

# The Effect of Dietary Protein and Insulin on Calciuria

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**Abstract** This review covers papers looking at the mechanism of dietary protein induced hypercalciuria and in particular looking at the mechanism of the effect that insulin has in varying calciuria. Consideration is also given to the role of pH, ammoniogenesis, gluconeogenesis, ammonia and dietary lipids. Examination of the mechanism of dietary protein induced hypercalciuria is shown to be significant and that insulin can have a significant effect on modifying the level of calciuria. The effect of insulin was shown to work via its inhibition of gluconeogenesis so that ATP is made more available for active transport of Ca. Consideration was also given to the role of pH that can result from a high protein meal and it was demonstrated to work by stimulating ammoniogenesis coupled to gluconeogenesis thus reducing the availability of ATP. The membrane transport of ammonia that is also produced by ammoniogenesis was examined to see if it could have a direct inhibitory effect on Ca transport. It is shown to change its molecular configuration to a non-polar form and diffuse through membranes in a manner that would have no effect on Ca transport. Dietary lipids are also considered in regard to possible effect on calciuria and they were shown to have no significant effect on calciuria.

**Keywords:** dietary protein, hypercalciuria, ammoniogenesis, gluconeogenesis, dietary lipids

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## 1. Introduction

In developed countries dietary protein has been linked to excessive loss of calcium in the urine and referred to as a possible cause for excessive osteoporosis and contribution to development of kidney stones. The mechanism by which dietary protein cause hypercalciuria will be discussed below with additional attention to the effect of insulin. The significance of the role that dietary protein has in the development of osteoporosis will be considered together with the occurrence and definition of osteoporosis and some other factors that have an effect on calcium loss. In particular a set of experiments looking at the involvement and mechanism of insulin on calciuria will be discussed

A positive calcium balance is necessary to achieve maximum peak bone mineralisation in young individuals and to prevent excessive bone demineralisation in older people leading to a condition known as osteoporosis. Calcium balance (b) is the difference between loss from the tissues which includes: endogenous loss to the gastrointestinal tract (e), urine (u) and integument (d), and the absorption of calcium from the intestine (a) and can be calculated from equation (1).

$$b = a - (e + d + u). \quad (1)$$

In normal individuals average values for the variables in this equation are: d, 1.6; e, 3.4; u, 5.5 (m.mol/day). Of these 'u' is the most variable ranging from 1.4 to 20 m.mol/day showing that the kidneys are the main homeostatic organ controlling blood calcium levels and

calcium balance. It is found that after the age of 35-40 years old a negative calcium balance (b) of about -0.5 to -1.0 % develops. Thus, calcium loss appears to be an inevitable consequence of ageing Matkovic, (1991). [1]

Absorption of calcium from the intestine (a) is affected mainly in older individuals because they are often vitamin D deficient. Vitamin D and PTH are the main regulators of the calcium balance. Ericksen and Glerup (2002) [2] The production of vitamin D by the skin using sunlight falls in elderly people, as they spend a lot time indoors. This vitamin D deficiency results in a fall in the concentration of calcium in the plasma and causes increase in the secretion of parathyroid hormone (PTH), which in turn causes more calcium to be resorbed from the bone. In other ages the exchange of mineral with the skeleton is also controlled by androgens, oestrogens, exercise and PTH.

An increase in dietary calcium intake seems beneficial up to age 40 years but to have little positive benefit after age 40 years Matkovic, (1991) [1]. However, the control of hypercalciuria may offer some opportunity for minimising the loss so that pathological conditions are postponed. Diet is one of many factors that are involved in producing hypercalciuria. However we need to understand the mechanisms by which diet can affect calcium loss so that dietary changes can be included with other life style modifications when trying to reduce idiopathic calcium loss. This paper will review examination of the calciuria produced by high protein and high fat intake as well as the role of insulin. The effect of high protein diets need particular attention at this time because of the current popularity of low carbohydrate diets such as those recommended by Robert Atkins [3] and

the CSIRO-Total-Wellbeing-Diet [4] which recommend high protein intakes.

There is also the danger that hypercalciuria could contribute to nephrolithiasis [5] Maalouf (2001) as well as osteoporosis Stefania S et.al. (2008) [6]. Nephrolithiasis, with a population incidence of up to 13%, results in significant morbidity and economic costs from medical treatment and time loss from work Kevin K. F and Bushinsky (2002) [7].

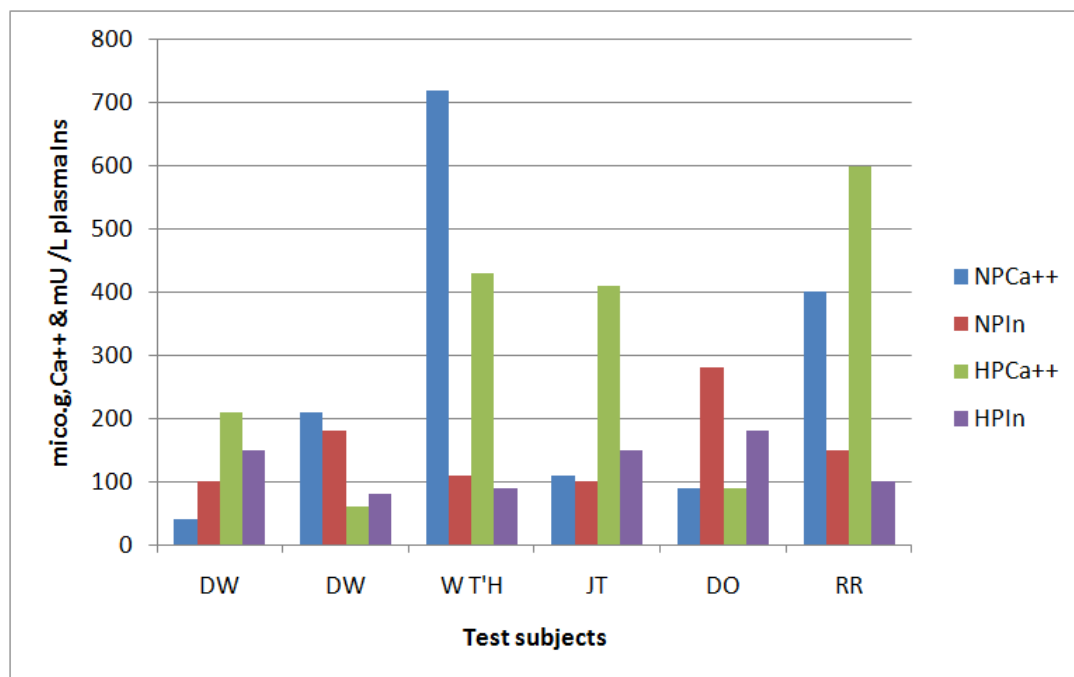
## 2. Hypercalciuric Effect of High Protein Meals

High protein diets have been proposed as the cause of the higher incidences of osteoporosis in western societies compared to less affluent countries (Abelow et. al., 1992) [8] and it has been suggested that metabolic acidosis may be the cause of protein induced hypercalciuria. This has been demonstrated with adult young women by Kaneko et. al. (1990) [8] and with rats by Fernandez-Repollet et. al. (1989) [10]. They suggested that acidosis resulted from oxidation of sulfur containing amino acids from protein. However when Funaba et. al. (1989) [11] studied the hypercalciuria of high protein diets on rats they found that calcium excretion could not be greatly reduced by adding extra dietary sodium bicarbonate and that calciuria did not correlate with net acid excretion. On the other hand Schneider and Menden (1988) [12] using long term

experiments (61 weeks) with rats found that high protein diets showed a positive correlation between renal hydrogen ion and sulphate excretion. However Schneider and Menden (1988) [12] found that increasing net acid excretion with extra phosphorus intake produce less hypercalciuria counteracting the suggestion that acid from increased dietary protein could be the only cause of hypercalciuria.

When high protein diets were consumed the increased ammoniogenesis appears to be associated with a reduction in sodium excretion (Schuette et. al., 1980) [13] indicating that sodium is being replaced by ammonium under increased acid load. Sodium is reabsorbed while protons are secreted into the urine via an anti-porter protein found in the brush border membranes. This is a secondary active transport using energy from the primary active, ATP driven sodium pump. Thereby lowering the pH of the urine

Because these studies have shown variation in results, the cause of the variation was considered by Brazier (2016a) [14] who examined the hypercalciuric effect of high protein meals and found that although on average, the high protein meals (HPM) caused more calciuria than normal protein meals (NPM), there was observed considerable variation between individuals. In fact two subjects (DW and W T'H) produced less calciuria after the HPM than the NPM as shown in Figure 1, which was the opposite of what was expected.



**Figure 1.** Total Calcium Excretion after NP and HP meals by Each Subject Some individuals show more and some show less calciuria after the HP meal, also plasma insulin levels are shown. (Mu/l) The unusually high insulin production levels of subject D.O. is seen to correspond with a much reduced level of calciuria and the opposite situation is seen in regard to subjects W.T.T. and R.R. From Brazier [14]

This study by Brazier [14] also recorded the levels of plasma insulin and this showed a considerable variation between individuals. The high level of calciuria corresponded with low levels of plasma insulin and low levels of calciuria corresponded with high levels of plasma insulin following both HP and NP meals.

Several papers from the Department of Nutritional Science at the University of California, Berkeley have

recorded protein induced hypercalciuria, Allen *et. al.* (1979 [15]; Margen, (1974) [16]; and Chu, (1975) [17]. Earlier workers; Hegsted, (1952) [18]; McCance *et. al.*, (1942) [19]; Adolf and Chen, (1932) [20] showed that calcium balance could be improved by a greater protein intake but these findings were obtained in relation to very low protein intakes. When the levels of dietary protein were above deficiency levels improved calcium

absorption was obtained by increasing the synthesis of the calcium binding protein and alkaline phosphatase necessary for calcium absorption, Kalk and Pimstone, (1974) [21].

High meat diets were postulated to induce hypercalciuria because of the acidifying action of their acid ash on the renal tubular fluids. The acid was suggested to be the results of oxidation of sulphur amino acids Wachman and Bernstein, (1968) [22]; Goulding and Malthus, (1970) [23]. This has been disputed by Margen *et al.*, (1970) [24] who used different amino acid mixtures and found no difference in renal calcium excretion. However, Benke (1978) [25] showed that the calciuria effect of protein depended on the source of the protein with lactalbumin producing maximum effect and a 70/30 mixture of beef/soy isolates having minimum effect. Whiting and Draper (1978) [26] have also obtained similar results that indicate the calciuria effect is proportional to the sulphur amino acid content of the proteins. Kaneko *et al.*, (1990) [9] correlated hypercalciuria with sulphur amino acid content in meat protein or soy protein and its amelioration with dietary potassium. Abelow *et al.*, (1992) [8] found a strong relationship between meat intake and hip fracture after examining cross cultural variation suggesting that the effect of metabolic acidosis on calcium balance should be further studied. Davis *et al.*, (1962) [27] and Nielsen *et al.* (1966) [28]; have shown that vegetable proteins (soybean protein) reduce calcium absorption due to the greater amount of phytic acid present than in meat proteins. However, Gregor *et al.* (1978) [29] showed that a 30/70% mixture of soy/meat proteins did not reduce calcium balance in adolescent girls.

### 3. Hypercalciuria and Lipids

When studying the calciuric effect of high dietary protein (HP) the meals are usually made isocaloric by adding edible oils and some glucose to the low protein meals BW. Brazier (2016a) [14] and Alien *et al.*, (1979)

[15]; Spencer *et al.*, (1978a) [30]; Kerstetter and Allen, (1990) [31] while keeping other ingredients constant.

The effect of high fat diets are significant these days because the high protein diets such as those promoted by Robert Atkins, [32] Grant, C, (2002) and the 'The CSIRO Total Wellbeing Diet, [4] Luscombe-Marsh *et al.*, (2005) [33] also recommend high fat as well as high protein intake. Wood *et al.* (1984) [34] showed with rats that an increase in amino acid content of their diet produce a linear relationship in regard to renal calcium excretion but there was no change when the low protein meals were made isocaloric with lipid or glucose. However there has been some evidence that lipids can change calcium uptake and excretion. It is one of the inevitable consequences of nutritional research that one component of a diet cannot be altered without affecting at least one other item.

Changing the fat content of a meal may modify the apparent effect of the protein induced changes by at least two different mechanisms. Hypercalciuria can be classified as either absorptive or renal Santos *et al.* (1987) [35], that is, in normal individuals a dietary induced calciuric change can result from changing either intestinal absorption or renal handling of calcium. In the later handling of calcium can be changed by affecting the glomerular filtration rate (GFR) or the fractional reabsorption (FR). There appears to be little published work on the effect of dietary lipid on any of these parameters on calciuria.

Individuals with fat malabsorption have been found to excrete higher levels of faecal calcium and less in calciuria Pike and Brown (1984) [36]; Lange (1979) [37]. Pike and Brown reported however that diets ranging from 1 % to 38% fat and equal calcium contents have shown equal calcium balances in healthy individuals. But this balance could have been achieved by changing calciuria. Lange (1979) [37] suggest that high fat diets could influence calcium balance by forming insoluble fatty acid/calcium complexes in the gut lumen. Potter *et al.* (1990) [38] has shown in man that calcium increases faecal loss of fatty acids by forming insoluble calcium complexes.

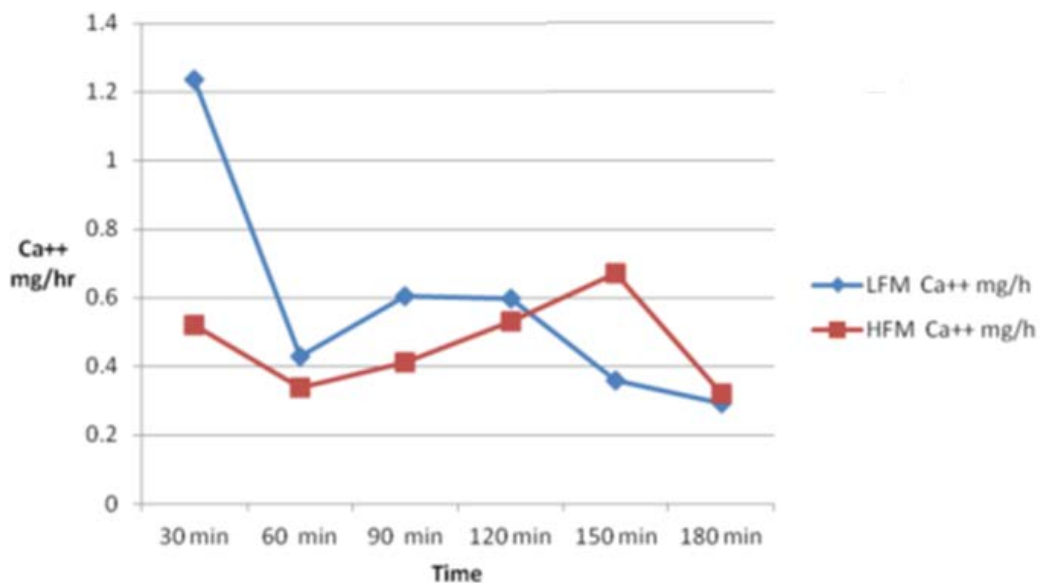


Figure 2. Rate of excretion of Calcium for high fat and low fat diets

Rate of excretion of calcium each time period t test are shown in Table A1 and indicate that only the 30 min time period shows significant difference. From Brazier [39].

The effect of high fat (HFM) versus low fat (LFM) meals was studied by Brazier [39]. Figure 2 shows the difference between the two meals at each time point. The average rate of calcium excretion in the low fat meal is more than twice that of the high fat meal, at the 30 minute at this point. However, calcium excretion levels out after this point remaining slightly higher in the low fat meal

until the 120 minute the high fat meal produces slightly greater calcium excretion. Figure 3 show that the low fat meal produced greater total excretion of calcium (3.512  $\mu\text{g}$ ) than the high fat meal (2.8099  $\mu\text{g}$ ). This result is due largely to the difference between the two meals at thirty minutes. The total calcium excretion difference was not significant between the two meals (0.05).

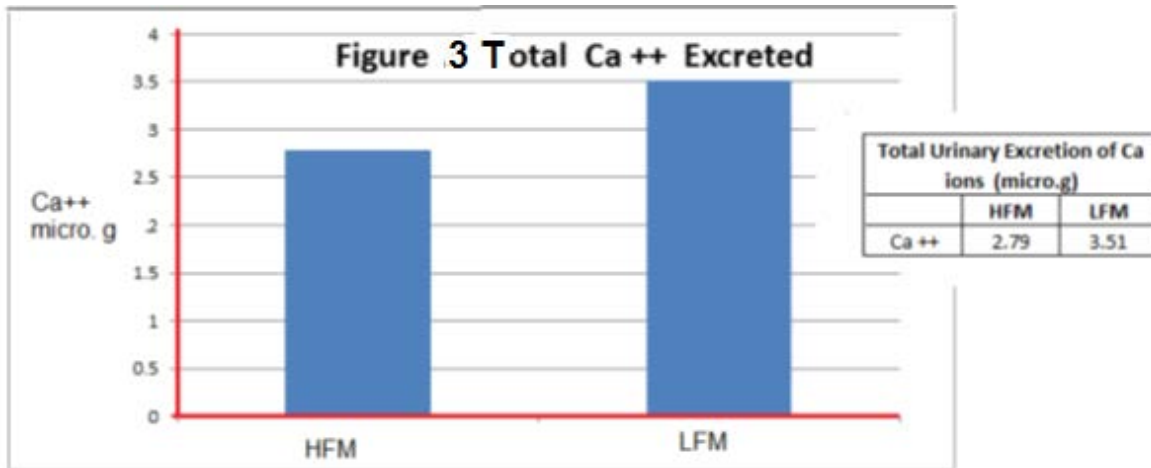


Figure 3. Total of excretion of urinary Calcium for high fat and low fat diets (From Brazier [39])

The large initial difference between the calciuric response of the low and high fat meals could be associated with nutrients found in the meals other than fat. The use of 'Skinny Milk' in the low fat meal, with its greater lactose content, compared to whole milk in the high fat meal, and may have contributed to the result. Lactose has been shown to facilitate calcium absorption [37] Lange, (1979). As more lactose was present in the low fat meal compared to high fat meal (HFM=11 g), calcium absorption may have been boosted. This combined with a low fat level could have contributed to the significant difference in the results during the first thirty minute period.

The results show that after the first half hour no significant differences between calciuric response of the low and high fat meals. Figure 2 clearly illustrates the large fall in calcium excretion after this period in the low fat meals. These results may be attributable to the rate of fatty acid digestion approached the rate of fatty acid absorption increasing so that there is less excess free fatty acid in the latter periods to complex  $\text{Ca}^{++}$ .

Free fatty acids not immediately absorbed can accumulate in the gut lumen and react with calcium ions to form insoluble calcium complexes. These compounds are not able to diffuse through epithelial cell membrane of the intestinal microvilli. Therefore, they are voided with faeces as has been reported by Potter (1990) [38] and Appleton (1991) [40] when they examined the FFA content of faeces after HF diets and LF diets.

In this study [39] the effect on calciuria of saturated fat meals (SFM) was also compared with unsaturated meals (USFM). This time the rate of excretion of urinary calcium was measured as the ratio to creatinine.

The results of the comparison between SFM and USFM showed that there was no recognisable pattern relating oil or solid fat diets with calcium excretion. However some individuals showed substantial difference between the two diets while others did not. For calcium excretion four showed SFM > USFM, three showed USFM > SFM and three showed USFM = SFM.

#### 4. Calciuria and Plasma insulin

The study regarding fat and calciuria above [39] showed a clear pattern in regard to insulinemia and renal calcium excretion rate. This was irrespective of the type of meal consumed. The insulin responses showed no correlation with meal type. When insulin showed a peak plasma concentration calcium / creatinine ratio was low and when urinary calcium /creatinine showed a peak the plasma insulin levels were low. This was a similar relationship to that observed with high protein meals [41]. Allen et. al. (1981) [8], Wood (1983) [42] and Howe (1990) [43] have reported correlations between the increased postprandial insulin release found with protein diets and the increased urinary calcium excretion. However the results by Brazier [39] showed the opposite response. No change was found in the levels of parathyroid hormone, active vitamin D or cyclic AMP by Schuette, et. al. (1980) [44].

The involvement of insulin and calciuria may be significant as indicated by several report of the difference in occurrence of osteoporosis in subjects with type I and type II diabetes mellitus. E.g., Leidid-Buckner and Ziegler (2001) [45]. They report that people with type I diabetes exhibit low bone density (i.e. less calcium) and people with type II diabetes have normal or greater bone density (i.e. more calcium). Osteoporosis Australia (2014) [46] suggest that although people with type II diabetes are more likely to have bone fractures than normal people this is probably due to increased falls and inactivity even though they have normal bone density.

An experiment by Brazier [47] aimed to take a more detailed look at the correlation of plasma insulin and postprandial calciuria revealed in experiments [14] and [39] but with all individuals consuming similar meals. Results for the study by Brazier [47] are shown in Figure 4 and Figure 5.

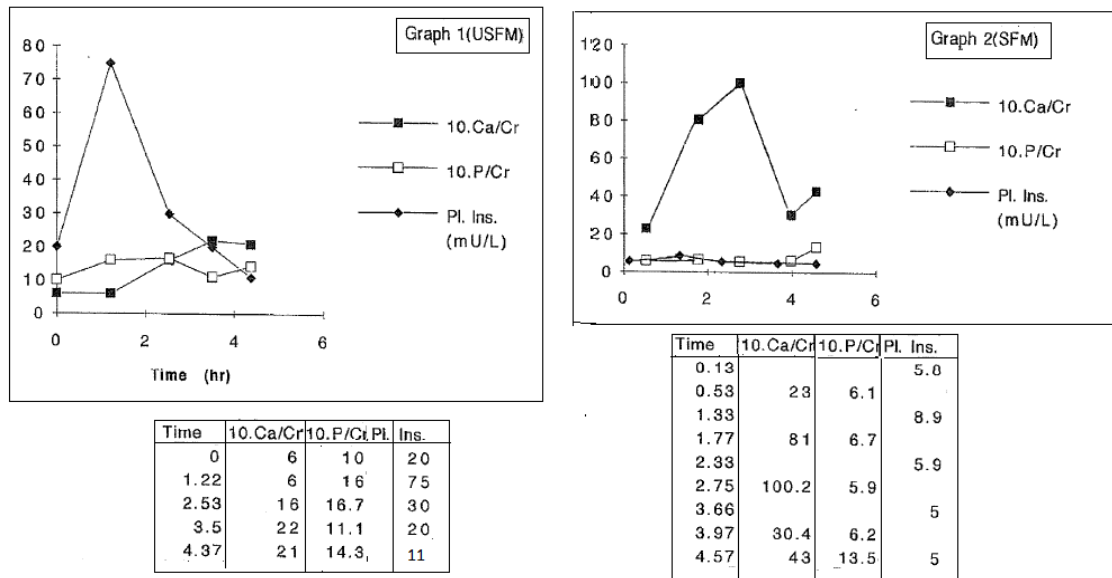


Figure 4. Displayed here is as an example of individual result that contribute to the correlation of plasma insulin and calciuria From Brazier [47]

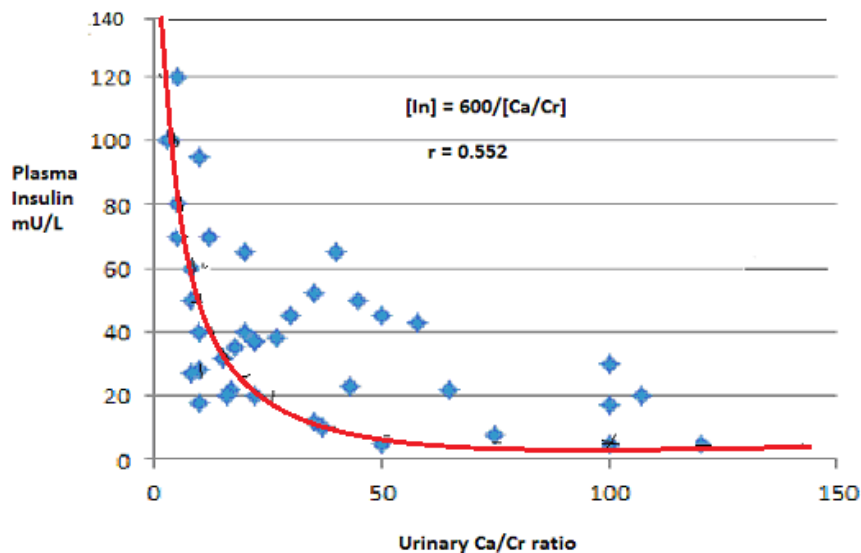


Figure 5. shows the Plasma Insulin concentrations in mU/L and the corresponding Urinary Ca/Cr ratios. The Pearson's correlation coefficient for the graph in Figure 5 is  $r = -0.552$  which indicates there is a definite inverse relationship. From Brazier [47]

Brazier's results [47] show a strong indication that insulin may have an effect that can greatly modify the hypercalciuria. In fact when looking at the individual results of each subject it appears that when a subject produces a high plasma insulin response the calciuria is markedly reduced. The effect of insulin could be because it causes increased protein anabolism thus reducing sulphate production and less acid excretion. The reduced calciuria could also be the result of interplay between insulin and the renin-angiotensin system or a direct effect of insulin on calcium transport in the kidney tubules.

These results show that insulin has a suppressing effect on calciuria which is the opposite effect suggested by Wood and Allen (1983) [42] and Howe (1990) [43]. This is shown clearly in Figure 4 and Figure 5. Reduced calcium excretion can be caused by reduced glomerular filtration rate or by increased fractional reabsorption. The effect of insulin on fractional reabsorption is a subject of a study by Brazier [48] which looks at the effect of insulin on membrane transport of  $Ca^{45}$  in isolated rat renal tubules and includes an additional study looking at possible biochemical causes this insulin effect.

The observations by Brazier [14] and [39] show that some young health individuals with no apparent pre-diabetes had exaggerated insulin responses. It may be that insulin influenced calciuria could be a contributor to idiopathic nephrolithiasis. i.e. low plasma insulin could contribute to nephrolithiasis.

### 5. Effect of Insulin on Membrane $Ca^{2+}$ Transport in Kidney Tubules.

An experiment by Brazier [47] attempts to examine the possibility that insulin effects fraction reabsorption by looking at a series of  $Ca^{45}$  isotope exchange measurements using desaturation analysis to test the hypothesis that insulin effects  $Ca^{45}$  transport by inhibiting the gluconeogenesis stimulated by reduced pH. This gluconeogenesis is normally linked to ammoniogenesis and the ammoniogenesis is stimulated to produce the  $NH_3$  required to neutralise the urine. The mechanism of the effect of pH on calcium membrane transfer rates has been

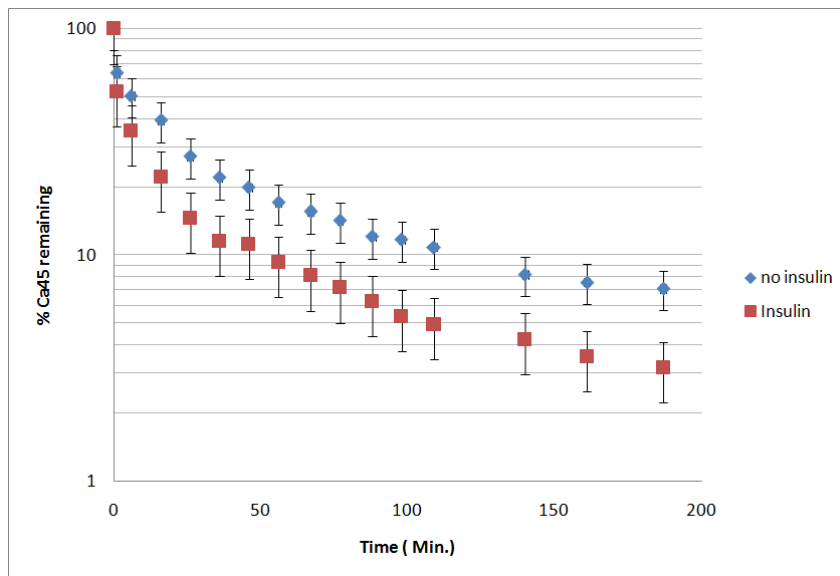
considered by Lemann (1967) [49] who suggested that metabolic acidosis has a direct effect on the metabolism of the renal tubular cells.

To test the above hypothesis the effect of various glutamine levels are tested to determine if the effect of pH is due to the ATP drain involved in ammoniogenesis /gluconeogenesis. This was described in a similar manner by Silva *et. al.* (1980) [50] who showed that sodium transport was inhibition by ammoniogenesis linked gluconeogenesis. The effects of a combination of insulin and glutamine are also compared with control tubules to see if insulin directly effecting renal calcium transport. Although Janda (1969) [51] observed increased calcium uptake when insulin was added to kidney slices, the use of isotope exchange by Brazier[48] allowed measurement of transmembrane movement under steady state conditions which should be a better measure of calcium reabsorption than the calcium uptake into tissue slices as done by Janda [51]. This is because tissue uptake does not differentiate between throughput and intercellular accumulations.

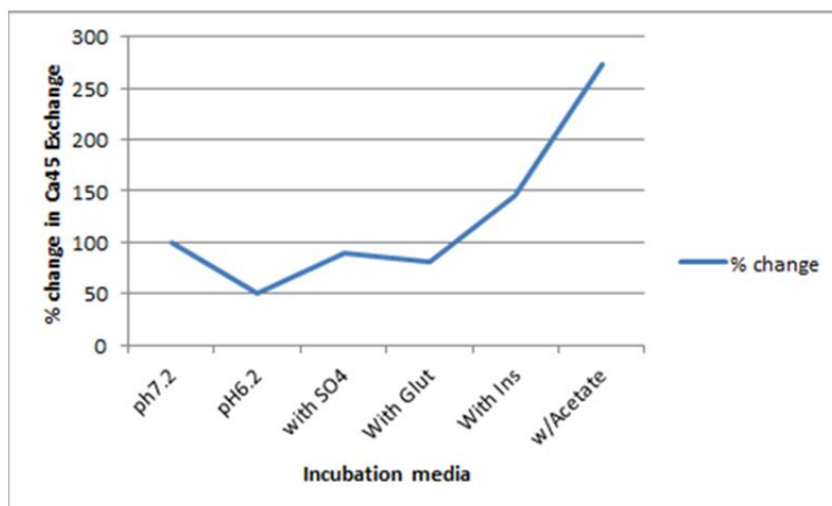
Kidney tubules are sometime reported as being insulin insensitive because they have a fast acting enzyme to

degrade insulin. But Mahler and Szabo (1967) [52] have shown that insulin insensitive tissues can react to insulin if insulin degradation mechanisms are impaired. The method used by Brazier's experiments [48] involved transferring tubules cells to fresh media every ten minutes so as to be similar to the continuous flow of *in vivo* conditions and thereby minimise the effect of insulin enzymic degradation.

In Brazier's [48] experiments a test which compared high pH with low pH indicated that reduced pH had a pronounced effect on the plasma membrane transport of calcium and a second test showed that the presence of sulphate had no significant effect. It has been suggested that metabolic acid affects calcium transport by changing the transferase stereochemistry, Studer and Borle (1979) [53], and it has also been suggested that sulphate may inhibit Calcium uptake by complexing with the  $Ca^{2+}$  ions Wlaser and Browden (1958) [54], the latter does not seem to occur in Brazier's study [48]. Metabolic sulphate probably has it effect by increasing plasma acid concentration and it is the pH drop rather than the sulphate that causes the reduced calcium exchange.



**Figure 6.** This graph regarding different insulin levels shows significant differences for high and low levels of insulin in regard to  $Ca^{45}$  transport. Error bars as Standard Errors. From Brazier [48]



**Figure 7.** Percentage change of  $Ca^{45}$  exchange rates From Brazier [48]

This graph shows the percentage change in  $Ca^{45}$  over the exchange rates in the pH7.2 set of media conditions.

This experiment [48] tested the hypothesis that ammoniogenesis uses mainly glutamine for deamination and thereby producing  $\alpha$ -ketoglutarate as a by-product. The  $\alpha$ -ketoglutarate is then usually removed by gluconeogenesis, the gluconeogenesis then uses up ATP making less ATP available for  $\text{Ca}^{++}$  absorption. When the cells were exposed to insulin the gluconeogenesis was inhibited and the  $\alpha$ -ketoglutarate was redirected into the citric acid cycle and extra ATP was produced. This allow an increase in  $\text{Ca}^{++}$  absorption as shown in Figure 7 Thus insulin had a direct effect of increasing calcium reabsorption indicating how it can causing less calciuria. These effects can be seen in Figure 6 that shows the  $\text{Ca}^{45}$  transport is much faster with Insulin compared to no Insulin. Figure 7 shows that the rate of transfer of  $\text{Ca}^{45}$  is reduced by lower pH and that inclusion of  $\text{SO}_4$  had no effect; whereas inclusion of glutamate reduced the rate of transfer and the addition of insulin and acetate greatly increased  $\text{Ca}^{45}$  transport. This later effect can be concluded as resulting from greater ATP availability. This hypothesis is also supported a later study by Brazier [55] that measured cellular ATP as well as ammoniogenesis.

It was also shown by Barzel (1971) [56] and Barzel and Jowsey (1969) [57] that ingestion of bicarbonate increased bone formation. Lemann *et al.*, (1965) [58] have suggested that acidosis has its effect because of the increased production of ammonium ion which may impairs calcium reabsorption

That increased GFR was sufficient to cause increased calcium loss without change to fractional reabsorption of calcium was shown in dogs by Massry and Kleemann (1972) [59]. However, Allen *et al.* (1979) [15] showed that there was a reduction in fractional reabsorption of

calcium in man, although Allen *et al.* [15] was not able to determine whether the reduction was due to saturation of the reabsorption process by the increased calcium load or if the fractional reabsorption rate was impaired.

## 6. Ammoniogenesis and Calciuria

The effect that ammoniogenesis and gluconeogenesis has on calcium transport is not clear. It could be that ammonia production or that ammonia itself has an inhibitory effect on calcium transport This dilemma is considered in and experiment by Brazier (2016) [55] or it could be that reduced supply of ATP caused by gluconeogenesis caused reduced calcium active transport in a manner similar to that described by Silva *et al.* (1980) [50] to explain the competition between sodium reabsorption and gluconeogenesis in kidney cells. The effect of small changes in ATP availability on calcium reabsorption was reported when humans were exposed to formic acid in the workplace. The action of formic acid is explained as a cytochrome oxidase inhibitor Lissivuori *et al.*, (1992) [60].

Observations in Brazier's experiments [14,39,47] & [48] of the effect on insulin on calciuria could be explained by its inhibitory effect on renal gluconeogenesis as per Hammerman, (1985) [61] making ATP more available for calcium uptake. The hypercalciuric effect of dietary caffeine reported by Whiting and Whitney (1987) [62] may be also be due to the stimulatory effect caffeine has on gluconeogenesis as described by Sach and Forster (1984) [63] and the resulting depletion of available ATP.

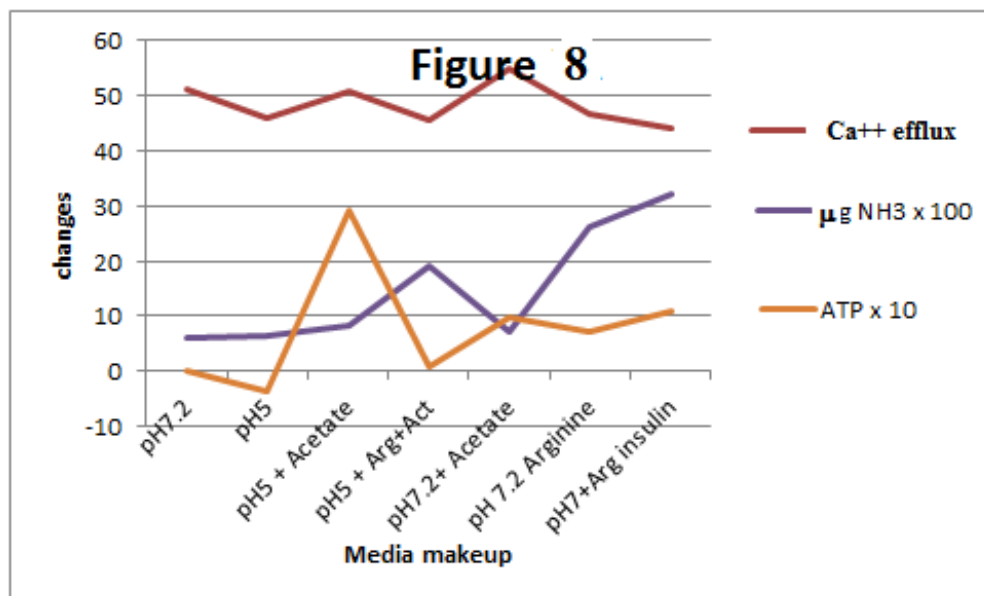


Figure 8. Changes in  $\text{Ca}^{++}$ ,  $\text{NH}_4$  and ATP. From Brazier [48]

This is a diagrammatic representation of the changes in  $\text{Ca}^{++}$  release, ammonia production  $\times 100$  and  $10 \times$  ATP of renal tubular cells content when incubated in different media.

In the Brazier's experiment [48] the calcium efflux from isolated kidney tubules was measured after one hour of incubation at  $37^\circ\text{C}$  using media with which included glutamine or arginine in combination with high pH (7.2) or low pH (5.2) and the with additions of either acetate or insulin. Measurements were also made of ammonia production and after incubation the remaining intracellular ATP.

In Figure 8 from Brazier [48] Calcium transport is shown to be affected by changes in pH. This effect may be due to the stimulation of ammoniogenesis caused by the increased hydrogen ion concentration. This increase in ammoniogenesis appears to result from activation of glutaminase and inhibition of citric acid cycle [7] Nissim, (1991). The corresponding change in calcium transport

may be due to reduced availability of ATP, Figure 8 does show some changes in intracellular ATP that may be due to ATP usage by the gluconeogenesis that is usually coupled with ammoniogenesis. Calcium fluxes involve an ATP driven active transport therefore any reduction in ATP could explain the reduction in calcium movement.

That reduced pH inhibits calcium transport across renal tubule membranes has been previously demonstrated by Studer and Borle (1979) [53] and that pH stimulates ammoniogenesis has been well established Tannen (1978) [64]. What has been demonstrated in this experiment [48] is the connection between the two effects of pH supports the observations of others and also shows that the effect of low pH on calcium transport is reversed by the addition of acetate. However when acetate is combined with arginine calcium transport is no increased

These observations could be explained if ATP levels controlled calcium transport. The increased ammoniogenesis in the low pH media would divert ATP away from calcium transport through its concomitant stimulation of gluconeogenesis and inhibition of the citric acid cycle as reported by Nissim (1991) [65]. The addition of acetate could have increased calcium transport by restoring the ATP availability by activation of the citric acid cycle. A similar explanation has been used to explain the effect of pH on renal handling of sodium by Silva *et al.* (1980) [50]. When arginine and acetate are both included in the media the reduced calcium efflux could have been because the extra ammoniogenesis was more than the energy yielding effect of the acetate.

The effects of acetate and arginine are observed to be similar in the pH 7.2 media as was observed in the pH 5 media, indicating the reduced pH is not essential to trigger the effect of either agent.

Measurement of ATP levels showed only significant differences for the pH 5 media and the pH 7.2 with arginine. These changes are not all consistent because a reduction would have been expected for the pH 5.0 media with arginine and an increase in the pH 7.2 media with acetate. It is not surprising the changes in ATP concentration are not observed as cells maintain such levels fairly constant. However it may not necessarily be the actual average level of ATP in a cell that is important in this consideration rather it may be the throughput or flux of ATP moving within the cell. The ATP concentration in the cytosol near the  $\text{Ca}^{++}/\text{ATPase}$  pump and the sodium/potassium pump could be reduced while ATP in some other part of the cytosol or mitochondria is maintained by intracellular compartmentalisation and intracellular membrane transport of ATP from the mitochondria.

The movement of calcium out of cells seems to involve three mechanisms one is an ATP driven pump that acts as an antiportal(PMCA) exchanging  $\text{Ca}^{++}$  for  $\text{H}^+$ . The other is a  $\text{Na}^+/\text{Ca}^{++}$  antiportal that exchanges 3  $\text{Na}^+$  ions for each  $\text{Ca}^{++}$  ion Racher, (1980) [67]. The third exchanges  $\text{Na}$  for  $\text{Ca}^{2+}$  and  $\text{K}^+$ . The  $\text{Na}^+/\text{Ca}^{++}$  antiportal is ATP dependant but not in a stoichiometric fashion. This establishes the concentration gradient that is used to drive  $\text{Na}^+/\text{Ca}^{++}$  antiportal. As the sodium flows back into the cell the calcium is expelled. As shown in Figure 9.

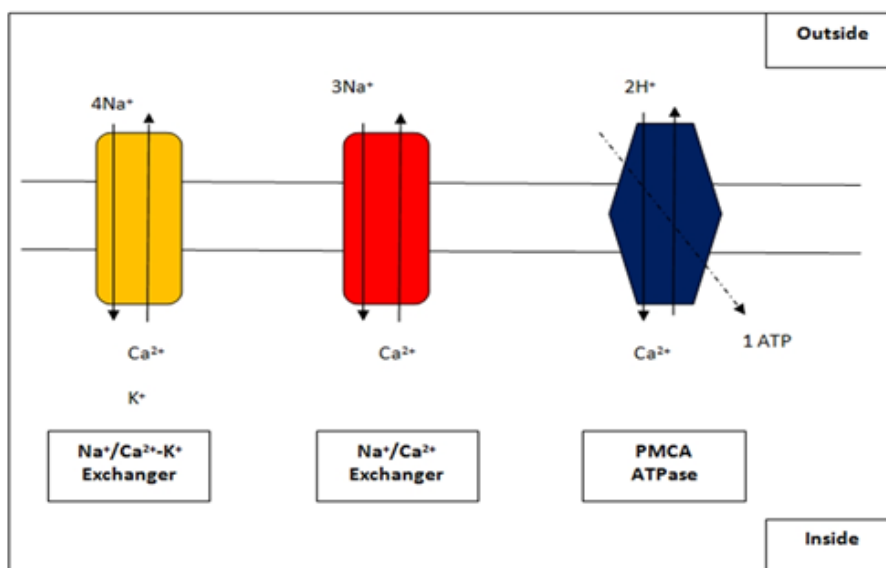


Figure 9. Mechanism of transport of  $\text{Ca}^{++}$  into cells

Three mechanisms one is an ATP driven pump that acts as an antiportal(PMCA) exchanging  $\text{Ca}^{++}$  for  $\text{H}^+$ . The other is a  $\text{Na}^+/\text{Ca}^{++}$  antiportal that exchanges 3  $\text{Na}^+$  ions for each  $\text{Ca}^{++}$  ion, the third exchanges  $\text{Na}$  for  $\text{Ca}^{2+}$  and  $\text{K}^+$ . From Richard Ali [69].

Even though the level of ATP in cells is known to be carefully maintained, variations in intracellular regions are used to explain the control of other cellular activities.

The fact that significant, although small, changes in ATP concentration were observed when cells were incubated in pH 5 media compared to pH 7.2 this may support the hypothesis that the ATP concentrations can be a limiting factor for  $\text{Ca}^{++}$  efflux.

In experiment [48] similar changes to calcium fluxes were observed when tubules were put into pH 5 media

with acetate and without acetate i.e., the calcium fluxes were reduced by the low pH and even more so by the low pH with arginine present. The inclusion of insulin however in the pH 5 medium alone and with arginine increased calcium fluxes but without changing the ammoniogenesis. These observations could further support the suggestion that ATP availability is a regulator of calcium efflux because insulin is known to be an inhibitor of renal gluconeogenesis Hammerman, (1985) [61] and



once gluconeogenesis is slowed ATP could be diverted to the ATPase transporters in the membranes.

Arginine was used in Experiment [48] and produced effects that could be related to the findings of Wood and Allen (1983) [67]. In order to evaluate the involvement of insulin in hypercalciuria Wood and Allen [67] infused rats with arginine and observed a consequential hyperinsulinemia and hypercalciuria and suggested that there could be a causal relationship between the two results.

When ammoniogenesis is uncoupled from gluconeogenesis ATP levels could increase due to  $\alpha$ -ketoglutarate being directed into the tricarboxylic acid cycle and producing extra ATP instead of causing a reduction of ATP. If this is so, under acid condition calcium transport could increase in the presence of insulin more than under neutral conditions:

The inclusion of pyruvate has a similar effect on calcium exchange and ammonia production as did acetate. The effect of these two agents can be explained in terms of their effect on the availability of ATP. As referred to above insulin could slow gluconeogenesis and divert ATP to calcium efflux. Pyruvate could provide ATP by entering the citric acid cycle. Figure 8 shows that there is significant difference between the calcium exchange and ammoniogenesis between media with glutamine and glutamine with insulin or pyruvate.

The negative effect that caffeine has been shown to have on calcium reabsorption by Massey and Opryszek (1990) [68], Massey and Hallingberg (1988a) [70] and (1988b) [71] and Whitney (1987) [72]. This could be due to its reported stimulation of gluconeogenesis Sashs and Forster, (1984) [63] and consequent reduction intracellular ATP.

## 7. Membrane Transport of Ammonia

The release of ammonia is closely related to reduction in calcium uptake it is possible that ammonia itself may interfere with the calcium transport a future experiment will considers some aspects of this possibility.

There is little reference in the literature as to membrane transport of ammonia. Gannon (1977) [73] describes ammonia transport as non-ionic diffusion. Non-ionic diffusion is used to explain diffusion of weak acids and bases across plasma membranes. In this explanation the ionised form of the material arrives at a membrane surface then it changes to its non-ionised configuration to allow diffusion through the nonpolar region of the phospholipid membrane. This description is satisfactory for weak acids and urea which have a non-polar configuration in their molecular form. However, ammonia in its molecular form has a dipole moment quite similar to water i.e. molecular ammonia is polar:  $\text{NH}_3$  has dielectric constant of  $D = 1.46$ ;  $\text{H}_2\text{O}$  has a dielectric constant of  $D = 1.84$  compared with  $\text{CCl}_4$ ,  $D = 0.0$  Zumdahl, Zumdahl (2013) [74]. Water is unable to diffuse across membranes by non-ionic diffusion and in fact requires protein bordered pores in membranes through which to pass. It seems impossible then for ammonia in its molecular form to diffuse as it is not nonpolar molecule. It may be that ammonia uses pores or transporters like water or it may be it alters its molecular configuration so as to produce an unknown nonpolar configuration. If the former is correct ammonia may compete with calcium for transport; but not so likely if the later idea is the true behaviour of  $\text{NH}_3$ .

Ammonia is very toxic due to the inability of cells to regulate its passage through membranes. If ammonia used pores or transporters, cells would have long ago evolved a means to regulate those apertures. But this has not happened so it may be that ammonia changes to a non-polar form. The experiment by Brazier [75] describes a spectrophotometric examination of ammonia in polar and non-polar environments to look for any change in its molecular properties.

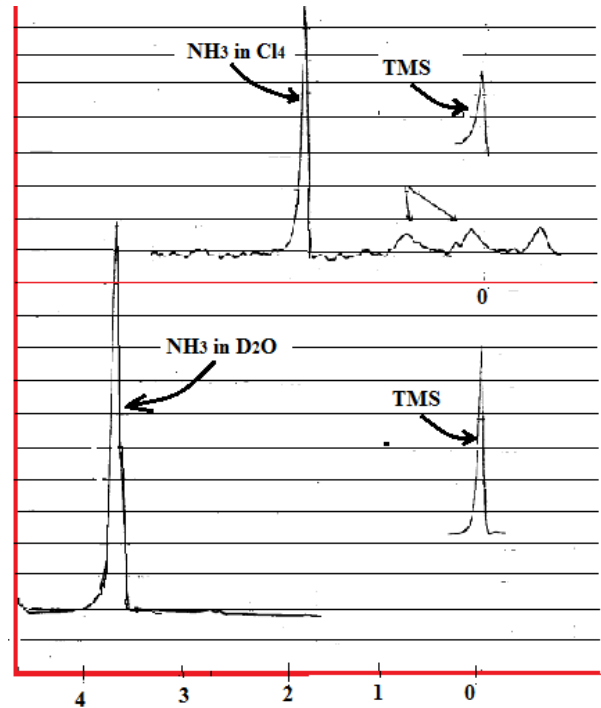


Figure 10. NMR of ammonia in  $\text{D}_2\text{O}$  and  $\text{CCl}_4$  From Brazier [75]

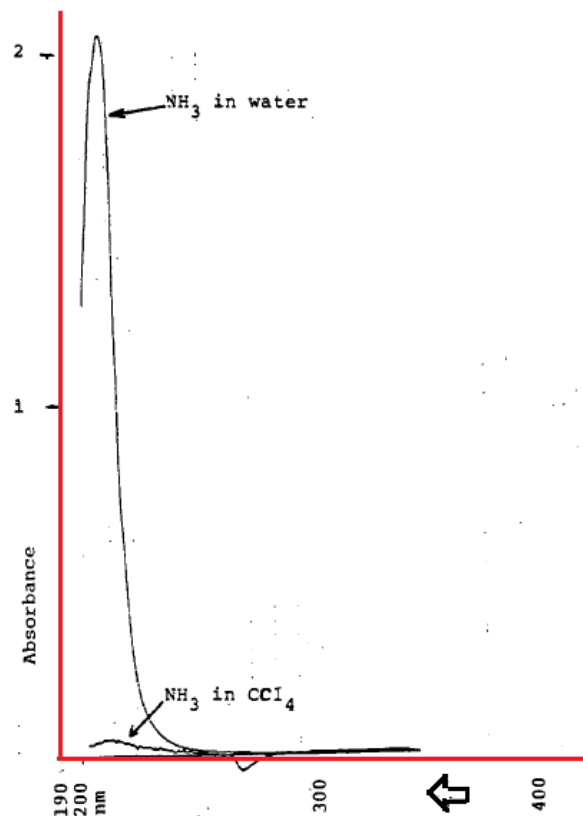


Figure 11. UV Spectra of ammonia in  $\text{D}_2\text{O}$  and  $\text{CCl}_4$  solvents. From Brazier [75]

For ammonia to pass through cell membranes by non-ionic diffusion it must change to a nonpolar configuration. The normal molecular form of ammonia in liquid and crystalline ammonia has a tetrahedral configuration with the three hydrogens extending down from the nitrogen to form a triangular pyramid Morris and Boyd (1983) [76] the apex of the pyramid is occupied by a lone pair of electrons. The negative charge on the lone pair of electrons and the positive charge in the three hydrogen nuclei are at opposite ends of the molecule and thus produce the dipole moment of  $D = 1.46$ . This configuration is explained by the electrons in the nitrogen being distributed into four equal tetrahedrally arranged  $Sp^3$  hybrid  $sp$  orbitals in the same manner as oxygen and carbon does in forming water and methane molecules respectively. As shown in Figure 12.

Spectroscopy of ammonia reveals that  $NH_3$  undergoes inversion i.e. turns inside out. There is only a small energy barrier of 2.5 kJ slowing this inversion and ammonia can absorb 210 nm light when it resonates between the two configurations.

In order to invert ammonia must pass through a higher energy intermediate where the lone pair of electrons are distributed equally on both sides of the molecule and the ammonia molecule

is in a planar configuration with equal bond angles of  $120^\circ$ . This intermediate configuration is most likely a transitory  $Sp^2$  hybridised form. Such a configuration is known to occur as the stable molecular form of  $BF_3$  with a dipole moment of  $D = 0$ .

The  $Sp^2$  planar form of ammonia is least stable in polar solvents and liquid ammonia because of the interparticle forces of the surrounding particles that tend to stabilise the polar configuration. However in nonpolar solvents the stabilisation of the tetrahedral configuration is no longer available and the planar form is likely to be stabilised by hydrophobic bonding.

The results of this experiment confirm that such a conformational change occurs because the absorbance of light at 210 nm is removed in the nonpolar solvent see Figure 11. Indicating the molecule no longer inverts and the disappearance of the NMR absorbance at 3.6 ppm and the appearance of a peak at 1.8 ppm and 0 ppm indicate that the H atoms have changed their orientation. See Figure 10.

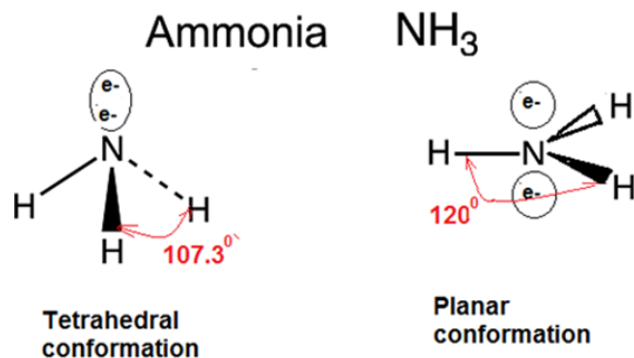


Figure 12. Alternative molecular configurations of ammonia

The tetrahedral configuration that ammonia has in water and the planar configuration it has in nonpolar solvents. From Brazier [75].

Membrane diffusion of ammonia probably involves ammonia changing from the polar tetrahedral form at the cell membrane surface and assuming the planar non polar form for its passage through the nonpolar region of the

membrane, reforming its polar configuration on the other side. Ammonia might be called an 'amphipolar' molecule because of its change from polar to nonpolar.

Because ammonia does not require transporters, pores or channels to cross membranes it is unlikely that it interferes with ions or polar molecules that do. It is therefore not likely that ammonia directly slows the passage of calcium into serial tubular epithelial cells.

The combined results of Brazier's experiments [14,39,47,48,57] and [75] all support the hypothesis that insulin has an important effect on calciuria and that it has this effect by inhibiting gluconeogenesis so that more ATP is made available for active transport of Ca.

## 8. Conclusion

Examination of the mechanism of dietary protein induced hypercalciuria was shown to be significant and that insulin can have a significant effect on modifying the level of calciuria. The effect of insulin was shown to work via it inhibiting gluconeogenesis so that ATP is made more available so that active transport of Ca increased. Consideration was also given to the role of pH that can result from a high protein meal and it was demonstrated to work by stimulating ammoniogenesis coupled to gluconeogenesis thus reducing the availability of ATP. The membrane transport of ammonia that is also produced by ammoniogenesis was examined to see if it could have a direct inhibitory effect on Ca transport. It is shown to change its molecular configuration to a non-polar form and diffuse through membranes in a manner that would have no effect on Ca transport. Dietary lipids are also considered in regard to possible effect on calciuria and they were shown to have no significant effect on calciuria.

## References

- [1] Matkovic, V. (1991). 'Calcium metabolism and calcium requirements during skeletal modelling and consolidation of bone mass', *Am.Jn.Clin.Nut.*, 54 (Suppl): 245S-60S.
- [2] Eriksen EF and Glerup H, (2002). 'Vitamin D deficiency and aging: implications for general health and osteoporosis.' *Biogerontology*, 3(1-2) 73-7.
- [3] Atkins International (2015), 'Atkins Low carb expert' <http://au.atkins.com/?gclid=CluQpcb0is0CFVgmvQodemcGRQ>.
- [4] Noakes M, Clifton, P, (2005), "The CSIRO Total Wellbeing Diet' CSIRO Total Wellbeing Diet series, Australia.
- [5] Maalouf NM, Moe OW, Adams-Huet B, Sakhaee K. (2011), 'Hypercalciuria associated with high dietary protein intake is not due to acid load'. *J Clin Endocrinol Metab.* Dec; 96(12): 3733-40.
- [6] Stefania, S, C. Cattelan, G. Realdi, and S. Giannini, 'Bone disease in primary hypercalciuria', *Clin Cases Miner Bone Metab.* 2008 May-Aug; 5(2): 118-126.
- [7] Kevin K. F and Bushinsky D. A., (2003) 'Molecular Mechanisms of Primary Hypercalciuria' *J Am Soc Nephrol* 14: 1082-1095.
- [8] Abelow, S.I., Holford, T.R and Insogna, K.L. (1992) 'Cross-cultural association between dietary animal protein and hip fracture: a hypothesis', *Calcif. Tissue Int.*, 50(1): 14-18.
- [9] Kaneko, K, Masaki, U., Aikyo, M., Yabuki, K, Haga, A, Matoba, c., Sasaki, H. and Koike, G. (1990) 'Urinary calcium and calcium balance in young women affected by high protein diet of soy protein isolate and adding sulfur containing amino acids and/or potassium', *J.Nut.Sci.Vitaminol.*, Tokyo, 36(2): 105-16.
- [10] Fernandez-Repollet, E., Van Loon, P. and Martinez-Maldonado, M. (1989) 'Renal and systemic effects of short-term high protein feeding in normal rats', *Am.J.Med.Sci.*, 297(6): 348-454.

- [11] Funaba, M., Yano, H. and Kawashima, R (1989) 'Effects of high protein diet and sodium bicarbonate supplementation on calcium metabolism in rats', *J.Nur.Sci.Vitaminol.*,Tokyo, 35(5): 495-503.
- [12] Schneider, W. and Menden, E. (1988) 'The effect of long term increased protein administration on mineral metabolism and kidney function in the rat. II. Kidney function and bone mineralization', *Z-Ernahrungswiss.*, 27(3): 186-200.
- [13] Schuette, S.A, Zemel, M.B. and Linkswiler, H.M. (1980) 'Studies on the mechanism of protein-induced hypercalciuria in older men and women', *J.Nut.*, 110(2): 305-15.
- [14] Brazier (2016a) 'Effect of Insulin Hypercalciuric Effect of High Protein Diets', *American Journal of Food and Nutrition*, Vol. 4, No. 1, 20-29
- [15] Allen, L.H., Oddoye, E.A and Margen, S. (1979), 'Protein-induced hypercalciuria: a longer term study', *American Journal of Clinical Nutrition*, 32: 742-49.
- [16] Margen, S., Chu, J.Y., Kaufmann, N.A and Calloway, D.H. (1974) 'Studies in calcium metabolism. I. The calciuretic effect of dietary protein', *Am.Jn.Clin.Nut.*, 27(6): 584- 9.
- [17] Chu, J.Y, Margen, S., Costa, F.M. (1975) 'Studies in calcium metabolism. II. Effects of low calcium and variable protein intake on human calcium metabolism', *Am.Jn.Cl.Nut.*,28(9): 1028-35.
- [18] Hegsted, D.M., Moscoso, M.I. and Colago, G. (1952) 'Study of minimum calcium requirements of adult men', *In.Nutr.*, 46: 181-201.
- [19] McCance, RA, Widdowson, E.M. and Lehmann, H. (1942) 'The effect of protein intake on the absorption of calcium and magnesium', *Biochem.Jn.*, 36: 686-91
- [20] Adolf, W.H. and Chen, S.C (1932) 'The utilization of calcium in soy bean diets', *In.Nutr.*,5: 379..
- [21] Kalk, W.J. and Pimstone, B.L. (1974) 'Calcium-binding protein and vitamin D metabolism in experimental protein malnutrition', *Brit.Jn.Phvs.*, 32(3): 569-78.
- [22] Wachman, A and Bernstein, D.S. (1960) 'Diet and osteoporosis', *Lancet*, 1: 958-59.
- [23] Goulding, A, Mathus, RS. (1970) 'Effects of protein content of diet on the development of nephrocalcinosis in rats', *AusUn.Exp.Med.Sci.*, 48: 313-20.
- [24] Margen, S., Kaufmann, N.A Costa, F. and Calloway, H. (1970) 'Studies in the mechanism of calciuria induced by protein feeding', *Fed.Proc.*, 29: 566.
- [25] Benke, S.S. (1978), 'Effect of dietary protein source on urinary calcium and zinc in adult male rats', *Fed Proc.*, 37(3) 372.
- [26] Whiting, S.I. and Draper, H.H. (1978) 'Studies on the calciuric effect of excess dietary protein', *Fed.Proc.*,37: 847.
- [27] Davis, P.N., Norris, L'C, and Krater, F.H. (1962) 'Interference of soybean protein with the utilization of trace minerals', *In.Nut.*, 77: 217-23.
- [28] Nielsen, FH., Sunde, M.L. and Hoekstra, W.G. (1966) 'Effect of dietary amino acid source on the zinc-deficiency syndrome in the chick', *In.Nut.*, 89(1): 24-34.
- [29] Gregor, J.L., Baligar, P., Abernathy, RP., Benett, O.A and Peterson, T. (1978) 'Calcium, magnesium, phosphorus, copper and manganese balance in adolescent females', *Am.Jn.Cl.Nut.*, 31(1): 117-21.
- [30] Spencer, H., Kramer, L., Osis, D. and Norris, C. (1978a) 'Effect of a high protein (meat) intake on calcium metabolism in man', *Am.Jn.Clin.Nut.*, 31(12): 2167-80
- [31] Kerstetter, J.E. and Allen, L.H. (1990) 'Dietary protein increases urinary calcium', *J.Nut.*, 120(1): 134-6.
- [32] Grant,C,(2002) *The Atkins Diet: An Unresolved Debate*, *Nutrition Noteworthy*, 5(1).
- [33] Luscombe-Marsh, Noakes M, Wittert GA, Keogh JB, Foster P, Clifton PM. (2005), 'Carbohydrate-restricted diets high in either monounsaturated fat or protein are equally effective at promoting fat loss and improving blood lipids.' *Am J Clin Nutr.* 2005 Apr;81(4):762-72.
- [34] Wood, RJ., Alien, L.H. and Bronner, F. (1984) 'Regulation of calcium metabolism in streptozotocin-induced diabetes', *Am.J.Physiol.*, 247(1 Pt 2): R120-3)
- [35] Santos, F., Suarez, D., Malaga, S. and Crespo, M. (1987) 'Idiopathic hypercalciuria in Children: Pathophysiologic considerations of renal and absorptive subtypes', *I.Pediatr.*, 110: 238-43.
- [36] Pike, R and Brown, M. (1984), *Nutrition: An Integrated Approach*, WHEy and Sons, Canada.
- [37] Lange (1979), *Review of Physiological Chemistry*, Lange Medical Publications, California.
- [38] Potter, S.M., Kies, e.V. and Rojhani, A (1990) 'Protein and fat utilization by humans as affected by calcium phosphate, calcium carbonate and manganese gluconate supplements', *Nutrition (Burbank)*, 6(4): 309-12.
- [39] Brazier BW (2016b) 'Effect of the Level of Dietary Fat and Fat Type on Postprandial Calciuria and Involvement of Insulin', *American Journal of Food and Nutrition*, 2016, Vol. 4, No. 2, 55-62
- [40] Appleton, G.U.N., Owen, R, Wheeler, E.E., Challacombe, D.N. and Williamson, RCN. (1991), 'Effect of dietary calcium on the colonic luminal environment', *Gut.*, 32(11): 1374-7.
- [41] Allen, L.H., Block, G.D., Wood, Rl. and Bryce, G.F. (1981), 'The role of insulin and parathyroid hormone in the protein induced calciuria of man', *Nut.Res.*, 1981, 1: 3-11.
- [42] Wood, RJ. and Allen, L.H. (1983) 'Evidence for insulin involvement in arginine and glucose-induced hypercalciuria in the rat', *J.Nutr.*, 113(8): 1561-67.
- [43] Howe, J.C. (1990) 'Postprandial response of calcium metabolism in postmenopausal women to meals varying in protein level/source', *Metabolism*, 39(12): 1246-52.
- [44] Schuette, S.A, Zemel, M.B. and Linkswiler, H.M. (1980) 'Studies on the mechanism of protein-induced hypercalciuria in older men and women', *J.Nut.*, 110(2): 305-15.
- [45] Leidid-Buckne, G and Ziegler, R (2001) 'Diabetes mellitus a risk for osteoporosis?'. *Experimental and Clinical Endocrinology & Diabetes* 109 (suppl 2): 493-514.
- [46] *Osteoporosis Australia (2014), 'Diabetes and osteoporosis Consumer Factsheet'*, 1st Edition 08/14.
- [47] Brazier BW (2016c), 'Calcium Exchange Rates in Rat Kidney Tubule Cells Affected by Insulin and pH', *American Journal of Food and Nutrition*, 2016, in press
- [48] Brazier BW (2016d), 'Effect of Serum Insulin on Calciuria Following Protein Meals in Humans', *American Journal of Food and Nutrition*, 2016, Vol. 4, No. 3, 63-67
- [49] Lemann, J., Litzow, J.R and Lennon, E.J. (1967) The effect of treatment of acidosis on calcium balance in patients with chronic azotemic renal disease, *In.Clin.Invest.*, 46(2) 280-6.
- [50] Silva, P., Ross, B. and Spokes, K. (1980) 'Competition between sodium reabsorption and gluconeogenesis in kidneys of steroid-treated rats', *Am.J.Physiol.*, 238(4): F290-5.
- [51] Janda, s. (1969) 'Mechanism of calcium transport in kidney cortex slices', *Physiologia Bohemoslovaca*, 18(5): 413-23.
- [52] Mahler, RJ. and Szabo, O. (1967) 'Insulin action upon insulin-insensitive tissue following impaired degradation', *Proc.Soc.Exp.Biol.Med.*, 125: 879-82.
- [53] Studer, RK. and Borle, AB. (1979) 'Effect of pH on the calcium metabolism of isolated rat kidney cells', *J.Membrane Biol.*, 48(4): 325-41.
- [54] Walser, M. and Browden, A (1958) Effect of Sulfate on Physical State and Renal Excretion of Divalent Actions, *J.Clin.Invest.*, 37, p.940.
- [55] Brazier BW (2016e), 'Ammoniogenesis, Gluconeogenesis and Calcium Exchange in Isolated Kidney Tubules', *American Journal of Food and Nutrition*, Vol. 4, No. 4, 93-102
- [56] Barzel, V.S. and Jowsey, I. (1969), 'The effects of chronic acid and alkali administration on bone marrow turnover', in *Clinical Science*, 36: 517-24.
- [57] Barzel, V.S. (1971) 'Alkali therapy in immobilization osteoporosis', *Isr.In.Med.Sci.*, 7(3): 499.
- [58] Lemann, J., Lennon, E.J., Goodman, AD., Litzow, J.R and Relman, AJ. (1965) 'The net balance of acid in subjects given large loads of acid or alkali', *In.Clin.Invest.*, 44: 507.
- [59] Massry, S.G. and Kleenman, CR (1972) 'Calcium and magnesium excretion during acute rise in glomerular filtration rate', *In.Lab.Clin.Med.*,80(5): 654-64.
- [60] Lissivuori, J., Laitinen, J. and Savolainen, H. (1992) 'Kinetics and renal effects of formic acid in occupationally exposed farmers', *Arch.Toxicol.*, 66(7), pp.522-4.
- [61] Hammerman, MR (1985) 'Interaction of insulin with the renal proximal tubular cell', *Am.J.Phvsiol.*, 249 (1 Pt 2), F1-11.
- [62] Whiting, S.I. and Whitney, H.L. (1987) 'Effect of dietary caffeine and theophylline on urinary calcium excretion in the adult rat', *J.Nutr.*,117(7): 1224-8.
- [63] Sachs, M. and Forster, H. (1984) 'Effect of caffeine on various metabolic parameters in vivo', *Z.Ernahrungswiss*, 23(3): 181-205.

- [64] Tannen, R.L. (1978) 'Ammonia metabolism', *Am.J.Physiol.*, 235(4): F265-77.
- [65] Nissim, I., Wehril, S., States, B., Nissim, I. and Yudkoff, M. (1991) 'Analysis of physiological implications of 2 oxoglutarate metabolism', *Biochem.J.*, 277(141): 33-8.
- [66] Racher, E. (1980) Fluxes of Ca<sup>++</sup> and concepts, *Fed.Proc.*, 39: 2422-26.
- [67] Wood, R.J. and Allen, L.H. (1983) 'Evidence for insulin involvement in arginine and glucose- induced hypercalciuria in the rat', *J.Nutr.*, 113(8): 1561-67.
- [68] Massey, L.K and Opryszek, AA (1990) 'Adaptation to dietary caffeine or calcium excretion in young women', *Nut.Res.*,10(7): 741-7.
- [69] Richard Ali, (2016) *Biology Notes Cell Biology Articles*, <http://www.fastbleep.com/biology-notes/31/174/1001>
- [70] Massey, L.K and Hallingberg, E.H. (1988a) 'Acute effects of dietary caffeine and aspirin on urinary mineral excretion in pre and post-menopausal women', *Nut.Res.*, 8(8): 845-51.
- [71] Massey, L.K and Hallingberg E.H. (1988b) 'Acute effect of dietary caffeine and sucrose on urinary mineral excretion of healthy adolescents', *Nut.Res.*,8(9): 1005-12.
- [72] Whiting, S.I. and Whitney, H.L. (1987) 'Effect of dietary caffeine and theophylline on urinary calcium excretion in the adult rat', *J.Nutr.*,117(7): 1224-8.
- [73] Gannon, W.F. (1977) *Review of Medical Physiol.*, 8th ed., Lange Med.Pub. Las Altos.
- [74] Zumdahl,S.S. and S.A.Zumdahl, (2013) 'Chemistry (AP Edition) 9th Edition, Brooks/Cole, Belmont Ca. USA.
- [75] Brazier BW (2016f) 'Membrane transport of ammonia', *American Journal of Food and Nutrition*, Vol. 4, in press.
- [76] Morris R T. and Boyd, R.N. (1983) *Organic Chemistry*, 4th ed., Allyn and Bacon Inc. Boston.