American Journal of Food and Nutrition, 2016, Vol. 4, No. 4, 93-102
Available online at http://pubs.sciepub.com/ajfn/4/4/2

DOI:10.12691/ajfn-4-4-2

Ammoniagenesis, Gluconeogenesis and Calcium Exchange in Isolated Kidney Tubules

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Abstract To study the connection between ammoniagenesis, gluconeogenesis and calcium exchange in kidney tubules, isolated kidney tubules were incubated in media with pH 5 and pH 7.2 and the exflux of calcium, ammonia production and cellular ATP levels was measure after a constant time period. Results indicate that calcium transport was reduced in low pH and by ammoniagenesis and increased my acetate and pyruvate and insulin inclusions. Results support the hypothesis that the calcium is controlled by ATP availability and that insulin can affect calcium transport by controlling ATP availability.

Keywords: *ammoniagenesis, gluconeogenesis, calcium exchange, hypercalciuri*

Cite This Article: B.W. Brazier, "Ammoniagenesis, Gluconeogenesis and Calcium Exchange in Isolated Kidney Tubules." *American Journal of Food and Nutrition*, vol. 4, no. 4 (2016): 93-102. doi: 10.12691/ajfn-4-4-2.

1. Introduction

This study is looking at the involvement and mechanism of the effect of insulin on calciuria. The involvement of insulin with 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. Eg, [1] Leidid-Buckner and Ziegler (2001). 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). [2] Osteoporosis Australia (2014) 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. This examination is also significant because of the increased use of high protein diets such as the diets recommended by Norma Atkins [3] and the Australian CSIRO. [4] Previous studies have shown that high protein diets produce hypercalciuria and such diets could be significant in the production of osteoporosis and nephrolithiasis.

The observations by Brazier [5] and [6] that some young health individuals with no apparent pre-diabetes had exaggerated insulin responses suggest that young people could be effecting their bone density in early life Another ramification could relate to the effect of calciuria on the development of nephrolithiasis [7] Frick, K.K. and D. A. Bushinsky, (2003),. It may also be that insulin is a contributor to idiopathic hypercalciuria [8] Worcester, E. M. and F. L. Coe. (2008).

The studies at this laboratory, regarding dietary protein induced hypercalciuria showed considerable variation in the induced calciuria [5] and studies regarding dietary fat and calciuria [6] also showed similar variation but both experiments showed strong inverse relationship between plasma insulin and calciuria. To determine if the effect of insulin were connected to glomerular filtration rate (GFR) or fraction reabsorption (FR) a third study involving the kinetic analysis of Ca45 desaturation curves [9] was conducted to measure the rate of transport of Ca through kidney tubule membranes and that experiment appeared to indicate a relationship between ammonia production and calcium transport and the Ca transport was an affected by insulin, glutamine and acetate.

As a result of these experiments [5,6] and [10] an hypothesis was suggested that insulin might inhibit Ca membrane transport of kidney tubules by inhibiting the gluconeogenesis that is connected to ammoniagenesis thereby making ATP more available for the active transport of Ca++.

This experiment aims to look in more detail at the effect of ammoniogenesis on calcium transport by using larger volumes of media so that ammonia can be measure along with the transported calcium and cellular ATP concentrations.

For some time dietary protein induced calciuria has been connected to increased renal acid and the reduced in pH has been known to increase renal ammoniagenesis [11] Tannen, (1978) and to use glutamine as the major substrate via the phosphate-dependent glutaminase pathway. In order for renal ammonia production to buffer urinary acidosis it is necessary to remove the H+ and NADH generated by the conversion of glutamine to α-ketoglutarate. (αKG)

e.g. glutamine + H_2O - glutamate + NH_3
and glutamate + NAD - KG + NADH + NH_3 + H+

Removal of intracellular αKΓ and hydrogen in tubular epithelium can be accomplished by either anabolism of αKΓ to glucose or catabolism to CO_2 and water via the mitochondrial citric acid cycle (CAC) [8] Tannen (1978). The connection between ammoniagenesis and gluconeogenesis has been demonstrated many times including [12] Nissim and States (1989) who were using cultured human renal cortical epithelial cells showed that reduced pH increased both ammoniagenesis and gluconeogenesis. See Figure 1.

There have been reports of an uncoupling of ammoniagenesis and gluconeogenesis by [13] Gougoux *et. al.* (1992) who showed that in the presence of 4-pentenoate glutamine uptake and ammoniagenesis was accelerated but gluconeogenesis suppressed. [11] Tannen (1978) also reviewed reports of ammoniagenesis and gluconeogenesis dissociation.

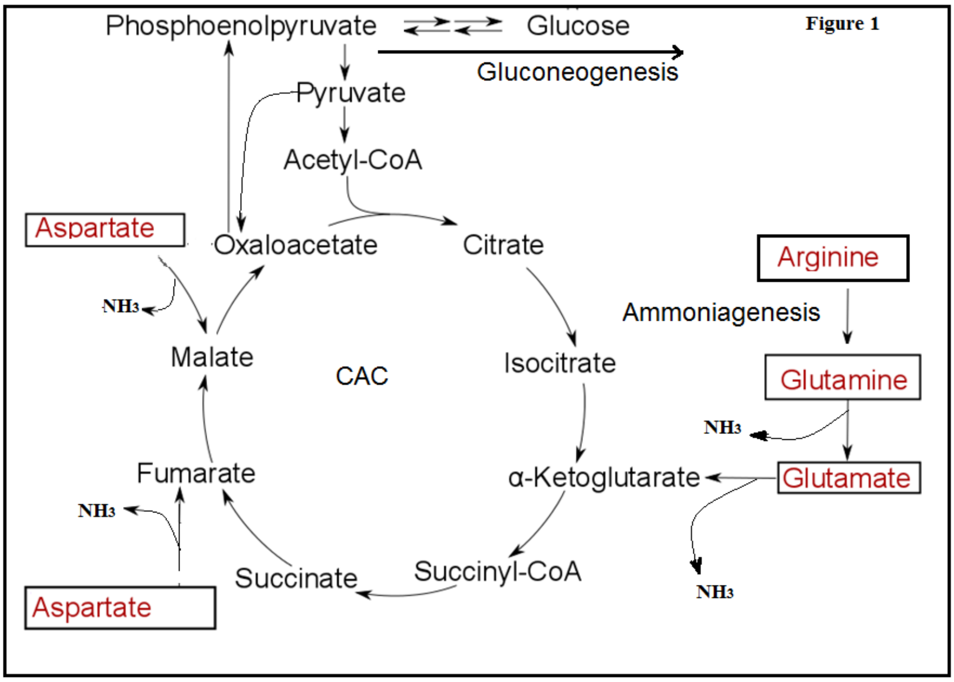

Figure 1. Relationship of ammoniagenesis and gluconeogenesis showing deamination of glutamine and aspartate as major sources of ammonia

The effect that ammoniagenesis 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 is considered in and experiment by [10] Brazier (2016) or it could be that gluconeogenesis has an effect by competing with calcium active transport for available ATP in a manner similar to that described by [14] Silva *et. al.* (1980). 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 [15] Lissivuori *et. al.*, (1992). Changes in ATP levels is reported in this experiment in Table 3A and Figure 3A The hypercalciuric effect of dietary caffeine reported by [16] Whiting and Whitney (1987) may be also be due to the stimulatory effect caffeine has on gluconeogenesis as described by [17] Sach and Forster (1984) and the resulting depletion of available ATP so less calcium is reabsorbed.

In this experiment calcium efflux from isolated kidney tubules is measured after one hour of incubation at 37°C using media with which glutamine or arginine in combination with high pH (7.2) or low pH (5.2) and the addition of either acetate or insulin. Measurements were also made of ammonia production and the intracellular ATP. remaining after incubation.

2. Method

Isolated renal tubules were prepared in a similar manner to those used by Brazier [9] in the Ca45 desaturation experiment so that the results can be correlated with the results form that report. The renal tubules were incubated in batches of six of media combinations per experiment as indicated in Table 1 and Table 2. Each experiment was then repeated five times with the same set of media combinations.

For each experiment six Sprague-Dawley rats weighing between 100g and 150g were sacrificed by stunning and neck dislocation. The kidneys were quickly removed and placed into ice cold normal saline. Each kidney was skinned and dissected to remove medulla and pelvic tissue then buttered through a 170-mesh sieve in 0.15 M saline then filtered through a 80-mesh sieve to remove tissue. The experiment was approved by the Deakin University Animal Ethics Committee.

Fragments and then through a 170-mesh sieve to remove glomeruli as described by [18] Price (1979). Fragments were washed with 0°C saline and centrifuged at low g, the supernatant decanted, resuspended and recentrifuged.

Tubules were incubated in media containing calcium for 60 min before being centrifuged and transferred into calcium free media with or without additions as indicated in Table 2.

The isolated tubules were divided equally by packed volume between six 50 cm^3 squat beakers and mixed with 10 cm^3 incubation media. The media were the same composition as that used in previous experiments [9] as per Table 1. When preparing media each dm^3 is made up with 500 ml containing the calcium chloride and 500 ml containing the phosphates salts. Equal aliquots of the two parts of the incubation media were mixed at less than 5°C just before each experiment then equilibrated in a water bath at 37°C for 10 min. This process was needed because the calcium and phosphate precipitated if they were mixed at ambient temperatures or stored overnight.

After incubations cell suspensions were filtered through acetate 'millipore' filters then the cells were washed with ice cold saline at the pump then the cells were either washed into a vial of cold TCA and sonicated or the filters and cells were dissolved in concentrated nitric acid.

Sonicated cells were used to assay ATP using a Sigma diagnostics enzymic ATP (No 366UV). Assay Aliquots of filtrate were used to determine effluxed calcium using atomic absorption spectrophotometry by the method of [19] Willis (1960) and for ammonia by the indophenol reduction method of [20] Chaney and Marbach (1962).

The amount of total calcium was obtained by multiplying the media calcium concentration by the media volume and adding it to total amount of calcium in the cell extract the effluxed calcium was then expressed as a percentage of the total calcium:

$$\%\ Efflux\ Ca = \frac{Media\ Ca}{Total\ Ca} \times \frac{100}{1}$$

Calcium exfluxed is recorded as percentage of the initial cell calcium and the ammonia produced is expressed as µg of ammonia per from of dry cell mass.

Table 1A. Incubation Media used in Experiment 1 - 3

Basal Media	pH 5.0	pH 7.2
KH_2P0_4	0.2 g/dm^3	0.2 g/dm^3
NaH_2P0_2	0.0	0.7934
Na_2HP0_4	0.36	0.00
$NaS0_4$	0.08	0.08
NaCl	80.0	80.0
CaCl	0.2 or nil	0.21 or nil
glucose	1.0	1.0
Addition to Basal Media (per drrr^3)		
Arginine	0.250 g	(Ar)
Glutamine	0.050 g	($gluNH_2$)
Insulin	100 ugm	(In)
Acetate	0.123 g	(Ac)
Pyruvate	0.125 g	(Py)

Media used for calcium efflux experiments 1 to 3 is shown. Two basal media with pH 5.0 and 7.2 were used with or without the additions that are show.

Table 1B. Media Combination Compared in Experiments 1 to 3

				Conditions				
Experiment		pH 5					pH 7	
Exp .1	nil	Ac	Ac		nil	Ac	Ac	
n=5			Ar				Ar	
Exp .2	nil	Ar	In	Ar	nil	Ar	Ar	
n=5				In			In	
Exp 3		$gluNH_2$	gluNH2	gluNH2	nil	gluHN2	$gluNH_2$	$gluNH_2$
n=5			In	Py			Py	In

Each experiment 1 to 3 compared the efflux of calcium from renal tubule and ammonia production while incubated in media with or without addition as indicated to basal media with either pH 5 or pH 7. Each experiment was repeated five times with the same set of media combinations and the difference of means results between combinations tested for significance with the student t-test. Average results are shown in Tables 2 A, 2B and 2C.

3. Results

After incubation of tubules the main changes in calcium efflux, ammonia production and intracellular ATP content compared to that of the pH 7.2 basal media is shown in Table 2A, 2B, and 2C. Results for the t-test of paired means and level of significance are also included in these tables for each experiment. The raw data for the corresponding set of six test are shown in Tables 3A, 3B. and 3C.

These results show that at low pH ammoniagenesis is increased and calcium transport is diminished and that the addition of arginine or glutamine increases ammonia production and decreases calcium fluxes in both neutral and acid conditions. The addition of acetate or pyruvate reduced ammoniagenesis and increased transport of calcium. However, the inclusion of insulin appeared to break the nexus between ammoniagenesis and the decreased calcium transport, because with addition of insulin calcium fluxes remained high even with either amino acid, or reduced pH, but with little change to ammoniogenesis.

Table 2A. Difference in Calcium Efflux, NH, Production and ATP content Between Each Medium and Basal Media

Experiment No. 1 Differences vs Basal Media (pH 7.2)						
	pH5			pH7.2		
Conditions Additions	Nil	Acetate	Acetate Arginine	Acetate	Acetate Arginine	Arginine
Ca^{2}+ Efflux % ±SD From Table 3A1	-10.16± 1.7 t=14;S	0.98±0.7 t=3.4 N	-10.9±1.3 t=21;S	+7.3±1.9 t=l.6 N	-9.1±1.9 11.6;S	-13±6 t=5.4;S
µg Ammonia Prod ±SD From 3A2	+.004±.003 3.25; N	+.024±.01 4.9 s	+.13±.01 t=25;S	+.012±.004 t=7.2;S	+0.2±.067 t=7.2;S	+0.26±.05 t=11.8;S
ATP in Cells ±SD From 3A3	-3.6±.5 t=14;S	+2.9±1.0 t=3.5;N	0±.07 t=O;N	0.96±0.8 t=2.6;N	0.7±1.7 t=0.9;N	1.1±0.64 t=3.7;N

Ave Ammonia Production in pH 7.2 with no additions = 0.060
Ave. percentage Ca++ released in pH 7.2 with no additions = 51.2
Differences are shown in percentage calcium effluxed and in total ammonia production for kidney tubules incubated in media with either pH 5 or pH 7.2 with addition of acetate, acetate and arginine or with no additions. Differences are also shown in the level of intracellular ATP compared to tubules incubated -in basal media at pH 7.2 the average ammonia production in the reference medium was 0.060 pgm!g of dry cell mass and the average percentage calcium released was 51.2.

Data presented in Tables 2A to 2C shows the mean differences between calcium exflux rates and ammonia production compared in each case with those rates obtained when tubules were incubated in pH 7.2 media with no additions.

The values are differences in percentage calcium exfluxed and μg ammonia produced per gram of cell mass.

The Tables 2A to D also show the student **t** test for each comparison and the corresponding levels of significance.

The Standard Deviation is calculated from the six experiments run for each set of media Using the formula

$$SD = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}.$$

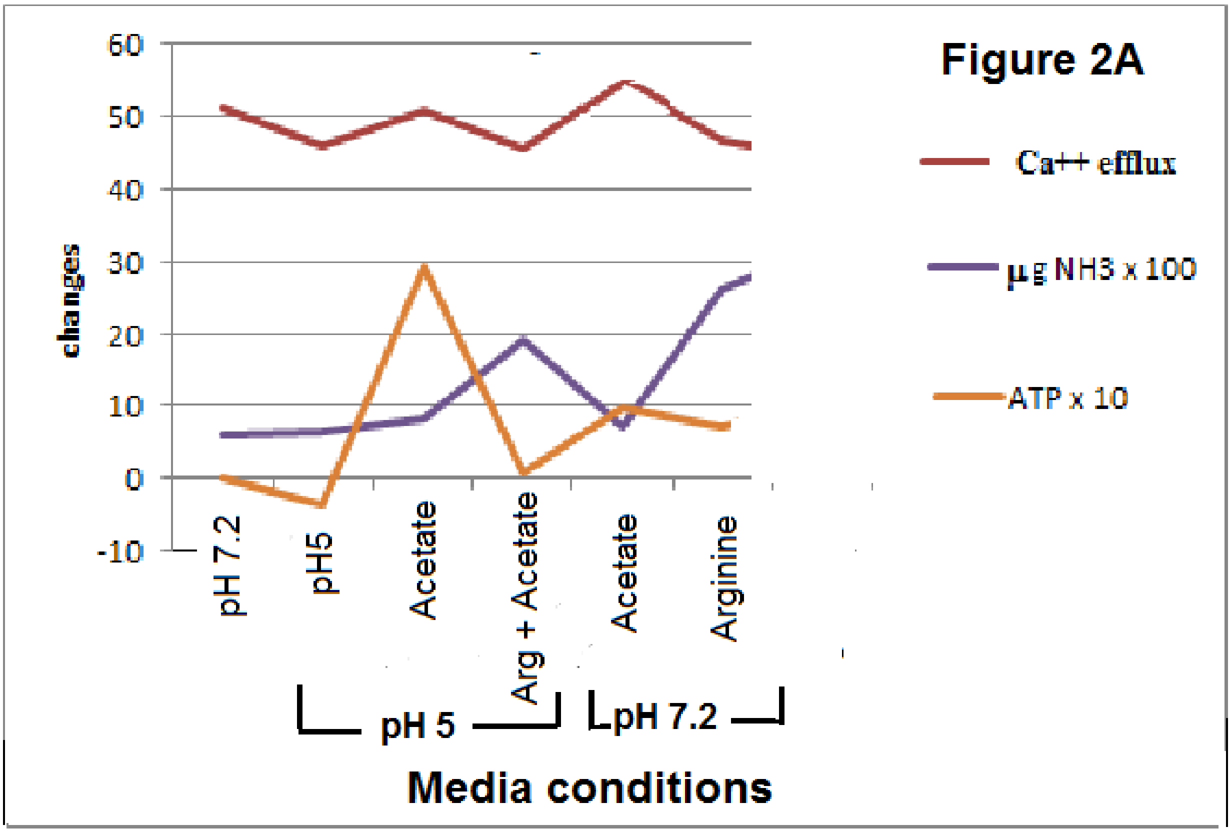

Figure 2A. Changes in Ca++. NH4 and ATP

This is a diagrammatic representation of the changes in Ca++ release, ammonia production x 100 and10 x ATP of renal tubular cells content when incubated in different media as indicated in Table 3A.

Table 2A and Figure 2A show the results of experiment 1. In Figure 2A the values of Ca are calculated by adding each % change to the Ph7.2 value of 51.2 μgm .The values of Ammonia are calculated by adding the change in μgm to the pH 7.2 value of 0.060 μgm. In table 2A and Figure 2A one can see that the change from pH7.2 to pH 5 results in a reduced calcium transport, increased ammoniagenesis and a reduced level of ATP. At pH 5 the addition of acetate restores the calcium transport to the original value, the ATP level is greatly increased and the ammoniagenesis remains high. The addition of Arginine greatly increases the ammoniagenesis and reduces calcium transport and offsetting the benefit of the acetate. Even at pH7.2 the addition of acetate greatly increases calcium transport and the inclusion of arginine increases ammoniagenesis and reduces calcium transport to the same level as pH5.

Table 2B. Differences in Calcium Efflux and Ammonia Production between Media and Basal Medium

Experiment No. 2 Differences compared to Basal Media (pH 7.2)							
	pH5				pH7.2		
Additions	Nil	Arginine	Insulin	Arg+ Insulin	Arginine	Arg + Insulin	Insulin
Ca Efflux % ±SD From Table 3B1	-10.1±2 t=12;S	-30±8.6 t=8.6,	-4.08±2.8 t=3.6 N	-0.38±0.6 t=1.7 N	-9.8±4 t=5.7 s	-6.12±2.3 t=6.4 s	-.02±8.02 t=3.3 N
Ammonia Product% From 3B2	0.005±.004 t=3.14 N	0.273±0.1 t=6.5 s	.004±.003 t=3.6 N	0.29±.04 t=1.8 N	0.12±.057 t=5.16 s	.002±.03 t=0.1 N	.017±.010 t=3.9 N
Ave Ammonia Production in pH 7.2 media with no addition = 0.072							
Ave. percentage Ca++ released in pH 7.2 with no additions = 50.37							

Differences are shown in the percentage calcium effluxed and total ammonia production for kidney tubules incubated in media with pH 5 or pH 7.2 with the addition of arginine, insulin, arginine and insulin or without additions. The tubules incubated in the reference media at pH 7.2 without additions produce an average 0.072 μgm/g of ammonia and released an average of 50.37 μgm/g of cellular calcium.

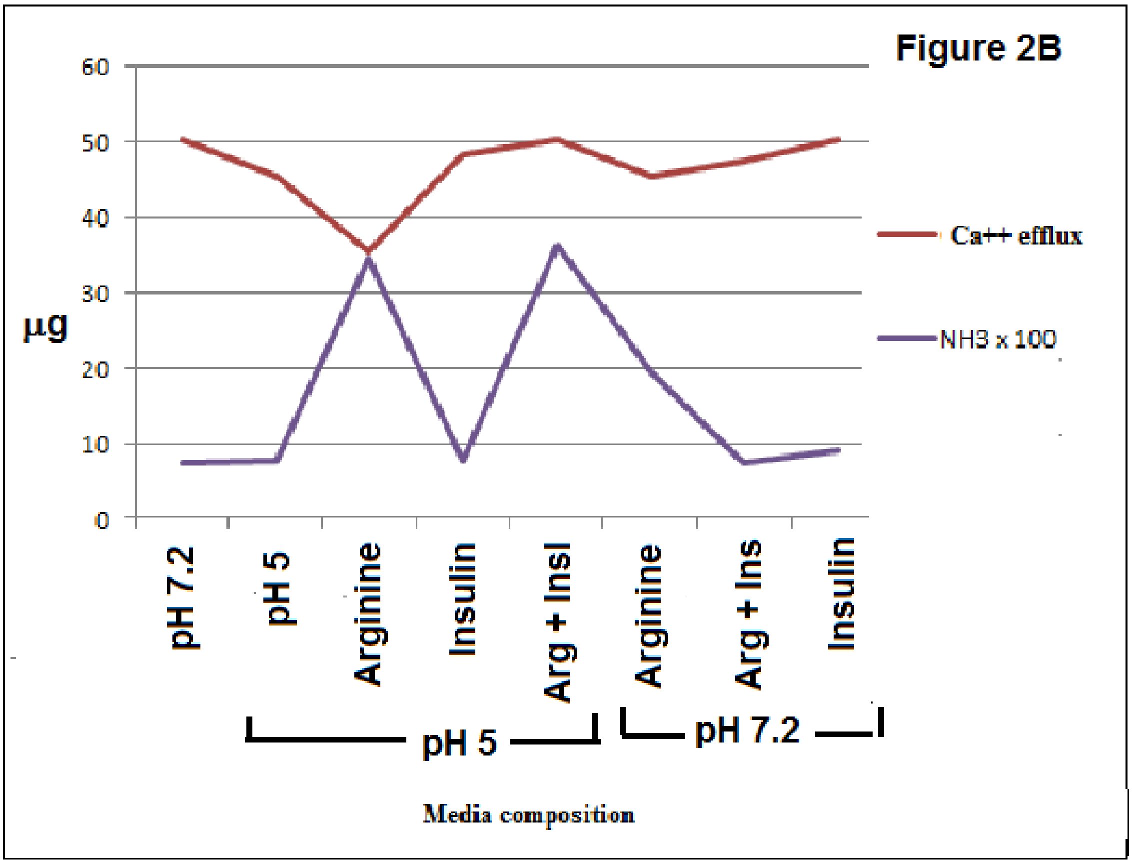

Figure 2B. Changes in Ca++ and NH4

This is a diagrammatic representation of the changes in Ca++ release, ammonia production x 100 of renal tubular cells t when incubated in different media as indicated in Table 2B.

Table 2B and Figure 2B show the results of experiment 2. In Figure 2B the values of Ca are calculated by adding each % change to the Ph7.2 value of 50.37 µgm .The values of Ammonia are calculated by adding the change in µgm to the pH 7.2 value of 0.072 µgm. In table 2B and Figure 2B one can see that the change from pH7.2 to pH 5 results in a reduced calcium transport, with a slight increase in ammoniagenesis. At pH 5 the addition of Arginine greatly increases the ammoniagenesis and markedly reduces calcium transport. The addition of insulin without arginine restore Ca transport to near Ph7 levels but the inclusion of arginine with insulin greatly increases Ca transport even with a large increase in ammoniagenesis At pH7.2 the addition of arginine alone reduces calcium transport and the inclusion of insulin with arginine increases calcium transport and when insulin is added alone calcium transport further increased with only a slight increase in ammoniagenesis.

Table 2C. Difference in Calcium Efflux and Ammonia Production Between each Media and Basal pH 7.2Medium

Experiment No. 3. Differences vs Basal Media (pH 7.2)						
	pH5.			pH7.2		
Additions	Glutamine	Glutamine+ Insuin	Glutamine +Pyr	Glutamine	Glutamine + Insulin	Glutamine + Pyr
Ca^{2}+ Efflux ±SD From Table 3C1	-31.7±10.2 t=7.6;S	-10.5±2.4 t=10.7;S	-3.9±2.65 t=3.6;N	-23.5±6.12 t=9.4;S	-4.3±3.2 t=3.3;N	-9.6±2.2 t=l0.7;S
Ammonia±SD Production From 3C2	.33±0.8 t=10;S	.13±.05 t=6.3;S	.15±.04 t=8.1 s	.24±.08 t=7.3;S	0.06±0.05 t=3.1 N	.098±.025 t=9.4 s

Differences are shown in the percentage calcium effluxed and total ammonia production for kidney tubules incubated in media with pH 5 or 7.2 with the addition of glutamine, glutamine plus insulin or glutamine plus pyruvate. The tubules incubated in the reference medium of pH 7.2 without additions produced an average 0.057 µgm/g of ammonia and released on average 51.43 µg/g of cell calcium.

Table 2C and Figure 2C show the results of experiment 3. In Figure 2C the values of Ca values are calculated by adding each % change to the Ph7.2 value of 51.43 µgm. The values of Ammonia are calculated by adding the change in µgm to the pH 7.2 value of 0.057 µgm. In table 2C and Figure 2C one can see that the change from pH7.2 to pH 5 with inclusion of glutamine results in a large reduction calcium transport with a large increase in ammoniagenesis. At pH 5 the addition of insulin greatly increases calcium transport with a reduction of ammoniagenesis and addition of pyruvate further increases calcium transport. In pH 7.2media the addition of glutamine increases Ca transport and ammoniagenesis and the addition of insulin with glutamine inhibits ammoniagenesis and slightly reduces Ca transport. The inclusion of pyruvate does not increase Ca transport at pH 7.2 and ammonia is only slightly increased.

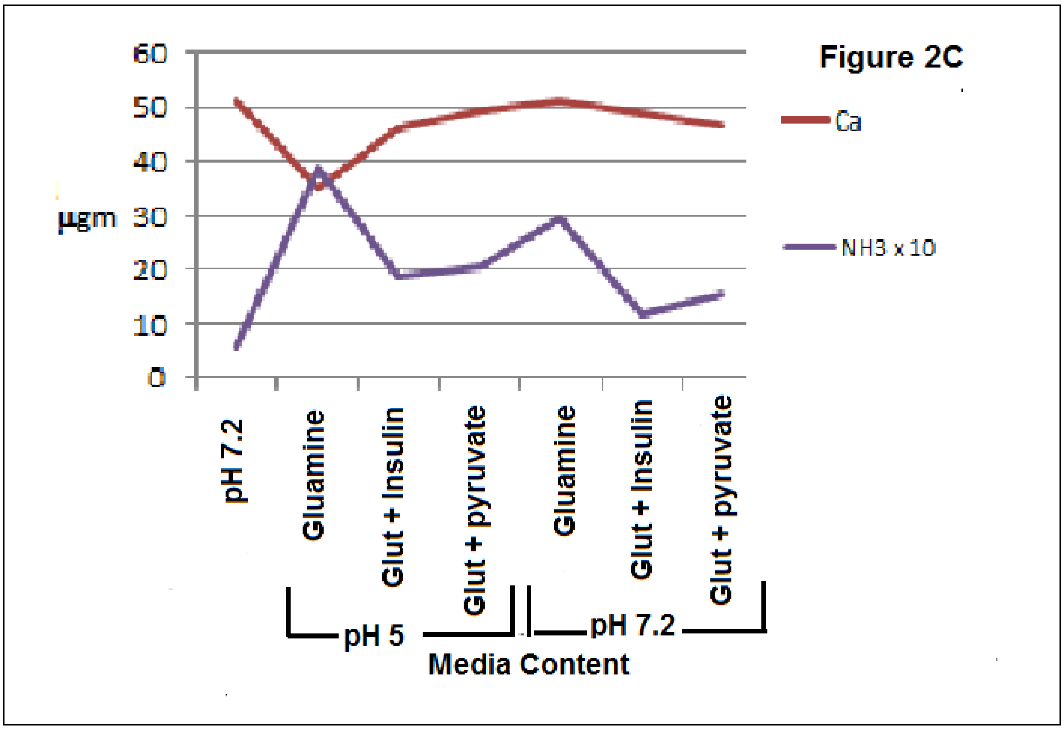

Figure 2C. Changes in Ca++ transport and NH4 production

This is a diagrammatic representation of the changes in Ca++ release, ammonia production x 100 of renal tubular cells content when incubated in different media as indicated in Table 2C.

Table 3A1. Full Resultsfrom Experiment 1a

Table 3A1	Exp1a Difference in ATP content microgram / g.Cell Mass Compared to pH 7.2 Basal Media					
pH	pH5			pH7.2		
Additives	Nil	Acetate	Ac.+Arg	Acetate	Ac.+Arg	Arg.
Diffs. D	-32	3.3	-0.01	-0.1	2.2	1.6
	-4.1	0.8	-0.02	0.1	1.3	0.8
	-2.7	0.5	0.1	-1.4	-1.6	0.7
	-3.6	4.8	-0.11	14	-0.5	0.2
	-3.9	4.5	-0.02	1.9	2.6	1.8
	-4	3.4	0	0.012	0.3	1.5
Ave.D=	-3.58	2.88	-0.01	0.319	0.72	1.1
St.Dv.	0.54	1.83	0.067	1.18	1.62	0.63
t,o--5	-16.2	3.86	-0.37	0.66	1.084	4.3
Sig.%	s. 01%	NS,>1%	NS,>1%	NS	NS	S,<l%

Differences in cell content of ATP(micro.g/g of cell mass)in tubules in media with Ph5.2or pH 7.2 with acetate, arginine, both or neither added are shown compared to the basal medium (pH 7.2). The average differences are shown together with the standard deviations and levels of significance. The average results calculated are used in Tables 2A.

Table 3A2. Full Resultsfrom Experiment 1b

Table 3A2	Exp 1b Difference Ammonia Production (micro g/g cell mass) Compared to pH 72 Basal media					
pH	pH5			pH7.2		
Additives	Nil	Acetate	Ac.+Arg	Acetate	Ac+Arg	Arginine.
Diffs. D	0.004	0.02	0.136	0.012	0.2	0.28
	0.006	0.01	0.144	0.011	0.21	0.18
	0.003	0.03	0.12	0.02	0.25	0.3
	0.01	0.02	0.13	0.007	0.18	0.19
	0.001	0.02	0.137	0.013	0.09	0.3
	0.002	0.045	0.11	0.012	0.3	*0.29*
Ave.D=	0.004	0.024	0.13	0.0125	0.205	0.26
St.Dv.	0.003	0.012	0.012	0.004	0.07	0.056
t,G--5	3.25	4.93	25.4	*7.2*	7.1	11.2
Sig.%	NS, >1%	S,<0.5%	S,<.O.l%	S,<0.1%	S,<.O.l%	S,<O.l%

Shown are differences in total ammonia production of tubules in media with acetate, arginine, both or neither added compared to their production in basal media together with the average values, standard deviations and levels of significance. The average results calculated are used in Tables 2A.

Table 3A3. Full Results from Experiment 1c

Table 3A3	Ex.1c Difference in percentage Calcium Ion Exchange Compared to pH 7.2 Basal media					
pH Additives	pH5 Nil	Acetate	Ac.+Arg	*pH72* Ac	Ac+Arg	Arg.
Diffs. (D)	-ll	-1	-11.2	5	-10	-13
	-10	-2	-9.6	2	-9	-20
	-13	0	-12.2	O.5	-11	-7
	-9	-0.9	-12.5	*0.2*	-7.5	-18
	-8	-O.5	-9.8	0	-6.3	-5.5
	-10	-l.5	-10.1	0.1	-ll	-16
Ave.D=	-10.2	-0.98	-10.9	1.3	-9.1	-13.25
St.Dv. t,O=S	1.72	0.71	1.26	1.96	1.92	5.91
	-14.5	-3.4	-21.3	1.6	-11.6	-5.5
Sig.%	S,<0.l%	NS, >1%	S,<0.l%	<0.1%	S,<0.1%	S,<.O.5%

Differences in percentage calcium exchange between tubules and media are shown for each test using Ph5or pH 7.2 media with acetate, arginine, both added or neither added compared to the basal medium with pH 7.2 together with the average values, standard deviations and levels of significance. The average results calculated are used in Tables 2A.

Table 3B1. Full Results from Experiment 2a

Table	Exp.2a	Differences in Ammonia Production (micro g /g cell mass)					
3 B1		Compared to pH 7.2		Basal	media		
pH		pH5				pH7.2	
Additives	Nil	Arg.	Ins	In-Arg	Arg.	Arg-In	Ins
	-0.006	0.24	0.002	0.01	0.16	0.01	0.002
	0.004	0.46	0.003	0.01	0.2	0.01	0.03
Diffs.	0.012	018	0.004	0.01	0.05	0	0.01
D	0.001	0.21	0.001	0.015	0.07	0.05	0.015
	0.002	0.23	0.01	0.02	0.1	-0.05	0.015
	0.005	0.32	0.005	011	014	-0.01	0.03
Ave.Dev	0.003	0.27	0.0042	0.03	0.12	0.002	0.017
St.Dev.	0.006	0.1	0.003	0.04	0.057	0.03	0.01
t,0=5	1.25	6.52	3.2	1.8	5.16	0.126	3.74
Sig.%	NS	S,<.0.5%	NS, >1%	NS	S,<.0.5%	NS	NS, >1%

Differences in total ammonia production of tubules in media with pH5 or pH7.2 and with arginine, insulin, both or neither added compared to production in basal medium together with the average values, standard deviations and levels of significance. The average results calculated are used in Tables 2B.

Table 3B2. Full Results from Experiment 2b

Table	EXP 2 b	Differences in Percentage Calcium Ion Exchange					
3B2		Compared to pH 7.2		Basal	Media		
pH		pH5					pH72
Additives	Nil	Arg	Ins	Arg-In	Arg.	Arg-In	Ins
	-10	-15	-4.1	0	-12	6.1	0.01
	-11	-35	0.01	-0.02	-15	7.1	0
Diffs.	-13	-33	-7	0.05	-7	5.9	0.05
D	-9	-32	-7.5	-1	-11	2.1	0.02
	-11	-27	-3.1	-1.2	-3	9.3	0.03
	-7	-40	-2.7	-0.01	-11	6.2	0.03
Ave.Dev=	-10.2	-30	-4.1	-0.36	-9.8	6.12	0.02
St.Dev.	2.04	8.62	2.82	0.57	4.22	2.34	0.02
t,0=5	-12.2	8.62	-3.53	-1.55	-5.71	6.41	3.26
Sig.%	S, <0.1%	S,<O.1%	NS, >1%	NS	S,<.0.5%	S,<.0.5%	NS, >1%

The Differences in percentage calcium exchange between media with ph5 or pH7.2 and arginine or insulin, both or neither added compared to basal pH7.2 media are shown together with the average values, standard deviations and levels of significance. The average results calculated are used in Tables 2B.

Table 3C1. Full Results from Experiment 3a

Table	Exp.3a	Ammonia Production (micro.g / g cell mass)				
3C1		Compared to pH 7.2		Basal	media	
pH		pH5			pH7.2	
Additives	GluNH	GluNH+ln GluNH+Py		GluNH	GluNH+ln GluNH+Py	
	0.32	0.1	0.15	0.27	0.11	0.07
	0.47	0.12	0.18	0.25	0.12	0.09
Diffs.	0.38	0.07	0.1	0.18	0.09	0.1
(D)	0.27	0.17	0.09	0.3	0.05	0
	0.25	0.2	0.2	0.11	0.11	0.1
	0.32	0.1	0.17	0.32	0.11	0
Ave.Dev«	0.335	0.13	015	0.24	0.098	0.06
St.Dev.	0.08	0.05	0.04	0.08	0.026	0.048
t,0=5	10.2	6.35	8.17	7.36	9.4	3.08
Sig.%	S,<O.1%	S,<.0.5%	S,<0.l%	S, <0.1%	S, <0.1%	NS, >1%

Differences in total ammonia production of tubules in media with pH 5 or pH 7.2 with glutamine or glutamine and insulin or pyruvate added compared to production in the basal ph7.2 medium are shown with the average values, standard deviations and levels of significance. The average results calculated are used in Tables 2C.

Table 3C2. Full Results from Experiment 3b

Table	Exp 3b		Calcium Ion Exchange		0/0	
3C2		Compared to pH 7.2		Basal media		
pH		pH5			pH7.2	
Additives	GluNH	GluNH+ln GluIH+Py		GluNH	GluNH+ln GluNH+Py	
	-36	-10	-5	-25	5	10
	-38	-9	-7	-21	7	8
Diffs.	-40	-11	-4	-27	2	6
D	-27	-15	-0.05	-13	-1	11
	-13	-8	1	-31	6	12
	-36	-10.5	-6	-24	7	10.2
Ave.Dev=	-31.7	-10.58	-3.51	-23.5	4.3	9.53
St.Dev.	10.17	2.42	3.26	6.12	3.2	2.18
t,0=5	-7.6	-10.73	-2.64	-9.4	3.3	10.72
Sig.%	S, <0.1%	S, <0.1%	<0.1%	S, <0.1%	<0.1%	S, <0.1%

Differences in percentage calcium exchange are shown between tubules in basal media and tubules in media with pHSorpH72 and either glutamine and insulin or glutamine and pyruvate added together with the average values and the standard deviations and the levels of significance. The average results calculated are used in Tables 2C.

Table 3C3. Full Results from Experiment 3c

Table 3C3	Exp 3c	Difference in Percentage Calcium Ion Exchange and Ammonia Production						
			Compared to Media with Glutamine					
pH		5				7.2		
Additions	Glutamine + Insulin		Glutamine + Pyruvate		Glutamine + Insulin		Glutamine + Pyruvate	
Prod.lExc	ammonia	calcium	ammonia	calcium	ammonia	calcium	ammonia	calcium
	0.12	26	-0.17	31	-0.2	30	-0.16	35
	0.15	29	-0.29	31	-0.14	28	-0.13	29
Diffs.	0.31	29	-0.28	36	-0.08	29	-0.09	33
D	0.1	12	-0.18	26.5	-0.3	12	-0.25	2
	0.05	5	-0.05	14	-0.1	37	0	43
	0.22	20.5	-0.15	30	-0.33	31	-0.21	35
Ave.Dev=	0.158	20.25	-0.187	28.08	-0.192	27.8	-0.14	29.5
St.Dev.	0.09	9.87	0.09	7.5	0.1	8.38	0.09	14.2
t,0=5	4.16	5.027	-5.13	9.12	-4.5	8.14	-3.85	5.08
Sig.%	S,<l%	S,<.0.5%	S,<.0.5%	S, <0.1%	S,<l%	S,<O.1%	NS, >1%	S,<.0.5%

Differences in percentage calcium exchange are shown when tubules in basal media are compared with tubules in media with pHs or pH72 with either glutamine and insulin or glutamine and pyruvate added and the average values and the standard deviations are indicated with the levels of significance. The average results calculated are used in Tables 2C.

4. Discussion

Calcium transport is shown to be affected by changes in pH. This effect may be due to the stimulation of ammoniagenesis to neutralise the acid. This increase in ammoniagenesis appears to result from activation of glutaminase and inhibition of citric acid cycle [21] Nissim, (1991). The corresponding change in calcium transport may be due to reduced availability of ATP, Experiment 1 does show a decrease in intracellular ATP that may be due to ATP usage by the gluconeogenesis that is usually coupled with ammoniagenesis. 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 [22] Studer and Borle (1979) and that pH stimulates ammoniagenesis has been well established [8] Tannen (1978). What has been demonstrated in this experiment is the connection between the two effects of pH. Experiment 1 shows that the effect of low pH is reduced and calcium transport is increased by the addition of acetate. Results show that with addition of acetate an increase in Ca transport is accompanied by increased ATP. However when acetate is combined with arginine calcium transport is again decreased. This may be because ammoniagenesis /glycogenesis is using the available ATP. Similar observations were reported by [21] Nissim (1991). A similar explanation has been used to explain the effect of pH on renal handling of sodium by [14] Silva *et. al.* (1980).

When arginine and acetate are both included in the media the reduced calcium efflux could be because the extra ammonia geneses override the energy yielding effect of the acetate. The acetate and arginine are observed to have a similar effect 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.

The movement of calcium out of cells seems to involve two mechanisms one is an A TP driven pump that acts as an antiportal exchanging $Ca^{++}$for H^{+}. The other is a Na^{+}/Ca^{++} antiportal that exchanges 3 Na^{+} ions for each Ca^{++} ion [23] Racher, (1980). The Na^{+}/Ca^{++} antiportal is ATP dependant but not in a stoichiometric fashion i.e., the energy is provided by the Na^{+}/K^{+} ATP antiportal when it pumps Na^{+} out and K^{+} in to the cells. This establishes the concentration gradient that is used to drive Na^{+}/Ca^{++} antiportal. As the sodium flows back into the cell the calcium is expelled.

In Experiment 2 similar changes to calcium fluxes were observed when tubules were put into pH 5 media with or without arginine 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 ammoniagenesis. 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 thereby making ATP more available. [24] Hammerman, (1985).

Arginine was used in Experiment 2 and glutamine in experiment 3 and they both produced effects that could be related to the findings of [25] Wood and Allen (1983). In order to evaluate the involvement of insulin in hypercalciuria [25] Wood and Allen infused rats with arginine and observed a consequential hyperinsulinemia and hypercalciuria and suggested that there could be a causal relationship between the two results.

The results of Experiments 2 and 3 show that arginine and glutamine both caused increased ammoniagenesis in acid and neutral conditions as well as reduced calcium effluxes. The calcium efflux was restored in the presence of insulin and with arginine the ammoniagenesis continued to be large.

When ammoniagenesis is uncoupled from gluconeogenesis ATP levels could increase due to α-ketoglutarate being directed into the tricarboxyclic 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: This was observed in Experiment 3 (Table 2C and Figure 2C).

In Experiment 3 glutamine is used instead of arginine because it is known to be a better substrate for ammoniagenesis [8] Tannen (1978). As expected the levels of ammoniagenesis were greater than with arginine and the reductions in calcium efflux were also greater than in Experiments 1 and 2. The inclusion of insulin again reduced the calcium exchange and the rate of ammoniagenesis was also reduced.

The inclusion of pyruvate and acetate had a similar effect on calcium exchange and ammonia production. 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.

A similar negative effect of caffeine has been shown to have on calcium reabsorption by [26] Massey and Opryszek (1990), [27] Massey and Hallingberg (1988a) and [28] (1988b) and [29] Whitney (1987) could be due to its reported stimulation of gluconeogenesis [17] Sashs and Forster, (1984) and consequent reduction intracellular ATP.

The release of ammonia is closely related to reduction in calcium transport and it is possible that ammonia itself may interfere with the calcium transport. Ammonia is known to readily pass though most cell membranes even though it has a dipole moment of 1.46, which is similar to water with a dipole moment of 1.87 and a nonpolar substance like CCl_4 has a dipole moment of 0.0. It may be that the polar molecule of ammonia could interfere with the Ca transporters. A future experiment will considers the mechanism of ammonia membrane transport and some aspects of this possibility.

5. Conclusion

These results support the original hypothesis “that insulin can inhibit Ca membrane transport of kidney tubules by inhibiting the gluconeogenesis that is connected to ammoniagenesis thereby making ATP more available for the active transport of Ca++.

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