M.Sc. Experimental Surgery

STUDIES ON EXPERIMENTAL PRODUCTION AND DISSOLUTION OF RENAL CALCINOSIS

by

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PREFACE.

The experimental work embodied in this thesis represents an attempt to contribute to the problem of renal calcinosis. The clinical importance of the condition lies in the fact that it leads to progressive renal insufficiency with resultant shortening of life span of the individual. Dissolution of the concrements appears to be the major goal of the research carried on in this problem, although basic studies on the pathogenesis of the condition are the primary requirements for such an achievement.

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CHAPTER I

INTRODUCTION:

Renal calcinosis is a well recognised entity to-day, occurring as a complication of a wide variety of clinical conditions. It is known to shorten the span of life of the individual in most cases by progressive renal insufficiency. There is an every day growing interest in the subject leading to case presentations and to accumulation of a voluminous literature on its clinical, pathological and experimental aspects. The experimental work has been concerned with the production of renal calcinosis, along with studies of its physiochemical and pathological nature.

To the present time there has been no experimental attempt to dissolve renal calcinosis, although a large number of chemicals, singly or in various combinations, have been used to dissolve formed calculi in the urinary tract. Our interest was directed to this problem by a publication of Clarke and Clarke, (1955), who used the di-sodium salt of ethylene-di-amine-tetra-acetic acid with remarkable success in clearing a patient of wide spread metastatic deposits of calcium in the kidneys. Thus we embarked on the project to obtain objective proof of the efficacy of the drug in animals with experimentally

induced renal calcinosis.

The studies outlined in this thesis can be divided into three parts. In the first part the entire subject of renal calcinosis is reviewed and a correlation of clinical and experimental data has been established. In the second part the various experimental methods of production of renal calcinosis are discussed and the advantages of the method employed in our study are outlined. Also, the method which was used to induce renal calcinosis has been described, as well as the methods used for dissolution. In the third part, the observations and results of the experiments carried out are reported and the attempts at dissolution of the concretions produced are accounted for.

The methods used in this work appear to be interesting from a physio-pathological and biochemical point of view and indicate that the studies on dissolution of renal calcinosis are a subject worthy of further investigation and research.

CHAPTER II

PATHOGENESIS OF RENAL CALCINOSIS:

HISTORICAL DATA:

Renal calcification as such has been known in medical literature for more than one hundred years. The vary first mention of the subject, as in so many other pathological conditions, is that made by Virchow who as early as 1855 published descriptions of eight autopsies in which he found bilateral calcifications located particularly in the renal papillae and occasionally in the renal cortex. All but one of his cases had died of generalised bone disease e.g. osteomyelitis, bone tuberculosis, spinal caries and osseous metastases from sarcoma of the cheek and epithelioma of the lip. The exception was a case of chronic nephritis without associated bone disease. Virchow pointed out that the kidneys, lungs and stomach were the most common sites for deposition of calcium in such cases. He therefore called the entity "Kalkmetastesan" or metastatic calcification. It is interesting to note that he stressed the importance of the increase of calcium salts in blood for this pathological deposition of calcium. Today over a century has passed and hypercalcaemia is still recognised as one of the main factors in the

pathogenesis of nephro-calcinosis. Virchow also stated that the presence of renal lesions was a secondary factor, which resulted in a faulty exretion of calcium salts.

During the last quarter of the nineteenth century works of various authors Chiari (1878), Hlava (1882), Kockel (1899), Kischenski (1900) and others revealed that extensive deposits of calcium could occur in the soft tissues including kidneys while there was no evidence of destructive bone lesion or hypercalcaemia. It was early in this century that Schmidt (1913) recognised the role of chronic nephritis as a primary factor in such calcification in the kidneys and called it as "Kalkgicht" or calcium gout. Schmidt observed that the loss of large quantities of albumin lead to the precipitation of the calcium fraction attached to albumin and remarked that albuminous substances and phosphates act as buffers to keep calcium salts in solution. Harbitz (1917) laid stress on the important role of disturbed acid-base equilibrium, due to impaired renal function, in the development of calcinosis without bone destruction.

Ever since Virchow published his manuscript under the heading of "Kalkmetastesan" it has been customary to distinguish two types of renal calcification.

(1) Dystrophic calcification:- In this calcium is deposited in the dead, injured or degenerated tissues. The damage in these may be due to circulatory, toxic, inflammatory or nutritional disturbances.

(2) Metastatic calcification:- Here calcium salts precipitate in essentially normal tissues. It is the second variety that we are mainly concerned with and evidently such precipitation can either be due to an excess of calcium or occur by a change in the physiochemical factors which keep the calcium salts in solution.

Kleinman (1928) postulated that all calcium deposits in tissues begin with the formation of crystals of tertiary calcium-phosphate. He suggested that deposition of such crystals is the result of a local or general condition producing a change in calcium and phosphorus concentration, and a variation in the degree of alkalinity of the blood serum and tissue fluid. It has long been recognised that local increase in the alkalinity of the tissues plays an important role in the precipitation of calcium salts. Such is in reality the case in both dystrophic and metastatic calcification, for the latter is known to occur commonly in stomach, lungs and kidneys, in which a relatively increased alkalinity exists. Reduction in local blood supply in the presence of hyper-

calcemia has been demonstrated to result in precipitation of calcium in tissues. (Leriche and Policard, 1929; Gray, 1935).

As the factors for ossification of bone per se or for ossifying cartilage and tissues became clearer in the early part of this century, the role of enzyme reactions in the abnormal calcification was suggested by many authors. (Robison, 1923; Martland, 1925). Kay (1926) showed that the kidney possesses a high phosphatase activity which is greater in the cortex than the medulls. There is no precipitation of calcium in the kidneys inspite of high phosphatase activity, when blood calcium level is normal, but in the presence of hypercalcemia renal calcinosis readily occurs. Indeed, the phosphatase content is markedly increased in generalised bone diseases e.g. osteitis deformans, osteitis fibrosa cystica, rickets etc. in which nephro-calcinosis has often been observed.

Most of the studies in the later part of the last century and in the early part of this century were based on material from autopsies and experimental production of renal calcinosis. It was only in 1934 that Albright et al suggested the term nephro-calcinosis and reported on such cases as found particularly in association

with hyperparathyroidism and other types of metabolic bone diseases. This led to the recognition of the condition clinically and soon was followed by many case reports and later by extensive publications on the subject by many authors both in U.S.A. and Europe. The medical literature of the last decade or so particularly contains numerous articles dealing with various aspects of the problem. This has undoubtedly clarified to a great extent the pathogenesis and clinical significance of this entity. It was in 1954 that Emmett and Mortensen made their remarkable contribution to the subject by collecting and analysing their series of 91 cases from world literature. This aroused fresh interest in the subject so that during the last two or three years many more case reports and papers have accumulated and the condition is being encountered and recognised more and more frequently.

TERMINOLOGY:

The term renal calcinosis may be defined as bilaterally occurring diffusely scattered deposits of calcium in the renal parenchyma due to various metabolic disorders. Until now the term has been loosely applied without precise definition. Thus to a pathologist it means histologically demonstrable renal deposition of calcium (Allen) which is rather a common lesion occurring in 25 to 40% of all kidneys removed at necropsy. Bell (1950) has specifically limited the use of the term nephro-calcinosis as applied to roentgenographically demonstrable renal calcification. Using this criterion Emmett and Mortensen could collect only 91 cases from the world literature to 1953, making this phenomenon rather rare. Only recent editions of medical dictionaries contain this term. Their definitions vary from a very general interpretation of nephro-calcinosis as simply meaning deposition of calcium in the kidneys, to a more precise but obviously too narrow meaning that it signifies deposition of calcium phosphate in the renal tubules caused by hyperparathyroidism.

The matter of terminology becomes even more complicated when we realise that renal calcinosis per

se is not a disease entity but only occurs in association with or as a complication of a wide variety of clinical conditions. Stout et al (1955) remarked that the term therefore has very little clinical meaning unless an etiological prefix is attached to it. They proposed the use of two words when possible, such as "metastatic nephro-calcinosis". However at present to most of us it designates renal deposition of calcium, the exact type, site, degree and extent of which unfortunately are variable and poorly defined.

Our definftion signifying renal calcinosis as bilateral widely scattered renal parenchymal deposits of calcium also fails to provide precise criteria for clinical or histological recognition. The first of these two measures by itself makes the term too narrow and by the second standard as such it becomes too wide in application. In general we agree with Bell's interpretation and which has been followed by Mortensen and Emmett in their excellent review of the subject, although we believe a more precise definition with more specific meaning as to the type, site, extent and degree of renal calcification is in order.

INCIDENCE:

The incidence of renal calcinosis has not been clearly determined. There is only one large series to our knowledge namely that of Emmett et al (91 cases). They have quoted the incidence as 1 in 3,000 autopsies and 0.5% of all cases of urolithiasis. This incidence of course depended on the roentgenographic demonstrability of the calcium deposits. In their series the largest number of cases were in the age group between 20 and 50 years. The youngest was a few months old infant while the oldest was 78 years of age. The incidence of this condition occurs equally in both sexes and in all races.

There have been various studies on autopsy material, which give some insight into the relative frequency of nephro-calcinosis. (Anderson 1939, 1940; Beer 1904, Huggins 1933, Anderson and MacDonald 1946, Randall 1950, etc. etc.). Stout et al (1955) while reporting their findings of 143 necropsies selected at random give an incidence of 54.4% definite microscopically demonstrable renal calcification. In 17% there were only a few areas, in 12.2% they found moderately numerous areas. Another 12.2% showed very numerous areas and 13% revealed innumerable areas,

with distribution typically similar to the lesion of lower nephron nephrosis, a remarkable coincidence in their opinion. The areas increase in frequency as the end of the papilla is reached. This led Stout and his co-workers to study the details of the clinical records of these patients. Eight cases selected at random showed the clinical picture of lower nephron nephrosis, so they postulated this lesion may be the precursor of nephro-calcinosis in a large number of cases.

Randall (1950) found an incidence of 19.6% of histologically demonstrable renal calcification in 1,154 routine autopsies. These he called calcified plaques in and on the papillae and postulated that certain bacterial toxins and local pathological processes in such papillae are primarily responsible for these calcifications, which ultimately may lead to calculus formation. Anderson (1940) presented a report of 960 autopsies, excluding those who died of hyperparathyroidism or upper gastro-intestinal upsets and mercury bichloride poisoning. He found areas of microscopic renal calcification in 15.5%; 12.9% were slight and 2.6% showed moderate calcification.

Pyrah and Raper (1955) studied the radiographs

of 216 supposedly normal kidneys, 21% revealed calcification in the renal parenchyma. Their pathologist Dr. Anderson made extensive studies of 103 pairs of autopsy kidneys, 22 pairs or 21% were shown positive for calcification.

For our own benefit and understanding a small survey of 110 routine autopsies was carried on at the Montreal General Hospital. 22% revealed a few areas of calcific deposits and 13% were classed as moderately heavy, while 4% of the kidneys were heavily ladden with precipitated calcium. Thus 39% of all kidneys removed showed calcification.

Recently there have been at least three reports of the occurrence of this entity in a family. Pitt et al (1955) reported a father and his three children, all with radiologically demonstrable renal calcification. Aponte (1954) had three cases of oxalate nephrocalcinosis in the same family. Schreiner in 1953 mentioned a father and his son having roentgenographically proven renal calcinosis. Their family history revealed that the father of his case and one of his brothers presumably suffered from it, but unfortunately they could not be persuaded to allow investigations.

The relative incidence of renal calcinosis will go hand in hand with the incidence of renal complications,

Primary Hyperparathyroidism — World Recorded Cases to 1947 (Norris)

Skeletal changes only	191
Skeletal changes plus renal calculi or nephrocalcinosis .	101
Renal calculi or nephrocalcinosis alone	17
Neither skeletal changes nor calculi	5
	314

Primary Hyperparathyroidism — Series Published after 1947

Type	Albright et al 1948	Burk 1948	Pyrah 1955	Lahey and Murphy 1953	Richardson 1953	Black 1955	Hellstorm 1954	Reinoff 1950	Fontani et al 1947	Schneider 1948	Total
Skeletal changes alone	11	3	13	16	..	25	13	7	11	2	101
Skeletal changes with nephrocalcinosis or renal calculi	24	7	14	9	7	36	10	2	..	2	104±7
Nephrocalcinosis or renal calculi alone	28		5	4	4	133	27	17	2	6	226±7
Neither skeletal changes nor calculi	1	..	..	..	..	13	..	1	..	..	15
Total	64	10	32	29	11	207	50	27	13	10	453

Fig, 1

of the various causative disorders. The accompanying table in figure no. 1 reveals this fact.

The importance of renal complications of hyperparathyroidism was first pointed out by Albright in 1934, based upon the findings of the frequency of the urinary symptoms occurring in these cases, with or without recurrent urinary lithiasis. These patients pass granular casts in their urine containing calcium phosphate crystals. We may be justified in presuming that some of these cases of hyperparathyroidism which do not have evident renal calculi or calcinosis but still have urinary symptoms and pass crystals in their urine, such patients may have obscure if not fully obvious nephrocalcinosis, that is, the calcinosis is not advanced or is not heavy enough to cast shadows in the X-ray film. Such damage may be reversible, but it is in those cases where urinary symptoms persist inspite of the removal of the parathyroid adenoma and where there is no demonstrable renal calcification, that one feels the relative inadequacy of the too narrow definition of renal calcinosis i.e. radiographic demonstrability (Bell 1950, Mortensen and Emmett 1954). Seriousness of the situation increases when these cases continue to have renal colic and repeatedly pass small stones. The seriousness of the situation becomes more obvious when we find that

most of these hyperparathyroid cases ultimately die of renal complications. In fact it has been stated repeatedly that the prognosis of hyperparathyroidism depends entirely on the renal complications rather than on its skeletal manifestations. Hellstorm (1950) had 8 deaths out of 21 cases operated on for parathyroid adenoma; all were renal deaths. He followed his cases from 5 to 24 years.

Here it will not be out of place to digress a little and look into the relationship of nephrocalcinosis to recurrent renal calculi. This relationship has been variously stressed by Bell (1950), Engel (1951, 1952), Mortensen (1953). Stewart of the Royal Infirmary, Bradford, has probably done the largest number of partial nephrectomies (152 in 1955) for recurrent renal lithiasis. A detailed study of the surgical specimens not only confirms the obsverations of Randall (1940) on calcified plaques leading to renal calculus formation but also revealed the presence of multiple focal areas of calcification in the segments of the kidneys removed. Carr of the Royal Infirmary, Bradford, has shown the presence of similar calcific areas or micro-liths deeper in the kidney substance of the involved segments. In his excellent review he postulated that in the ordinary course of events such

microliths are removed by the intact lymph stream. It is in those cases in which this mechanism of removal fails that recurrent lithiasis develops. Black in 1954 stated that 15% of all cases of recurrent renal lithiasis have hyperparathyroidism, which is a disease most commonly associated with nephro-calcinosis. The incidence of recurrence of all renal calculi varies anywhere between 10 to 25% in various clinics and in various parts of the world.

From the above discussion we may infer that the incidence of nephro-calcinosis is relatively much greater than the number of times we actually diagnose it by radiography. It therefore follows that as the sensitivity of radiography or of our other diagnostic methods increases more and more cases will come to light.

PHYSIOLOGICAL CONSIDERATIONS:

The calcium, phosphorus and proteins of the blood exist in intimate relationship with each other. About 60% of the serum calcium is bound with the plasma proteins chiefly with albumin and in this form it is non-diffusible through celloidin membrane. This fraction is physiologically unimportant and varies in amount with the concentration of plasma proteins. The rest of about 40% serum calcium is wholly or mainly in ionised form and is mostly diffusible, so is physiologically important. The concentration of inorganic blood phosphate bears an inverse relationship to the concentration of ionised calcium, so that these ions are in equilibrium with calcium phosphate of the blood, the product of the two being normally 60 in children and less (40) in adults (where the values are expressed in mg./100 ml.). Both these ions, calcium and phosphate, are in equilibrium with calcium phosphate according to the well established formula $Ca^{++}_{3} \times (P\equiv)_2 = k$, where k is constant only for a fixed pH and is known as solubility product constant. The value of 'k' varies according to pH level of plasma, being higher when pH falls and is lower with the increase of pH. It may however be noted that the equation is dependent

upon all of the blood inorganic phosphate remaining in a diffusible form, which is probably not always the case. It has been shown (Grollman, 1927) that normally in mammals all of the inorganic blood phosphate is diffusible, but with the rise of calcium level to 12 mg./100ml. or more significant amounts of phosphate become non-diffusible, and are postulated to form a colloidal complex of calcium phosphate in loose combination with serum albumin.

The excretion of calcium and phosphorus is controlled by many factors. Normally over 60% of the absorbed calcium is excreted through the intestines where as the greater part of the phosphorus is lost by way of the urine. Renal threshold of calcium reabsorption (Albright 1942) is at a serum level of 7 mg./100 ml. so that normally this ion is continuously being excreted through urine and loss increases proportionately with the rise of serum calcium level.

The excretion of phosphorus through the kidneys has been shown to be intimately related to the activity of the parathyroid glands. In hyperparathyroidism, therefore, there is intensified phosphaturia, while hypoactivity of the parathyroids results in decreased phosphate excretion. As the excretion of phosphorus increases the level of phosphate in the blood falls

which leads to a proportionate rise of calcium level in order to keep 'k' constant. This obviously sets up an increased calcuria. Conversely with lower excretion of phosphate the amount of calcium lost in urine also decreases. In uraemia, however, despite the slow excretion of phosphorus by decreased glomerular mass, the calcium loss may still be rapid in order to counter-act the acidosis present in this condition. This is due to the inability of the tubules to manufacture enough ammonia to cover the acid products of metabolism and thus a fixed base like calcium is called upon to do so. Calcium is particularly suitable being bivalent and large reservoirs are available in the bones.

ETIOLOGY:

Obviously nephro-calcinosis is a secondary manifestation of a primary disorder. The following list of such primary disorders has been modified from that published by Goldstein and Abehouse (1938).

Nephro-calcinosis has long been classified into two types for purposes of description as already mentioned.

1. Metastatic Calcification (Type 1).
    A. Conditions associated with generalised bone disease.
        a. Osteitis fibrosa cystica.
        b. Osteomalacia, osteitis deformans.
        c. Rickets.
        d. Osteosclerosis.
        e. Multiple myeloma.
        f. Multiple fractures.
        g. Osteoporosis.
        h. Primary osseous neoplasm and neoplastic osseous metastasis.
    B. Diseases of the Gastro-intestinal Tract.
        a. Pyloric obstruction due to peptic ulcer or carcinoma.
        b. Intestinal lesions.

c. Prolonged intake of alkalies and excessive amounts of milk.

d. Cirrhosis of liver.

e. Dysentery.

f. Undetermined gastro-intestinal diseases with dietary deficiency.

g. Ingestion of certain drugs eg. vitamin D and Diamox.

C. Endocrinal Diseases.

a. Hyperparathyroidism.

b. Adrenal dystrophy.

c. Prolonged action of excess testosterone (Allen 1951).

d. Hypothyroidism (Naylor 1955).

D. Certain Systemic Diseases.

a. Sarcoidosis.

b. Tuberculosis.

c. Other diseases requiring prolonged recumbancy.

2. Dystrophic Calcification (Type 11).

A. Bilateral renal diseases.

a. Acute or chronic nephritis.

b. Nephrosis.

c. Primary sclerosis (arteriosclerosis).

d. Experimental Nephritis.

e. Chronic pyelonephritis.

f. Bilateral renal lithiasis.

g. Renal tubular acidosis or renal rickets.

h. Certain poisonings eg. heavy metals like mercury, lead etc., sulphonamides, para-amino-salcylic acid etc.

i. Lower nephron nephrosis (McLetchie 1943, Stout et al 1955).

B. Acute or chronic localised infections eg. renal tuberculoma, pyonephrosis, hydronephrosis, various renal cysts or tumours, benign or malignant parasitic lesions like echinococcal disease or bilharziasis, vascular lesions, infarcts, injuries, recurrent renal lithiasis etc.

This list may still be incomplete but it signifies how a wide variety of conditions of entirely different etiology may lead to a common lesion through the operation of many different mechanisms. The pathological lesions thus produced seem to be identical, but it appears that there is no common denominator that can be assigned an etiologic role in all these conditions.

However, we may list some of the known important factors which may operate singly or in combination to produce renal calcinosis. Such factors are:-

A. Hypercalcaemia.

B. Increased local alkalinity in the renal tissues.

C. Increased activity of alkaline phosphatase.

D. Damage to the renal tissues direct or indirect by local reduction in blood supply may also play some role.

As to how these factors work singly or in combination we shall discuss in some detail in the mode of development of renal calcinosis.

The cause of renal calcinosis is thus a matter of recognizing the underlying lesion. Analysis of 91 cases by Emmett et al (1954) reveals that three fourths of their collection were associated with one of the following three disorders, namely, hyperparathyroidism, renal acidosis and chronic pyelonephritis. The other less frequently associated disorders were chronic glomerulonephritis, recurrent renal lithiasis, idiopathic hypercalcuria, sulphonamide toxicity, vitamin D intoxication, poisoning with heavy metals, multiple myeloma, sarcoidosis etc. All these causes

have been suggested and should be considered in the differential diagnosis of the cause of radiographically demonstrable nephro-calcinosis. A few more conditions may also be enumerated for they commonly produce histologically demonstrable calcium deposits, but are not likely to be recognised radiographically, namely hypochloraemic alkalosis, and osteolytic or osteoporotic lesions of the bones.

MODE OF DEVELOPMENT OF RENAL CALCINOSIS:

Barr (1932) may be credited for our present day conception of calcification of healthy tissues. In his exhaustive review he has outlined the factors that govern this phenomenon. Although during the last 25 years there have been many excellent reviews of the subject but no factual information has been added. The works of Mulligan (1947), Albright and Reifenstein (1948), Greenspan (1949), Vaughan et al (1947), Goldstein and Abehouse (1938), Mortensen and Emmett (1954), and Pyrah (1955), Abehouse and Goldfarb (1955) are commendable.

Goldstein and Abehouse presented at least twelve theories that have been advanced over the years to explain the mechanism of renal calcification. To those may be added the more modern ideas based on local tubular disorders in the kidneys leading to a disturbance in citrate and carbonic anhydrase mechanisms. A study of all these well compiled works demonstrates the uncertainty and dissatisfaction of the authors with the etiologic concepts and suggests that several different physiologic mechanisms may be responsible for the production of this lesion. The alterations in the factors, physical or chemical

which control the solubility of calcium salts in renal blood, glomerular filterate, renal tissue fluid and urine, may well be conceded to be responsible for the metastatic type of calcification.

Even more difficult to explain is the nature of dystrophic calcification. Although it has been long recognised that degenerating, devitalized or necrotic tissues are frequently the site of calcification, still the explanations offered seem merely theoretic in nature.

Our present concept of the pathogenesis of nephro-calcinosis as it occurs in association with a variety of causative disorders may now be outlined.

Primary Hyperparathyroidism.

Even though the concept of the mode of action of parathormone has undergone a radical change from the days of Collip (1925) to the more modern concept laid down by Albright (1934), the outcome of excessive production of the hormone remains the same. Hyperparathyroidism leads to hyperphosphaturia, hypercalcuria, hypercalcemia and hypophosphatemia. With the exception of the latter all the other conditions favour renal calcification on a simple basis of exceeding the solubility product of calcium phosphate or

calcium carbonate, in urine, in the blood flowing through the kidneys or in the fluid of renal tissues. In the later stages of the disease when renal insufficiency usually occurs there is retention of phosphates leading to hyperphosphatemia which makes the precipitation of calcium even more likely. The possibility of injury to the renal tubular cells from excessive circulating parathormone or hypercalemia was first suggested by Anderson in 1939. Only recently experimental studies of Baker et al (1954) seem to support Anderson's suggestion. These authors gave to the animals large doses of parathormone with or without tolouidine blue. This dye seems to exert a protective influence on the groumd substance. The animals not receiving the dye uniformly developed renal calcinosis in the presence of excess parathormone, While the majority of the animals given similar amounts of parathormone along with the dye did not show any evidence of calcification.

These factors of hypercalcemia, hypercalcuria, hyperphosphatemia and renal damage are indeed important but they certainly are not a complete answer to nephro-calcinosis in hyperparathyroidism. They may all exist without calcification. This

statement is well supported by the clinical observation that only 15% of the patients with well advanced, easily recognisable primary hyperparathyroidism have nephrocalcinosis. It therefore signifies that there must be some other factor or factors yet undetermined which influence the development of nephrocalcinosis. In 1934 and again in 1935 Albright et al discussed this pathogenesis in detail in their review of renal complications in 83 cases of hyperparathyroidism. They have stressed the intimate relationship between the calcium deposits in the intrarenal urinary passages and the fine granular casts in the urinary sediment of these patients. This probably shows that initially such deposition starts in the tubular lumina and later works its way towards the interstitial tissues. Also these granular casts have been considered to be directly related to calculus formation in hyperparathyroidism. In reality they are microscopic calculi lying within the tubules. In their opinion the same factors i.e. concentration in the urine of calcium, phosphates and hydrogen ions governs the formation of calcium casts as well as stones and even nephrocalcinosis.

It may be added here that a similar patho-

genesis may be operating in other degenerative and destructive bone lesions. In some of these cases markedly increased alkaline phosphatase activity may be an additive factor. Values of serum alkaline phosphatase may exceed 40 units per 100 ml. of blood in osteitis deformans. Moreover, that there is excess parathormone in circulation in most of these cases can be shown to be due to the common association of these lesions with secondary hyperparathyroidism.

Renal Acidosis:

This type of nephrocalcinosis has been described in literature under at least twelve different names, signifying a rather wide disagreement regarding whether the renal tubular insufficiency is or is not associated with glomerular insufficiency. It is commonly believed that in this condition kidney tubules are unable to make or excrete ammonia. This results in persistently alkaline urine and systemic acidosis.

Albright in 1946 described the following sequence: 1. Inability of the tubules to make ammonia or to excrete acid urine due to renal tubular disease. 2. Shortage of base to excrete mineral acids. 3. Increased loss in urine of calcium acting as base.

4. Tendency to low serum calcium level. 5. Secondary hyperparathyroidism to meet this tendency. 6. Hypophosphatemia. 7. Leading to low phosphate rickets.

Farrell writing in Guy's Hospital Report in 1953 collected 78 cases of this syndrome from the world literature and discussed the pathogenesis in detail. He postulated that in these patients there may be an inability to re-absorb sodium bicarbonate, which results in the same effects i.e. persistently alkaline urine and systemic acidosis. Greenspan (1949) has suggested the possibility of chronic inactivation of carbonic anhydrase which can be produced experimentally by administering sulpha drugs. The basic problem in this syndrome still remains unexplained, that is the inability of the kidney to produce acid urine.

Renal acidosis was first described by Lightwood in 1935 who observed the typical renal lesion in eight autopsies of children and correlated this finding with the clinical records. Later this disease has appeared in the literature under several names, eg. nephrocalcinosis infantum with hyperchloremic acidosis. (Boutourlin and Young 1949), nephrocalcinosis

with rickets and dwarfism (Albright et al 1946), persistent acidosis (Lightwood 1935), Lightwood syndrome (Lightwood et al 1936), concommitance of chronic acidosis with late rickets (Boyd 1942), idiopathic renal acidosis (Stapleton 1949) etc.

Baines, Barclay and Cook (1945) observed a 29 year old woman who had typical metabolic findings i.e. hyperchloremia, hypercalcemia, hyperphosphaturia and alkaline urine, but no skeletal changes. These authors emphasised that it is not essential to postulate the parathyroid mechanism to explain the low serum phosphate concentration for the chronic acidosis in itself is sufficient to produce hyperphosphaturia which may bring about a lowered serum phosphorus. Haldane has shown that acidosis produced by ammonium chloride will increase phosphate excretion and he believes that this hyperphosphaturia is concerned with the buffer action of urinary phosphates, allowing the kidneys to excrete more acid with less base.

Whatever may be the sequence of events and however one may try to explain the etiology or pathogenesis of this syndrome, the result is persistent hypercalcemia with hypercalcuria and alkaline

urine. Both these conditions make an ideal situation for precipitation of calcium by exceeding the solubility product of calcium phosphate and calcium carbonate.

Chronic Pyelonephritis:

There have been several reported instances of extensive calcification of the kidneys in chronic pyelonephritis (Albright, Diens and Sulkowitch 1938, Wohl 1942, Ewell 1941, Grossman and Allyn 1941 etc.). Albright et al reported two cases in 1938 in which the infecting organism was H influenzae. The blood calcium, phosphorus and proteins were normal, P.S.P. excretion was good, but urine specific gravity was only 1008. The pathogenesis of renal calcinosis in chronic pyelonephritis is even more poorly understood. It is commonly noted in these cases that there is present a chronic mild but persistent infection with urea splitting organisms. It has been suggested that these organisms growing in the tubules or interstitial tissues of kidneys liberate ammonia which results into a local increase in pH. This alkaline environment decreases the solubility of existing calcium salts sufficiently to allow or facilitate their precipitation. Further, presence of necrosis caused by bacterial invasion or diminution

in blood supply by fibrosis and sclerosis of blood vessels, may result in deposition of calcium of the dystrophic type. Similarly one may think of the possibility of tubular damage due to chronic infection, which may give rise to systemic acidosis or there may be disturbance of carbonic anhydrase mechanism leading to acidosis again.

All these factors although probably operating singly or in combination, in patients with chronic pyelonephritis, seem rather inadequate to explain the development of nephrocalcinosis; for the complication is infrequent as compared to the relatively high incidence of the disease itself. It would therefore seem that the final factor for precipitation of calcium salts is yet unknown; for if nephrocalcinosis were to occur primarily on the basis of such factors, it would be a very common phenomenon, instead of a most unusual complication of chronic pyelonephritis.

The above described conditions are the common entities with which nephrocalcinosis is commonly associated. It occurs in many other conditions though less commonly or even rarely but the mechanism of production appears basically the same. In degenerative

and metastatic bone lesions apart from hypercalcemia increased alkaline phosphate activity has already been suggested to be a potent factor. Kay (1926) had conclusively shown that in the kidney tissue there is much more alkaline phosphatase than in ordinary tissues. He also pointed out that it is maximal at the cortico medullary zone with more in the cortex than in the medulla. Similarly in vitamin D intoxication, hypercalcemia is the most important factor; so also in multiple fractures, prolonged recumbency and in sarcoidosis which has recently been shown to be one of the primary causative conditions (Davidson 1954).

The deposition of calcium in acute or chronic glomerulonephritis or in various forms of toxic nephritis or that associated with various drug intoxications eg. sulphonamides (Engel 1951), mercury, para-amino-salicylic acid (Cowie 1954) etc., seems to be related to the amount of tubular as well as glomerular damage found in these cases. This damage ultimately leads to advanced renal insufficiency which may result in accumulation of phosphate in the blood. This in turn disturbs the calcium phosphate solubility product, even though the concentration of calcium is

normal or low. In the relatively alkaline renal tissues the precipitation would most likely occur. On the other hand it is quite possible that dystrophic calcification may occur in the severely damaged, fibrotic or hyalinised glomeruli and tubules.

Engel (1951) considered the following factors responsible for the production of nephrocalcinosis in sulphonamide intoxication. 1. Sulphanilamide inhibits carbonic anhydrase. 2. Pitts and Alexander were able to produce blood acidosis and urinary alkalosis by administration of sulphanilamide to dogs. 3. Hyperchloremic acidosis has been reported by Leutseher and Blackman after therapeutic doses of sulphathiazole. 4. Antopole et al were able to produce chronic calcifying necrosis confined to the distal tubules after severe transient sulphonamide toxicity in animals. Finally they remarked that in pathological studies by Lucke and French 153 soldiers were shown to have died of acute toxic lesions due to various sulphonamide drugs.

Severe and prolonged upper gastro-intestinal obstruction with vomiting has long been known to result in calcification in and around the tubules (Nazari 1904). It has been suggested that the pathogenesis of these changes is dependent upon the marked

alkalosis present in them. According to this concept the calcium precipitation results from continued excretion of acid urine in spite of a systemic alkalosis. While there is a great loss of chloride with prolonged vomiting there is also appreciable loss of sodium. This if continued may lead to a fall in the level of the blood sodium. When this occurs, there is a call upon the kidneys to conserve the decreasing supply of body base. In order to accomplish this the kidneys must excrete acid urine inspite of alkalosis. It may here be argued that the kidneys should not preferentially conserve sodium especially when the body is even more depleted of chlorides. The most likely explanation of this is the fact that the loss of acid radicals represented by the chlorides can be somewhat compensated for by an increase in the bicarbonate content of the blood, while there is no suitable ion to compensate the sodium loss. So in an effort to conserve sodium the kidney uses the bivalent calcium as a base to excrete the mineral acids. This increases the amount of calcium in urine, which in the presence of marked alkalosis may precipitate in the tissues. That such is the sequence of electrolyte changes has been

shown by Martz(1940).

Massive alkali therapy is known to have resulted in marked calcium deposition, even though in these cases there was no reason for the urine to turn acid for there was always excess of base due to the nature of this alkalosis. Here the precipitation of calcium may be attributed to the concentration of solutes in the collecting tubules, plus of course the continued alkalosis. This variety of renal calcinosis has been well recognised by various authors in prolonged alkali therapy in cases of peptic ulcer. (Wermer, 1953). The incidence of renal calcinosis in these cases increases with the intractability of the ulcer and with the presence of pyloric obstruction. A detailed study of the subject was presented by Becker et al in 1952.

More recently the important role of urinary citrates as complexing agents for calcium salts has been studied in great detail by many authors. (Kissin and Locks 1941, Shorr et al 1942, Scott and Huggins 1943, Abehouse 1956). The effect of the drug Diamox (acetazol-amide) acting as carbonic anhydrase inhibitor as well as lowering the citrate output in urine was explored by the Harrisons in 1955. They produced

nephrocalcinosis in rats using Diamox along with alkaline ash or acid ash diets. The mechanism of production of renal calcinosis has been discussed by them in great detail. There have been no reports on nephrocalcinosis following prolonged Diamox therapy but several case reports have appeared in the literature during the past few years in which patients had renal colic and calculus formation attributed to long continued ingestion of Diamox. (Persky et al 1956, Becker 1955). The action of various hormones affecting the citrate metabolism has been studied by Shorr et al (1948) and Pincus et al (1951) but there has been no clinical evidence to present of a hormonal imbalance of this type being directly responsible for the production of renal calcinosis. Estrogens seem to increase citrate output whereas testosterone decreases the amount of urinary citrate. Cortisone causes a fall in the level of serum citrate and calcium, whereas ACTH raises their levels in blood when injected intramuscularly.

In conclusion it may be added if hypercalcuria per se is a cause of nephrocalcinosis, it would seem logical to assume that the mechanism is that the solubility product of calcium salts in urine is

exceeded, resulting in simple precipitation. This process is enhanced in the presence of increased alkalinity in the renal tissues.

PATHOLOGY:

Detailed necropsy findings have only been described in a few cases of nephrocalcinosis. An excellent review with extensive bibliography and findings of their own cases was presented by Mortensen and Baggenstoss in 1954. Most of the subject matter condensed here is drawn from their study.

The size of the kidney is usually normal but may be decreased slightly. Occasionally the kidney may be small and contracted. It is very common to find in such cases that one or the other complication is usually associated eg. chronic pyelonephritis, arteriosclerosis or other vascular lesions secondary to hypertension. The capsule mostly strips with ease though often it may be adherent. The surface of the kidney is generally finely granular due to the areas of hypertrophy surrounded by areas of fibrosis. Occasionally nodules of hypertrophic renal tissue may be noticed.

On section probably the most constant and characteristic finding is the gritty sensation imparted to the cutting knife. The cut surface on gross inspection shows deposits of calcium in the medulla, most marked in the tips of the pyramids. The calcification is whitish or pale grey in appearance and is

scattered as streaks, flecks or sand-like dots. Evidence of other associated disease or complication may be present. Calices and renal pelvis are usually normal unless residual deformities from previous infection or stones are present.

Microscopically scattered calcium deposits form the most constant finding. The authors Mortensen and Baggenstoss however remark that it must be admitted that one cannot differentiate by histological examination alone, a kidney, that will cast recognisable roentgenologic shadows from one that will not. Deposits are more common in the medullary region towards the tips of the pyramids than in the cortical zone. The basement membrane of the collecting tubules was the commonest location in their experience. The other sites in order of frequency were medullary interstitial tissue, intracellular, intraluminal, in the convoluted tubules, cortical interstitial tissue, lumen of collecting tubules, glomeruli and walls of renal blood vessels. Calcified casts were noticed in 40% of cases in the convoluted tubules and in 30% of cases in the collecting tubules. The granules of calcium were deposited directly in the nuclei but extended also to the cytoplasm of the epithelial cells

of the distal convoluted tubules. The nuclei were often sharply outlined by calcific rings and the cellular debris was desquamated into the tubular lumens as calcareous casts.

This calcification is a bland process generally provoking no noteworthy inflammatory reactions. Although areas of fibrosis, scarring and hypertrophy were frequently noted along with occasional infiltration of chronic inflammatory cells. Evidence of associated disease such as glomerulonephritis or chronic pyelonephritis can be recognised. It is not possible to distinguish the causative disorder from the histological picture of the kidneys alone, although a diagnosis of the primary disease can be made after correlation with clinical and other necropsy findings in 85% of cases.

There is no pathognomonic group of findings, gross or histologic, without radiography to make an unequivocal diagnosis of nephrocalcinosis. It is not always possible to readily distinguish grossly the white solid material comprising calcium deposits from uric acid deposits, hypertrophic tubules, diffuse fibrous streaks or inflammatory nodules seen in chronic pyelonephritis. Microscopically the VonKossa's special

stain does not necessarily prove that the deposits are due to calcium. Chemical analysis is by far the best method to confirm the presence of calcium. Finally as yet there are no gross characteristics or histological criteria or specifications as to the chemical percentage or amount of calcium that will be revealed radiologically. The problem becomes even more complicated when we still cannot distinguish between the presence of parenchymal calcifications that do not cast an identifiable radiographic picture from those that will show the pattern of nephrocalcinosis (Mortensen 1954).

CHAPTER III.

CLINICAL FEATURES AND CLINICAL SIGNIFICANCE:

There is no definite set of signs or symptoms which can be attributed to renal calcinosis. The picture is primarily that of the causative disorder. The latter when removed in certain cases leaves some residual clinical features. These usually consist of a general malaise, muscular atony and lassitude. Headaches, loss of appetite and other symptoms of impaired renal function eg. polyuria, polydypsia, a urine of persistently low specific gravity etc., are usually present. Both these sets of symptoms may be attributed to the two conditions which are most commonly related to nephrocalcinosis i.e. hypercalcemia and increasing renal insufficiency.

If the case is complicated renal stone formation and chronic infection, bouts of acute pain, chills, fever, pyuria etc. may occur. Some cases have been known to pass small calculi or gravel repeatedly. After the disease has been diagnosed radiographically these symptoms are generally progressive and death is usually due to ultimate renal failure. But it may well be added that the signs and symptoms may not be proportional to the severity of calcium deposition revealed by radiography.

Radiologically two or three varieties are usually seen. The most common and striking type is that in which calcium deposits occur in radial streaks in the pyramids. In the other varieties deposits of calcium are scattered as small blobs or dots throughout the renal parenchyma. In still other cases only portions of the kidney may be involved. These pictures may occasionally be associated with renal stone formation. There is apparently no correlation between the primary causative lesion and the type of radiological picture. A kidney may be riddled with deposits of calcium microscopically or even grossly, and still do not throw any shadow on the X-ray film.

As to the clinical significance of this entity we concur with the ideas expressed by Mortensen and Baggenstoss (1954) in their excellent review of the subject. Histologically demonstrable calcium deposits are so common that no definite clinical significance can be attached to them. In the first place unless such a lesion is so extensive and is of a certain particular type (Mortensen et al 1954) that it can be demonstrated radiologically, the lesion cannot be recognised clinically. Secondly the relationship

between the lesion and renal functional impairment is not established. Time and again one comes across deposits of calcium in the renal parenchyma along with other pathologic lesions so that the functional impairment in such cases could easily be accounted for on the basis of the latter. Finally occasionally one finds rather marked degree of renal calcinosis at autopsy, yet those kidneys functioned normally in life, caused no symptoms and there was no clinical indication of their being laden with so much calcium.

On the other hand nephrocalcinosis as defined by Bell, being clinically recognisable by its characteristic radiographic appearance, is of great interest and significance to the clinician. From the previous statement on the incidence of such a lesion we concluded that it is very rare, having been found only once in every 20,000 clinical examinations at the Mayo Clinic.

After reviewing the clinical features in 91 collected cases of this disorder, Mortensen and Emmett remarked that because nephrocalcinosis as such probably does not cause any symptoms and produces no clinical signs, the diagnosis must entirely depend upon radiography. The manifestations of the

primary disorder also help in suspecting or looking for such a lesion, particularly in the presence of decreasing renal efficiency. Improved simple techniques for renal biopsy will not only reveal further useful information in these cases but also may even confirm the diagnosis when the radiological picture may still be obscure or doubtful. Lastly as in the case of any other relatively uncommon disorder the diagnosis directly depends up the suspicion index of the clinic for the particular condition.

PROGNOSIS:

The data available in the literature are so limited that no definite rules can be given. In general it is agreed that once the condition is diagnosed the lesion is usually progressive with increasing renal damage leading to ultimate death in uraemia. By the time the lesion has reached a stage of producing its characteristic radiological picture, the lesion is irreversible and may even be progressive in spite of removal of the primary cause. Mortensen's study of 91 cases reveals that the ultimate life span in these cases is rather short, only 50% surviving more than three years and only 25% living more than 5 years after a diagnosis was made radiologically. On the other hand there are known patients with rather advanced nephrocalcinosis who lived for 16 years or more with good renal function. Thus there appears to be no correlation between renal function and radiographic picture to throw any light on prognosis. It should also be pointed out here that so far there is no definite treatment available and all that can be done for the patient is to make an early diagnosis and eradicate the primary causative lesion in the hope

that the lesion may regress or may at least become stationary.

Nephrocalcinosis when associated with kidney disease itself carries a rather grave prognosis. The outlook of the patient is certainly poor in the presence of any renal lesion major or minor.

The interrelationship between nephrocalcinosis and renal lithiasis has been a subject of increasing interest since Albright's publication in 1934 and later that of Randall in 1938. There seems to be an increasing belief in the concepts that there is no essential difference between nephrocalcinosis and multiple renal lithiasis (Engel 1952, Carr 1954) and that renal lithiasis is a natural sequaela of nephrocalcinosis and will be observed in all cases if looked for carefully (Engel 1952). These concepts, although lacking positive evidence,, do create pessimism concerning prognosis.

Finally it may be remarked that although the prognosis depends on many factors as for example the primary causative lesion, the presence or absence of renal complications etc., in general it is poor and the span of life is considerably reduced in renal calcinosis.

CHAPTER IV.

EXPERIMENTAL ASPECTS OF RENAL CALCINOSIS:

Renal calcification and deposits of calcium elsewhere in the body have been a subject of experimentation for almost a century. Metastatic calcification of the kidney has been reported to occur under a variety of experimental conditions (Follis 1948), including nutritional deficiency of magnesium (Heller and Dicker 1947), chloride (Cuthburtson and Greenberg 1945, Cuttins et al 1948), potassium (Follis et al 1942, Orent-Keiles and McCollum 1941, Schrader et al 1937, Wachstein 1944), linoleic acid (Burr and Burr 1929 and 1930) amd choline (Wachstein 1944). Tubular degeneration followed by calcification has also been produced by the excessive feeding of inorganic phosphates (MacKay and Oliver 1935, McFarlane 1941, Heller and Dicker 1947), experimental uric acid nephritis (Dunn and Polson 1926), heavy metal poisoning (Hepler and Simonds 1945), and in the use of other forms of toxic nephritis (Goldstein and Abehouse 1938).

The earliest available references to the experimental production of renal calcinosis are those of Virchow (1855) and Litten (1881). Litten ligated the

renal artery on one side and noted calcification within 24 hours. VonKossa studied the subject of metastatic calcification in great detail and continued Litten's experiments (1901). He ligated both artery and vein alomg with the ureter on one side. The animal died in 22 days when calcification was found just under the capsule. It is indeed amusing to read his work. Particularly the way he explains the mechanism of this calcification is fascinating. He discusses in detail the role of mercury, phosphorus, oxalic acid, lead etc. in the production of renal calcification which in most cases depends on the time factor, the dose of the chemical and the local resistance of the kidney tissues.

Nephrocalcinosis has also been produced by vitamin D intoxication (Pyrah 1949, Shohl et al 1930, Dixon and Hoyle 1928), excess calium intake, acid and alkaline ash diets and various combinations of these, (Chown 1936 and 1937, Duguid 1938, Mulligan 1947, Rabl 1923), administration of parathyroid extracts (Cantarow 1938, Becker 1954, Hueper 1927), production of metabolic alkalosis which can be induced by upper gastrointestinal obstruction (Pyrah 1949, Zeman et al 1924, Kirsner and Knowlton 1941, Kirsner 1941, Mulligan

and Stricker 1948) or by prolonged administration of alkalies. More recently Selye (1956) produced renal calcinosis by feeding rats 1% sodium di-hydrogen phosphate in their drinking water. The effect of this was appreciably enhanced by the use of DOCA and nullified by cortisone and other gluco-corticoids. Metabolism of citrates has been studied in great detail with special attention to their potency in maintaining calcium salts in solution. Basing their ideas on the work of Kissin and Lock (1941), Shorr (1942) etc., the Harrisons (1955) started to study the effect of diamox which greatly diminishes the citrate output in the urine. This resulted in moderately heavy deposits of calcium in the rat kidneys.

A vast variety of experimental methods are thus available to induce renal calcification. Our initial problem was to produce metastatic calcium deposits in the renal parenchyma by a method which would satisfy the following criteria.

1. It should be simple and cheap so that studies on dissolution can be carried on, at some measurable scale.

2. The method should be uniformly successful in producing renal calcinosis.

3. The deposits should be stable and not spontaneously reversible.

4. The induction of calcinosis should not cause profound local or systemic damage.

5. It should induce calcinosis within a reasonable period of time.

Keeping these ideas in view several methods of producing nephrocalcinosis were studied in detail. In the following pages we will outline the advantages or disadvantages encountered in these methods.

1. Experimental Hyperparathyroidism:

A chronic hyperparathyroidic state can be easily produced in various animals by parathyroid extract or parathormone. This method is known to give uniform results. The drug is given bi-weekly or three times a week intramuscularly. A total dose of 1200 to 1600 U.S.P. units is believed to be sufficient for an average sized young rat. Parathormone is easy to handle though the potency varies from batch to batch. Our main objection to its use was the formidable cost, which would have prevented the carrying out of dissolution studies on any scale yeilding statistically significant results. More over calcium deposits are liable to occur in other organs. This might have

interfered with the dissolutory effect of the dissolving drug and the results would have been fallicious as regards its effect on renal calcinosis. Finally the renal calcinosis produced by excess parathormone may lessen or disappear after the drug is stopped. Clinical observations in man substantiate this idea. Removal of a parathyroid adenoma is occasionally followed by disappearance or marked reduction in renal calcification. These considerations led us to reject this method of inducing renal calcinosis.

2. Experimental Hypercalcemia:

This has been induced in several ways and in itself leads to renal calcification if continued long enough. Hypercalcemia can be induced either by excess calcium intake or by hypervitaminosis D. The former method is somewhat uncertain and takes a longer time. Excessive doses of vitamin D in young growing rats in the presence of normal calcium intake uniformly results in renal calcinosis. This type of induction is distinctly reversible, so that the calcium deposits disappear after vitamin D is discontinued. Toxic doses of vitamin D are liable to cause renal damage. Hence this method too was not suitable for our purpose.

3. Acid or Alkaline Ash Diets:

This method has been used with rather varying and inconsistent results. Also the possibility of reversibility in this type of induction is even greater.

4. Diets Low in Various Minerals Such as Magnesium, Potassium, Chloride etc.:

In these cases metastatic deposits of calcium are believed to occur through interference with the growth of bone or by restriction of normal bone metabolism. The resulting dietary depletion is liable to produce many other undesirable effects. Evans and MacPherson (1956) were able to produce heavy deposits of calcium by feeding rats a potassium free diet in 3 to 11 days, but most of the animals were moribund by that time. Similarly depletion of magnesium or chloride, when prolonged leads to marked inanition. The unsuitability of this method is obvious.

5. Metabolic Alkalosis:

This can be produced in several ways. A chronic obstruction of the upper gastrointestinal tract causes marked alkalosis. Thus ligation of the pylorus or duodenum has been tried. The animal dies in 5 to 6 days. Calcium deposition in the renal tissue was produced in rats by Thorn, Clinton, Farber and Edmonds

(1946) following repeated short exposures to reduced atmospheric pressure, causing metabolic alkalosis. Calcium was deposited between collecting tubules at the cortico-medullary junction. The reversibility of these deposits is suspected. More over this method requires more elaborate apparatus and technique. The uncertainty of production is another undesirable factor.

Administration of large doses of alkalies in food or in drinking water may also produce such effects but the process is again inconsistent and rather time consuming.

6. Feeding of Sodium Di-Hydrogen Phosphate:

Recently Selye (1956) reported the production of nephrocalcinosis in rats by giving 1% sodium di-hydrogen phosphate instead of water. He also studied the effects of gluco-corticoids and des- oxycorticosterone acetate, on calcium deposition by this method. The method is a simple one but this did not come to our notice until our program was already under way.

7. Administration of Diamox with High Calcium or High Phosphorous Diet:

Kissin and Locks, Shorr et al and Huggins and

Scott, accumulated evidence that the citrate content of urine has a great deal to do with the solubility product of calcium salts in the urine. Citrate metabolism was studied by them in great detail. An excellent review of the subject will be found in Abehouse's (1956) publication. All these data appear to show that by lowering the amount of citrate in urine and kidney tissues one could expect that the solubility product of calcium salts might be so disturbed, that these salts would precipitate. Problem was now to acheive a lowered citrate output in urine.

The Harrison's (1955) produced convincing evidence that the drug diamox (acetozolamide) distintly lowers the citrate output in urine in rats. They observed deposits of calcium in the kidneys when animals receiving diamox were fed special diets containing relatively large proportions of calcium and phosphorus. Their article aroused considerable interest and led us to select this method because of its uniform success, simplicity and cheapness. The deposits of calcium in the renal tissues can be obtained within a reasonable period of time without any local or systemic damage. Obviously our only

problem was whether these concrements are spontaneously reversible or not. The work of the Harrisons is remarkable for they observed two distinct varieties of renal calcinosis, obtained merely by varying the proportions of calcium and phosphorus in the diet. It may be mentioned here that vitamin D intake also influences the citrate output in urine; an increase vitamin D intake being associated with increased urine citrates, Evan and MacPherson (1956), disagreeing with the Harrisons, carried out a series of experiments with detailed bio-chemical studies on the relationship between citrate output and production of renal calcinosis. They found that citrate has little, if any, influence on the solubility of calcium salts in urine. In their opinion, the action of diamox in producing renal calcinosis depends probably on increased calcium absorption from the bowel. Both lowered urine citrates and increased calcium absorption may well be responsible for diamox induced calcinosis.

Whatever may be the mechanism we had gratifying results and shall describe this method in detail in the next chapter.

CHAPTER V.

MATERIALS AND METHOD OF EXPERIMENTAL PRODUCTION OF RENAL CALCINOSIS:

Animals:

In this experiment young male rats of Royal Victoria Hospital hooded strain were used. Their average age was 3 to 4 weeks and their initial weight varied from 100 to 180 gms. Animals were divided in two groups - control and acetazolamide treated. All the rats were put on the following diet mixture.

Diet - Composition:

Casein	180 gms.
Corn starch	450 gms.
Dextrose	250 gms.
Peanut oil	80 gms.
Brewers' yeast	50 gms.
Salt mixture	50 gms.
Vitamin A concentrate	10,000 units

- Ingredients of the Salt Mixture:

NaCl	5.250 gms.
KCl	6.000 gms.
KH_2PO_4	15.500 gms.
Ca_3PO_4	7.450 gms.

$CaCO_3$	10.500 gms.
$MgSO_4$ (anhydrous)	4.500 gms.
$K_2Al_2(SO_4)_2 \cdot 4H_2O$	0.005 gms.
$CuSO_4 \cdot 5H_2O$	0.020 gms.
NaF	0.029 gms.
KI	0.003 gms.
$FePO_4 \cdot 4H_2O$	0.735 gms.
$MnSO_4$ (anhydrous)	0.010 gms.

This salt mixture is the Wessen modification of Osborne and Mendel salt mixture (1932). It was obtained from the Nutritional Bio-Chemical Corporation, Cleveland 28, Ohio.

The mixture contains 7 mg. of calcium and 5.6 mg. of phosphorus per gram. When the said mixture is used in the proportions described the ratio of calcium and phosphorus in the diet is apparently the same as in cow's milk. Our animals being exclusively fed on this diet naturally received more calcium and phosphorus per day than they would from the standard Purina meal diet. For the acetazolamide treated group 250 mg. diamox (acetazolamide) were thoroughly mixed

with each 100 gms. of diet. The control group received the same diet with salt mixture but without diamox. This diet is deficient in vitamin D, so a single large dose of 5,000 units of vitamin D concentrate was injected into the calf muscle of each rat at the beginning of the experiment. All these animals were kept in separate cages and water and food was supplied ad libitum.

This dietary regime was continued for three weeks when a portion of the lower pole of the left kidney was removed from each rat. Following this biopsy all the animals were fed on standard Purina meal diet.

TECHNIQUE OF BIOPSY:

The animal was anaesthetised by an intraperitoneal injection of nembutal (1 min. per 100 gm. body weight). This dose was usually sufficient but was increased as necessary. The effect lasted for 45 minutes to one hour. The animal was then tied to a board lying flat on its back. The abdomen was opened by a left paramedium incision. The bowels were gently displaced to the right and packed in position, thus exposing the left kidney. After mobilizing the kidney it was held between the thumb and index finger of the operator's left hand. Most of the lower pole of the kidney was then removed by a sharp knife and a gentle pressure kept on the rest of the kidney by the thumb and index finger for one or two minutes. In the meantime a small piece of gelfoam was applied to the cut surface. This very nicely adhered to the kidney tissue and stopped the bleeding quite effectively. The kidney was then dropped back and the pack was removed. Viscera were replaced and the abdomen was closed in two layers using interrupted fine #00000 silk sutures.

It may be added here that no special aseptic technique is essential in this animal surgery. Only

scrupulous cleanliness and gentleness are necessary. The abdomen was shaved and prepared with 1% mercurochrome.

The tissues removed were kept in neutral formalin. No antibiotics were used pre- or postoperatively.

METHODS OF EVALUATION OF THE DEGREE OF CALCIUM DEPOSITS:

Three methods were available - radiological, histological and biochemical.

Several attempts to demonstrate these calcium deposits on a plain X-ray film were unsuccessful. It is quite possible that the calcinosis was not heavy enough to cast a shadow or the type of calcification might have been radio-lucant. Therefore the other two methods were used.

Histologic Evaluation:

The tissue was processed, blocked and sections, six microns, in thickness were prepared. They were stained with eosin and haematoxylin. Duplicates of each section were stained by VonKossa's special technique for demonstration of calcium particles (Lillie 1948). The technique is described below:

VonKossa's Calcium Stain:

Fixation: Absolute ethyl alcohol or formalin fixed tissue. Alcohol (ethyl) preferred. We used neutral formalin and alcohols and paraffin.

Embedding: Paraffin.

Sections cut at 6 microns.

Solutions: Silver nitrate solution (5% aqueous).

Sodium thiosulfate solution (5% aqueous).

Nuclear fast red (1% aqueous).

We used the following instead of Nuclear fast red.

Mayers alcoholic carmine (Mallory, page 79).

Carminic acid	2 grams.
Aluminum chloride	1 gram.
Calcium chloride	8 grams.
Alcohol (ethyl) 70%	200 c.c.

Dissolve cold or with gentle heat; allow the solution to settle and then filter.

Stain for 15 to 30 minutes or longer and without washing in water, differentiate in 70% alcohol (ethyl), to which was added 2.5 per cent glacial acetic acid if a more purely nuclear stain was desired.

Staining in bulk requires 24 to 48 hours.

Technic:- as follows:

1. Toluol (or xylol) 2 changes.
2. Absolute alcohol (ethyl) 2 changes.
3. 95% alcohol (ethyl) 2 changes.
4. Distilled water 2 or 3 changes.
5. Silver nitrate solution (filter before using the 5% silver nitrate solution) 30 to 60 minutes. We stained our slides from 2 to 3 hours. Exposed to direct sunlight (and an electric lamp on the staining dishes).

6. Rinse in distilled water.
7. Sodium thiosulfate 2 to 3 minutes.
8. Wash well in distilled water.
9. Counterstain with Mayers alcoholic carmine for half an hour.
10. Differemtiate in 70% alcohol (ethyl) to which is added 2.5% glacial acetic acid.
11. 95% alcohol 2 changes.
12. Absolute alcohol (ethyl) 2 changes.
13. Toluol or xylol 2 changes.
14. Mount from tolnol or xylol with permount.

Results:- Calcium salts appear black.

The degree of calcium deposits was designated as +, ++, +++, ++++ depending upon the size and number of areas in the particular slide. The results were charted accordingly.

Biochemical Evaluation:

Portions of the removed kidney tissues were subjected to biochemical analysis to estimate the amount of calcium. This estimation had to be limited to a fewer number of specimens because of the limitations of our laboratory. The technique followed was that of Larson and Greenburg (1938). The amount of calcium was expressed in mg. per 100 gms.

of fresh kidney tissue. Results were charted and compared with those of histologic evaluation.

Following the biopsy the surviving animals of both the control and acetazolamide-treated groups were put on the standard Purina meal diet. All animals were sacrificed after a six week period. The kidneys were removed and subjected to the same scrutiny to check on the stability of the calcium deposits.

Complete autopsies were preformed on all those rats which died during or after the biopsy or during the course of the experiment otherwise. Attempts were thus made to determine the cause of death in each case.

MATERIALS AND METHODS OF EXPERIMENTAL DISSOLUTION OF RENAL CALCINOSIS:

Animals:

After being satisfied with the quality and type of calcification produced in the previous group of animals, a fresh and larger set of the same strain, age and weight was taken. Renal calcinosis was induced similarly and its presence verified and evaluated both by histologic slides and biochemical analysis. These animals were then divided into four groups. One was a control group and the other three received courses of different drugs. Details of dosage, mode of administration with pharmacological actions are given in the subsequent paragraphs.

Ethylene-Di-Amine Tetra acetic Acid (EDTA):

This is a synthetic amino acid originally produced by a German firm, I.G. Farber in 1937, under the name 'calsol' for use in dye industry. (Gehres 1951). In 1938 Enders prepared and investigated the properties of two new compounds, Trilon A and Trilon B. The latter was the sodium salt of EDTA. This is the compound which has long been used in the United States under the trade name Versene. Various complexes of this were prepared by Pfieffer et al in

1942 and by Britzinger et al in 1942 and 1943. The properties of this compound were studied by Schwarzenbach et al in 1947 and 1948. Versene was a trade name used by the Bersworth Chemical Co., Framingham, Mass., it is the tetra-sodium salt of EDTA. The various related compounds are sold under a number of trade names viz. Calgon, Sequestrene, Complexen III and more recently, Ederate of the Abbott Laboraties.

The dissolution of the concrements depends upon the complexing, sequestering and chelating properties of these compounds. The difference between these properties is essentially a matter of chemical structure of the compound. A complex is any material which will inactivate metallic ions eg. ammonium cyanide, citrate and tartrate etc. All chelating and sequestering agents are also complexing agents, but the reverse may or may not be true. Sequestration is the ability to form soluble non-ionic complexes which are undissociated and do not possess hetrocyclic ring structure. Thus a sequestering agent is a compound which will inactivate a metallic ion and at the same time keep it in solution eg. ammonia, cyanide, versene, tri-glycine, hexa-

meta-phosphate etc.

In Webster's chemical dictionary chelation is defined as the chemical process by which a group or compound possessing two valencies i.e. principal or residual or both, attaches itself to a central metallic atom, so as to form a hetro-cyclic ring structure within the molecule. A chelating agent is thus a compound which has the ability to form soluble non-ionic compounds with polyvalent metallic ions, the properties of which are completely changed in solution. Chelated metallic ion can no longer be precipitated from solution by the usual means. Commonly used chelating agents are tri-glycine, versene, di-methyl glyoxene etc.

Thus EDTA is a compound with both complexing and chelating properties. Complexes of EDTA are formed with the rare earths and Ba, Ca, Cd, Co, Cr, Cu, Fe, Li, Mg, Mn, Ni, Pb, Sr, Pi, U, Zn, and Zr. In these chelates the strength of binding varies with the cation. Lead, mercury, ferric iron and copper are very strongly bound. Zinc, ferrous iron and cobalt are more weakly bound. Calcium and magnesium are held still more weakly and sodium, potassium and lithium very weakly bound.

Absorption and Excretion:

Foreman and Trujillo (1954) studied metabolism of C-14 labelled EDTA in humans following intravenous, intramuscular and oral administration, and skin application. They found that almost all of parentrally given drug could be accounted for in the urine within 24 hours. These investigators concluded that their studies indicated that calcium EDTA passes through the body unchanged and is chiefly excreted via the kidneys. Following injection the drug mixes with all the body water but does not go into the red cells and passes slowly into spinal fluid compartment. The drug is poorly absorbed from the bowel (maximum 5%) and practically not at all through the skin.

Clarke and Clarke (1955) pointed out that EDTA is not metabolized in the body and is excreted entirely in the urine. In the rat it passed through the body with a turnover time of approximately 50 minutes with less than 0.1% oxidized and expired as CO_2. Only the skin retained any concentration after 6 hours and no more than 0.5% of the dose.

Uses:

EDTA and its salts have been widely used in

the rubber, dye and textile industries, in the separation of metals, paints and varnishes, in detergent mixtures and for the clarification of soaps, and removal of traces of metals. EDTA has been widely employed in the general field of water softening and the reserve alkalinity of tetra-sodium versenate is very useful in producing solutions with controlled composition and pH. In the past few years there has been increasing use (Cooper 1955) of the drug in heavy metal poisoning eg. lead, mercury or radio-active fission products of yttrium, zirconium, radium, plutonium etc. Other chemical uses are in hypercalcemia and as an anticoagulant for it takes up the ionised calcium of the blood. It has also been used in various strength to dissolve urinary calculi, but being irritant to the mucosa of the urinary tract it has been discontinued. This property of taking up calcium suggested a new field of usefulness in the treatment of such conditions as arteriosclerosis, metastatic calcification of various types, arthritis, coronary artery disease, calcification of heart valves and scleroderma. Its use in treating these and similar conditions remains, however on an experimental basis.

The tetra sodium salt of EDTA is distinctly alkaline while the di-sodium salt is acid. Solubility of the salts increases with alkalinity. The di-sodium calcium salt is neutral. When the tetra-sodium and di-sodium salts are employed for taking up calcium the following complexes are produced.

If tetra-salt is used:-

$Ca^{++} + Na_4$ EDTA $= Na_2$(Ca EDTA) + 2 Na

If di-salt is used:-

Ca + Na_2H_2 EDTA $= Na_2$(Ca EDTA) + 2H

Toxicity:

Evidently the toxicity of the drug would increase with fall in pH i.e. di-sodium salt is more toxic than tetra-sodium salt. But it is no more toxic than any other alkaline solution of equal pH. The following observations are recorded (Abehouse and Weinburg 1951).

1. Bersworth Chemical Co. - There were no ill effects in the personnel for over 15 years of mass production. The pH range of their products was pH3 to pH13.

2. Compounds have been used both in industry and research without complaints.

3. Patch test with pH7 to pH8.5 at 0.5% concentration shows no irritating effects.

4. Intradermal 2% tetra sodium salt of EDTA is no more irritating than normal saline control.

5. Oral feeding: no ill effects were observed from 2 to 5% solution given to rats in place of drinking water, for a period of one year. (Rubin).

6. Dykernoff used the drug parenterally up to 80 mgm./kgm. body weight in rabbits without any untoward reaction. Doses of 125 mg. and over per kgms. body weight caused tetany. It is the rapidity of the intravenous or intra-peritoneal injection that accounts for the development of tetanic spasm in some cases.

As the drug is coming into wider use nephrotoxic hazards of uncontrolled therapy have been described by Foreman et al (1956), Dudley and Riches (1955). Clarke and Clarke have noted local burning sensation at the site of injection, most marked with potassium salts. Mild nausea, diarrhoea, occasional crampy abdominal pain, toxic reactions on the skin and mucous membranes have also been described. But it was seldom necessary to interrupt the therapy.

Dosage and Mode of Administration:

Dose and course have been standardized during the last few years. Foreman et al (1956) thus recommends 70 mg./kg. body weight given daily for 5 days followed by rest for 2 days. This is carried on for 3 weeks after which an interval of one full week is given without any injection. This forms one course and may be repeated as necessary. Drug is usually given intravenously well diluted either in 5% glucose in water or in saline. The solution is otherwise very irritating.

The solution of EDTA used in the present work was prepared as follows:- 50 gms. of di-sodium (or tetra-sodium) salt (Abbott) was weighed into a 500 ml. beaker and approximately 300 ml. of distilled water were added. This mixture was stirred and heated gently until the salt was dissolved completely. 20% solution of sodium hydroxide (or N/10 hydrochloric acid for tetra-sodium salt solution) was used as necessary to adjust the pH to 7.25 to 7.35. This solution was then made up to 500 ml. by addition of distilled water. It was filtered and bottled in rubber stoppered injection vials of 20 ml. capacity. These vials were then autoclaved and stored. All the

water used in this process was distilled, sterile and pyrogen free. All the glassware was cleaned by conventional methods and finally given 3 rinses with sterile, pyrogen free distilled water. A more dilute solution (0.1%) was made at the time of injection, using normal saline as diluent.

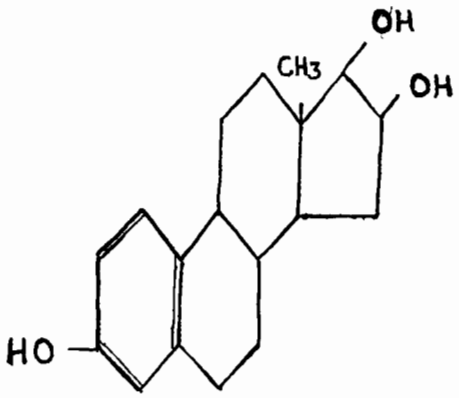
Standard courses as outlined by Foreman et al and described earlier in this chapter were administered. The optimum human dose was given namely 7 mgm./100 gms. body weight. The same dosage was maintained with both di-sodium and tetra-sodium salts of EDTA. We gave three such courses to begin with, during which time the rats were kept on the standard Purina meal diet.

After this therapy the left kidney of each of these animals and that of the controls was removed. A period of about two weeks was allowed for post-operative recovery. All the animals were then given a more intensive course over a period of five weeks with a daily dosage of 10 mgm./100 gms. body weight. Also during this period of five weeks all the rats receiving the two salts of EDTA along with the controls were put on a diet free of calcium (obtained from Rolston Purina Co. Ltd. of Canada). At the end of

this time right nephrectomies were performed and the animals sacrificed. Histological sections were prepared and stained as described on page 69. Biochemical analysis in these groups of animals were done on only a few samples. The technique adopted was different than the one we used previously in the induction experiment. A detailed description will be found in an article published in 1952 by Dahl and Dole who modified the Sobels (1939) technique.

Oestriol:

This is one of the three natural oestrogens which are substituted derivatives of oestratriene, derived from oestrane. It has the same basic four ring structure of the phenanthrine nucleus of steroid hormones. It is a tri-hydroxyl compound with the three OH groups in the 3, 15 and 17 positions.



Oestriol along with the other two natural oestrogens, viz. oestrone and oestradiol, is found in the ovary, placenta, adrenal cortex, testis and in the urine of normal male and female adults. There is a large excretion in the urine of pregnant women.

Apart from many other actions of oestrogens in the mammalian female (in relation to oestrus, menstrual cycle, breast, uterus and its adnexa, parturition, pregnancy, anterior pituitary and other hormones), it has long been known that they increase solubility of calcium salts in urine probably by increasing citrate output. Also the output of calcium itself is increased when there are excessive amounts of oestrogens in circulation. This phenomenon was studied very extensively by Shorr et al (1942).

Without going into further details of pharmacology and therapeutics of oestriol we will now outline the mode of administration and the type of courses that were given to one of the groups of animals with experimentally induced renal calcinosis.

Course No.1:-

Three weeks daily injections of 1.3 micrograms of oestriol were given intraperitoneally. Animals

were kept on standard Purina meal diet. This course was followed by removal of the upper pole of the left kidney.

Course No.2:-

Same as course no.1 followed by removal of the remaining portion of the left kidney.

Course No.3:-

Five weeks daily injections of 1.5 micrograms given intraperitoneally. During this time the animals were fed a calcium free diet. Following this period they were sacrificed and the right kidney removed.

METHODS OF EVALUATION OF THE EFFECTS OF THE DRUGS USED FOR DISSOLUTION OF RENAL CALCINOSIS:

The same technique as outlined previously was used i.e. histological grading and biochemical estimation. All the tissues removed during the course of drug therapy were sectioned and stained with haematoxylin and eosin, as well as by VonKossa's special technique for calcium. Only a few representative tissues were chosen for biochemical analysis. Observations and results are tabulated in the following pages.

The animals which were lost during the course of these experiments were autopsied and an attempt was made to determine the cause of death in each case.

These studies of dissolution were made on a fresh colony of 50 rats after inducing renal calcinosis as described earlier. The initial biopsy after three weeks treatment with acetazolamide revealed calcium deposits in all animals. Two of the rats were lost during or soon after biopsy. The remaining 48 animals were divided into the following four groups.

Group 1:-

There were 30 animals in this group and each of them received the previously outlined courses of

di-sodium EDTA.

Group 2:-

This consisted of ten animals, all of which received similar courses of tetra-sodium EDTA.

Group 3:-

This contained only 4 animals which received courses of oestriol.

Group 4:-

The four animals of this group served as controls. They did not receive any drug whatsoever, although they were fed on the same diets as the rest of the drug treated animals i.e. the Purina meal diet during the first three courses and the calcium free diet during the five weeks period of intensive drug therapy.

CHAPTER VI.

OBSERVATIONS AND RESULTS OF EXPERIMENTAL PRODUCTION OF RENAL CALCINOSIS:

All the animals were weighed at the beginning of the experiment and there after every Monday morning. It was noted that the young growing rats did not gain weight normally during the time they were fed the special diet containing diamox. As soon as they were started on the standard Purina meal diet the gain in weight was rapid. In general all the animals remained active and in satisfactory health. Only two of them had pneumonia which cleared by itself.

Out of the total of 25 rats three or 12% developed middle ear disease. This was recognised by the gait of the animal which moved about with head turned to the opposite side. Another test (Farris 1949) to confirm this diagnosis is to let the rat hang freely in the air from its tail. The one with middle ear disease revolves violently in an effort to balance itself, the balance being lost due to the involvement of the labyrinth. These animals have been known as "twisters". A normal rat remains quite steady and does not swing in either direction. These rats were not given any therapy for middle ear involvement and they carried on normally otherwise.

Intraperitoneal nembutal used in the standard dose seemed to be a safe and potent anaesthetic. It is prepared by Abbott Laboratories and contains pentobarbitol gr.1 per c.c. with alcohol 10% and propyl glycol 20%. Induction was relatively quiet and the course of anaesthesia quite smooth. Occasionally the rats developed partial obstruction of their larynx and trachea by excessive secretions leading to coarse and noisy breathing. These animals can easily be lost if the respiratory passage is not cleared. We used a small bulb syringe attached to its nozzle was a 2 to 2½" piece of rubber tubing of the size of a 10 F. catheter. The distal end of this rubber tubing was bevelled. This slid into the larynx very easily when the mouth was opened and the tongue displaced to one side. This simple maneuver saves the animal's life and operative procedure can be continued smoothly.

We lost 8 animals, or 32% of a total of 25 rats, during biopsy or soon after it. These may be accounted as operative or post-operative deaths. There seem to be several reasons for such a high mortality. The first was my inexperience in handling the animals. Another possible explanation was the severity of cold

weather. It was indeed bitterly cold when these biopsies were carried out. Over dose of the anaesthetic agent might have been an important factor. Ineffective suction and inadequate clearing of the trachea was responsible in a few of the early cases. In one of the animals shock and haemorrhage were responsible for death. Haemorrhage from the site of biopsy continued until the animal bled to death into its own peritoneal cavity. Another explanation may be that those animals whose kidneys were heavily laden with calcium were poor surgical risks, for all the three animals with histological grade 4 calcification did not even wake up from anaesthesia.

No asepsis was observed during operation but an attempt was made to maintain cleanliness. It is amazing how these rats escaped all septic complications. We did not have any post-operative peritonitis. Only 4 rats had local stitch abscesses which healed promptly after drainage. We did not use any antibiotics pre or post-operatively.

Pathologic Study:-

Gross:-

There was no appreciable change in the size of the kidneys. The surface was uniformly smooth and

the contour regular. The capsule stripped with ease. Only rarely i.e. in three of the 20 acetazolamide treated animals, did we encounter some gritty sensation while splitting the kidneys into two halves by a razor blade. Occasionally the cortico-medullary zone looked somewhat prominent and had a pale grey appearance. The calyces and renal pelvis showed no abnormality.

Microscopic:-

The calcium deposits are stained black by the VonKossa's staining technique and appear somewhat dark blue with the routine eosin haematoxylin stain. The calcification was seen chiefly at the cortico-medullary zone (Figure 2). The precipitated material was intratubular although the tubules were disrupted at many places. There was no histological evidence of renal tubular cell injury except at the site of calcium precipitation where the tubules were widely dilated and often ruptured. No sign of any inflammatory reaction was observed in the interstitial tissues even in the routinely stained sections. The calcium precipitates were found sharply localised at the cortico-medullary junction with some scattered foci in the inner medullary zone. In two cases we noted the

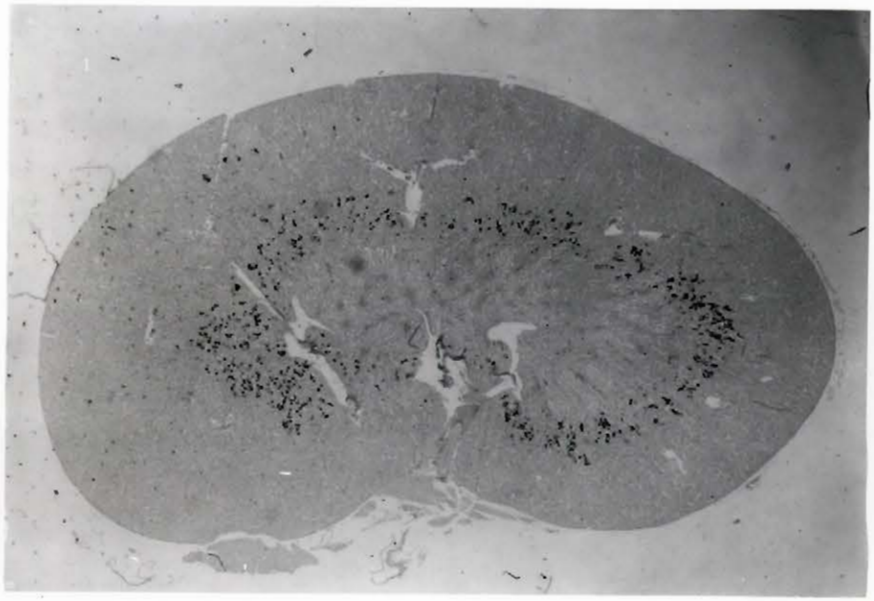


Fig. 2. Zonal Distribution of Experimentally Induced Renal Calcinosis.

concrements lying at the renal papilla and in one of these it had actually eroded into the renal pelvis (Figure 3 and 4) confirming with Randall's theory of renal calculus formation. In this last instance picture was striking in that there were no calcium deposits in the cortico-medullary zone.

The proximal and distal convoluted tubules in the cortex were free of calcium as were most of the collecting tubules in the renal pyramids. The histological pattern suggests that most of the precipitation of calcium is in the terminal or straight segment of the proximal convoluted tubule. There may also be some precipitate in Henle's loop and rarely in the collecting tubules. In none of the animals did we find any calcium deposits in the cortical zone and the glomeruli were uniformly free.

There were no remarkable changes in any other organs studied at autopsy of those animals which were lost during or after the biopsy.

Finally when all the animals were sacrificed at the end of six weeks there was no alteration in the kidney at necropsy and no gross abnormalities were noted in other organs. Figures 5, 6 and 7 are from rat no. 3. Figures 5 and 6 are of the same section

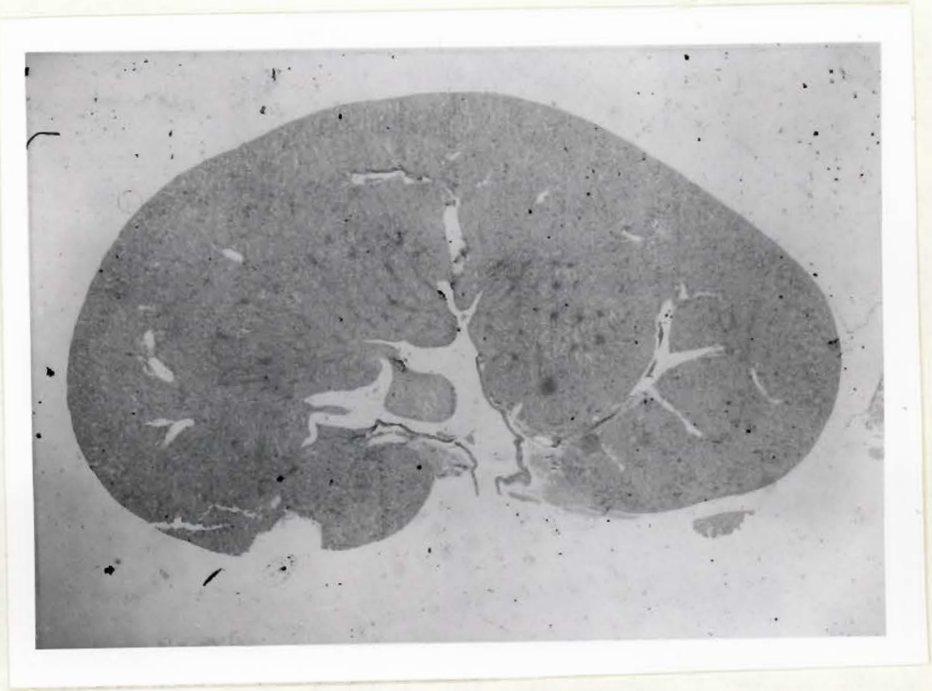


Fig. 3. Section of the whole kidney. Animal no. 19.

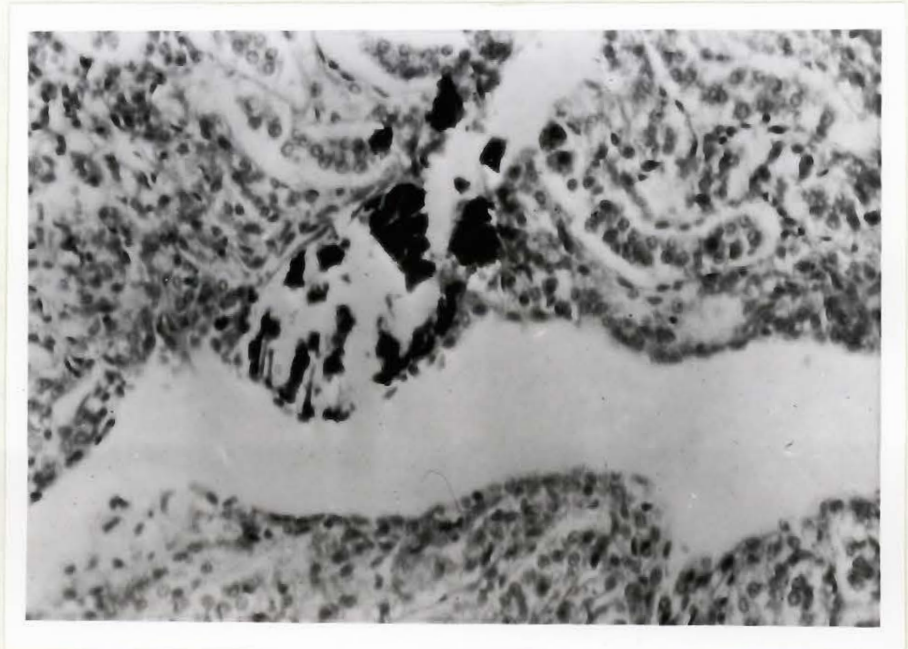


Fig. 4. Low Power photomicrograph of the same section as in fig. 3. Showing deposition of calcium on the tip of a papilla.

under low and high power. This was from biopsy and figure 7 is a section from the autopsy kidney of this animal showing no change in the calcium deposits.

There were 25 animals in all and 5 of these were kept as controls, that is no diamox was added to their diet. The kidneys of these 5 control animals were uniformly free of calcification at the time of biopsy as well as later when they were sacrificed.

The remaining 20 animals in this group were acetazolamide treated. At the time of biopsy the histological grade of calcification was classified as follows:-

Histologic Grade of Calcium Deposits.	No. of Animals.	Percentage of Total.
Negative	2	10%
±	2	10%
+	3	15%
++	5	25%
++ +	5	25%
++++	3	15%

We lost 7 rats out of the acetazolamide treated

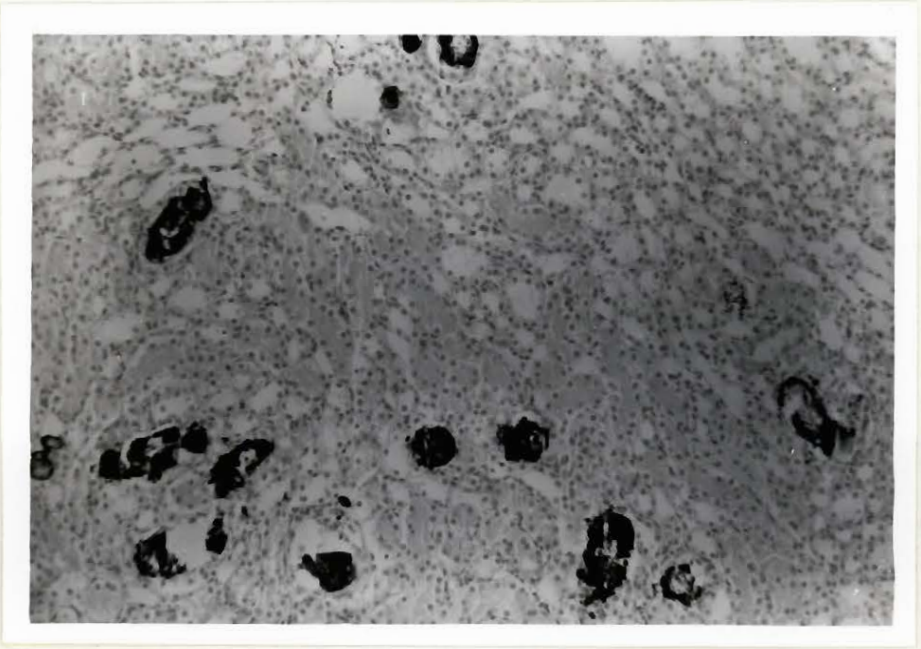


Fig. 5. Low power section. Animal no. 3. (Biopsy)

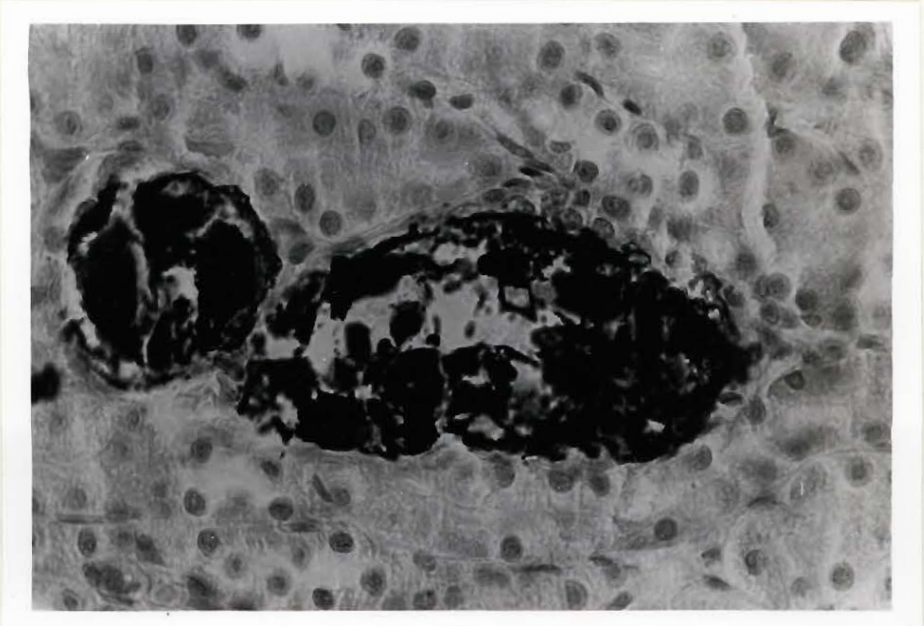


Fig. 6. Same section as in fig. 5, under high power.

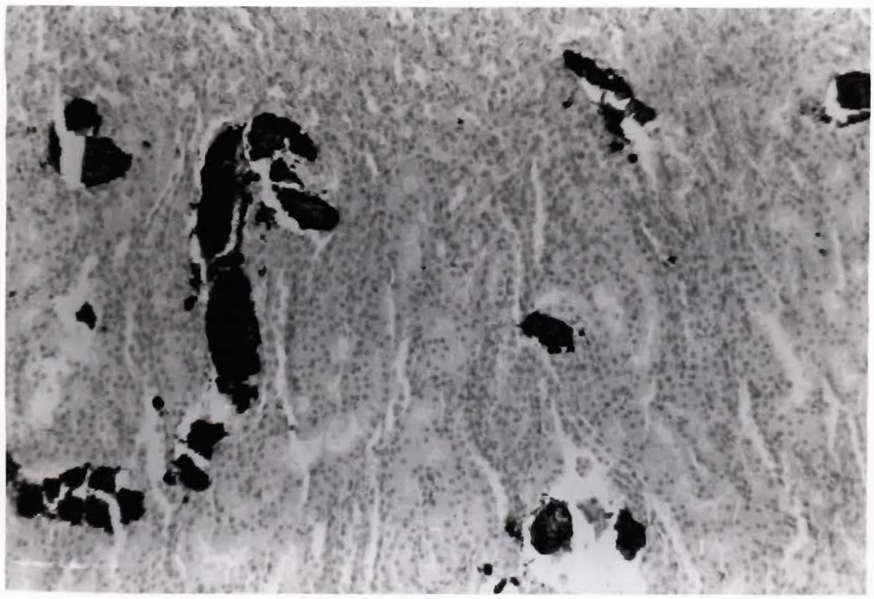


Fig. 7. Final slide of animal no. 3. (L.P. section.)

group during or soon after biopsy. The remaining 13 animals showed the following histological results after a period of 6 weeks:-

Grade of Calcification.	No. of Animals.	Percentage of the Survivals.
Negative	0	0.0%
±	1	7.7%
+	3	23.0%
++	6	46.2%
+++	3	23.0%

Analysis of the results shown in the tables (Figures 8 and 9) reveals that this method was uniformly successful in producing renal calcinosis except for rat no.1, which was negative at biopsy and remained doubtful after six weeks, when both the kidneys were examined. Animal no.9 was negative at biopsy, and proved to be two plus finally. This signifies the inadequacy of the biopsy tissue. Similarly no.7 was doubtful at biopsy and was found to be positive when the whole kidney tissue was subjected to histological study.

The histological survey of the final slides did

Serial No. of animal	Initial weight in gms.	3wks. After Exptl. Diet.			6wks. After ordinary Diet.			Remarks
		weight in gms.	Hist.Report of Biopsy	Amt. of calcium per 100gms D/W	weight in gms.	Hist. Report of Autopsy	Amt. of calcium per 100gms D/W	
1	110	120	-	..	240	±	..	No change
2	115	130	±	..	250	+	..	No change
3	95	110	+++	726mgm.	180	+++	281mgm.	No change
4	90	100	++	26mgm.	200	++	26mgm.	No change
5	130	150	++++	..				Died during Biopsy
6	140	170	+++	..				Died during Biopsy
7	135	150	±	860mgm.	230	++	194mgm.	Increased
8	95	110	+	..				Died during Biopsy
9	120	130	-	611mgm.	210	++	249mgm	Increased
10	110	120	++	180mgm.	190	++	188mgm.	No change
11	120	130	++	..	200	+	..	Decreased
12	120	140	+++	712mgm.	250	+++	248mgm	No change
13	130	150	+	260mgm	300	+	164mgm	No change

Fig. 8

Serial No. of animal	Initial weight in gms.	3wks. After Exptl. Diet.			6wks. After ordinary Diet.			Remarks
		weight in gms.	Hist.Report of Biopsy	Amt. of calcium per 100gms. D/W	weight in gms.	Hist. Report of Autopsy	Amt. of calcium per 100gms. D/W	
14	125	140	++	370mgm.	250	+++	277mgm.	Increased
15	100	110	+	..	170	++	..	Increased
16	130	150	+++	1790mgm.	240	++	203mgm.	Decreased
17	90	110	++++	..				Died during Biopsy
18	90	100	++++	485mgm.				Died during Biopsy
19	100	130	++	..				Died during Biopsy
20	110	120	+++	..				Died during Biopsy
21c	120	130	-	..	200	-	..	No change
22c	130	140	-	..	210	-	..	No change
23c	110	115	-	..	190	-	..	No change
24c	140	160	-	..	240	-	..	No change
25c	110	120	-	116				Died during Biopsy
N	120	160	-	56mgm.	260	-	60mgm.	No change

Fig. 9

not add to the previously recorded description of biopsy sections except that the size of the concretions appeared larger with further dilatation of the involved tubules. The tubular epithelial cells in the vicinity of calcium deposits were destroyed and in places the deposits were extending into the interstitial tissues. It may be emphasized again that in the final section there was no local reaction to the deposited calcium. No inflammatory or other histological changes were seen.

This led us to the conclusion that this method of production of experimental renal calcinosis was quite suitable being relatively easy, quick and cheap. Above all we were satisfied that these deposits were quite stable and did not disappear spontaneously when the animals were left on the standard Purina meal diet. The stage was set for our further studies on dissolution.

During this experiment biochemical estimation of calcium was done in a few selected specimens but due to inexperience and faulty technique the results were quite inconsistent. However, we have charted them. At least they show that there was an unequivocal increase in the amount of calcium in the

acetazolamide treated kidneys as compared to the untreated kidneys or otherwise normal rat renal tissue. Therefore during the studies on dissolution a different technique was adopted for determination of the amount of calcium as described by Dahl and Dale (1952). It may well be added here that normally there is very little calcium in the rat kidneys. The amount is sometimes so small as to be difficult to estimate by ordinary methods. The range of calcium content in the acetazolamide treated rats of the Harrisons (1955) was 27 to 355 mgm./100 gms. of kidney tissue.

OBSERVATIONS AND RESULTS OF ATTEMPTED DISSOLUTION OF RENAL CALCINOSIS:

Before describing the observations and results in detail some general remarks are in order. All animals were weighed once a week and it was noted that none lost weight during the drug therapy, except those animals which became victims of pneumonia or middle ear disease. These two diseases are quite common in rats. Of the 48 animals of this lot 8 (16.6%) had middle ear disease and at least 10 (20.8%) were detected to have developed signs of pneumonia. No treatment of any sort was given to these apparently sick animals nor was it considered necessary to discontinue the attempts at dissolution of renal calcinosis, on account of these complications.

There were no significant complications attributable to repeated surgery performed on this colony of rats, except for the development of a few localised stitch abscesses. There abscesses promptly responded to incision and drainage.

Only one animal was lost soon after the initial biopsy due either to overdose of anaesthesia or choking in its own secretions. Another developed pneumonia five days after the initial biopsy. It

subsequently died. This left us with a total of 48 animals out of the lot of 50 in which renal calcinosis was induced. They were then divided into various groups as already mentioned. Now I shall deal with the results of each group separately.

Group 1:-

There were 30 animals in this group. All received di-sodium EDTA. At the end of the total therapy only 23 were living. There were no deaths to the end of the second course. In general they all stood the therapy very well. The only untoward or toxic reaction to the drug was occasional diarrhoea. This would disappear by itself at the end of the course, so no particular attention was paid and injections were continued without interruption.

It may be recorded here that a freshly prepared solution of the di-sodium salt is less irritating than tetra-sodium EDTA, especially if the injection leaks into the tissues of the abdominal wall. Not infrequently it was found that by a rapid intraperitoneal injection the animal went into tetanic convulsions which lasted from a few seconds to a minute or so. This effect was certainly more marked with tetra-sodium EDTA.

Account of the Animals Lost During This Therapy:-

We lost 7 rats out of the 30 in this group. Four (nos. 42, 47, 63, 48) died of urinary tract calculi with associated complications. Three of these (nos. 63, 47, 48) had calculi in the kidney with hydronephrosis. The fourth (no. 42) had multiple small calculi in the bladder and developed acute urinary retention with bilateral hydronephrosis. Animal no. 63 developed acute infection along with hydronephrosis which probably lead to multiple papillary necrosis or to so called necrotizing papillitis, in the only remaining right kidney. The left kidney had been removed earlier after the third course of di-sodium EDTA. The other two rats, nos. 47 and 48, had small concretions obstructing the ureter. There was also a stone present in the kidney of no. 47. Resultant hydronephrosis of the only kidney led to death in uremia.

One animal i.e. no. 62 died soon after injection from penetration and laceration of a large blood vessel by the injecting needle. Acute hemorrhage with associated shock was responsible for the death of this animal. Animal no. 80 finally succumbed to chronic peritonitis. The cause of peritonitis was

an overlooked sponge in the peritoneal cavity left during initial biopsy operation. It is interesting that it lived for almost 18 weeks, in satisfactory health.

No definite cause of death could be determined in the case of rat no. 41. Probably it died of heat and prostration in the very unfavourable environment of a rather long continued spell of moist heat. Signs of early pneumonia were detected microscopically in the lungs of this animal.

All these animals which died provided excellent opportunity for studying the rest of the viscera, apart from the kidneys, during the course of the experiments. No calcification other than that in the kidneys could be detected in the remainder of the organs, even the lungs and stomach (which are the other two most common sites for metastatic calcification) were free from calcium deposition. Abdominal viscera and chest organs were examined routinely in each autopsy. No remarkable changes were noted in these organs except microscopic evidence of pneumonia was present in 5 animals. Pneumonia was detected grossly in one animal only.

Finally we may add that four of these seven deaths

were due to complications of formed calculi. The remaining three died of other causes. The chemical composition of these calculi was as follows:-

When hydrchloric acid was added to ashed stone (powdered) effervescence, was noted under the microscope indicating the presence of carbonates. Phosphate test using ammonium molyledate - positive. Magnesium test using ammonium phosphate and nitric acid and potassium thiocyanate - traces. Calcium precipitation of calcium ions using ammonium oxalate - positive.

Pathology:-

Gross appearance of the removed kidneys:-

Ther was no enlargement of the kidneys at the time of biopsy or at left nephrectomy. Marked hypertrophy was noted in the right kidney at the end of the experiment. The surface was usually smooth and the capsule stripped with ease. The site of initial biopsy on the lower pole of the left kidney was found to have healed remarkably well. though occasionally parts of intestines, omentum or spleen were adherent to that site. One of the rats developed hydronephrosis with a stone blocking the uretero-pelvic junction of the left kidney. By the time of left nephrectomy a large pyonephrosis had developed in it.

A gritty sensation was frequently noted on cutting through the kidney substance. This was demonstrable in about 40% of the kidneys studied. The separating zone between the cortex and medulla was often very prominent and in at least 30% of the animals greyish white or pale grey streaks or specks could be identified by a hand lens at the cortico-medullary zone. The cut surface was otherwise smooth, shiny and normal in appearance. Signs of associated infection with stone formation were recognised in the kidneys where this complication developed.

Histologic Appearance of the Removed Kidneys:-

Histologic survey of the slides of this group as well as of the other groups was made by comparing the biopsy slides with those made after 3 courses of the drug and those prepared still later after right nephrectomy.

On the basis of the microscopic studies alone the results seem to be disappointing. The drug did not appear to have had any appreciable solvent effect as used in these experiments. Histologically the concretions in general were large and at least in 8 animals they were more numerous and perhaps heavier in the final sections than those in the biopsy

slides. The tubules were more widely dilated and the cell lining was completely disrupted in places, so that the deposits were seen to be growing into the interstitial tissues (Figures 10, 11 and 12). It was noteworthy again, that there was no reaction of any sort in the vicinty of the calcium deposits except in those kidneys where some complications developed. Marked inflammatory reaction with polymorphonuclear leucocytes and focal necrosis with abscess formation were the common features associated with those complications.

Results have been condensed in the table shown in figures 13 and 14, where an attempt has also been made to compare the histological gradings with the biochemical evaluations.

The following table gives a picture of the effect di-sodium EDTA based on histological findings alone.

Time of Evaluation.	%age in Grade +	%age in Grade ++	%age in Grade +++	%age in Grade ++++
Initial Biopsy.	33%	20%	20%	27%
After 3 Courses.	20%	27%	27%	26%
After Final Course.	11%	11%	41%	37%

This table shows that the numbers of grade 3 plus and grade 4 plus increased in spite of therapy. Five animals (17%) of this group of 30 rats developed renal calculi within a relatively short period after induction of renal calcinosis i.e. 9 to 15 weeks. Four of these five actually died of complications resulting from calculus formation.

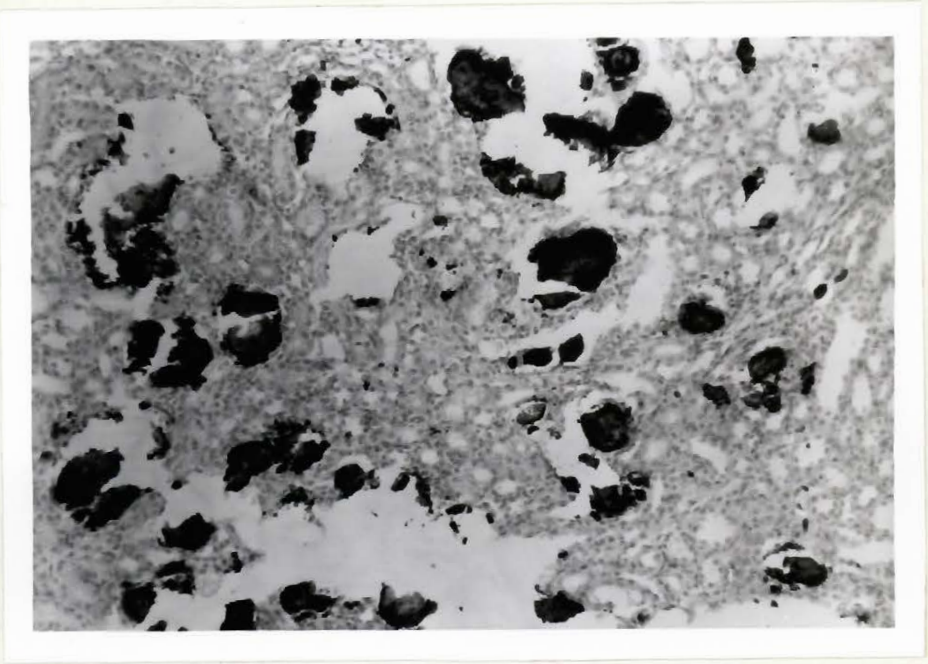


Fig. 10. Animal no. 52. Biopsy. Low power section.

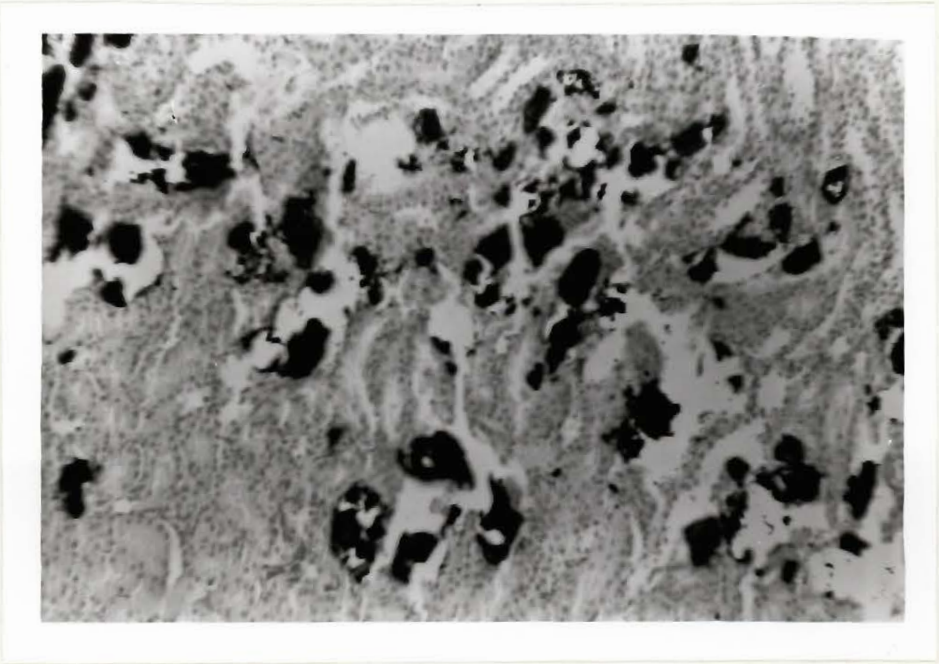


Fig. 11. Animal no. 52. After 3 courses of di-sodium EDTA. (L.P. section.)

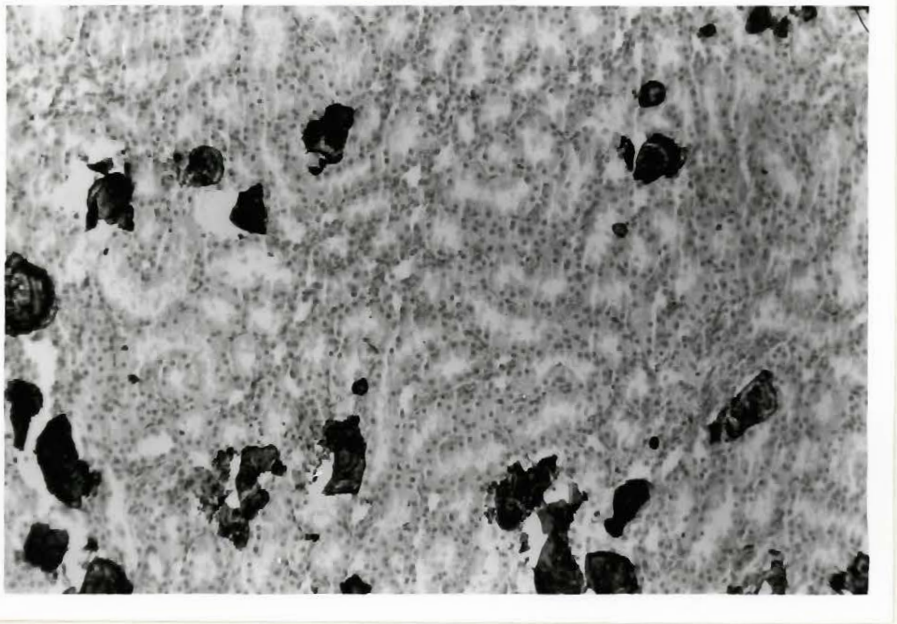


Fig. 12. Animal no. 52. After final course of di-sodium EDTA. (L.P. section.)

Chemical Analysis:-

Biochemical estimation was done in 10 cases and comparison was made between the amounts of calcium noted at biopsy with those found after three courses of di-sodium EDTA therapy , and then finally after right nephrectomy. In the tables shown in figures 13 and 14 the heading biochemistry stands for the amount of calcium in mg. per 100 gms. of fresh kidney tissue. Of the 10 serial estimations shown, there was an appreciable decrease in the amount of calcium during this therapy in 5 instances, no change in another two and a distinct increase in the amount of calcium in three rats.

In the tables the results of chemical analysis have been tabulated side by side with the histological gradings. Apparently there is a correlation between the two in some but in others no relationship can be seen.

In rat nos. 51 and 52 there was no apparent change in the histological grade whereas a progressive decrease was noted chemically. The reverse seems to be true for rat no. 70, in which there was marked increase in the amount of calcium as estimated chemically. The histological grading being the same in all three specimens.

Results of Di-Na EDTA Therapy.

Serial No. of animal	Initial weight in gms.	Biopsy after Exptl. Induction of nephrocalcinosis			Left nephrectomy after three courses of Di-Na EDTA			Right nephrectomy after the final course of Di-Na EDTA			Remarks
		Weight	Histology	Bio-chem.	Weight	Histology	Bio-chem.	Weight	Histology	Bio-chem.	
31	120	140	+++	59mg.	200	+++	355mg.	220	+++	121mg.	Improved
32	150	170	++++	268mg.	280	++++	430mg.	280	++++	168mg.	Improved
39	160	180	+++	..	270	+++	..	300	+++	..	Not improved
40	150	170	+	..	270	+	..	270	++	..	?Increased
41	160	180	+	..	270	+	..	250	++	..	?Increased
42	190	200	++++	..	260	++++					No change
45	180	210	+	73mg.	310	++	79mg.	300	+++	121mg.	Increased
46	180	220	++++	466mg.	320	++++	356mg.	325	++++	525mg.	No change
47	120	130	+	..	250	++			++++		Increased
48	160	180	++++	..	280	++++			++++		No change
49	170	190	+++	..	300	+++	..	300	++++		?Increased
50	170	200	+	..	340	+	..	340	+		No change
51	160	190	++++	357mg.	270	++++	253mg.	300	+++	201mg.	Improved
52	165	190	++++	425mg.	325	++++	251mg.	280	++++	247mg.	Improved
53	170	200	+	..	320	+++	..	325	+++	..	Increased

Fig. 13

Results of Di-Na EDTA Therapy (contd.).

Serial No. of animal	Initial weight in gms.	Biopsy after Exptl. Induction of nephrocalcinosis			Left nephrectomy after three courses of Di-Na EDTA			Right nephrectomy after the final course of Di-Na EDTA			Remarks
		Weight	Histology	Bio-chem.	Weight	Histology	Bio-chem.	Weight	Histology	Bio-chem.	
54	160	210	+++	..	340	+++	..	340	++++	..	?Increased
57	150	180	++	..	270	++	..	285	+++	..	?Increased
58	150	180	++	..	280	++	..	270	+++	..	?Increased
61	150	190	++	..	290	++	..	290	++	..	No change
62	140	170	+	..	255	+	..	...	.	..	No change
63	160	190	+++	..	320	+++	..	...	.	..	No change
64	140	170	+	..	280	+	..	290	+++	..	Increased
67	160	200	++++	..	300	++++	..	300	++++	..	No change
68	130	150	++	..	250	++	..	260	+++	..	?Increased
69	175	220	+	30mg.	330	+	66mg.	360	+	60mg.	No change
70	140	160	++++	167mg.	240	++++	89mg.	250	++++	294mg.	Increased
75	160	190	+++	342mg.	280	+++	345mg.	290	+++	240mg.	Improved
76	150	190	+	61mg.	320	+	48mg.	340	+++	362mg.	Increased
79	150	170	+	..	270	+	..	290	+	..	No change
80	140	175	++	..	270	++	..	270	++	..	No change

Fig. 14

Group 2:-

There were 10 animals in this group which received injections of tetra-sodium EDTA. At the end of the last course 7 were living and the remaining three died during the course of therapy. In general we found that this salt of EDTA was more toxic and most of the rats developed diarrhoea quite frequently. The injections were also irritating so that signs of disapproval were noticed as painful convulsions, moaning, groaning etc. A rapid injection caused tetanic convulsions more often than the di-sodium EDTA. There were no other toxic reactions such as skin changes, loss of appetite, lassitude and loss of weight. The animals maintained their health satisfactorily. No treatment was given for diarrhoea and nor was it considered necessary to discontinue the drug at any time.

Account of the Animals Lost in Group 2:-

There were three deaths. Animal no. 43 died close to the end of the second course and the other two (rat nos. 44 and 66) were lost at the end of the third course. Animals 43 and 44 were victims of the complications of formed calculi. No. 43 developed acute urinary retention with marked bilateral hydro-

nephrosis and multiple calculi in the bladder measuring 3 to 4 mm. in diameter. The calculi were pale, hard and of various shapes. (The bladder along with calculi is shown in figure 15). The surface was smooth, or finely granular. The composition of these calculi was the same as noted earlier in the records of Group 1. Animal no. 44 had calculus dust blocking most of the length of the right ureter, with hydrnephrosis in that kidney. It also had multi-lobular pneumonia, which was really responsible for its death. Hydronephrosis was a contributory factor.

Animal no. 66 was found to be losing weight and became rather inactive during the later part of the third course of therapy. It practically stopped eatting and appeared sick. There were signs of pneumonia noticeable clinically. It died in 4 days, when fluid and antibiotic therapy was instituted. At autopsy no cause could be determined on gross examination. The only change found was in the kidneys, in which 3 to 4 small, irregular, purplish, pulpy and soft areas were present. These areas were somewhat depressed and could be easily spotted. No other changes were found in the rest of the viscera except generalised

Fig. 15. Bladder full of stones. Animal no. 43.

marked congestion was present in the lungs.

Microscopic study revealed early pneumonia infilteration in both lungs. The areas spotted in the kidneys during gross examination showed hydropic changes in the cortical tissues. The cells lining the glomeruli and those in the proximal convoluted tubules were swollen. The nuclei were displaced to one side and in places the cell membrane was broken. These areas did not take the staining properly and appeared rather bizarre and somewhat disorganised. No inflammatory reaction was detected in the vicinty of these changes. The scattered deposits of calcium were still intact and there were no other remarkable changes in the kidneys. Liver, spleen and other organs were not remarkable.

In conclusion there were two deaths (20%) related to urinary calculi and associated complications. The third (10%) was probably due to the toxicity of the drug. Hydropic changes in the kidney have been described as nephrotoxic hazards of EDTA by Foremen et al (1956).

Histologic Evaluation of the Effect of Tetra-Sodium EDTA:-

Histologic survey of the slides of the remaining seven animals revealed practically the same findings as recorded in Group 2. Results of animal no.65 are shown in figure nos. 16, 17 and 18. The details of the results have been tabulated in figure 19. The following table shows the percentages of histologic grades of calcium noted during the course of therapy.

Time of Evaluation.	%age of Grade +	%age of Grade ++	%age of Grade +++	%age of Grade ++++
Initial Biopsy.	50%	20%	20%	10%
After 3 Courses.	30%	40%	20%	10%
Final Course.	29%	29%	29%	14%

This table reveals that there was no dissolutory

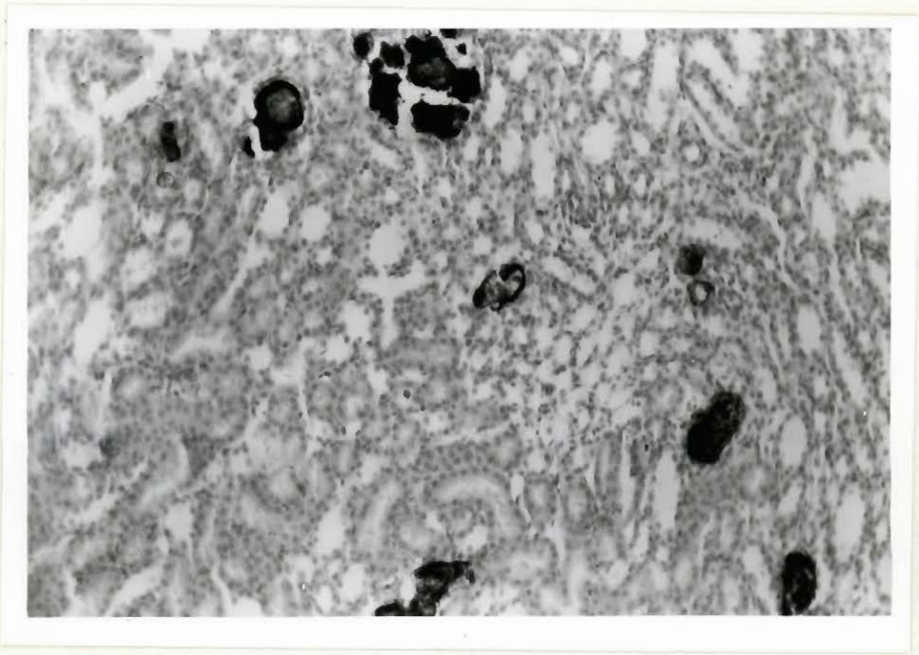


Fig. 16. Animal no. 65. Biopsy. Low power section.

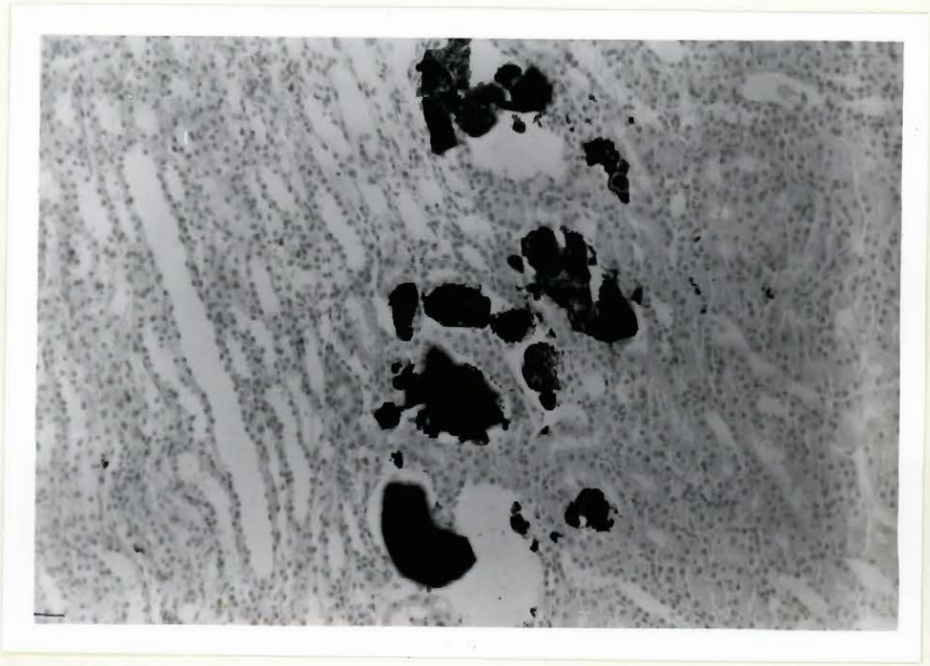


Fig. 17. Animal no. 65. After 3 courses of tetra-sodium EDTA. (L.P. section.)

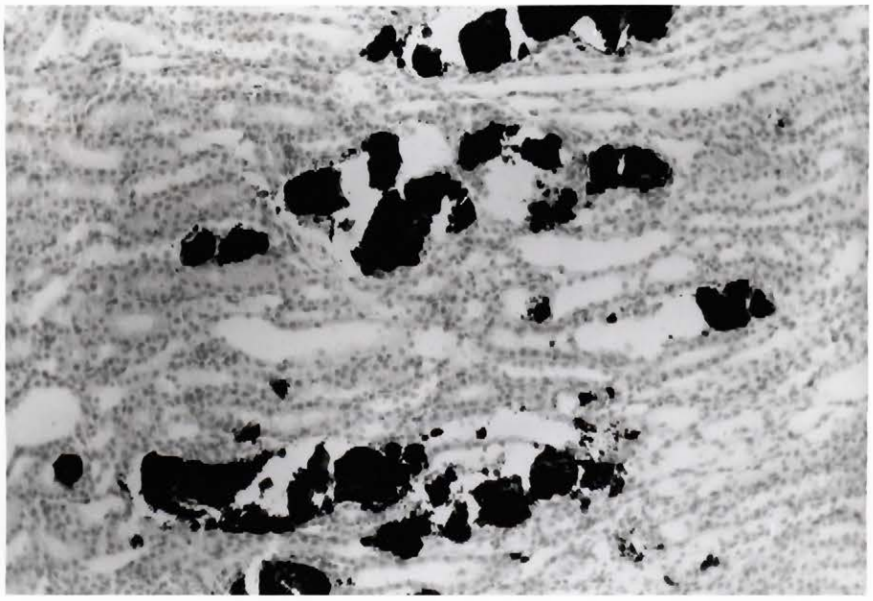


Fig. 18. Animal no. 65. After final course of tetra-sodium EDTA. (L.P. section.)

Results of Tetra-Na EDTA Therapy as Compared to The Control Group of Animals.

Serial No. of animal	Initial weight in gms.	Biopsy after Exptl. Induction of nephrocalcinosis			Left nephrectomy after three courses of Tetra-Na EDTA			Right nephrectomy after the final courseof Tetra-Na EDTA			Remarks
		Weight	Histology	Bio-chem.	Weight	Histology	Bio-chem.	Weight	Histology	Bio-chem.	
33	160	190	+	..	270	+	..	300	+	..	No change
34	130	150	+	..	260	+	..	280	+	..	No change
35	150	170	++++	277mg.	270	++++	292mg.	285	++++	..	No change
36	150	170	++	51mg.	250	++	44mg.	280	++	87mg.	No change
43	140	170	+	..	235	++	..				No change
44	175	210	+++	..	275	+++	..				No change
55	140	170	++	48mg.	310	++	87mg.	325	++	64mg.	No change
56	160	195	+	30mg.	260	++	64mg.	265	+++	101mg.	Increased
65	180	230	+++	126mg.	320	+++	109mg.	350	++++	136mg.	Increased
66	150	180	+	..	230	+					No change
71c	190	210	+++	..	280	+++	..				No change
72c	180	220	+++	117mg.	300	+++	90mg.				No change
73c	170	210	++	374mg.	310	++	295mg.	310	+++	374mg.	No change
74c	160	190	++++	..	280	++++	..	245	++++	..	No change

Fig. 19

effect of this drug based on histologic findings alone. Although we can infer that there was no definite increase in the calcium deposits as it happened in Group 1.

Chemical Analysis:-

The results of chemical estimation of the amount of calcium in kidney tissues seem to be even more inconsistent. Analysis was done in 5 animals. Only one of these showed a definite decrease in the amount of calcium, whereas no change occurred in two of these. In the remaining two we found some increase in the amount of calcium inspite of the drug therapy. Again no correlation can be established between the two methods of evaluation viz.; histologic and chemical. The results have been tabulated side by side in figure 19.

Group 3:-

In this group we had only four animals, therefore no significant results can be drawn. One of these animals died after two courses of oestriol. No definite cause of its death could be determined. This animal was also lost during the heat wave. Autopsy findings were not contributory.

In general we observed that there were no calculi formed in these animals, although histologically no dissolutory effect was exerted by oestriol therapy. Another significant effect of oestrogen therapy was that these animals were somewhat slow to recover, and heal after operative procedures. Wound disruption and wound infection were more common. No toxic manifestations attributable to oestrogen therapy were noted. There was no tendency for the deposits to increase in these animals. The number was too small for the results to be significant. No chemical analysis was done in this group.

Group 4:-

This consisted of four animals in which renal calcinosis had been induced similarly as in the other groups. They served as controls and were studied at similar stages of the experiment along with the other animals under therapy. The control animals maintained satisfactory health throughout. One of them was lost after three months of induction due to pneumonia and another animal died during the heat wave. Autopsy revealed no remarkable changes in these animals.

Histologically the calcium deposits did not show any tendency to regression, instead appeared to have increased in size and number (figure nos. 20, 21 and 22). The tubules containing these deposits were more widely dilated and their disruption was noted similarly as in other groups. The number of deposits had significantly increased in two of these rats. Chemical analysis in two of these animals again failed to correlate the histologic appearances and chemical estimation of the amount of calcium in renal tissue. The results have been tabulated along with those of tetra-sodium EDTA in figure 19.

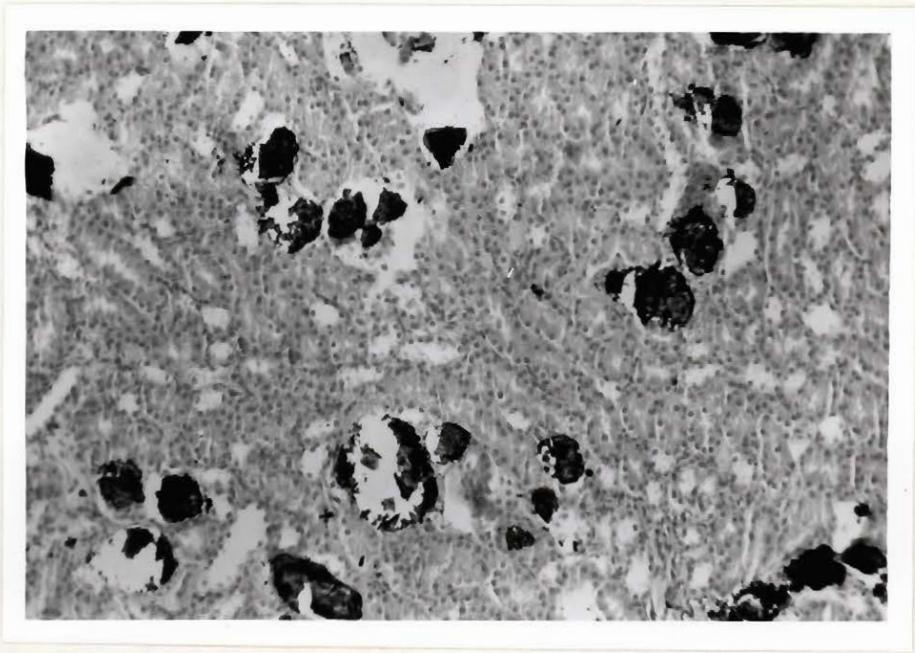


Fig. 20. Animal no. 72. (control). Biopsy. Low power section.

Fig. 21. Animal no. 72 (control). Section taken after 3 courses of therapy to other animals. (L.P.)

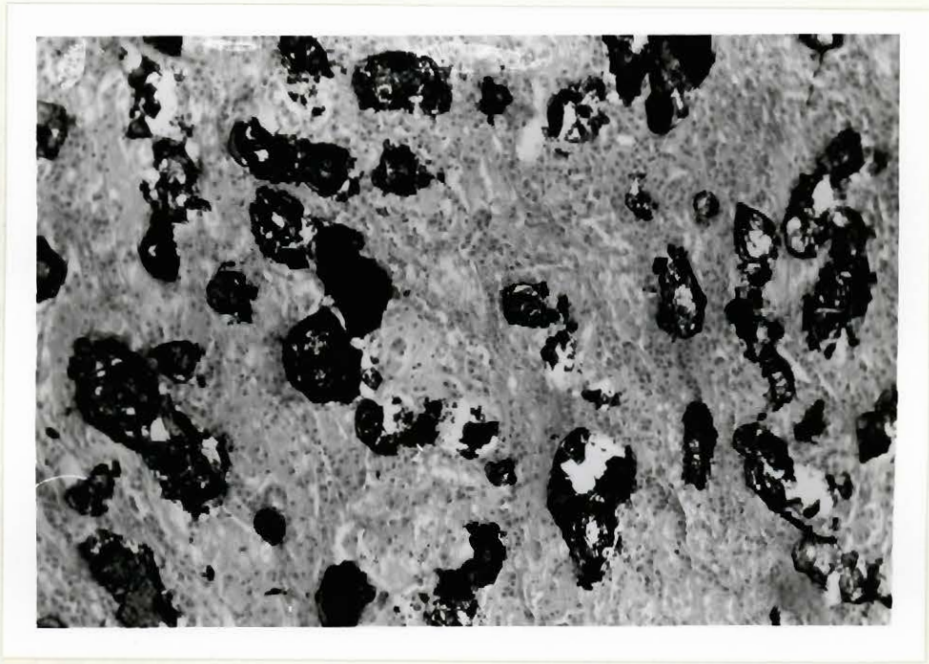


Fig. 22. Animal no. 72 (control). Section taken at the end of the experiment. (L.P.)

CHAPTER VII.

DISCUSSION:

The search for an objective proof for any scientific assumption or an experience is certainly the basis for most experimental work. The success or failure of any experiment directly depends on the type and number of odds encountered in it. Elimination of such odds in the initial planning and during the course of the experiment carries one a long way on the right road to success. But it is indeed the failures which teach us the very minor details of the odds in which lies the further progress and success of the experiment.

To begin with our main problem was to find a suitable method which would be uniformly successful in producing renal calcinosis. This calcinosis may be radiographically demonstrable, but the most important criterion for our purpose was that it should be relatively stable and not disappear spontaneously.

The method which we used was the same as used by the Harrisons (1955) in their study of the drug diamox (acetazolamide). After producing the renal calcinosis we put our animals on the ordinary Purina meal diet for six weeks, when we proved to our satisfaction that these calcium deposits were relative-

ly stable and did not disappear spontaneously. Only, of course, to our disappointment these deposits could not be demonstrated radiologically. So the calcinosis in this respect fell short of Bell's (1951) definition and precise criterion of radiographic demonstrability. However, the calcium deposits produced in our animals, being stable could still be used for dissolution studies, even though we had to resort to other methods of evaluation of the degree of calcification. We used histologic studies and biochemical analysis for this purpose. Clinical use of these means, to present time would be very difficult, but these methods opened the avenues for us and trials of dissolution could be made in animals. Moreover clinical data reveal that by the time nephrocalcinosis has reached the stage of X-ray demonstrability, it is already irreversible and this of course might make it unsuitable for our purpose of initiating investigations on dissolution. Therefore the histologic studies and biochemical analysis were our only choice.

Dissolution studies were carried on using two compounds of EDTA viz. the di-sodium and tetra-sodium salts. Also oestriol was given to a small

group of animals on a more empirical basis. All our attempts to dissolve the calcium deposits met with failure.

EDTA and its compounds form a group of powerful drugs in the field of sequestration and chelation. Our failure or poor results per se cannot be attributed to them. It is quite possible that the dose used was insufficient or was not given for an adequate period. The mode of administration also was not entirely satisfactory. In human cases the drug is given intravenously, in a slow drip, spread over a period of 4 to 5 hours daily. This provides some lasting period of concentration of the drug in the body tissues. A rapid intraperitoneal injection in rats is known to be excreted very quickly. Over 95% of the dose given was recovered from the urine within 50 minutes (Clarke and Clarke 1955). This allows a relatively short period for satisfactory drug action. It was impossible to give daily doses by intravenous drip to rats in numbers sufficiently great to yield statistically significant results.

It is interesting to point out that during the course of therapy with di-sodium or tetra-sodium EDTA, seven rats of the 40 (18%) developed renal calculi and died of complications associated with them. On the

other hand there were no calculi in the eight rats in the control and oestriol groups. One may be justified in asking if EDTA had anything to do with this. I am at a loss to suggest any explanation but this observation is significant in that so many animals with experimentally induced renal calcinosis developed renal calculi. Obviously the control and oestriol groups are too small to permit any conclusions.

As yet there does not seem any accurate method of evaluating the degree of renal calcification. Chemical analysis is by far the most reliable technique, but this too has many limitations. The amount of calcium in the deposits is relatively small, and in our animals it was localised to the cortico-medullary zone. It is easy to imagine that if the piece of kidney taken for analysis includes mostly the cortex, it will contain hardly any calcium, whereas if it happened to include a large part of the cortico-medullary zone the amount of calcium found would be much greater. Thus a comparison of the effects of therapy based on this can be misleading unless care is exercised in obtaining pieces of kidney tissue of the same dimensions. It should include the whole thickness of the kidney tissue and not be taken from

cortex or medulla alone. Another odd which we had to face was that all these tissues were preserved in neutral formalin to avoid the acidic action of formalin on the calcium content of the kidneys. Inspite of this precaution of initial neutralization, we found that most of the solutions turned acidic after sometime. Not infrequently calcium could be detected in the formalin itself. Therefore we feel that chemical analysis should be done immediately after the removal of the tissue. One of our rats had multiple calculi in the bladder; most of these were dissolved by formalin in about 6 weeks. To avoid this error we suggest that if analysis cannot be done immediately, the tissue should be preserved in alcohol. Apart from these limitations, the chemical estimation itself has its own sources of error. These errors are increased when only small pieces of tissue are available for analysis, particularly those obtained by biopsy.

Similarly evaluation by histologic studies is even more vague and fallacious. Grading of calcific deposits is certainly a very crude method and would differ from one person to another. Again the type of section and the site from which it is taken are

important factors which bear on the results obtained for the zone of calcium is limited. This method depends chiefly on comparison of heaviness of the color of the deposit. Staining with dyes cannot differentiate moderate or small increases and decreases in the amount of calcium. The unreliability of this method is increased by the lack of complete specificity of VonKossa's stain for calcium. Other metals can take up this stain.

We chose rats for these experiments for various reasons. First, the experiments could be made on some statistically significant scale. Second, we had at hand a satisfactory method for production of renal calcinosis. Finally we were not sure of diamox or any other method which would work so well in other animals, in which case one might expect different results.

Whatever might have been the drawbacks and the limitations of evaluation, we are bound to conclude that these experiments do not demonstrate that EDTA compounds are effective in the dissolution of renal calcinosis. However, they also do not prove them entirely inefficient.

Reviewing the whole experiment we feel that

dissolution of renal calcinosis is a very interesting research problem and with its multiple odds throws a great challenge before us. The importance of this work is realized when more and more clinicial cases are coming to light. The relationship of renal calcinosis to recurrent urolithiasis is being appreciated in the urological field. Thus there is a growing need for the solution of this problem. This may be one reason for the selection of this work by the American College of Surgeons for presentation before their Surgical Forum at the October 1957 session. Even though our experiments failed to yield conclusive results, they do seem to pave the way for further work on the dissolution of renal calcinosis. It is quite possible that conclusive results may be obtained by changing the dose, course, mode of administration and even the type of experimental animals along with other additional therapeutic means eg. calcium free diets, feeding of sodium phytate to prevent absorption of calcium from the bowel, maintaining blood pH slightly on the acid side in alkalosis and vice versa, use of cortisone or other gluco-corticoids etc.

We feel strongly about the importance of the mode of administration of EDTA compounds. Slow intravenous drip certainly seems to be the method of choice.

CHAPTER VIII.

SUMMARY:

This study consisted of production and dissolution of renal calcinosis.

A brief review of the entire subject of nephrocalcinosis has been presented.

Various criteria with respect to experimental production required for dissolution studies have been laid down.

Several possibilities of experimental production of renal calcinosis have been discussed and historical aspect of the subject has been reviewed.

Experimental induction of renal calcinosis was obtained by the drug diamox mixed with special diet. Details of this mehtod along with advantages and disadvantages have been outlined.

Results and illustrations of experimentally produced renal calcinosis have been embodied.

In this experiment calcium deposits were not reversible spontaneously.

Dissolution studies were made on a larger colony of rats. Animals were divided into 4 groups. One group received di-sodium EDTA, another received tetra-sodium EDTA and the third group was given

injections of oestriol, while the animals in the fourth group served as controls.

Pharmacology and therapeutics of the drugs used have been discussed.

Results of attempted dissolution as evaluated by histology and biochemistry have been tabulated.

There was no definite dissolutory effect in any group, still no conclusions could be drawn.

A brief discussion of the odds encountered in the experiment has been presented and some suggestions for further research have been made.

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