CHAPTER 5

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Studies presented in this dissertation were designed to study the effect of age, and the inter relationship of various types of stones formed by stone forming subjects (SF) vis-a-vis non stone forming subjects (NSF). The urinary excretion pattern of lithogenic as well as inhibiting substances were studied in detail to locate the high risk period during the day as well as in the course of the year, amongst stone formers and how such patterns differ from those of non-stone forming subjects. Characterization of the various inhibitors and the inhibitory potency of the biological solvent/solute (urine) was also attempted. Isolation and base analysis of inhibitory RNA from the urine of stone formers and non-stone formers was performed and its inhibitory activity was assessed to detect any difference between the two groups.

The results obtained from the present studies have been discussed under the following headings:
1. Chronological studies of the bio-excretion of various lithogenic substances and inhibitors with respect to age and types of stones formed.
2. Circadian and circannual studies of urinary excretion of lithogenic substances and inhibitors from the urine of stone formers and non-stone formers.
3. Characterization of the urinary inhibitory potency and isolation of RNA from the urine of stone formers and non-stone forming subjects.
5.1 CHRONOLOGICAL STUDIES OF THE BIO-EXCRETION OF VARIOUS LITHOGENIC SUBSTANCES AND INHIBITORS WITH RESPECT TO AGE AND TYPE OF STONES:

5.1.1 Chemical Composition:

Studies on 120 calcium oxalate stone formers showed that on an average kidney stone formers excrete high uric acid, calcium and oxalate (Table 1). On the contrary phosphate excretion was found to be decreased amongst SF as compared to NSF subjects. Moreover, the ratio of Ca/PO₄ excretion was found to be higher amongst SF (0.22) as compared to that of non-stone formers (0.15). This increase in the ratio (by a factor of 1.5) suggested the availability of free calcium in the urine which may associate with oxalate. On the other hand Ca/Ox ratio was found to be lower amongst SF (8.14) as compared to that of NSF (11.2), also by a factor of 1.5. This factor is found to be approximately similar to that of Ca/PO₄ ratio. Studies by several workers (Eanes, 1976; Tung & Braw, 1983; Doi and Eaves, 1984; Cheng, 1985) have shown that basic calcium phosphate is thermodynamically more stable by virtue of its lower surface tension as compared to calcium oxalate. Increase in the Ca/PO₄ ratio and a decrease in the Ca/Ox ratio amongst SF suggest the formation of greater amount of calcium oxalate (insoluble) as compared to calcium phosphate. Increase in the Ca/PO₄ ratio and decrease in the Ca/Ox ratio seems to be a prerequisite for the initiation of calcium oxalate crystallization. The above results have also been confirmed by the oral
phosphate supplementation to calcium oxalate stone formers, which resulted in a decreased urinary calcium and decreased urinary supersaturation of calcium (Robertson et al, 1976a; Klein & Griffith, 1982; Dik et al, 1990).

Urinary uric acid was also observed to be excreted in greater amounts amongst SF as compared to control subjects. The role of uric acid in the epitaxial growth of calcium oxalate has been well established. Lonsdale (1968a,b) showed the relevant lattice similarities between the crystals of uric acid and calcium oxalate. Coe & Kavalich (1974) and Ettinger (1990), established a strong association between hyperuricosuria and hyperuricemia with calcium oxalate stone formation. The hypothesis proposed by them was that, in the patients with hyperuricosuria, the urine becomes supersaturated with respect to sodium urate to cause the nucleation of the urate phase. Subsequently, these urate crystals serve as nucleation agents for calcium oxalate in urine which may not be sufficiently supersaturated for the nucleation of calcium oxalate crystalline phase to occur in the absence of suitable seed materials (Fellstrom et al, 1981, 1983).

The renal handling of uric acid involves a four component system: (1) complete filtration by the glomerulus, (2) reabsorption in proximal tubule, (3) secretion of an amount of uric acid equal to 50% of the filtered urate load, and (4) reabsorption of approximately 80% of the secreted urate at a postsecretory site (Williams and Wilson, 1990). This reabsorbed material must be
transported away from the medulla. High concentration of uric acid in the medullary tissue is transported away by erythrocytes in the normal subjects. However, it has been observed that erythrocytes of stone formers cannot take up the same amount of uric acid. Thus concentration of uric acid in the tubular fluid increases and leads to the formation of uroliths (Atsmon et al., 1963; Gutman and Yur, 1968; Coe and Kavalich, 1974; Fellstrom, 1983, 1984). The above hypothesis was supported by the studies of Van et al. (1977) and Simmonds et al. (1976), who showed a lower activity of adenine phosphoribosyltransferase amongst SF RBC's. Low activity of this enzyme prevents the conversion of adenine to nucleotides and this adenine is oxidised by xanthine oxidase to 2,8-dihydroxy adenine resulting in hyperuricosuria amongst patients.

Uric acid is a degradation end product of purine metabolism. Ribose-5-phosphate and the oxidative pentose shunt play important roles in the regulation of purine biosynthesis. Ribose-5-P\(\text{O}_4\) is converted to 5-Phosphoribosyl-1-pyrophosphate (PRPP) in the presence of PRPP synthetase (Fig. 33). In recent years abnormalities in these enzymes have been discovered in subjects with hyperuricosuria due to:

i) Deficiency of hypoxanthine-guanine phosphoribosyl transferase (HGPRT), and

ii) Superactivity of phosphoribosyl pyrophosphate (PRPP) synthetase.
PENTOSE PHOSPHATE SHUNT $\rightarrow$ RIBOSE - 5 - PHOSPHATE + ATP

PRPP SYNTHETASE

5 - PHOSPHORIBOSYL - 1 - AMINE (PRPP)

PRPP AMIDOTRANSFERASE

5 - PHOSPHORIBOSYL - 1 - PYROPHOSPHATE + GLUTAMINE (PRPP)

NUCLEIC ACIDS

GUANYLIC ACID (GMP) $\rightarrow$ INOSINIC ACID (IMP)

PRPP SYNTHETASE

GUANOSINE

PRPP INOSINE + ADENOSINE

GUANINE

PRPP SYNTHETASE

HGPRT

5 - PHOSPHORIBOSYL - 1 - AMINE

DE NOVO SYNTHESIS

INHIBITED AMP DEAMINASE

NUCLEIC ACIDS

INTER - CONVERSION

2-8 - DIHYDROXY ADENINE

XANTHINE

XANTHINE OXIDASE

URIC ACID

FIG. 33 PATHWAYS OF PURINE METABOLISM IN LIVER
HGPRT deficiency causes hyperuricosuria mainly by accumulation of the substrate PP ribose-PO₄ and by a decreased feedback inhibition of purine biosynthesis. Superactivity of PP ribose-PO₄ synthetase leads to the increased availability of PP ribose-PO₄ for purine biosynthesis and its overproduction as shown in the metabolic chart (Fig. 33).

The above mentioned enzyme abnormalities result in an increased availability of Ribose-5-PO₄ (which is a carbohydrate precursor for glycolate and oxalate) and may explain the simultaneous occurrence of hyperuricosuria and hyperoxaluria in some patients (Fig. 34). Fructose-6-PO₄ has been shown to result in the formation of oxalate through the metabolic cycle. On the other hand, fructose-6-PO₄ has also been shown to stimulate the human purine synthesis thus leading to hyperuricosuria (Kari et al., 1975; Perheentupa et al., 1967). However, whether this increased rate of purine synthesis was a result of direct stimulation by a fructose metabolite or was secondary to fructose induced purine metabolite depletion is still not clear (Kari et al., 1975; Henri et al., 1979). Moreover, glycine which is another potent precursor of oxalate synthesis is also synthesized de novo during the conversion of 5-Phosphoribosyl-1-amine to inosinic acid during the purine metabolism in the liver.

Another hypothesis related to the occurrence of hyperuricosuria amongst urinary SF is that of abnormal adenine monophosphate deaminase (AMP). Henri et al. (1979)
FIG. 34 PATHWAYS OF CARBOHYDRATE METABOLISM TOWARDS HYPERURICOSURIA AND HYPOXALURIA
showed that amongst the various enzymes of purine metabolism AMP deaminase is the most regulated. At physiological concentrations it is 95% inhibited by organic \( \text{PO}_4 \) and Guanosine triphosphate (GTP) which act as potent inhibitors whereas adenosine triphosphate (ATP) acts as a stimulator of AMP-deaminase. Hyperuricosuria is caused when the inhibition of AMP-deaminase is affected, either by decreased GTP or inorganic phosphate ions. In the present studies the decreased excretion of inorganic \( \text{PO}_4 \) ions amongst SF may contribute partly to the decrease in \( \text{PO}_4 \) caused in the liver cells, thus leading to hyperuricosuria associated with hyperoxaluria. The exact mechanism can only be elucidated with the detailed study of such ionic metabolites in hepatic tissue. Such conditions of decreased AMP deaminase have also been reported after fructose infusion which also creates hyperoxaluria in experimental animals (Rofe et al. 1980; Thom et al. 1981). So the association of hyperoxaluria along with hyperuricosuria can only be explained by the detailed studies of these enzyme abnormalities amongst SF. These abnormalities may not be severe in nature but may be manifested at the subclinical level. Thus their correlation studies vis-a-vis idiopathic urinary stone formation may lead to some conclusive findings.

Urinary excretion profile also revealed decreased amount of citrate, pyrophosphate and \( \text{Mg}^{++} \) ions in stone formers (Table 1 & 2). This decreased urinary excretion has
been reported by many other authors as well (Robertson et al. 1971a; Marshall et al., 1972; Williams et al., 1981). Citrate is known to affect calcium oxalate crystallization and some workers have recognised calcium citrate ion pairing as a contributing factor (Meyer et al., 1980) towards the prevention of calcium oxalate crystallization. In addition to controlling the free calcium by complex formation, it has also been shown to be most potent inhibitor of calcium oxalate crystal growth (Tiselius et al. 1981a). The synthetic phosphocitrate was found to be an exceedingly potent inhibitor of the growth of hydroxyapatite seed crystals in a medium supersaturated with respect to Ca$^{++}$ and phosphate (Tew et al., 1980). Further William and Sallis (1981) observed that phosphocitrate inhibited the growth of crystals by 66% as compared to pyrophosphate (44%) and helps to reduce the urinary calcium excretion amongst stone formers (Yasukawa et al., 1987).

Pyrophosphate has been proposed to be a natural urinary inhibitor for preventing calcium oxalate lithiasis. Results of most in vitro studies (Meyer 1985; Sidhu, 1987) agree that pyrophosphate does affect calcium oxalate crystallization. It delays the various processes involved in the formation of solid phases viz. epitactic or heterogenous nucleation, crystal growth and aggregation. These various effects appear to be related to the binding of the inhibitor on to the crystals, thereby retarding growth and aggregation (Fleisch et al., 1981). Decreased pyrophosphate excretion in the urine of stone formers
agrees with the findings of earlier workers who established the role played by this metabolite in the prevention of stone formation (Russel and Hodgkinson, 1966; Baumann et al, 1978; Wikstrom et al, 1984; Elisabet et al, 1988).

A relatively high level of magnesium retards or prevents renal stone formation, whereas deficiency of magnesium in the body or a low level of urinary magnesium encourages stone formation (Pyrah, 1979). High concentrations of magnesium affecting the solubility of calcium oxalate have been reported by Chulkartana et al (1971). Magnesium forms a stable complex with oxalate thereby decreasing the availability of free oxalate to complex with calcium ions for crystal growth. Hallson et al (1982), demonstrated that low magnesium urine yielded significantly more crystals, whereas high magnesium in the urine yielded less crystals than normal magnesium urine. Magnesium often supplemented with Vitamin B6 has been suggested as therapy to prevent the recurrence of urolithiasis although its mechanism of action has not yet been defined (Gulati et al, 1988).

Urinary excretion of trace metals showed decreased excretion of zinc and iron amongst SF (Table 2). Several in vitro experiments have shown that certain trace metals have an effect on the calcium oxalate crystallization (Eusebio and Elliot 1967; Sutor, 1969; Sutor and Wooley, 1970). Further, Meyer and Angino (1977a) studied the trace metal
content of urinary stones composed of calcium oxalate by emission spectroscopy. The trace metals found were iron, copper, zinc, tin and aluminium. The same group of authors also reported the inhibitory potential of copper, zinc, tin and aluminium. In the present study decrease in the urinary excretion of zinc may contribute to the formation of calcium oxalate crystals. Similarly, Meyer and Thomas (1982) showed that metal citrate complex inhibited the calcium oxalate crystallization. The effect of citrate complexes were compared with those of two other chemically similar ions i.e. Al (III) and Cr (III). The citrate iron complex in solution was found to be an effective inhibitor of calcium oxalate growth of a specific reaction which was not present in the other two similar metabolic ion complexes. It may be speculated that Fe (III) citric acid system might possibly have a selective biological role in regulating the growth of calcium containing urinary calculi (Meyer and Thomas, 1982).

Urinary excretion of electrolytes showed no significant change in any of its parameters (Table 2). The pattern of excretion was found to be similar to that of control subjects. However, detailed circadian cyclic studies revealed some statistically significant changes amongst stone formers as compared to control subjects which will be discussed separately (see section 5.2.4).

Urinary inhibitory activity showed a statistically significant decrease amongst SF as compared to non-stone forming subjects (Table 3). Based on the assumption that
various inhibitors in the urine affect calcium oxalate crystal growth by reversibly combining with the growth sites on the crystal, an expression for inhibitory activity was derived from Langmuir adsorption isotherms by Coe et al (1980). However, in the present study the range of inhibitory activity amongst SF (0.16-3.10) and NSF (0.95-5.10) showed wide variations. Similar variations have also been reported by other workers (Fleisch et al, 1978; Grases et al, 1988), who correlated that urine contains certain inhibitors (like pyrophosphate, citrate, glycoproteins etc.) which affect crystal formation and growth by blocking the growing sites, whereas, aggregation inhibitor (GAGS) change the surface zeta potential (Kohri et al, 1989). The effects of the multiple urinary components are roughly additive and each contributes partly to the solubilization of calcium (Finlayson, 1974; Drach, 1978; Werness et al, 1981; Azoury et al, 1985, 1987; Matti, 1989). Thus the lower inhibitory activity observed amongst SF may be due to a deficiency of several inhibiting species normally present in the urine (Nakagawa et al, 1983; Tiselius et al, 1987).

5.1.2 Effect of age on the excretion profile of lithogenic substances:

The maximum risk group amongst SF was found to be between 35-45 years followed by 25-35 years (Fig. 6). Combined, they constitute 67% of all stone episodes. The present studies are in conformity with the previous reports (Fetter and Zimskind, 1961; Drach et al, 1978; Churchill et
which showed a rapid increase of stone incidence, starting from 25-35 years, with a peak rise in the forties. Since the age of the patient is recorded at the time of the stone episode (i.e. when the stone formation has already occurred), the triggering or initiation of the formation of calcium oxalate stone must have taken place on or before the age of 25 years which is followed by high occurrence of stone formation. The above hypothesis was supported by the observations, of excretion profiles of lithogenic substances amongst such patients (Table 4). These patients revealed that calcium excretion was significantly increased only in the age groups of 15-25 years. In the subsequent age groups there was no significant variation in calcium excretion. However oxalate excretion was found to increase gradually and significantly with advancing age, from 15-40 years. This observation suggests the possible initiation of crystallization in the early years of 20-35 years and subsequently its high incidence at 35-45 years. Maximum incidence of calcium oxalate stones between 25-40 years range has also been reported by Marshall et al. (1975) and Fellstrom (1989). Almost identical results were obtained in case of uric acid excretion amongst SF. However oxalate and uric acid showed another peak between 55 and 65 years (Table 4). The presence of a second peak in the stone incidence has been recently reported (Matti et al. 1988; Gault et al. 1989), the cause of which has not yet been elucidated. In our studies also a second peak of excretory
lithogenic substances has been observed during the same period.

Phosphate excretion was observed to be low in the first two age groups i.e. 15-25 and 25-35 years (Table 4). As observed earlier the range of 15-35 years age has been speculated as the most probable age for the initiation of the process of stone formation. This tendency for decreased phosphate excretion coincides with the decreased excretion of crystal inhibitors of pyrophosphate and polyphosphate origin. Moreover these patients with decreased phosphate excretion also showed a gradual increase in the excretion of oxalate and uric acid, reflecting high risk and low protection against stone formation amongst SF as compared to control subjects. Recently, Gault et al (1989) have also shown a similar strong relationship between oxalate to phosphate weight ratio in patients with kidney stones.

5.1.3 Excretion profile of lithogenic substances amongst various types of stone forming patients:

As shown in the Fig. 7, the majority of the stones formed were found to be of mixed origin (66%), followed by pure uric acid (21%) and pure calcium oxalate (13%). As seen in the Table 5, amongst pure calcium oxalate stone formers, uric acid was found to be excreted in significantly high quantities followed by oxalate. Similarly, amongst pure uric acid stone formers, oxalate was found to be excreted in high quantities followed by uric acid. Moreover stones of a very pure nature were found
to be very small in size and crystalline in nature as compared to stones of a mixed nature. Mixed stone formers which constituted the bulk of the stone formers showed a significantly higher excretion of calcium, oxalate and uric acid, with no variation in phosphate excretion. It is very well established that a majority of the urinary stones (CaOx) contain phosphate though in trace quantities (Chambers et al., 1972; Borghi et al., 1990). Several authors have reported that the nuclei of most calcium stones are frequently composed of apatite or uric acid (Prien et al., 1968; Chambers, 1972) and they act as nidus, stimulating the precipitation of calcium oxalate and uric acid (Crassweller et al., 1978; Hesse et al., 1979). These reports suggest that the initiation of crystallization may start with the first step of availability of pure phosphate, uric acid or oxalate crystals. Further growth on these crystals lead to the formation of pure or mixed type of stones depending upon the urinary fluid environment. The concept of the formation of mixed calcium oxalate stones has been further confirmed by the studies of Tozuka et al. (1981) and Iwata et al. (1985), who showed that there are many pores in the core area of calcium oxalate and uric acid and apatites are commonly present at the walls of these pores though in tracer quantities. It is the process of binding of the rosette shaped phosphate crystals with the binding sites on these crystal pores which stimulate the formation of these biological stones (Hesse et al., 1979). The above results might indicate that the calcium
phosphate in oxalate stones is not simply present as a passive component but rather it plays an active role in its attachment with the available pores, thus playing an important role in the stimulation or inhibition of such stones (Takasaki et al, 1989).

5.2 CIRCADIAN BIOLOGY IN STONE FORMERS AND NON-STONE FORMERS:

5.2.1 Chronometric studies of urinary lithogenic substances:

It has been well established that biological rhythms vary from region to region according to the social setup and environment. In the present study also, the patients and control subjects (NSF) were specifically instructed to maintain the constant routines of their life with fixed quantities of food and water intake.

Cosinor analysis of urine volume revealed no biological rhythm in SF as compared to control subjects who showed an acrophase at 263° with elevated urine volume (Fig. 8). Such rhythms have previously been demonstrated in urine flow amongst healthy male adults (Lavie et al, 1977). The higher mean urine volume and amplitude suggest a direct relationship between the mesors and amplitudes of these curves. Such a relationship in healthy subjects has been reported previously also (Goldsmith et al, 1965; Mills, 1973; Hiller et al, 1980). The low mean volume excretion and amplitude amongst SF suggest a higher supersaturation of urinary solutes, as compared to control subjects where a significant dilution of these solutes
takes place during the day time (i.e. the time of the acrophase).

Significantly, higher excretion of calcium and oxalate amongst SF during the evening hours well coincides with the decreased urinary volume (Figs. 9 & 10). Though the rhythm amongst these patients was not found to be significant, but, high mesor values of calcium and oxalate accompanied by decreased urinary volume contribute to an additive effect in the stimulation of homogenous calcium oxalate crystallization (Hiller et al, 1980; Azoury et al, 1987). Similar results were also obtained by Touitou et al (1983) who showed a statistically significant rhythm for all variables except urea, in healthy subjects. Such rhythms were found to be absent amongst SF though their mean urinary excretions were found to be higher. However, a periodicity of 8 h (ultradian rhythm) was demonstrated for calcium and oxalate with their peaks at 2\(^{00}\), 10\(^{00}\) and 18\(^{00}\)h. The presence of ultradian rhythm of calcium amongst SF has also been observed in our laboratory (Wangoo et al, 1989) with an acrophase at 2\(^{00}\), 10\(^{00}\) and 18\(^{00}\) h. Moreover, a lack of rhythmicity amongst SF as compared to the cohorts of stone formers has recently been shown (Sidhu et al, 1989; Wangoo et al, 1989b).

Kanabrocki et al (1983) also reported a maximum urinary excretion of calcium during 16\(^{00}\) h and lowest at 04\(^{00}\)h amongst normal subjects, which coincides well with our own data. Moreover, it was also observed that the
periods of high urinary excretion for calcium and oxalate were overlapping suggesting that this could be a very high risk period for supersaturation and crystallization. Kinoshita et al (1987) recently showed a diurnal variation in plasma oxalate during oxalate restriction and loading. They also showed oxalate clearance during the day to be higher amongst human subjects. No significant difference in the oxalate clearance behaviour amongst SF and NSF was noted by them. Recently Rathore et al, 1989 showed the higher excretion of lithogenic components during the day time, however concentration of these components was found to be higher during night time.

A high mean excretion and low amplitude of uric acid excretion observed amongst SF (Fig. 11) further enhances the high risk of calculogenesis during the day-time. The greater sensitivity of the renal reflex mechanism during the day as compared to that of night, has been observed in humans by many workers (Moore et al, 1983; Mills et al, 1987;). On the contrary phosphate behaves differently than the other urinary constituents, for it has been shown (Min et al 1966; Halberg et al 1969; and Minors et al, 1976) that the acrophase of the cosinor curve, which best described the rhythm of phosphate excretion, was exactly opposite to that of the other urinary lithogenic constituents. In the present study the acrophase was found to be between 0900h-1200h (168°) amongst (NSF) (Fig. 39a) which is about 6 hours earlier than that of calcium and oxalate (240°) (Figs. 36a & 37a). Similar results have been
reported previously by Minors et al (1982), who reported an acrophase for phosphate at different times of mid-wake condition to be between 08:00h-12:00h amongst normal subjects. However, amongst idiopathic urolithic patients, phosphate excretion showed a marked circadian rhythm at 02:00h (Fig. 39a). Subsequently its nadir was located at 14:00 and this was exactly the range when the maximum risk of calculogenesis was predicted in terms of volume and excretion of calcium, oxalate and uric acid (Figs. 35a, 36a, 37a & 38a). The above discussion shows that at the time of the high risk period (in terms of excretion of lithogenic urinary substances) the circadian cycle of phosphate excretion is at its minimum. Moreover, amongst control subjects the acrophase of phosphate excretion precedes that of calcium and oxalate, thus providing the possible protection against lithogenesis.

Exactly similar results have been reported by Touitou et al (1983) who studied the alteration in circadian rhythmicity in calcium oxalate and renal stone formers. They showed a minimum excretion of phosphate during 14:00h with maxima during the night amongst calcium oxalate SF. The same group of workers showed maximum phosphate excretion at 14:00h amongst control subjects in the month of January. In our present study, the maxima for phosphate excretion amongst NSF subjects has been observed at 11:00h (Range 10:00h-14:00h) and amongst SF at 02:00h (Fig. 39a) thus corroborating the previous findings. Similar
statistically significant rhythms for all variables (calcium, oxalate, uric acid, phosphate and glycolic acid) except urea was validated in healthy subjects (Touitou et al, 1983).

5.2.2 Chronometric Studies of Urinary Inhibitors of Calcium oxalate Crystallization:

The acrophase for pyrophosphate excretion amongst NSF was observed in the morning hours at 0330h (Fig. 40a) suggesting that maximum protection provided by pyrophosphate is during the early morning hours of the circadian cycle. A urinary bladder filled to capacity during the morning hours has increased chances of the precipitation of lithogens due to their high concentrations during that period as compared to the full day span when increased excretions are voided at short intervals. This justifies the presence of pyrophosphate acrophase during the early morning hours. The high risk factor in the early morning hours and protection by various inhibitors have been studied and reported by many workers (Ogawa et al, 1983; Ahlstrand et al, 1984; Schulz et al, 1984).

Rectangular and polar co-ordinate representation of circadian rhythm amongst NSF for citrate excretion and the overall inhibitory potential of urine showed the same range of acrophase timings i.e. 216° (1424h) and 215° (1420h) respectively (Figs. 41a & 42a), suggesting that the inhibitory potential of citrate contributed additively to the overall inhibitory potential of urine which has its acrophase during the afternoon hours (Wangoo et al, 1989c).
This is exactly the period during the day span when maximum protection is required as reflected by the excretion profiles of various lithogenic substances. (Figs. 35a, 36a, 37a & 38a). Recently Li et al (1987) reported a significantly lower (negative) inhibitory activity in the afternoon and evening urine samples of SF indicating an increased tendency towards crystallization as compared to normals. In the present study, NSF subjects were found to be well protected during that phase of the day as compared to SF who were found to be at higher risk (increased excretion of lithogens) and low protection (minimum level of inhibitors/inhibitory activity) against calcium oxalate crystallization. Further, the results obtained also suggest that normal subjects are protected by inhibitors of crystallization during the whole circadian cycle i.e. from midnight to early morning they have an acrophase of pyrophosphate, and during the day time they are protected by citrates and other inhibiting macromolecules which contribute effectively towards the overall inhibitory potential of urine.

5.2.3 Chronometric studies of metallic ions:

Trace metal circadian biology of metallic ions in urine has been well reported (Vokac et al, 1981; Kanabrocki et al, 1983). However, not many reports are available on these variations with respect to urinary stone formation. Bach et al (1978) and Vahlensieck et al (1982) were some of the prominent workers who reported the role of magnesium in
urolithiasis and how this metallic ion varies in controls and stone forming subjects. The excretion curve by Vahlensieck et al (1982) showed an acrophase between 20°h-23°h for controls as well as for SF. However, in our present study the acrophase for controls was found to be at 01°h, (Wangoo et al, 1989a), while SF showed a lack of rhythmicity and the mean excretion levels were found to be significantly decreased (Figs. 16, 43a). The lack of circadian rhythmicity in the various urinary, and serum parameters amongst idiopathic SF has been reported from various laboratories (Touitou et al, 1983; Kinoshita et al, 1987; Sidhu et al, 1989). On the contrary, the acrophase of serum magnesium was located at 10\{12\}h in elderly females, 11\{35\}h for elderly males and 11\{36\}h for young males (Touitou et al, 1978). This suggests the accumulation of blood magnesium during daytime, which is cleared during the night hours as reflected in the present study with a urinary peak of magnesium at 01°h (Fig. 43a).

The urinary copper and iron excretion showed no significant rhythm amongst patients and normal subjects. The best fitting cosine curve of copper showed acrophase at 302° amongst NSF subjects as compared to 27° amongst SF (Fig. 17). But, the data obtained above was not significant to draw the confidence region at 90% level. However, a significant decrease in the iron excretion amongst SF (Fig. 18) justifies the previous findings by Kimuro et al (1989) who showed that magnesium deficiency
(known to be one of the causes of idiopathic urolithiasis) results in the accumulation of iron in the body tissue and thus results in its decreased excretion through the urine. On the contrary zinc which is a very essential metal ion for growth and is involved in RNA and protein synthesis, was found to show altered circadian rhythmicity. The cosinor rhythmometric data (Fig. 44a) revealed a presence of rhythm in SF whose time of peak excretion (212°) was found to be just opposite to that of NSF (30°). Rectangular and polar representation of the data revealed a significant 90% confidence region. The maxima in NSF was found to occur just after midnight at 30° and this phase of time was found to be the nadir (minimum) amongst SF. On the contrary Kanabrocki et al (1983) showed the acrophase of urinary zinc excretion to be around noon (1200h). This contrasting difference of acrophase observations (for magnesium and zinc) of metallic ions by Kanabrocki et al (1983) may be due to the fact that they studied only non-dialysable magnesium and zinc ions as compared to the other authors who studied the total metal ions excreted in the urinary system. Variations in the zinc ions excretion amongst idiopathic SF are further supported by other studies (Underwood, 1971; Spencer et al, 1974; Kanabrocki et al, 1983) which showed changes in the zinc concentration amongst patients with impaired renal function. However, the mechanism by which an altered circadian rhythmicity of metallic ions amongst SF affects calcium oxalate crystallization is still open to speculation.
5.2.4 Chronometric studies of urinary electrolytes:

Although circadian variations in the urinary excretion of electrolytes have already been reported (Fiorica et al., 1968; Moore and Burr, 1973; Shane and Jones, 1975; Bhattacharya, 1979), but the only study on circadian rhythm of electrolytes amongst SF has been carried out by Yuan et al. (1983). In the present study circadian analysis of electrolytes (Na\(^+\), K\(^+\)) revealed similar behaviour amongst SF as well as control subjects (Figs. 20 & 21). The peaks of their excretion maxima, which was found to be between 8h-13h in case of sodium excretion and 13h-16h in case of potassium excretion is similar to the earlier reports by Touitou et al. (1983) who showed the circadian peaks between 8h-13h for sodium and 11-14h for potassium both amongst SF and control subjects. Minors et al. (1982) also showed that the range of excretion of urinary electrolytes is twice the amplitude of the cosine curve. Later, Muratani et al. (1985) reported the electrolyte acrophase amongst NSF subjects between 13-15h. On the contrary, Kanabrocki et al. (1983) showed the acrophase of urinary electrolytes i.e. for sodium between 13h-18h and for potassium between 9h-15h. This difference in their observations as compared to the other reports may be due to the effect of dialysis. Kanabrocki's group studied the circadian rhythm of non-dialysable urinary electrolytes as compared to the other authors who studied these electrolytes in ionic form.
Though in the present study the circadian pattern of electrolytes was found to be similar amongst SF and NSF (Figs. 20 & 21), but when the same data was expressed in terms of $\text{Na}^+/\text{K}^+$ ratio, there was a significant circadian fluctuation amongst SF as compared to control subjects (Figs. 22 & 47a). In spite of the non significant mean excretion, the acrophase was found to be around $16^{30}\text{h}$ for NSF as compared to that of SF which showed an acrophase at $05^{00}\text{h}$. This contradictory behaviour between SF and NSF subjects may be significant in the etiology of the role played by electrolytes in urinary calculus formation.

5.3 CIRCANNDAL BIOLOGY IN THE URINARY EXCRETION OF LITHOGENIC AND INHIBITING SUBSTANCES IN STONE FORMERS AND NON-STONE FORMERS:

The incidence of urinary calculi has been related to high summer temperature (Prince et al., 1956), the peak incidences being in July-August. These observations were further confirmed by various other authors (Rivera, 1973; Elliot et al., 1975). However, most of the reports deal with the incidence of urinary calculi in different climatic conditions and very few reports are available on the analysis of risk factors for urinary calculi (Robertson et al., 1975). Detailed analysis of the excretory profiles of various urinary variables vis-a-vis the climatic conditions throughout the year may help to establish the role of climatic conditions in the etiology of the formation of urinary calculi (Hallson et al., 1977).

in the months of December-February in healthy, adult males, but the data was not analysed by the cosinor rhythmometric technique. In the present study cosinor rhythmometric technique revealed a peak excretion from November-February amongst stone formers and NSF subjects (Fig. 23). Stone forming subjects also showed a significant circannual rhythm for oxalate excretion with its peak in July-August (Fig. 24). High oxalate excretion in the month of June-August has also been previously reported by various other authors (Hallson et al., 1977; Elomma et al., 1982) amongst stone forming patients. Contrarily, Touitou et al. (1983) showed the absence of such rhythmicity amongst control subjects, who showed a uniform excretion of oxalate levels throughout the year as observed in the present study.

On the contrary no reports are available about the circannual rhythms of urinary inhibitors of calcium oxalate crystallization amongst patients and control subjects. Touitou et al. (1983) studied control subjects in the months of January and March and showed that there was no significant rhythmic change in phosphate excretion. In the present study also a lack of rhythmicity was observed in phosphate excretion amongst control subjects. However, stone forming patients revealed a seasonal rhythmic cycle of phosphate excretion with its minima in June-July (Maxima in February) (Fig. 50a). Exactly similar results with their minima in June-July (Maxima in January) were obtained for the urinary inhibitors, viz., pyrophosphate (Fig. 51a) amongst SF with no significant rhythm in NSF subjects.
Citrate was the only variable which showed circannual variation amongst NSF as well as SF. Amongst NSF subjects maximum citrate excretion was observed to be in July-August, thus providing an enhanced protection in this season. However, amongst stone formers, citrate excretion was found to be minimum with well defined confidence regions, thus exposing the section of the population with high risk of calculogenesis during the months of June-July (Fig. 52a).

The overall inhibitory activity of urine amongst SF and NSF subjects showed similar trends as observed in the citrate excretion, with its maxima in July-September amongst NSF subjects and minima amongst SF in August-September. In the present study the climatic factor of July-September seems to be the high risk period. It is during this period that high lithogenic oxalates and low pyrophosphates, citrates and inhibitory activity is observed, thus contributing additively to the risk of formation of urinary calculi. Schulz et al (1984) have also showed increased risk of calculogenesis in the month of May-July, though these studies were confined to entirely different regions and, thus results from one centre cannot be extrapolated to another belt region where dietary habits and climatic conditions could be different. In the Indian subcontinent these months (June-August) coincide with the monsoon season, with heavy rains and hot (42°C temperature) summers. Such correlation studies between hot humid summer...
vis-a-vis the urinary excretion profiles of promoters/inhibitors of crystallization for a larger stretch (of one decade or so) may give some concrete information on the role played by the environmental factors in idiopathic urolithiasis.

5.4 CHARACTERIZATION, ISOLATION AND BASE ANALYSIS OF RNA LIKE INHIBITORS FROM THE URINE OF STONE FORMERS AND NON-STONE FORMERS:

Centrifugation of urine showed no significant effect on inhibitory activity, whereas there was decrease (10-30%) in the inhibiting activity of urine amongst both the groups of subjects after dialysis (Table 13). This decrease was significant and consistent among NSF. On the contrary, stone formers with initial very low inhibitory activity showed negative inhibition (promotion) in their inhibitory index thus suggesting that low molecular weight ions which contribute 10-30% of the inhibitory activity are lost with dialysis. Inhibitory activity in such subjects is mainly due to the low molecular weight inhibitors viz., pyrophosphates, citrates etc. Moreover, after dialysis the urine of SF showed negative inhibitory activity suggesting the presence of a dynamic equilibrium of inhibitors and promoters in the urine solution i.e. the promotory effect in SF superimposes and dominates the inhibitory activity of urine. Such negative inhibitory activity in SF has previously been reported (Li et al, 1987; NanCollas et al, 1989; Koide et al, 1989).

The enzymatic treatment of urine from SF and NSF
revealed no significant effect by arginase digestion. However, pronase reduced the inhibitory activity of urine amongst NSF (Table 14). On the contrary, the SF who showed the negative inhibitory activity (promotion) exhibited an increase in their inhibitory activity against COM crystallization after the treatment of pronase. This supports our previous observations that, apart from the presence of some inhibiting protein molecules present in healthy subjects, there are some promoting molecules also present in SF which get inactivated by pronase action resulting in an increase in the inhibitory activity of urine. Similar trends in the urine samples have been observed by NanCollas et al (1989) who showed that protein mixtures in SF and NSF are capable of dual action (inhibition and promotion) in stone formation. Koide et al (1989) have also showed the clinical presence of promoters or some blocking substances of the inhibitors present in the healthy urine thus confirming the dynamic state of the urinary activity. However, ribonuclease digestion showed a very specific effect on the inhibitory activity of SF and NSF. Ribonuclease digestion resulted in an overall decrease in the inhibiting activity irrespective of the urine from SF and NSF (Table 14). Such a decrease in the inhibitory activity of urine by ribonuclease digestion has been reported previously (Ito and Coe 1977; and Schreir et al 1981; Mandel et al, 1987; Brown et al, 1989; Kleboth et al, 1989). They attributed this decrease to the digestion and inactivation of RNA or RNA substances present in the urine.
In vitro studies on the effect of various polynucleotides (RNA, polycytidylic acid, polyadenylic acid) showed an increase in the inhibitory activity at the concentration of 50 ug and this effect was concentration dependent (Fig. 30).

On the contrary DNA showed no effect at this concentration (50 ug) but stimulated the COM crystallization at higher concentration (Fig. 30). In vitro studies, (Martin et al, 1984) have also shown the phase stabilizing effect of RNA and other polynucleotides in calcium oxalate crystallization. This phase stabilizing effect helps to retain supersaturated solution without permitting precipitation thus adding to the inhibitory potential of urine.

The specific effect of ribonuclease enzyme in SF and NSF (Table 15) in decreasing the inhibitory activity of urine and high activity of RNA (in vitro) (Fig. 30) may play some active role in calcium oxalate crystallization. The isolation of RNA from the urines also revealed the decreased yield of RNA from SF as compared to control subjects (Table 16 & 17). Moreover, the inhibitory potential of this RNA was found to be significantly decreased amongst SF as compared to NSF subjects (Table 18). It suggests that ribonucleic acid in the urine inhibits the formation of COM crystals, presumably by promoting the conversion to COD crystals which are not active in the formation of calcium oxalate stones. The
mechanism behind this inhibiting behaviour is not yet very clear though some authors have reported its effect through crystal membrane interaction (Mandel et al, 1987) while others have shown it as an adsorption inhibitor (Robertson et al, 1986).

Base analysis of this isolated RNA revealed a decrease in adenine and guanine contents (Table 19). This observation is important in view of the change of potency of RNA towards prevention of calcium oxalate crystallization amongst SF as compared to NSF subjects. The decrease in potency seems to be achieved through certain unknown changes in the purine bases. Moreover, the nucleotides base ratio of this RNA was found to be 1:2.2:1.6:1.3, for adenine, guanine, cytosine, and uracil respectively. This ratio nearly coincides with the molar ratios of cytoplasmic RNA thus suggesting the possible source of this RNA from the cell cytoplasm (Cantarow and Schepartz, 1967). In SF this ratio was found to be slightly changed i.e. 1:2.2:2.0:1.5 with decreased purine contents. It is likely that these RNA fragments might be representing non-complimentary regions of whole RNA such as tRNA which makes bulk of cytoplasmic RNA and does not show complimentary base ratio (Cantarow and Schepartz, 1967). Schreir et al (1981) showed that these fragments, rather than complete chains of RNA are existent in the human urine. Later, Martin et al (1984) showed the possibility of RNA citrate complexes in the human urine. Same group of workers in another study, Martin et al (1985), showed that
RNA fragments in the urine are responsible for the inhibition of calcium oxalate crystallization. A difference in the inhibitory potency and base analysis of isolated RNA from SF and NSF subjects suggested the presence of some variations in the urinary RNA of SF vis-a-vis NSF subjects. Such structural variations have been observed in the case of nephrocalcin, a protein inhibitor of calcium oxalate crystallization, which has been found to be excreted both in SF and NSF (Nakagawa et al, 1983). Same workers showed the absence of T-carboxyglutamic acid (GLA) (Nakagawa et al, 1985, 1987) in nephrocalcin obtained from the urine of SF as compared to control subjects. The defective glycoprotein with very weak amphiphillic properties was isolated from the kidney tissue of SF (Hess et al, 1989) but the mechanism of induction of such defects in these inhibitors amongst SF is still not known.

Increased activity of GAGS in vitro and decreased inhibitory activity of urine after hyaluronidase digestion (Fig. 32) confirms the earlier findings of inhibitory potential of GAG biomolecules excreted in the urine of SF and NSF subjects. Recently Rodgers (1989) showed that chondroitin sulphate A is a nucleation inhibitor and not a growth inhibitor. Another group (Angell et al, 1989) showed that GAGS and GAG-like substances adsorb to CaOx crystals thus neutralizing the binding sites on these crystals which may give rise to the aggregation of calcium oxalate crystals. The same group of authors also showed that this
adsorption phenomenon adheres to Langmuir adsorption isotherm principles.

In vitro effect of various polypeptides showed that polyaspartic acid and polyglutamic acid are inhibitory in nature, however polyglutamic acid showed stimulation at higher concentrations (Fig. 31). The results obtained in the present study are in confirmation with earlier reports (Ito & Coe, 1977; Azoury et al, 1985; Kohri et al, 1989, 1990). The mechanism underlying this inhibition was thought to be, that aspartic acid has a strong negative charge and it combines with calcium in the urine and further, more and more aspartate, glutamate and oxalate act competitively with each other (Kohri et al, 1989). However, Hussain et al (1989) have shown that there is no significant difference in the excretion profile of glutamic acid in the urine of SF and NSF subjects.

Since small molecular weight inhibitors (citrate, pyrophosphate etc.), glycoproteins and RNA-like substances contribute additionally towards the inhibitory potential of the urine, the detailed mechanism of the induction of such defects needs to be studied as a future prospective in the development of some therapeutic alternative against calcium oxalate crystallization.