids may act as chelating agents to increase the solubility of calcium salts (McGeown, 1959). The presence of amino acids increases the solubility of calcium substances in some types of urinary lithiasis. Shaker et al., (1983) have estimated urinary amino acid levels in stone formers and normal controls by paper chromatography. The data revealed that there was a significant decrease in amino acid excretion in all the stone formers and the individual amino acid pattern varied according to the type of stone formed.

Another calcium binding amino acid, \( \gamma \)-carboxyglutamic acid (GLA) identified by Hauschka et al. (1976) in renal cortex of chicken, rat and rabbit has been observed to play a vital role in urolithiasis. Lian et al. (1977) were the first to analyse kidney stone matrix for
its content of GLA. Joost et al (1981) have reported 2-3 times higher excretion of GLA in calcium stone formers than normal subjects. Nakagawa et al (1981, 1983) have shown two residues of GLA in the principal glycoprotein inhibitor of calcium oxalate crystal growth isolated from normal human urine.

2.6.3 Large Molecular weight inhibitors:

Certain acidic peptides containing a number of aspartic acid and glutamic acid residues, as well as ribonucleotides and deoxyribonucleotides, are examples of large molecular weight anionic materials which can be expected to arise during the course of cell metabolism and thus exist in urine in reasonable amounts. Smith et al, 1973 showed that passage of urine through anion exchange resin or treatment with acetylpyridinium chloride (a substance that precipitates anionic material from aqueous solution) removes much of its inhibitory activity. Ito and Coe (1977) showed that poly-L-aspartic acid and poly-L-glutamic acid inhibited calcium oxalate crystal growth at $5 \times 10^{-6}$M. Gastric pepsin, a common endogenous acidic protein also significantly inhibited the calcium oxalate crystal growth. Various types of such large molecular weight inhibitors are discussed as follows:

i) RNA and like substances

ii) Role of Glycosaminoglycans

iii) Glycoproteins
2.6.3.1 Ribonucleic Acid and like substances:

Ito and Coe (1977) demonstrated that RNA and four synthetic homopolyribonucleotides were strongly inhibitory towards COM crystallization whereas, DNA was shown to have an inhibitory effect much less than that of RNA. Schrier et al. (1981) have reported that 20-40 percent of the inhibitory material in normal urine is RNA or RNA-like material (fragments of RNA). Further they suggested that these fragments do not contain full complement of purines and pyrimidines bases, but they were highly acidic. Scurr and Robertson (1985) have investigated the mode of action of polyanionic inhibitors of calcium oxalate crystallization in urine. Martin et al. (1985) studied the effect of RNA and other potent inhibitors on the conversion of COM to COD and showed that RNA, at a concentration of 20 mg/L accounted for 16-20 percent conversion of COM to COD only, preceded by pyrophosphates and citrates which accounted for 45% conversion.

The preferential formation of COD over COM in the presence of CaOx crystal growth inhibitors may be the result of inhibition of phase transformation of the COD into the more thermodynamically stable COM or the preferential inhibition of COM crystal growth. Initially, Tomazic and Nancollas (1979) suggested that surface adsorption of inhibitors was higher for COM than COD, a situation that could allow the preferential adsorption of the inhibitors on the COM phase. Robertson et al. (1986) and Mandel et al (1987) studied the interaction of COM and
ribonucleic acid (RNA) by adsorbing RNA on the COM surface. The result showed that at low RNA concentration i.e. 0.2 mg% and less, polymeric bridges occurred resulting in slight aggregation of crystals, but at higher concentrations (0.6 mg% to 50 mg%) the denser polymer layers at the surface prevented aggregation by steric repulsion. The authors also suggested that at lower concentration aggregation results from Ca binding by RNA which reduces its net charge and steric repulsion, whereas, at higher concentration the inhibiting property of RNA is regained.

Measurement of rate of growth and aggregation of calcium oxalate crystals showed the order of potency as RNA>GAGS>THG>PPi and this was postulated to be directly related to a decrease in zeta potential produced by these inhibitors from near zero to values between -30 and -40 mV. Recently Mandel et al (1989) Brown et al (1989) and Kleboth et al (1989) have shown an important role of RNA as an inhibitor of CaOx crystallization.

2.6.3.2 Role of glycosaminoglycans (GAGS):

Commercial preparations of chondroitin sulphate and heparin were reported to be potent inhibitors of calcium oxalate crystallization in vitro (Robertson et al, 1976; Sallis and Lumely, 1979; Fellstrom et al, 1984; Sidhu et al, 1989). Urine contains about 3 to 5 mg/day of acid mucopolysaccharides of which the major components are chondroitin sulphate A and C (Foye, 1982). Varalakshmi et
al (1977) have reported lowering of certain species of acid mucopolysaccharides in stone formers. Bichler et al., (1982) observed decreased GAGS excretion only in cases of struvite calculi, while the patients with calcium oxalate calculi showed no change in GAGS excretion. Recently, Nikkila (1989) have also reported a significant decrease in the amount of GAGS excretion in patients with staghorn calculi and in those forming struvite calculi.

Foye (1982) have suggested that the average sulphate content of normal urinary mucopolysaccharides (MPS) was one sulphate group per disaccharide unit, whereas, the average sulphate content of MPS from stone forming urine was two sulphate groups per disaccharide unit. It was found that MPS from stone forming urine formed insoluble calcium salts in standard calcifying solutions, whereas, the calcium salts of MPS from normal urines remained soluble. The difference was explained by the ability of more highly sulphated MPS to undergo cross linking through calcium, for making the polymers insoluble (Foye, 1982). Martin et al. (1984) have shown that the inhibitory potential of urine collected from the urinary bladder is higher than that of the urine collected from the ureters and have speculated it to be due to increased GAGS and RNA-like material present in the bladder urine. Recently, Hautmann et al. (1984) found a steep increase of GAGS from the cortex of the papillary tips in the renal tissue obtained from healthy and stone forming kidneys. These GAGS protect the papillary tips from
calcification because even in healthy subjects there is supersaturation of calcium oxalate in the interstitium of the papilla.

2.6.3.3 Glycoproteins:

Acidic polypeptides present in urine have also been reported to play a major role in the inhibition of calcium oxalate crystallization. Nakagawa et al (1981) isolated four acidic polypeptides from human urine which inhibit the growth of calcium oxalate crystals. The same group of workers in 1981, isolated a glycoprotein with a molecular weight of $1.33 \times 10^4$ daltons, from human kidney tissue culture medium. This protein had a carbohydrate content of 38.4 percent and was very rich in acidic amino acids and also contained two residues of GLA ($\gamma$-carboxyglutamic acid). Kinetic studies of COM crystal growth, suggested a surface inhibition mechanism where the inhibitor binds to specific growth sites of the crystal. Nakagawa (1983, 1987) isolated a similar glycoprotein from normal human urine and reported that 90 percent of the COM crystal growth inhibition in human urine is due to this nondialysable macromolecule.

Lopez et al (1984) raised antibodies against this purified crystal growth inhibitor (Mol. wt = 14 KD) by streptovidin-ELISA. The antibody reacted with the crystal growth inhibitor (CGI) at 1:32,000. Complete ELISA inhibition was produced by Tamm Horsfall glycoprotein (THG) only after treatment with EDTA. By immunodiffusion against
anti-CGI and anti-THG, native CGI and THG were found to be identical and both were localized by immunofluorescence in the distal segments of the nephron. The EDTA induced cleavage from THG of fraction similar to CGI suggests a close relationship between the two proteins which may play an important role in the pathogenesis of urolithiasis (Lopez et al., 1984). Recently Nakagawa et al. (1987) isolated nephrocalcin, a glycoprotein inhibitor of COM crystal growth from normal human urine. The nephrocalcin obtained from the patients of stone formers was found to be deficient in gamma carboxy glutamic acid residues as compared to non-stone formers (Hess et al., 1989).

2.7 THE MATRIX : PROMOTION OF CRYSTALLIZATION :

The exact influence of matrix upon formation and crystallization of urinary calculi has been hotly debated for years. Extensive investigators have characterized matrix as a derivative of several of the mucoproteins of urine (Malek and Boyce, 1973). The only difference in the composition of the matrix protein and the urinary uromucoid is the absence of sialic acid residues in the former, which has been postulated to be due to cleavage of the acid residues of uromucoid by the renal sialidase (Malek and Boyce, 1973). Initially Watanable (1972) showed that matrix functions, to bridge stone crystals together. However, Finlayson (1974) suggested that simple co-precipitation cannot explain all the interactions observed between stone crystals and the matrix and suggested that polymerization
of mucoids occurs to form the stone matrix. Moore and Gowland (1975) conducted extensive studies into immunologically distinct reactants of stone matrix and urinary uromucoids. They found three to four antigenic proteins unique to stones and they detected these stone specific antigens in the urine of 85 percent of stone formers, but none in the normal individuals.

Resnick and Boyce (1979) showed that 85 percent of the active calcium oxalate stone formers had low molecular weight urinary proteins present in their urine, whereas none of control subjects or inactive or struvite stone formers had these proteins. Paternain et al. (1980) have reported isolation and characterization of a mucoprotein possessing mineral nucleating activity. This protein has a molecular weight of 51,000 daltons, isoelectric point of 2.8, its hexouronic and sialic acid content is quite small while its glycine and glutamic content are high.

The role of urinary uromucoids (Tamm Horsfall Mucoprotein) as promoters of calcium oxalate and calcium phosphate crystal formation has been highly emphasized by the Rose's group (Hallson and Rose, 1979; Rose and Sulaiman, 1982, 1984). Ultrafiltration of urine (mol. wt. cut off 10,000 daltons) leads to a large reduction in calcium oxalate and calcium phosphate crystallization, which is restored by addition of human urinary Tamm Horsfall protein. They proposed that urine concentration leads to uromucoid precipitation, providing nuclei for heterogenous mineralization with calcium oxalate or calcium
phosphate. These partially mineralized uromucoids may become attached high up in the urinary tract where they become nuclei of stones. Later, sialic acid may be split off from the uromucoid, thus converting it into stone matrix. However, Nan Collas et al. (1989) suggested that various protein fractions and other components present in the urine mixture are capable of dual actions in stone formation. He reported that human serum albumin, polyglutamate and polyaspartic acid are capable of nucleating COM at particular concentrations. These results show that the relative roles of different processes during stone formation are still unclear. However, electron microscopical research clearly indicates that matrix influences the orientation of subsequently formed crystals i.e. there is a definite promoting effect on nucleation of whatsoever type of stone formed (Nancollas et al., 1989).

2.7.1 The role of epitaxy:

It is very well known that lattice similarities are present between uric acid, calcium oxalate and calcium phosphate crystals and epitaxial induction does occur among them (Lonsdale, 1968). Precipitation of sodium urate is induced both by hydroxyapatite and calcium oxalate (Pak et al., 1976). The precipitation of calcium oxalate can be induced from metastable solution by hydroxyapatite (Meyer et al., 1975), brushite (Pak et al., 1976) and urate (Coe et al., 1975). The effect of calcium oxalate on calcium phosphate precipitation is not as efficient as the reverse
(Meyer et al, 1975). This may be because hydroxyapatite is not the first salt to form when calcium phosphate precipitates, but is preceded by other phases, the nature of which is quite controversial (Meyer et al, 1975).

Epitactic mechanism of precipitation provides an explanation for the well-known fact that most stones consist of a mixture of salts. This could also correlate with the clinical findings that hyperuricosuria and hyperuricemia are strongly associated with calcium oxalate stone formation (Coe and Kavalich, 1974). In urine sample containing many Wedellite crystals, a growth of group of crystals of parallel orientation could be seen. This growth of crystals is not only orthorhombic and parallel in orientation but they are even in one line. Similarly, in urine supersaturated with respect to uric acid there is a nucleation of sodium urate which serves as a nuclei for calcium oxalate crystallization. Besides, epitactic heterogenous nucleations, it was also proposed that monosodium urate exists in a colloidal form in urine which can adsorb urinary GAGS, thus further emphasizing the relationship between urinary urate and calcium oxalate lithiasis (Zarwekh et al, 1983). However, Iwata et al reported the basic mechanism of stone formation to be crystal adhesion. At the time of epitaxial growth, these crystals are randomly oriented and there is a free space between these crystals. Consequently, the initial stone construction was found to be brittle and it later grows
epitaxially due to the presence of various binding sites on the crystal surface (Iwata et al., 1989).

2.8 MEASUREMENT OF INHIBITORY ACTIVITY OF URINE AND CRYSTALLIZATION INDICATORS:

One of the earliest techniques used to test urinary inhibitory activity made use of a system described in 1930's for studying the calcification of cartilage (Howard and Thomas, 1959; Thomas and Howard, 1959). Epiphyseal cartilage from rachitic rats was incubated in vitro in a supersaturated salt solution and the precipitation of calcium phosphate studied. Urine or substances to be tested were added to the incubation fluid. Since the cartilage is enzymatically very active it is likely to destroy certain inhibitors and lead to erroneous results (Bird and Thomas, 1953).

A commonly employed method involves determination in vitro of the minimum product of calcium x phosphate (formation product) or calcium x oxalate, necessary for the formation of a solid phase within a preset time period under defined conditions (Robertson, 1973). Formation product can also be determined in the presence of a nucleating agent eg. collagen, elastin or crystals. Though very useful in investigating the effect of individual compounds, the determination of the formation product presents problems when used to test urine, because in urine one must measure the thermodynamic product of ionic activities and not just simple calcium x phosphate or calcium x oxalate (Pak et al., 1971; Pak and Chu, 1973).
Another approach which measures mainly crystal growth, is to measure the kinetics of precipitation of mineral after addition of a seed to trigger the reaction. Calcium phosphate (Nancollas and Mohan, 1970) or calcium oxalate monohydrate (COM) seed crystals (Robertson et al., 1973; Ito and Coe, 1977) are added to a solution with a defined supersaturation and calcium, phosphate or oxalate in the solution are measured with time. Inhibition by urine or the additive is measured by adding this to the system and to express the difference between uptake in a inhibited system and uptake in a control system, to which only saline is added (Meyer and Smith, 1975; Will et al., 1976, Ito and Coe, 1977; Baumann and Wacker, 1979).

Techniques have also been devised to measure aggregation in vitro (Fleisch and Monod, 1973; Felix et al., 1977). Disaggregated oxalate crystals are incubated in a slightly supersaturated solution and the development of crystal clusters with time are measured. When urine is added to the system, aggregation is strongly inhibited. Recently, an attempt has been made to separately assess calcium oxalate crystal growth and aggregation using a coulter counter (Ryall and Marshall, 1981; Ryall et al., 1984; Dik et al., 1990).

As most of the techniques described use diluted urine, it has been a big question whether the results obtained at various urine dilutions can be extrapolated to whole urine (Fleisch, 1978). Hallson and Rose (1978) have
described a technique which utilizes whole urine. The urine samples were subjected to rapid evaporation to a fixed osmolarity, at a fixed temperature (37°C) and a fixed pH and the crystals thus obtained were quantitated microscopically or by radio-isotope methods.

A gel method for measuring crystallization inhibitor activities has been recently established (Schneider et al, 1983; Paul et al, 1984). A microslide is uniformly coated with one per cent agar and a hole made is loaded with starter solutions (calcium chloride and ammonium oxalate) and test substances or urine. The density and breadth of the resulting crystal streak measured photometrically, are good indicators for the crystallization inhibitory activity of the test substances. Recently Rao et al (1988) studied the calcium oxalate crystal growth in polyacrylamide gels prepared with a 10% monomer concentration and found it most suitable for diffusion controlled crystal growth studies.

2.9 CHRONOBIOLOGICAL RHYTHMS AND FACTORS AFFECTING UROLITHIASIS:

During the last few years much has been learnt about the rhythmic nature of living organisms. At present there is sufficient evidence to support that oscillation is a fundamental characteristic of all living systems. Besides characterizing all organisms within the plant and animal kingdoms, rhythms can be found at all levels of organization within an organism. Rhythms, endogenous and exogenous may be affected by various factors which are discussed as follows:
2.9.1 Geographical Factors:

Anderson (1969) compared the incidences of stones in different countries during different periods. He divided his studies in 3 areas:

a. Developing countries of South East Asia represented by India and Thailand.

b. Modern industrialized countries represented by Norway and Great Britain.

c. Countries with an intermediate development along the Mediterranean Sea eg. Sicily, Israel and Egypt.

He concluded that a direct relationship exists between the incidence of renal calculi and different lifestyle of inhabitants.

Finlayson (1974) reviewed several geographic surveys and observed that the United States has a relatively high incidence of urinary calculi. Other incidence areas are the British Isles, Scandinavia, the Mediterranean countries, Northern India, Pakistan, Northern Australia and Central Europe. Low incidence areas include Central and South America, most of Africa and those areas of Australia populated by Aborigines. In India, the pattern of incidence of renal stones was reviewed by Anderson (1969) and Colaba Walla (1971). They demarcated two stone belts in India. One belt starts from Amritsar in the north and extends to the north west, including Delhi and Agra and ends in U.P. The other belt starts on the west coast at Jamanagar and extends inwards towards central India to
2.9.2 **Climatic Factors:**

The incidence of urinary calculi has been related to high summer temperature in South-Eastern United States, the peak incidence being during July, August and September, the months with the highest average temperature (Prince and Scardino, 1960). Elliot *et al* (1975) observed that peak incidence occurs during periods of above average temperature and below average rainfall.

Robertson *et al* (1975) observed higher urinary Ca\(^{+2}\) values in normal subjects and idiopathic stone formers during summer than in winter. The peak values occurred in the month of June. The increase in the urinary excretion of Ca\(^{+2}\) with increased exposure to sunlight was attributed to the vitamin D stimulation of calcium absorption from intestine. The increased urinary excretion of oxalate during summer was explained on the basis of an increased intestinal absorption of Ca\(^{+2}\) as a result of vitamin D stimulation, leaving in the intestine a reduced content of Ca\(^{+2}\) and larger amounts of free oxalate for absorption and subsequent urinary excretion.

2.9.3 **Nutritional Factors:**

In Europe, North America and Australia, not only is the incidence of renal calculi higher but the animal protein and the fat content of diet are approximately 5 times and sugar content 10 times greater than in Africa, where calcium stones are very rare. Use of refined
carbohydrates and animal proteins have been shown to increase urinary Ca$^{2+}$ and oxalate excretion (Thom et al, 1978; Rao et al, 1984; Conyers et al, 1985). The mechanism of this increase in urinary calcium (Ca$^{2+}$) is not clearly understood but it may be due to diminished Ca$^{2+}$ reabsorption in the distal tubule. Oreopoulos et al (1981) observed that when Ca$^{2+}$ intake was restricted to 570 mg/day, bone mineral decreased in kidney stone patients. The increased Ca$^{2+}$ absorption found in majority of idiopathic stone formers and its stimulation by available carbohydrates in the diet could mean a greater excretion in the urine of normal persons and kidney stone patients (Thomas et al, 1972; Hodgkinson, 1974). Blacklock and McLeod (1974) observed that sucrose can cause a significant increase in intestinal Ca$^{2+}$ absorption in idiopathic stone formers as well as in normal subjects. Pansu et al (1976) suggested that this may be due to sugar induced increased permeability related to cellular and physicochemical mechanisms which are involved in the regulation of osmolarity. Conyer et al (1985) have observed that sucrose diet does not increase the risk of urolithiasis in humans but fructose rich diet does so in rats.

2.9.4 Water Intake:

It has been confirmed that increased water intake and increased urinary output decreases the incidence of urinary calculi. Finlayson (1974) demonstrated that increased urine flow causes reduction in the oxalate
concentration of the urine. For a significant effect, a urine output of more than 3600 ml per day was found to be essential. Hosking et al (1983) showed that only increased water intake could prevent new stone formation by 60% in patients suffering from idiopathic urolithiasis.

2.9.5 Concentration of ions in the urine:

The concentration of various ions in the urine may play an important role in stone formation. It has been observed that when the saturation of urine reaches the formation product value with respect to the salt, a measurable crystalluria results. Thereafter, the amount of crystalluria becomes proportional to the degree of supersaturation (Hodgkinson and Pyrah, 1958). Hypercalciuria is a common abnormality found in 50-70% of patients with nephrolithiasis (Pak et al, 1980). It has been suggested that hypercalciuria plays a less critical role than hyperoxaluria, because an increased Ca$^{2+}$ concentration may be less effective than increased oxalate concentration in raising the urinary saturation of calcium oxalate (Nordin et al, 1973). Antonacci et al (1985) observed higher supersaturation values when these two disturbances occurred together, hyperoxaluria being an important cause of supersaturation in Ca$^{2+}$ stone formers even though it occurs less frequently.

2.9.6 Occupation:

Lonsdale's study (1968) indicated that urinary
calculi are more likely to be found in individuals who have sedentary occupations. Its incidence was very low in individuals doing manual work. It may be possible that physical strenuous nature of the manual work results in disruption of the crystal aggregation phenomenon in the urinary tract. Wisniewski (1981) also correlated occupation with the incidence of urinary calculi and observed increased incidence in the management class and low incidence in farmers, fishermen, miners and unemployed men, but not among unemployed women.

2.9.7 Enzymes:

Gasser et al (1973) observed that the lysozyme (muramidase) activity is increased in the urine and serum of patients with urinary calculi. The renal failure is known to increase the activity of this enzyme not only in urine but also in serum. Stone formers with normal kidneys also show higher activities of this enzyme. However, it cannot be determined whether lysozymuria is a primary or secondary phenomenon in calculogenesis.

Thind et al (1977, 1985) investigated the role of glyoxylate and glycolate oxidase, lactate dehydrogenase and alkaline phosphatase in urolithiasis. This study was conducted on human kidney biopsies. It was found that the activities of glyoxylate and glycolate oxidase did not differ significantly between the normal subjects and the stone formers. However, as compared to the normal persons, the alkaline phosphatase activity was found to be lower and
that of lactate dehydrogenase higher, amongst stone formers. Later Thind et al. (1985) also showed the mechanism of glyoxylate oxidation and enzymes of oxalate biosynthesis in thiamine deficient rats.

2.9.8 Circadian rhythms and their role in urolithiasis:

Although currently, there is a great deal of interest in rhythms with higher and lower frequencies than circadian, but most rhythms which have been studied are with periods of approximately 22-24 hours. Circadian fluctuations of most physiological variables are not apparent in the same sense as are the respiratory, sleep-wake or menstrual cycles. Circadian rhythms only become overt when they are properly measured at frequent intervals along a 24 h time scale. Because of their somewhat invisible nature, and the rationalization on the part of some scientists that circadian variation represents merely a minor fluctuation around a daily mean, these rhythms have been ignored in experimental design. By overlooking these biological rhythms, one is simply ignoring the scientific facts of biological life (Koopman et al., 1985).

Admittedly, the circadian rhythms of hormones that modify the renal excretion of water, sodium and chloride are dominated by postural changes (Donckier et al., 1986). The highest values of their excretions are found nocturnally (Cugini et al., 1985). However, potassium, calcium and phosphate have been studied in more detail. Potassium is believed to move from the intracellular to extracellular compartment during the morning hours under
the influence of cortisol (Minors and Waterhouse, 1984). Plasma potassium concentration is maximum at about noon but the amplitude of the rhythm is small. This is because maximal urinary loss of potassium takes place at this time (Minors and Waterhouse, 1984).

The cyclic excretion of a wide variety of constituents in the urine has been described by many authors (Kanabrocki et al, 1983; Koopman, 1985). In most cases peak excretions occurred diurnally with the exception of phosphate which excreted nocturnally in great amounts. Some rhythms for example, for those of flow and the excretion of sodium, potassium, chloride and urate are of considerable amplitude. Their nocturnal rates of excretion have been found to be less by 50 percent than during the day time.

The mechanisms of circadian rhythms in renal excretion are complex with several factors responsible. First the amount of material lost in the urine is often a very small percentage of the initial filtered load and the quantitative account of the mechanism responsible for the formation of urine is not yet of sufficient accuracy. Secondly, several interdependent factors are believed to be involved (Koopman, 1985). These include neural and mechanical factors i.e. glomerular filtration, tubular function and humoral factors and the amount of the substance in the body (influencing blood volume). Undoubtedly, dietary factors and postural changes are
important exogenous influences, though the mechanisms by which these effects are exerted are unknown (Muratani et al., 1985). Postural or dietary changes are not wholly responsible for renal circadian rhythms since the rhythms continue when diet and posture are controlled (Muratani et al., 1985). The role of the nerves to the kidney in generating rhythmicity has often been speculated upon, but the experimental data are rare and inconsistent (Koopman, 1985).

As the biological system is rhythmically changing, it follows that the organism is biochemically a different entity at different circadian phases. Therefore, it reacts differently to the same stimulus at different time (Gupta and Gupta, 1980).

Among exogenous rhythms, it is well known that circadian fluctuations may arise from region to region with the change of food habits, climate and living style. So it is desirable to study the circadian and circannual rhythmic behaviour of the subjects with respect to urolithiasis, to point the particular time of the day, or the year, as high risk periods in the formation of uroliths. These high risk periods may vary from region to region, culture to culture and race to race. The circadian variation of wide variety of urinary constituents in normal population has been studied by various authors and significant rhythms have been located in serum calcium and in the excretion of sodium, potassium, chloride and urate (Minors et al., 1974; Kanabrocki et al., 1983).
The role played by these circadian rhythms in the formation of urinary stones has not been much exploited except for a few reports (Vahlensieck et al., 1982; Minors et al., 1982; Touitou et al., 1983). The acrophase and the amplitude of the urinary constituents of stone formers and non-stone forming subjects were found to vary in different regions depending upon their environmental and social factors and dietary load (Schulz et al., 1984; Li et al., 1987; Kinoshita et al. 1987). Moreover, most of the patients studied by various authors were kept on standard diets under hospital conditions. In fact, the patients on standard diets and particularly under hospital conditions do not constitute the real and original group of idiopathic stone formers in the particular region because standard diets and hospitalization segregate them from their natural existence in the particular belt of idiopathic disease (Koopman et al., 1985; Muratani et al., 1985; Waterhouse et al., 1987). Thus it is desirable to study such patients under routine conditions and lifestyle.

The therapeutic implications of these rhythms are of great importance. Any altered circadian rhythm can act as a marker for various idiopathic diseases. Based on the findings it can be said that abnormal circadian rhythmicity and illness are associated, and subtle changes in the chronobiology might be a predictor of the illness rather than a mere concomitant of it. One implication of this is that there is a need for individuals to measure
continuously their own circadian rhythms whilst they are healthy, so that subtle changes (possible indicators of forthcoming illness) can be assessed accurately. This technique of autorhythmmetry has been advocated by Halberg (1973, 1983) and Halberg et al. (1983). Moreover, knowledge of circadian rhythms also leads one to consider if treatment might be affected at particular time of the day, as three times a day or at meal times might not be the only, or even the best, treatment for various diseases (Minors and Waterhouse, 1987).

Thus exploitation of chronobiology in medicine promises to be useful in diagnosis and in the better understanding of the cause, mechanism, management and last but not the least, the prevention of different diseases.