THE RELATIVE IMPORTANCE OF URINARY pH AND URINARY CONTENT OF CITRATE, MAGNESIUM AND CALCIUM IN THE PRODUCTION OF NEPHROCALCINOSIS BY DIET AND ACETAZOLAMIDE IN THE RAT

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SUMMARY

1. The association of varying levels of urinary pH, urinary citrate and urinary calcium and magnesium excretion rates with kidney citrate, calcium and magnesium concentrations in experimental nephrocalcinosis was examined in twenty-four rats in a 3 x 2 multifactorial experiment with four replicates. All rats received the same synthetic diet for 6 weeks before being killed in the seventh week. In addition the rats received calcium supplements as calcium chloride (diet A), calcium carbonate (diet B), or as an equal mixture of calcium chloride and carbonate (diet N), the content of calcium being kept constant at 6.3 mg per g of diet for all rats. Half of the rats also received 2.5 mg of acetazolamide per g of diet.

2. Diet A produced a systemic acidosis and the most acid urinary pH. Diet B plus acetazolamide produced a more severe systemic acidosis and the most alkaline urinary pH. Urinary magnesium, citrate and calcium excretion rates were generally reduced below normal. Urinary excretion of magnesium and calcium were significantly higher in those rats on diet A than in those on diet B, while urinary citrate excretion was highest in the latter. Acetazolamide caused a further increase in urinary calcium excretion but a decrease in urinary magnesium and citrate excretions.

3. Acetazolamide significantly reduced plasma calcium but elevated plasma magnesium. The changes produced in plasma and urinary calcium and magnesium in the present study were consistent with an action through systemic acidosis for calcium and through urinary pH for magnesium, both being effected at a tubular site.

4. Variation in diet alone as well as acetazolamide administration were significantly associated with variation in the degree of nephrocalcinosis ($P < 0.05$ and $P < 0.005$ respectively). Acetazolamide increased nephrocalcinosis by a factor of at least 10. Analysis of covariance showed that acetazolamide was no longer associated with significant nephrocalcinosis when its effects on urinary pH and magnesium were...
removed from its effect on nephrocalcinosis. Removal of the effect of acetazolamide on urinary citrate excretion did not alter the effect of acetazolamide in producing nephrocalcinosis. Although urinary citrate was reduced to below 10% of normal whenever nephrocalcinosis was severe, it was also reduced to below 10% in rats on diet A which had normal kidney tissue calcium content, the most acid urinary pH and the highest urinary magnesium.

5. Elevation of urinary pH and reduction in urinary magnesium excretion were therefore considered to be of major importance in the causation of experimental nephrocalcinosis; reduction in urinary citrate excretion appeared to be only of secondary importance.

Medullary nephrocalcinosis in renal tubular acidosis has generally been attributed to the constantly elevated urinary pH in the renal tubules. Calcification was considered to be due to the effect of pH on the solubility of octocalcium phosphate and hydroxyapatite. Following the work of Harrison & Harrison (1955) on rats given acetazolamide, the emphasis shifted to the urinary content of citrate as being more important in causing nephrocalcinosis. This possibility was supported by the study of Dedmon & Wrong (1962) who showed that urinary citrate concentration was low in patients with renal tubular acidosis. The same authors, however, also noted that calcification occurred in a number of patients in spite of a normal urinary citrate excretion.

From the wilderness of the aetiology of nephrocalcinosis and nephrolithiasis, the following factors have emerged as of major importance: magnesium deficiency (Cramer, 1932; Brookfield, 1934; Greenberg, Lucia & Tufts, 1938; MacIntyre & Davidsson, 1958), a high phosphorus diet (MacKay & Oliver, 1935; Faragalla & Gershoff, 1963), a high calcium diet (Harrison & Harrison, 1955; Fourman, 1959), a low phosphorus diet (Faragalla & Gershoff, 1963), vitamin B₆ deficiency (Agnew, 1951; Andrus, Gerschoff, Faragella & Prien, 1960; Faragalla & Gershoff, 1963), a reduced citrate excretion in the urine (Harrison & Harrison, 1955), a high urinary pH (Addis, Mackay & Mackay, 1926; Robertson, Peacock & Nordin, 1967), a reduced urinary content of magnesium (Smith, Baxter, Lindner & Ginn, 1962; Evans, Forbes, Sutton & Watson, 1967), the presence of a systemic acidosis or alkalosis (possibly Györy, Edwards & Shannon (1968a) and Koburg, Wienart & Goebel(l959)) and water restriction (Smith et al., 1962). In man the lack of a specific peptide inhibiting calcification has been considered to be of importance (Howard, Thomas, Barker, Smith & Wadkins, 1967). It is apparent therefore, that no single factor can solely be held responsible for the development of nephrocalcinosis under experimental conditions. An impression that single factors could be of overwhelming importance would however be gained if only single factors were looked at.

It was therefore thought desirable to elucidate further the relative importance of urinary citrate excretion and pH in the production of nephrocalcinosis with a multifactorial experimental design. This design is particularly well suited to detecting simultaneously interacting factors. For this purpose rats were given a synthetic diet together with acetazolamide and other treatments to produce changes in systemic acid-base balance and thereby obtain varying combinations of levels of urinary pH and citrate excretions. In a preliminary study (Györy, Edwards & Shannon, 1968a) the experiment of Harrison & Harrison (1955) was reproduced as far as was possible from the published data, using their diet C (high phosphorus and low calcium) as the basic diet. In addition, four different treatments were given (acetazolamide,
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hydrochloric acid, calcium chloride and sodium citrate) to sixteen rats in a $2^4$ multifactorial experiment. Surprisingly, not only did nephrocalcinosis develop in those eight rats receiving the acetazolamide but also in those rats not given the acetazolamide, although it was more severe in the former. All rats also developed interstitial fibrosis and tubular atrophy throughout the kidney. From this preliminary study it became apparent that the sustained ability to lower urinary pH was probably of importance in reducing the severity of the nephrocalcinosis. Because of the development of severe tissue damage however, no definite conclusions could be drawn. A second study was therefore undertaken with a different diet, namely the one used by MacIntyre and Davidsson (1958).

The results obtained from these studies lend support to the concept that a chronically alkaline urinary pH is a key factor in the production of experimental nephrocalcinosis. A reduced urinary content of magnesium is also probably of importance. On the other hand, changes in urinary citrate or calcium excretion had no significant effect on the development of the disease. Chronic systemic acidosis may have played an important role in the production of kidney tissue damage.

MATERIALS AND METHODS

The diet employed was that used as the control diet by MacIntyre & Davidsson (1958). The major constituents were: Casein (fat and vitamin free) 180 g, cane sugar 680 g, olive oil 80 g, and cod liver oil 20 g mixed with 50 mg of vitamin E. The salt mixture added was as described by the authors except for calcium and the phosphate content. Phosphate was kept at 6.6 mg/g of diet to conform to the phosphate content of the diet used in the preliminary study. The basic diet contained by analysis, 0.5 mg of magnesium, 1.7 mg of sodium and 1.1 mg of potassium per g of diet. The trace element mixture and the multivitamin preparation of MacIntyre & Davidsson (1958) were also used.

Male albino Wistar rats were used in a $2 \times 3$ multifactorial experimental design. Two rats each received the basic diet plus either calcium chloride (diet A), calcium carbonate (diet B), or an equal mixture of calcium chloride and carbonate (diet N), so that all the rats received 6.3 mg of calcium per g of diet by analysis. One of each pair of rats receiving any one of these three diets would also be given acetazolamide, 2.5 mg/g of diet. There were thus six rats each receiving different treatments and four replicates were carried out, producing a total of twenty-four rats.

The first two replicates of rats were started 2 weeks before the second two replicates of rats. Rats were weighed weekly from then onwards.

The growth graph of all groups of rats is shown in Fig. 1 and, as can be seen, by the end of the third week on the synthetic diet body weight began to decrease, an effect already noted with this diet (MacIntyre & Davidsson, 1958). Mean initial weight of all rats was 100 g and at killing it was 169 g. Mean body weight at death of those rats not receiving acetazolamide was 183 g and of those receiving it was 155 g. All rats in the multifactorial design received the synthetic diet during weeks 1, 2, 3, 5, 6, and 7. In addition, data from periods on normal rat cube diet during weeks 0 and 4 were obtained for comparison and for evaluation of reversibility of treatment effects. Twenty-four hour urine collections were carried out once a week, and it was so arranged that each rat had its 24 h urine collection at the same time each week and following the same number of days on any one diet. The 24 h urine collections were made after a subcutaneous injection of 500 mU of vasopressin tannate in oil (Parke-Davis), in
metabolic cages modified slightly from that described by Hanna & Alcock (1961). The modification consisted of adding a small rectangular external compartment which would hold a metal food container sunk into the bottom. The food was covered with a heavy perforated metal plate with holes sufficiently large to enable the rat's snout and tongue to obtain food but to prevent it from grabbing or spilling the food with its paws. The square compartment had glass bars running through it along a diagonal plane, which could be removed when filling the metal container with food, but which would restrict the space sufficiently so that only the rat's head would fit near the food. Drinking water was also allowed during the 24 h collection and was fed by a glass tube into one side of the small compartment. In this manner continued treatment with the diet and drinking water was ensured during the collection, and contamination of the urine with food and water was avoided. The urine collection chamber was a cylindrical separating funnel with a tap at one end. The funnel was truncated to provide a collection chamber of about 8 cm height and filled to 3 cm with paraffin and a crystal of Thymol was added as preservative. Above the paraffin was fitted a grid of copper wire with relatively large holes but small enough to prevent faeces from falling through. In this manner urine could be collected without faecal contamination and rapid urine collection without excessive exposure to the atmosphere was ensured. If occasional faecal particles slipped through the grid, they

Fig. 1. Change in body weight of rats during the 7 weeks of study. The acute changes in week 4 were caused by a return to normal laboratory cube diet for 1 week. A, N and B represent rats receiving diets A, N and B respectively, without acetazolamide (solid lines), and with acetazolamide (interrupted lines). Each point represents the mean of four rats.
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floated on the paraffin and did not reach the urine. At the end of the collection the urine could easily be run out into measuring cylinders or syringes and topped with paraffin. Bladder emptying was ensured at the beginning and end of each collection period by placing a nose cone saturated with anaesthetic ether over the animal's nose and face while in the cage.

On the urines thus collected, urinary pH, volume, osmolality, citrate, calcium, magnesium, and creatinine excretions were measured.

During the seventh week the rats were killed with light intraperitoneal pentobarbitone anaesthesia followed by laparotomy and aortic exsanguination. On the aortic blood the following measurements were made: pH, $P_{CO_2}$, whole blood base excess, standard bicarbonate, citrate, calcium and magnesium.

The kidneys of the experimental animals and normal controls were quickly removed, cleaned of excess fat and blood and one half of one kidney placed in a tared glass container with formol-saline for histological examination. The other three halves of the two kidneys were quickly placed into a tared graduated centrifuge tube containing a small volume (2–3 ml) of ice cold 10% trichloroacetic acid and quickly weighed and then placed into an ice bath until homogenization could be carried out within 10 to 20 min. Kidney tissue citrate, calcium and magnesium contents were measured on the final tissue extract.

Laboratory methods.

Arterial pH, $P_{CO_2}$, whole blood base excess and standard bicarbonate concentrations were measured by a micro method (Siggaard-Andersen, Engel, Jorgensen & Astrup, 1960). Osmolality was measured on an Advanced Instruments Osmometer. Urine pH was measured with a glass electrode and a Radiometer pH meter, urinary phosphorus by the method of Negrin (1964), calcium and magnesium in urine, plasma and kidney tissue on a Techtron atomic absorption spectrophotometer with a diluent containing 0.1 M KCl, 0.1 N HCl in 6% butanol. Citrate in urine, plasma and kidney tissue was measured by the method of McArdle (1955).

Kidney tissue was homogenized in ice cold 10% trichloroacetic acid with a Turrax homogenizer in graduated centrifuge tubes and the homogenizer shaft washed three times with 10% trichloroacetic acid and added to the kidney homogenate. The mixture was vigorously shaken for 60 s on a vortex mixer and left to stand overnight. Next day the tube was mixed again on a vortex mixer for 60 s, volume made up to 35 ml with 10% trichloroacetic acid and mixed well and centrifuged at 3000 r.p.m for 20 min. Twenty-five ml exactly of the supernatant was then pipetted into a round bottomed stoppered centrifuge tube pre-marked to a 5 ml volume, and evaporated down to 5 ml on a water bath with dry nitrogen blowing over the top. This was then used for the citrate estimation. The remaining kidney extract was used to measure the calcium and magnesium content of kidney tissue. The extraction of calcium and magnesium by this method was checked against duplicate kidney slices ashed at 600° in platinum crucibles and subsequently determined by atomic absorption, identical values being obtained by both methods.

RESULTS

The analysis and design of a multifactorial experiment is based on the analysis of variance and covariance. Thus from a relatively few but randomly chosen number of animals the effects of treatments can be satisfactorily distinguished. In the present experiment the estimations on plasma and kidney tissue were made at the time of killing and on urine prior to killing.
The results have been analysed in two ways shown in Tables 1 and 2. In Table 1 the results of the experimental group of rats have been divided into two sections, one receiving the various synthetic diets alone (without acetazolamide) and the other receiving acetazolamide as well. This was done in order to compare the results with those obtained from rats on a normal laboratory cube diet.

This division was also made necessary by the relatively marked effect of acetazolamide on many of the variables, especially kidney tissue calcification. In a combined analysis the large differences in the means thus obtained produced a large variance masking significant effects. Such inhomogeneous variance was tested for and the analysis carried out accordingly. The significance of any differences between the experimental groups and the normals, shown by the asterisks, was tested by using a combined standard error of the differences of the means (not shown). In addition in Table 1 the effects of the three synthetic diet types are shown separately in the two experimental subgroups. Significant variation caused by variation of the diets is shown by the significance of the 'F' values (asterisks in parentheses). Standard error of the difference in means between any two diet effects is also shown separately for each subgroup (SE $\bar{x}$). For a comparison with blood and kidney tissue values the mean urine data of weeks 5 and 6 were used.

Table 1 shows that the diet caused a decrease in urinary calcium and magnesium excretion, but no significant change from the normal of plasma citrate, calcium and magnesium nor in kidney tissue magnesium or urinary concentrating ability. The individual diets produced changes according to their effects on systemic acid-base balance. This general tendency was also maintained when the rats received acetazolamide as well, and was confirmed when the results of all rats were analysed in a combined analysis of variance as shown in Table 2. The first part of Table 2 shows the effect of variation of diet on the variables in all rats. The second part of Table 2 shows the effect of acetazolamide alone on these variables; while the third part shows the presence or absence of any synergism between diet and acetazolamide.

The synthetic diet used in the present study did not produce the interstitial and tubular damage produced in the preliminary study (Györy et al., 1968a). However, it was not entirely satisfactory. The rate of growth on the synthetic diet (weeks 0–3 and 4–7, Fig. 1) was much reduced below that on cube diet (week 34, Fig. 1), an effect already noted for this diet with the same calcium content by MacIntyre & Davidsson (1958). Both urinary calcium and magnesium excretion were markedly reduced below the amounts excreted by rats on the cube diet, or when on the same synthetic diet but force fed (Alcock, 1965). These reductions are possibly in part due to reduced food intake. The inorganic phosphate content of 6·6 mg/g of the diet was moderately increased above the normal, and this also possibly contributed to the generally low level of urinary calcium and magnesium excretion (Alcock & MacIntyre, 1962).

Kidney tissue values

The degree of kidney calcification was defined by wet kidney tissue content of calcium ($K_{\text{Ca}}$) and confirmed by histology. The normal values for $K_{\text{Ca}}$ obtained in the present study agreed with those published by MacIntyre & Davidsson (1958) assuming a rat kidney water content of 77% (Skelton, 1927). Their normal for $K_{\text{Ca}}$ was 0·06 SEM 0·003 mg/g kidney wet weight. $K_{\text{Ca}}$ found by Fourman (1959) in the normal rat kidney was in the range of 0·047–0·156/g wet weight. Acetazolamide treatment caused a highly significant increase in $K_{\text{Ca}}$. on the
Table 1. Summary of means of blood and kidney tissue values at time of killing and of urinary values during weeks 5 and 6 from rats on the synthetic diet with and without acetazolamide separately and from rats on normal laboratory cube diet

<table>
<thead>
<tr>
<th>Whole</th>
<th>Plasma</th>
<th>Urine</th>
<th>Kidney tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>blood</td>
<td>base</td>
<td>Calcium</td>
<td>Magnesium</td>
</tr>
<tr>
<td>excess</td>
<td>(mEq/l)</td>
<td>(mg/100 ml)</td>
<td>(mg/100 ml)</td>
</tr>
<tr>
<td>Normal cube diet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>0.7</td>
<td>(0.05)</td>
<td>(0.05)</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Synthetic diet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>without acetazolamide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet A</td>
<td>-1.7</td>
<td>23.6</td>
<td>9.7</td>
</tr>
<tr>
<td>SEM</td>
<td>0.7</td>
<td>(0.05)</td>
<td>(0.05)</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>with acetazolamide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet A</td>
<td>-9.2++</td>
<td>22.4</td>
<td>9.17</td>
</tr>
<tr>
<td>Diet N</td>
<td>-9.9+++</td>
<td>18.0+++</td>
<td>9.44</td>
</tr>
<tr>
<td>SEM</td>
<td>1.1</td>
<td>0.085</td>
<td>0.085</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

SEM § represents standard error of the difference of the means within the experimental group. * = P<0.05, ** = P<0.01 and *** = P<0.001 refers to levels of significance of the difference between the means of rats on normal cube diet and of those on the various synthetic diets, in parentheses for 'F' values of analysis of variance. † Significance probably related to differences in weight of the rats (see text).
TABLE 2. Summary of the results of the combined analysis of variance on the results of all rats

<table>
<thead>
<tr>
<th>Whole blood base excess (mEq/l)</th>
<th>Plasma</th>
<th>Urine</th>
<th>Kidney tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Citrate (µg/ml)</td>
<td>Calcium (mg/100 ml)</td>
<td>Magnesium (mg/100 ml)</td>
</tr>
<tr>
<td>Diet A</td>
<td>-5.5</td>
<td>22.8</td>
<td>9.41</td>
</tr>
<tr>
<td>Diet N</td>
<td>-6.4</td>
<td>19.3</td>
<td>9.52</td>
</tr>
<tr>
<td>Diet B</td>
<td>-4.2</td>
<td>23.0</td>
<td>9.24</td>
</tr>
<tr>
<td>SEM</td>
<td>0.99</td>
<td>3.1</td>
<td>0.14</td>
</tr>
<tr>
<td>'F' value of diet effect</td>
<td>2.54</td>
<td>0.92</td>
<td>1.86</td>
</tr>
<tr>
<td>Without drug</td>
<td>-1.7</td>
<td>21.6</td>
<td>9.53</td>
</tr>
<tr>
<td>With drug</td>
<td>-8.9</td>
<td>21.9</td>
<td>9.26</td>
</tr>
<tr>
<td>SEM</td>
<td>0.81</td>
<td>2.5</td>
<td>0.12</td>
</tr>
<tr>
<td>'F' value of drug effect</td>
<td>79.2***</td>
<td>0.006</td>
<td>5.38*</td>
</tr>
<tr>
<td>'F' value of diet with drug effect (synergism)</td>
<td>0.05</td>
<td>0.74</td>
<td>0.81</td>
</tr>
</tbody>
</table>

The diet means were summed across 'with and without acetazolamide' treatments, and drug means were summed across all three diets. Urinary values represent results obtained during weeks 5 and 6 of the study. * = P<0.05, ** = P<0.01, *** = P<0.001.
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average by a factor of 50 (Table 2). The nephrocalcinosis in individual rats receiving acetazolamide varied so widely that in the combined analysis no statistically significant effect of diet alone could be demonstrated (Table 2). When however the non-acetazolamide treated rats were analysed separately for diet effect as shown in Table 1, KT_Ca was significantly affected by variation in diet alone. KT_Ca in rats receiving diet N was reduced below the normal and had significantly lower KT_Ca than those receiving either diet A (P<0.05) or diet B (P<0.025).

There was no significant effect of the various diet treatments on kidney tissue magnesium (KT_Mg), either with or without acetazolamide (Table 1). Acetazolamide however did significantly increase KT_Mg (Table 2), and those rats with the highest KT_Ca (diet plus acetazolamide) also had the highest content of KT_Mg.

The normal kidney tissue citrate (KT_Cit) levels in the present study agree well with those of Crawford, Milne & Scribner (1959) and Gordon (1963). KT_Cit was significantly elevated above the normal by acetazolamide treatment (Table 2) and the group receiving diet B plus acetazolamide had the highest content of kidney tissue calcium and also the highest content of kidney tissue citrate. However, diet alone also had an effect on KT_Cit (Tables 1 and 2), the more carbonate in the diet the higher the kidney tissue content of citrate. In addition the combined effect of diet and drug on KT_Cit was not simply additive but synergistic (Table 2). The acid producing diet also produced a significant reduction in KT_Cit in the absence of acetazolamide when compared to the normal (Table 1), as was shown by Crawford et al. (1959).

Both KT_Mg and KT_Cit contents were associated to a high degree with KT_Ca (Fig. 4), the positive linear correlation being highly significant between KT_Ca and KT_Cit and KT_Mg with a coefficient of correlation of 0.9229 and 0.9344 respectively (P<0.001 for both).

Blood values

Blood acid-base values of normal rats in the present study were pH 7.42 SEM 0.01, P_CO_2 40.7 SEM 1.9 mmHg, standard bicarbonate 25.2 SEM 0.5 mEq/l and whole blood base excess +1.6 SEM 0.6 mEq/l. All three groups of rats receiving the experimental diets without acetazolamide were mildly acidotic when compared to the normal, but only in those receiving diets A and N was this difference statistically significant (Table 1). Acetazolamide produced a significant systemic acidosis in all rats (Tables 1 and 2), but there was no significant difference attributable to diet when on acetazolamide. There was no synergism between diet and drug (Table 2).

Plasma citrate values of the control rats agreed well with those found by Simpson (1963) and Zelewski, Zyduwo & Purzycka (1962) and were somewhat higher than those found by Crawford et al. (1959). The plasma citrate values in all groups except one (diet N plus acetazolamide, Table 1) were well within the normal range.

Normal plasma calcium was 9.9 SEM 0.3 mg/100 ml and was somewhat lower than the values reported by MacIntyre & Davidsson (1958) and by Alcock (1965). Varying the diet did not affect plasma calcium values (Table 2). With acetazolamide, rats on diets A and B had plasma calcium values that were significantly lower than those on the normal diet (Table 1).

Normal plasma magnesium was 1.45 SEM 0.04 mEq/l and was somewhat lower than those published by MacIntyre & Davidsson (1958) and Alcock (1965). Varying the diet had no effect on plasma magnesium values (Table 2), but acetazolamide significantly increased plasma magnesium for those rats on diets A and B (Tables 1 and 2).
Urinary values

Urinary excretion of citrate, calcium and magnesium were corrected to the excretion of 1 mg of creatinine in the urine to minimize collection errors. There was a highly significant positive linear correlation between body weight of rats and creatinine excretion, both while the rats were on the normal laboratory cube diet and when on the synthetic diet, the coefficient of correlation being 0.9520 \((P<0.001)\) and 0.9057 \((P<0.001)\) respectively. The slope of the regression equation of creatinine excretion on body weight was 0.0503 in the former and 0.0323 in the latter, the difference not being statistically significant \((P>0.05, n = 152)\). Thus, the correction to 1 mg of creatinine also corrected for differences due to body weight of rats. Acetazolamide caused a slight fall in creatinine excretion in excess of decrease in body weight and this had a small effect on the differences in excretion rates found between acetazolamide and non-acetazolamide treated rats. Thus differences found between the two groups would actually be somewhat larger if excretion rates had not been corrected to 1 mg of creatinine.

Urinary citrate excretion was markedly reduced below normal in those rats receiving diets A and N (Table 1), but was not significantly reduced below the normal in those on diet B. The variation in diet produced a statistically significant variation in urinary excretion both in those rats not receiving acetazolamide as well as in those receiving acetazolamide (Table 1). Acetazolamide itself reduced urinary citrate significantly (Table 2). Thus, an acid diet such as diet A reduced urinary citrate while acetazolamide itself produced a further drop in urinary citrate excretion, there being a synergistic (Table 2) rather than just an additive effect between acetazolamide and an acid diet.

Urinary calcium excretion was significantly reduced below the normal in all rats receiving the synthetic diet, and was significantly affected by variation in diet both in the acetazolamide and non-acetazolamide treated rats (Tables 1 and 2), the effect being a decrease in excretion when a more alkaline diet (diet B) was given than when an acid diet (diet A) was given \((P<0.05)\). Acetazolamide caused an increase in urinary calcium excretion in all groups (Table 1) but not to normal levels. The effect of diet and acetazolamide was not synergistic (Table 2), only additive. Faecal excretion of calcium was also measured on one occasion during week three on the synthetic diet and was as follows: diet A 220, diet N 545, diet B 325, diet A plus acetazolamide 700, diet N plus acetazolamide 1150 and diet B plus acetazolamide 1500 \(\mu\)Eq day\(^{-1}\) 100 g rat\(^{-1}\). Thus urinary excretion of calcium was augmented by acetazolamide whereas plasma calcium was reduced. In the absence of measurements of glomerular filtration rates, filtered loads could not be estimated, but the available data would favour a tubular site of action of acetazolamide.

Urinary magnesium excretion (Fig. 2) was also generally and significantly reduced below the normal in all rats on the synthetic diet. Variation in diet caused a significant change in urinary magnesium both in the presence or absence of acetazolamide (Tables 1 and 2) and was highest in those rats on diet A and lowest in those on diet B \((P<0.05)\). However, acetazolamide very markedly reduced urinary magnesium excretion in all groups (Table 1), an effect entirely different to that on urinary calcium excretion. The effect of the combination of drug and synthetic diet on urinary magnesium excretion was not simply additive but truly synergistic (Table 2). Faecal excretion of magnesium measured once during week three was as follows: diet A 28.8, diet N 60.0, diet B 32.9, diet A plus acetazolamide 78.2, diet N plus acetazolamide 134.2 and diet B plus acetazolamide 158.0 \(\mu\)Eq day\(^{-1}\) 100 g rat\(^{-1}\).

Variation in diet caused significant change in urinary pH in both the acetazolamide and
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Fig. 2. Change in urinary magnesium excretion of rats during the study. Notation otherwise as in Fig. 1.

the non-acetazolamide treated rats (Table 1), the mean urinary pH values for the various diets being significantly different from each other \( P < 0.01 \) in both groups. Urinary pH was reduced below the normal by both diet A and diet N but elevated above the normal by diet B. Addition of acetazolamide elevated urinary pH in all diet groups, the combination of acetazolamide and diet being additive only and not synergistic (Table 2). The weekly urinary pH graph is shown in Fig. 3 for each individual diet and treatment with and without acetazolamide. As can be seen the trends were well maintained throughout the study and rats on diet A plus acetazolamide had practically normal urinary pH values. Also apparent from Fig. 3 is that rats on diet B and diet N plus acetazolamide had very similar urinary pH values.

Urinary osmolality following subcutaneous injection of vasopressin but with free access to fluids was 1776 mosmol/kg water in normal rats of average weight 100 g. There was a significant positive linear correlation in the normal rats between urinary osmolality and weight \( r = 0.6316 \ P < 0.01 \), the smaller the rat the smaller the urinary osmolality under these conditions. There was no significant difference between the urinary osmolalities of normal rats and rats on any of the treatments during week 4 when they were again placed on cube diet, even taking the weight differences into consideration \( P > 0.05 \). There was thus no concentrating disability present after 3 weeks of treatment, where at least a small degree of nephrocalcinosis might be expected. During weeks 5 and 6 the osmolalities of rats not receiving acetazolamide was not significantly different from normal (Table 1) and there was no difference in urinary osmolalities between any of the diet groups without acetazolamide. The addition of acetazolamide, however, did
cause a significant decrease in urinary osmolality (Table 2) in all rats. There was true interaction or synergism between diet and acetazolamide (Table 2), and those rats receiving diet B plus acetazolamide had the lowest urinary osmolalities.

**Histology**

Histologically, sections from half the rats in this series (those receiving acetazolamide) showed calcific deposits in a zone along the corticomedullary junction. Many other deposits of similar shape and appearance were probably also situated in tubules but tubular epithelium could not be seen around them and it had presumably degenerated completely around these deposits. All these deposits stained strongly with von Kossa's method; many were laminated. With rare exceptions there was no reaction around deposits other than attenuation or disappearance of the epithelium. Although one could not be certain, most of the intratubular calcification appeared to affect the distal part of the proximal tubular lumen.

Fig. 4 presents in diagrammatic form the data in Table 1 that has a major bearing on the aetiology of nephrocalcinosis. It shows the combinations of important factors probably responsible for the nephrocalcinosis. It is for example evident that urinary citrate excretion was not of primary importance in the prevention because rats on diet A excreted only as much citrate as did those on diet B plus acetazolamide. The severity of nephrocalcinosis was, however, in the reverse order, rats receiving diet B plus acetazolamide having the highest $KT_{Ca}$, at least
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FIG. 4. Bar diagram summarizing the changes produced by three different diets and acetazolamide. Urinary values are the combined averages for weeks 5 and 6 of the study immediately before killing during week 7, for which the blood and kidney tissue values are shown. The first clear bar under each variable represents the normal values obtained in rats on normal laboratory cube diet, other values are as in Table 1. The other clear bars show the results in rats receiving the synthetic diet without acetazolamide, while the shaded bars immediately following the respective clear bars show the results in those rats on the synthetic diet plus acetazolamide. UpH = urinary pH, UV_{Cit}, UV_{Mg}, UV_{Ca} = urinary excretion of citrate, magnesium and calcium respectively. The ratios of urinary citrate to calcium, and magnesium to calcium represent molar ratios. KT_{Ca}, KT_{Mg}, and KT_{Cit} = kidney tissue calcium, magnesium and citrate concentrations respectively. Base excess = whole blood base excess of aortic blood. Units of measurements are as shown in Table 1.

A = diet A, N = diet N and B = diet B.

10 times that found in rats on diet A. Even in the group receiving diet B, with the highest urinary citrate excretion, but also the highest urinary pH in the non-acetazolamide group, KT_{Ca} was the highest and was significantly increased above the kidney calcification in rats on diet N, with a significantly (P<0.01) lower level of urinary citrate excretion.

Fig. 4 also shows that a relatively alkaline urinary pH was usually associated with a high level of KT_{Ca} content. For instance, those rats receiving diet N plus acetazolamide and diet B plus acetazolamide had the highest urinary pH values as well as the greatest degree of nephrocalcinosis. Of particular interest are the urinary citrate and pH values in those rats on diet A and diet B plus acetazolamide (Fig. 4), because in both the urinary citrate excretion rates were low but the urinary pH values were widely differing (acid in the former and alkaline in the latter) as were the KT_{Ca} contents. However, urinary pH was not the only major factor in favouring the development of nephrocalcinosis. Those rats receiving diet A plus acetazolamide and those receiving diet N or the control cube diet all had very similar urinary pH values and yet, of these, only those rats receiving diet A plus acetazolamide had significant nephrocalcinosis.
The other factor which has unexpectedly emerged from this study as having a significant association with the development of nephrocalcinosis, is hypomagnesuria. There was a close association between a reduced magnesium excretion and the development of kidney tissue calcification (Fig. 4). Acetazolamide markedly reduced urinary magnesium excretion in all rats, an effect already noted for acetazolamide in man by Barker, Elkinton & Clark (1959). The rats showing the lowest urinary magnesium excretion (acetazolamide plus diet A, N and B and diet B alone), also showed the highest level of $KTCa$ content.

Fig. 4 also shows that under these experimental conditions the rate of urinary calcium excretion had no constant association with the degree of kidney calcification.

The association of excretory patterns and nephrocalcinosis shown in Fig. 4 has been confirmed by an analysis of covariance performed between $KTCa$ and urinary pH, urinary magnesium, urinary citrate and urinary calcium separately as shown in Fig. 5. This shows the comparison of the statistical significance at the 5% level (interrupted lines) of acetazolamide and diet effects on $KTCa$ (first bars). Fig. 5 also shows the remaining effect of acetazolamide on $KTCa$ after the separate removal of its effect on urinary pH, urinary magnesium, citrate and calcium excretion as performed by an analysis of covariance.

The significant role of urinary pH in relation to $KTCa$ was confirmed by this analysis of covariance. Removal of the effect of acetazolamide upon urinary pH completely abolished the effect of acetazolamide on kidney tissue calcification. A similar, but less pronounced effect of diet alone on urinary pH and $KTCa$ in those rats not receiving acetazolamide is shown in the second part of Fig. 5. Removal of the effect of acetazolamide on urinary magnesium, abolished the effect of the drug on kidney tissue calcification. Removal of the effect of diet on urinary magnesium excretion from its effect on $KTCa$ produced the greatest drop in the effect of diet on $KTCa$ (Fig. 5), approaching but not reaching significance. The significance of the drug action on $KTCa$ was not removed when its effect on urinary citrate excretion was excluded. The second half of Fig. 5 shows a similar analysis of diet effect alone on $KTCa$ in those rats not receiving acetazolamide. When the effect of diet on urinary citrate excretion was removed, the effect of diet on $KTCa$ was hardly altered, showing that variation in urinary citrate excretion had no significant effect upon the development of nephrocalcinosis in this group. Removal of the effect of acetazolamide or diet on calcium excretion did not alter their separate effect on $KTCa$ (Fig. 5). This was not entirely surprising as the primary abnormalities involved in the nephrocalcinosis in this study were not of excess calcium excretion but of other factors involved in the calcification processes.

**DISCUSSION**

The results of the present study show that induction of systemic acidosis increases, while induction of alkalosis or a reduction in the existing acidosis decreases both urinary calcium and magnesium excretion in the rat. In addition acetazolamide caused a further significant increase in urinary calcium excretion and a decrease in plasma calcium. The effect of acetazolamide on urinary calcium excretion was independent of urinary pH (coefficient of correlation between urinary pH and calcium excretion was $0.27$, $P<0.05$) and was therefore mediated through the acid base changes of the blood. The site of action of the acidosis has been postulated to be at a tubular level in man (Lemann, Litzow & Lennon, 1967) and proven in the sheep (Stacy & Wilson, 1970), and although the data from the present study were insufficient for a definite
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Effect of Acetazolamide on kidney tissue calcification ($K_{Tc}$), with and without removal of its effect on:

- Effect of diet on kidney tissue calcification ($K_{Tc}$), with and without removal of its effect on:

![Graph comparing the effect of acetazolamide and diet on kidney tissue calcification](image)

**Fig. 5.** Comparison of the effect of acetazolamide (shaded bars) and diet alone (clear bars) on kidney tissue calcification. The height of the bars represents, as a percentage, the square of the non-centrality parameter*, a significance value similar to Fisher's F value. The level of significance at a probability of 0.05 is indicated by the interrupted lines. The first bars ($K_{Tc}$, shaded and clear) represent the effects of acetazolamide or the three different diets alone respectively on kidney tissue calcification, both being above the interrupted lines and therefore significant ($P<0.005$ and $P<0.05$ respectively). The bars underneath $\text{UpH}$ (urinary pH), $\text{UVMg}$ (urinary magnesium excretion), $\text{UVcit}$ (urinary citrate excretion) and $\text{UCa}$ (urinary calcium excretion) represent the non-centrality parameter after the effect of either acetazolamide or diet on these values had been removed from the effect of acetazolamide or diet on kidney tissue calcification (analysis of covariance). It can be seen that when the effect of acetazolamide on urinary pH and urinary magnesium excretion was removed, this drug was no longer associated with significant kidney calcification. When the effect of acetazolamide on urinary citrate and calcium excretion was removed from its effect on kidney calcification, a significant effect of the drug in causing nephrocalcinosis still remained. The trend with diet alone was similar but not pronounced.

* The square of the non-centrality parameter (as a percentage) is obtained by expressing the difference between the mean square of the acetazolamide or diet effects and the mean square of their respective errors (divided by 12 for the acetazolamide and by 8 for the diet group), as a percentage of the mean square of their respective errors.
conclusion to be reached, they would be in accord with a tubular site of action rather than through an increased load caused by acidosis. Acetazolamide had quite a different effect on magnesium excretion, markedly reducing urinary excretion, while significantly increasing plasma magnesium levels. It is not known whether this action of acetazolamide is direct. However, in the present study there was a high degree of negative correlation between urinary pH and magnesium excretion (coefficient of correlation = 0.79, \( P < 0.001 \)). This suggests that acetazolamide decreases urinary magnesium excretion through its effect on urinary pH. The simultaneous increase in plasma magnesium levels and reduction in urinary excretion of magnesium suggests that the site of action of acetazolamide is at a tubular level. This contrasting effect of acetazolamide on the urinary excretion of calcium and magnesium was intriguing, and was possibly related to the contrasting effect produced by this drug upon blood acid-base status and urinary pH. It would be of interest to examine whether potassium deficiency, which also produces changes in blood and acid-base status and urinary pH but in the opposite direction, could effect a similar divergence of urinary calcium and magnesium excretion. Heidland, Röckel, Maidhoff & Hennemann (1970) have shown in man that urinary magnesium excretion is affected by changes in urinary pH rather than by changes in systemic acid-base balance. They showed that various diuretics induced a reduction in urinary magnesium excretion in association with an elevation of urinary pH, but irrespective of the systemic acid-base changes produced.

In the present study, elevation of urinary pH also led to reduction in urinary magnesium excretion, and it is therefore impossible to determine which factor was of more importance in the production of nephrocalcinosis. Magnesium deficiency alone has been found to induce nephrocalcinosis. Thus, Cramer (1932) first noted calcification of the kidneys of rats given a magnesium deficient diet. This was further confirmed on a number of occasions (Brookfield, 1934; Greenberg et al., 1938; MacIntyre & Davidsson, 1958). MacIntyre & Davidsson (1958) also showed that hypercalcaemia was induced by magnesium deficiency, and they thought that this was probably the cause of the nephrocalcinosis. In the present study the diet had an adequate content of magnesium by analysis and there was no hypercalcaemia, but rather hypocalcaemia, in those rats receiving acetazolamide. The effect of the latter in inducing nephrocalcinosis was therefore entirely different and is shown in this study to be connected with its effect on the urinary content of magnesium and pH. The mechanism of the involvement of magnesium in the precipitation of calcium salts is unknown although the association of a lowered urinary excretion of magnesium and the higher incidence of nephrolithiasis in man has also repeatedly been noted (Smith et al., 1962; Koburg et al., 1959) and has recently been described in patients with renal tubular acidosis (Robertson et al., 1967). It is therefore likely that hypomagnesuria on its own does play an important role in the development of nephrocalcinosis.

Comparisons between the molar ratios of urinary citrate to calcium and urinary magnesium to calcium (Fig. 4) are of interest, and have been stated to be important in connection with nephrocalcinosis and nephrolithiasis (Harrison & Harrison, 1955; Oreopoulos, Soyannwo & McGeown, 1968). Although the ratio of urinary citrate to calcium was considerably lower than normal in all rats receiving acetazolamide with a high degree of kidney tissue calcification, this ratio was also reduced to similarly low levels in those rats receiving diet A alone with a normal kidney tissue calcium content. There was no obvious correlation between the reduction of the citrate to calcium ratio and the degree of kidney calcification. There appeared a
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A much closer correlation between the reduction in ratio of urinary magnesium to calcium and the severity of nephrocalcinosis. This ratio was reduced in all rats receiving acetazolamide and was highest in those with the lowest $KT_{ca}$. The adverse effects of a high urinary pH on the development of kidney calcification and stone formation have been described (Addis et al., 1926; Koburg et al., 1959; Oreopoulos et al., 1968). Rats maintained on alkaline diets developed significantly more nephrocalcinosis and stones, producing haematuria and hydronephrosis, than did rats receiving hydrochloric acid or calcium chloride supplements, although these latter showed other histological damage (Koburg et al., 1959). These authors also found that, in the presence of water restriction, either systemic alkalosis or acidosis produced a rapid increase in tissue calcification. A distinction between the effects of systemic acidosis or alkalosis and those of changes in urinary pH produced by the various treatments on nephrocalcinosis and tissue damage has not been made in these studies. By employing acetazolamide to induce acid base changes such a distinction is made even more difficult. In the preliminary study a high degree of positive correlation was demonstrated between kidney calcification and systemic acidosis in those rats on acetazolamide ($r = 0.8427$, $P < 0.01$). In the present study, non-calcinotic interstitial changes, when occasionally found, could only be seen in those rats on acetazolamide who were also severely acidotic. In the preliminary study rats on acetazolamide were much more acidic than the non-acetazolamide treated rats in the present study, and were found to have kidney tissue damage and nephrocalcinosis. This tends to support the hypothesis that chronic systemic acidosis may produce histological damage to kidneys and, in consequence, significantly affect kidney tissue calcification.

Very few studies however have been performed on the effect of acidosis on the structure of the kidney. Seegal (1927) produced severe transient tubular epithelial damage by feeding rabbits and dogs hydrochloric acid, ammonium chloride and calcium chloride; these changes were abolished if the serum bicarbonate concentration was allowed to return to normal for 2 days prior to killing. Ewing (1908) produced proteinuria and hyaline casts in the urine of rabbits injected with hydrochloric acid subcutaneously. Kidney histological changes were similar to those found by Seegal (1927), Goto (1917) and MacNider (1923). In the present study both the elevation in urinary pH and reduction in urinary magnesium excretion were significantly associated with the production of kidney tissue calcification, whereas urinary citrate and calcium excretions were probably of less importance. An association between systemic acidosis and kidney tissue damage and nephrocalcinosis can only be suspected from the available data, but should be kept in mind as a working hypothesis.

It is concluded from the present study that an elevation of urinary pH is of major importance in the production of experimental nephrocalcinosis, but a reduction in urinary citrate excretion is of only secondary importance. In view of the close correlation between urinary pH and urinary magnesium excretion, the separate importance of the two could not be defined with certainty.

In summary, it is surmised that various factors interact at all times, either one or more becoming of prime importance as the conditions vary from moment to moment in the urine. Primary determining factors seem to be urinary pH and urinary content of magnesium, an alkaline urinary pH favouring calcification and an acid pH preventing calcification. Within each of these primary extremes a low urinary content of magnesium would favour calcification and a high urinary content of magnesium would prevent calcification. Urinary citrate may be playing a secondary role, in that a low urinary citrate would only cause calcification if the
first two conditions were unfavourable to calcification. These considerations have important implications in the therapy of patients prone to develop nephrocalcinosis, such as patients with renal tubular acidosis. These patients have a constantly elevated urinary pH, a chronically reduced citrate excretion in the urine and a lowered urinary excretion of magnesium (Robertson et al., 1967). Attempts at returning systemic acid base balance and urinary content of citrate to normal with simple alkalis in these patients may actually harm them by increasing urinary pH even further, and might explain the singularly low rate of success with this therapy in reducing existing nephrocalcinosis (ten cases out of a total of 137 over the last 30 years). The suggestion therefore that magnesium supplements should be given a therapeutic trial in patients with renal calculi and nephrocalcinosis should be considered, especially as magnesium administration has been found to increase urinary citrate excretion (Götz & Womersley, 1963). Also the incorporation of a carefully controlled amount of inorganic basic phosphate into alkalinizing mixtures would play a double role of increasing the excretion of pyrophosphate (Fleisch, 1965) which has been shown to be an inhibitor of calcification, and of keeping urinary pH at its minimum possible (Györy et al., 1968b).

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