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Calcium Exchange Rates in Rat Kidney Tubule Cells Affected by Insulin and pH

B.W. Brazier*

Bendigo Kangan Institute, Bendigo, Victoria, Australia
*Corresponding author: sail_bad@yahoo.com

Abstract Calcium exchange rates in rat kidney tubule are measured by measuring the loss of Ca^{45} isotopes from tubule cells when the tubules are placed in Ca^{45} free media that was adjusted to different pH sand when different additives of sulphate, acetate, glutamate and /or insulin were included. The exchange rates showed decrease with low pH and glutamine but increased with addition of acetate and insulin. The effect of insulin is considered in relation to its observed effect on protein induced postprandial calciuria.

Keywords: *hypercalciuria, insulin, calcium exchange rates, gluconeogenesis, ammoniogenisis*

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1. Introduction

In a previous experiment at this laboratory plasma insulin has been linked with dietary protein induced hypercalciuria [1] Brazier (2016a). The postprandial calciuria was observed to show considerable variation between the test subjects. This variation appeared to correlate with plasma insulin levels. In another experiment [2] Brazier (2016b) where dietary fat was tested for variation in calciuria the main correlation was found to be between insulinemia and calciuria. This was also observed by [3] Allen *et. al.* (1981) and [4] Wood and Allen (1983) and [5] Howe (1990) who indicate that insulin may be involved in increasing calciuria. However in the experiments looking at the effect of dietary protein,the indication wasthat plasma insulin reduced calciuria. Another study by [6] Brazier (2016c) showed that plasma insulin had an inverse relationship with calciuria. 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 ofinterplay between insulin and the renin-angiotensin system or a direct effect of insulin on calcium transports the kidney tubules.

Urinary sulphate has been implicated in reduced calcium reabsorption [7] Wachman and Bernstein (1960) and reports have indicated a correlation between the sulphur content of dietary protein and the increase in excreted calcium [8] Walser and Browden, (1958, [9] Whiting and Draper, 1981, [10] Zemel *et. al.* 1981). Whether sulphate had a direct effect on reabsorption or if the effect was a result of reduced pH was not determined. However pH has been shown to significantly reduce membrane transport of calcium in kidney tubules [11] Lemann *et. al.*, (1961), and [12] Studer and Borle, (1979). When protein derived sulphate reduces the pH of urine the tubules are stimulated to produce ammonia to neutralise the acid. This experiment will test the hypothesis that this ammoniogenesis uses mainly glutamine for deamination thereby producing α-ketogluterate as a by-product. The α-ketogluterate is then usually removed by gluconeogenesis. The gluconeogenesis then uses up ATP making less ATP available for Ca++ absorption. If the cells are exposed to insulin the gluconeogenesis is inhibited and the α-ketogluterate is redirected into the citric acid cycle and extra ATP is produced. This would allow an increase in Ca++ absorption. Thus insulin would have a direct effect of increasing calcium reabsorption thus causing less calciuria.

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., [13] 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). [14] 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. The observations by Brazier [1] and [2] that some young health individuals with no apparent pre-diabetes had exaggerated insulin responses. If that insulin influenced calciuria it could be a contributor to idiopathic hypercalciuria and nephrolithiasis. [21] Worcester (2008) This experiment 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 determine if the gluconeogenesis stimulated by reduced pH is the causative factor in reduction of calcium reabsorption and to see if insulin has a direct action on this process. The mechanism of the effect of pH on calcium membrane transfer rates has been considered by [11] Lemann (1967) 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 [15] Silva *et. al.* (1980) who showed that sodium transport was inhibition by ammoniogenesis linked gluconeogenesis. The effect of a combination of insulin and glutamine is also compared with control tubules to see if insulin directly effecting renal calcium metabolism. Although [16] Janda (1969) observed increased calcium uptake when insulin was added to kidney slices, the use of isotope exchange studies here allow measurement of transmembrane movement under steady state conditions which should be a better measure of calcium reabsorption than calcium uptake into tissue slices as done by Janda [16]. 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 [17] Mahler and Szabo (1967) have shown that insulin insensitive tissues can react to insulin if insulin degradation mechanisms are impaired. The method used in these experiments 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.

2. Method

The exchange rate between the cytosol of isolated kidney tubules and incubation medium were calculated from Ca^{45} desaturation curves after the method of [18] Uchikava and Borle (1978). The kidneys from two 150-200 g male Sprague-Dawley rats were buttered through a 170-mesh sieve in 0.15 M Saline at 5°C and the mixture filtered through an 80-mesh sieve as described by [19] Price (1979). The homogenate was centrifuged at 20 g at 5 °C for 1 minute and the supernatant discarded. The tubules were washed four times in phosphate buffer then divided into 2 cm^3 aliquots to provide about 2 mg protein per ml. The tubules were allowed to equilibrate in incubation media for one hour at 37 ° C before labelling for 60 minutes with 1 μCu, $Ca^{45}cm^3$. The cells were washed then suspended in non- radioactive media for 5 minutes then centrifuged and suspended in fresh media each 10 minutes for 250 minutes. Each supernatant collected was retained and the fluxed radioactivity measured using a refrigerated scintillation counter and PPO toluene scintillant as described by [20] Borle (1975). The specific activity of the tubule cells initially and after each washing was calculated by the method of Borle (1975) to produce the radioactive desaturation curves similar shown in Figure 4A to 4E.

For each experiment media was prepared and refrigerated the day before. On the day of each experiment, rats were killed at about 0800 hrs. It took two hours to isolate the tubules then the process involving saturation with Ca^{45} and then the subsequent desaturation involved continuous activity as each test tube had to be handled separately as media was transferred into the scintillant. The desaturation stage was finished by 1700 hrs. The scintillation counting was then done overnight.

The method of analysis used by [21] Uchikawa and Borle (1978) involved plotting the percentage Ca^{45} remaining in the sells against time and drawing by hand a line of best fit. Values of cell content and time were then read off at 10 minute intervals and used for the mathematical analysis of the curves.

The assumption was made that the calcium pool sizes are: S1 indicates the interstitial space, S2 indicates the cytosol and S3 the mitochondria, and P20 and P30 indicate exchange between medium and compartments S2 and S3 respectively. S2, S3 P20 and P30 are calculated using the formulae used by [21] Uchikawa and Borle (1978) and using the desaturation equations:

$$r(t) = b_3\exp(-l_3t) + b_2\exp(-l_2t) + b_1\exp(-l_1t) \quad (1)$$

$$r_2(t) = b_3\exp(-l_3t) + b_2\exp(-l_2t) \quad (2)$$

$$r_3(t) = b_3\exp(-l_3t) \quad (3)$$

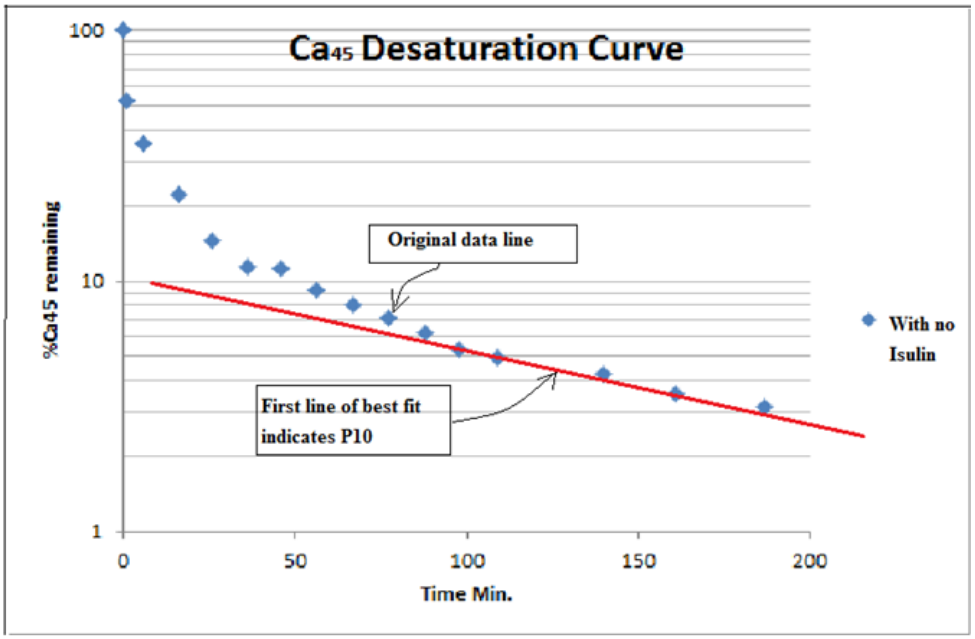


Figure 1. Step one in determining the curvilinear line of best for the multi term exponential curve for the experimental data set

Analysis of each desaturation curve involved three stages. First a line of best fit was drawn using the last few points on the graph. The gradient of this line of best fit was used to calculate the P10 from the third term of equation (1) as in Figure 1 and using the formulae in Table 3. A second curve is then produced by subtracting the third term from the equation. The last few points on this second curve was used to produce a new line of best fit and its gradient used to calculate P20as in Figure 2. This value was used to remove the second term from Equation (2) above to produce a curve of equation(3) A third line of best fit was then used to calculate P30 as per Figure 3. As this method allows some human interpretation of the original data in drawing each line of best fit by hand, in this experiment the raw data was used each time by a computer to calculate a least squares line of best fit. After obtaining values for P01, P02 and P03 a new theoretical cure was produced was produced. The goodness of fit of the theoretical curve to the original experimental results was then determined by the computer programed using a Chi Square test. If the Chi Square value was less 10, the choice of points used to determine the three components of the curves were varied and a new theoretical curve produced and tested with Chi Squared. This was repeated over and over until a resulting theoretical curve was produced that had a close fit with the original data.I.e. with a Chi Squared value of less than 10. The values of P20 were then used to indicate the Ca^{45} exchange rate from the cytosol to the medium for each set of test conditions.

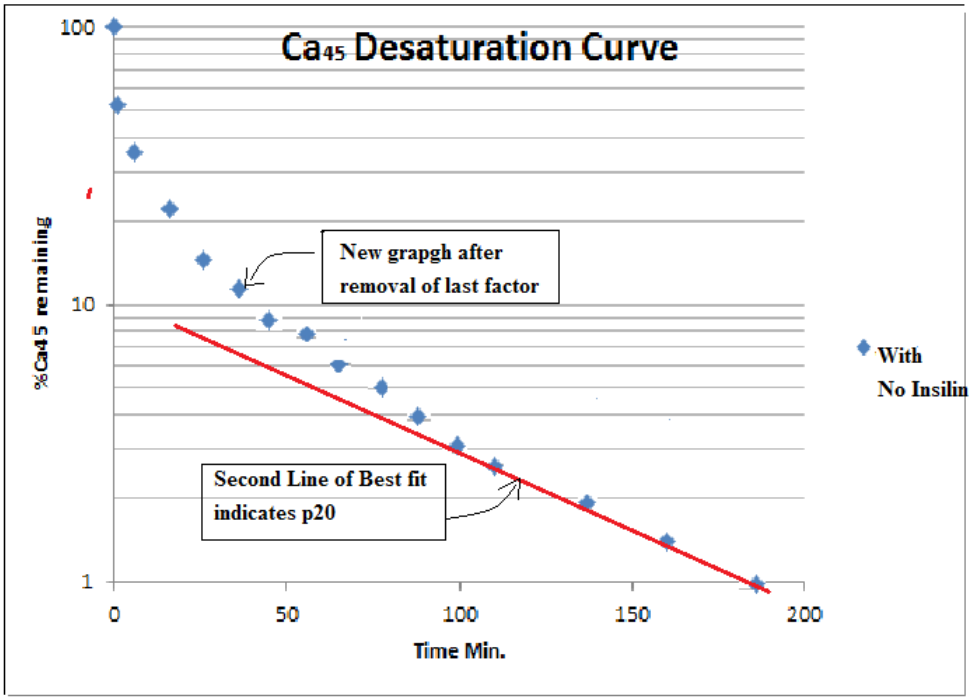


Figure 2. Step two in determining the curvilinear line of best for the multi term exponential curve for the experimental data set

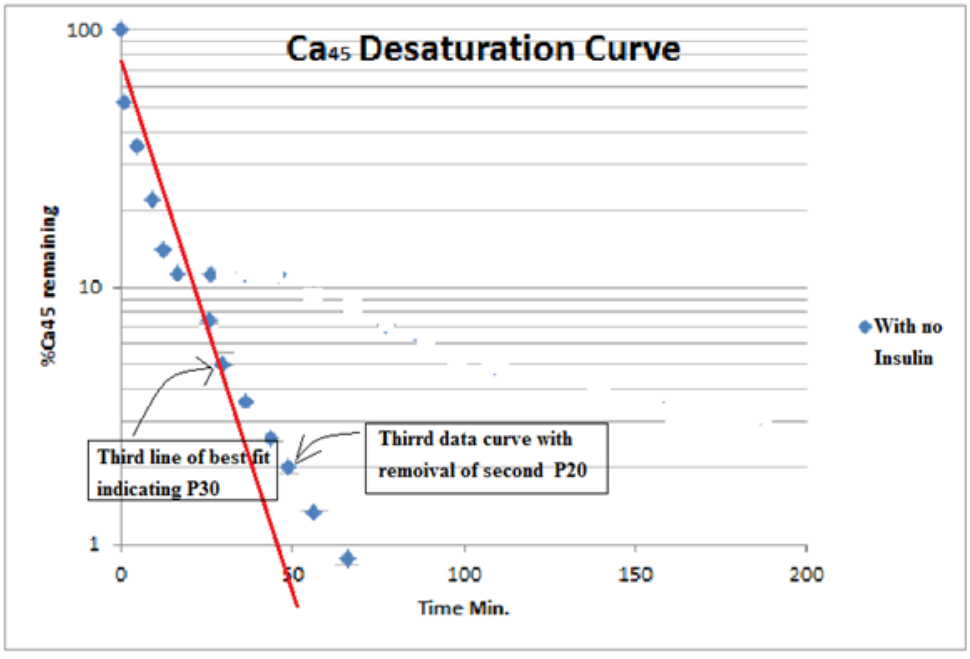


Figure 3. Step three in determining the curvilinear line of best for the multi term exponential curve for the experimental data set

The media used for each experiment are shown in Table 1. In each set of experiments two media variations were compared against each other using kidney tubules from the same batch of homogenate. For each pair of media the experiment was repeated six times. In each experiment there were three 4 ml aliquots of media/cell preparation for each media type. Cells and media were incubated in 10 ml centrifuge tubes while bubbling oxygen through the media from 1 mm diameter tubes to provide aeration and agitation. The significance of the differences between pairs of media tested was determined by the paired student t- test.

Table 1. Media Used in Calcium Desaturation Experiment

Basal Media	pH 7.2	pH 6.2
NaCl	8 g/dm^3	8 g/dm^3
KCI	0.20	0.20
$MgCl_26HzO$	0.10	0.10
$CaCl_2$	0.21	0.21
Na_2HP0_4	1.15	0.36
KH_2P0_4	0.20	0.20
glucose	1.00	1.00
NaH_2P0_4	0.00	0.79
Na_2S0_4	0.08	0.08
Additions		
High Sulphate Media	pH 6.2 basal media plus 160 mg $Na_2S0_410H_20$ per dm^3	
Glutamine Media:	Sulphate media plus 50 mg L.glutamine per dm^3(Sigma grade 3)	
Insulin Media:	Glutamine media plus 100 m□ /ml Sigma porcine crystalline insulin Cat. No. 13505. Lot NO.69C-0417	
Acetate Media:	Insulin media plus 123 mg/drn^{3}sodium acetate	

Desaturation of renal tubules labelled with Ca^{45} was accomplished by incubating tubules at 37 °C in media with either of the two pHs shown with or without the additions of extra sulphate, glutamine, insulin or acetate that are described in this table.

Table 2. Sets of Media used in Paired Desaturation Experiments

Experiment	Medium 1	Medium 2
3A	pH 6.2	vs. pH 7.2
3B	pH 6.2 + sulphate	vs. pH 6.2 no sulphate
3C	pH 6.2 sulphate glutamine	vs. pH 6.2 sulphate no glutamine
3D	pH 6.2 sulphate glutamine insulin	vs. pH 6.2 sulphate glutamine no insulin
3E	pH 6.2 sulphate glutamine insulin acetate	vs. pH 6.2 sulphate glutamine insulin acetate

Each desaturation experiment compared a pair of media, Medium 1 against medium 2 as shown, and each comparison was repeated six times. The composition of these media is shown in Table 1. The average results of analysis of desaturations curves produced are shown in Tables 4A to 4E.

2.1. Cell Potassium and Cell Viability

Atomic absorption was used to measure the cell potassium and calcium content of tubules before and after desaturation experiments

3. Results

3.1. Exchange Rates

The rates of exchange of calcium between the incubation medium and the metabolic compartments and the size of the calcium pools in each compartment is shown in Tables 4 A to E for each pair of media used. The assumption was made that the calcium pool sizes are: S1 indicates the interstitial space, S2 indicates the cytosol and S3 the mitochondria, and P20 and P30 indicate exchange between medium and compartments S2 and S3 respectively. S2, S3 P20 and P30 are calculated using the formula used by [20] Borle (1975).

$$S_0 \underset{P10}{\overset{P01}{\rightleftarrows}} S_1 \underset{P21}{\overset{P12}{\rightleftarrows}} S_2 \underset{P32}{\overset{P23}{\rightleftarrows}} S_3 \; S_0 \underset{P30}{\overset{P20}{\underset{S_1}{\rightleftarrows}}} S_2 \; S_2$$

3.2. Calculations

Calculation of P01, P02 and P03 were done using the results from the graphical analysis and the formulae in Table 3.

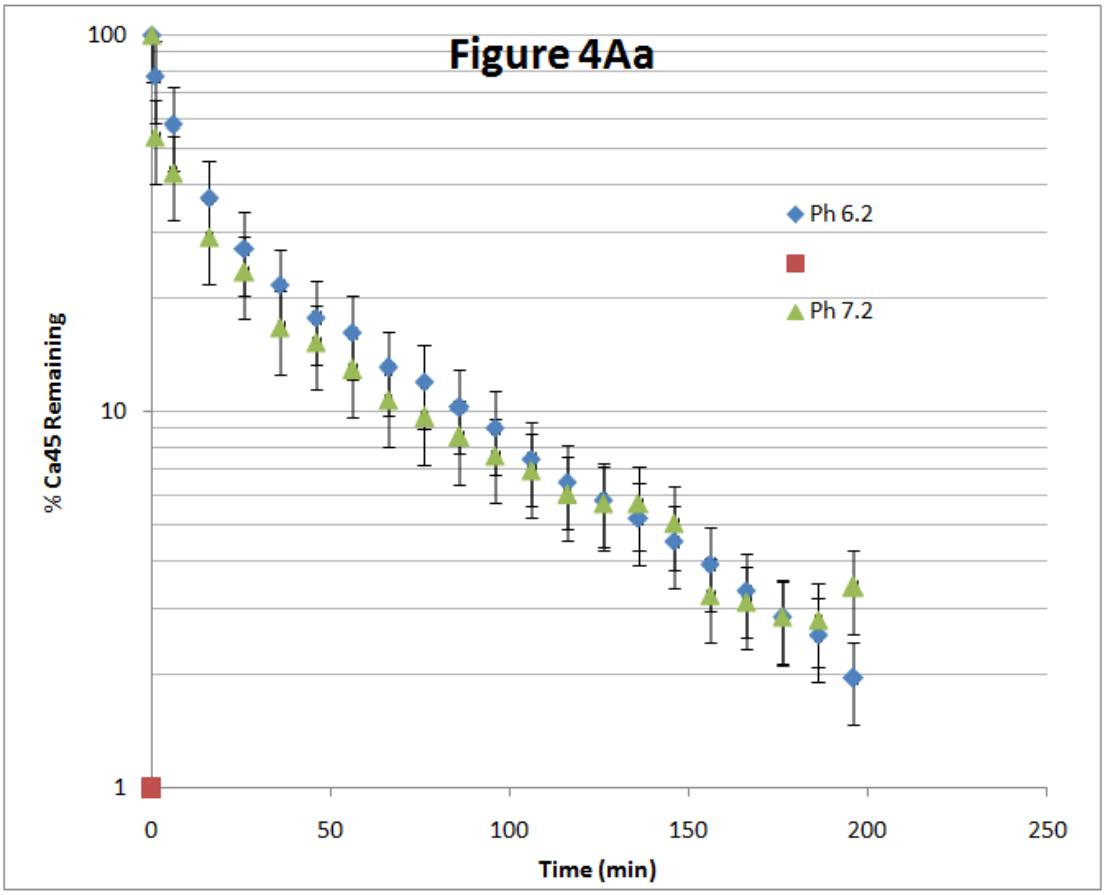
Table 3. Solutions for calculation of calcium fluxes (P10,P20 and P30) and pool sises (S1,S2 and S3) And rate cosnstants from Ca^{45} desaturation curves

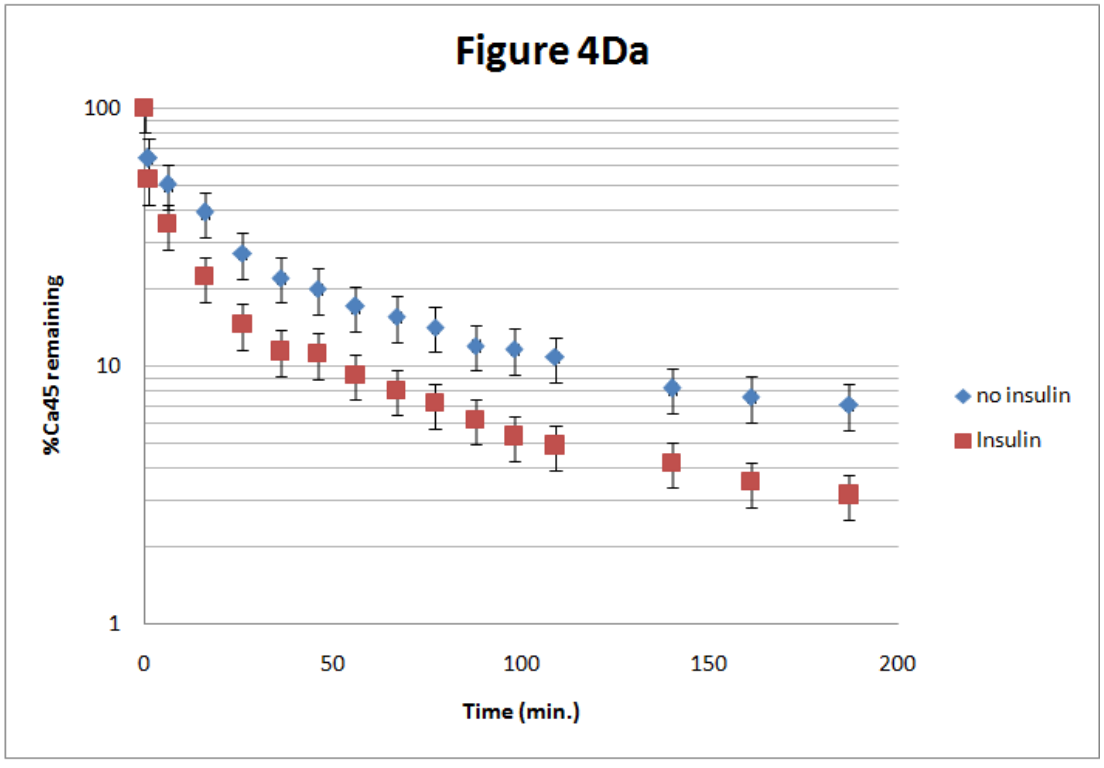
$Ci = bi/(1-\exp(-liT))\ \ (I = 1,2,3)$	(4)
$Si = Ci$	(5)
$P10 = l_1S_1$	(6)
$P20 = C_2l_2 + C_3l_3$	(7)
$S_2 = (P20)^2/((C_2l_2)^2 + (C_3l_3)^2$	(8)
$S_3 = C_2 + C_3 - S_2$	(9)
$P23 = S_2S_3l_2l_3/P20$	(10)
$P30 = S_2.S_3.l_2.l_3/P12$	(11)

Example desaturation curves for pairs of media are shown in as Figure 4Aa and Figure 4Da. The Curves for different pH, different sulphate levels, and different glutamine levels looked similar to the pH graphs shown in Figure 4Aa. The graphs regarding different Insulin levels and acetate levels show significant differences for high and low levels and look similar to the insulin graphs shown in Figure 4Da.

Each of the experiments in Table 2 were repeated 6 times for each of the sets of media. Desaturation curves for the six repeated experiments for each pair of media are averaged to give a pairs of graphs similar to those shown in Figure 4Aa to 4Ea. These averaged results do not show significant differences when the standard errors are compared for each time period. However when the exchanged rates are calculated for each experiment separately and the results compared using the t-test, significant difference are observed for all pairs of media except high and low sulphate. Assuming a steady state of

efflux equals influx the low pH medium showed a significant decrease (P>O.1) in calcium exchange (P20) rate indicating less tubular calcium exchange with the medium compared to high pH medium (Table 4A and Figure 4A). With the P20 in Table 4C the inclusion of glutamine with the low pH medium decreased the calcium exchange rate even more, and the addition of acetate produced a greater exchange rate revering the effect of low pH and made equal to the pH 7.2 medium,as shown in Table 4E. The presence of insulin with the glutamine/pH 6.2 also increased the cytosol/medium exchange rate as shown in Table 4D. However, the different rates of cytosol/medium exchange for the high and low sulphate experiment were not significant (Table 4B). The paired student t-test test was used to determine the significance of the difference between the groups of results for the P20 exchange rates.





The inclusion of glutamine in the low pH medium reduced the calcium exchange rates further but not as much as the difference between high and low pH. Glutamine was included in the media (pH 6.2) used for the insulin experiment (Table 4D) and it can be observed that insulin masks the effect of glutamine.

4. Potassium and Total Cell Calcium

Measurements of cellular potassium and calcium show no loss of potassium of total calcium from cells

Table 4A/1. Intracellular Calcium Pool Sizes and Exchange Rates in Renal Tubules Incubated in Media with pH6.2 or with pH 7.2

Experiment Number	Media Conditions	Ca++ Pool Size (n.mol /mg cell prot.)		Ca++ Exchange Rate (pmol/mg prot./rnin.)	
	pH	S(2)	S(1)	P(20)	P(30)
1	7.2	2.66	0.27	0.038	0.0029
	6.2	1.79	1.88	0.026	0.0016
2	7.2	3.95	2.52	0.086	0.0081
	6.2	139	10.49	0.021	0.0073
3	7.2	4.74	L09	0.119	0.0056
	6.2	~.36	2.41	0.108	0.0081
4	7.2	8.94	1.03	0.153	0.0008
	6.2	1.08	3.21	0.083	0.0071
5	7.2	1.57	0.58	0.068	0.0019
	6.2	1.79	187	0.016	0.0016
6	7.2	4.51	1.72	0.161	0.0054
	6.2	4.61	3.92	0.051	0.0007

Results of graphical analysis of the six desaturation curves from Table 4A/3 in Appendix1 [22} Brazier (2016c)have been used to calculate the six sets of kinetic data shown. The. S1 and S2 are taken as intracellular Ca ++ pool sizes for cytosol and mitochondria respectively and the P20 and P30 are taken as the exchange rates between the incubation medium and the cytosol or the medium and the mitochondria respectively.

Table 4A/2. Difference in Paired Calcium Exchange Rates (P20) for Media with pH6.2 and pH 7.2

Experiment No	Exchange rates			*T-test (Paired Differences)*	
	pH7.2	pH6.2	Differences		
1	0.038	0.026	0.002		
2	0.086	0.021	0.066	t	3.33
3	0.119	0.108	0.011	P (1 tail)	0.0103
4	0.153	0.083	0.021	Critical t	2.015
5	0.068	0.026	0.042	P(2 tail)	0.0207
6	0.16	0.051	0.111	Critical t	2.571
Average	0.104	0.052	0.052		
% change			-50.58%	Total % change	-50.58%

Student t-test of paired results are shown comparing the Ca^{45} exchange rate measured in tubules incubated in media with high or low sulphate levels. The P20 exchange rates compared were assumed to be between the cytosol and the medium.

Figure 4A

Time	Ph 6.2		Ph 7.2	
0	100	SE	100	SE
1	77.36	4.86	53.66	2.88
6	57.85	4.65	43.08	4.48
16	36.82	5.45	29.03	5.58
26	27.06	5.21	23.41	5.31
36	21.6	4.46	16.77	4.54
46	17.81	4.51	15.25	4.90
56	16.2	4.43	12.93	4.85
66	13.06	4.02	10.77	4.63
76	11.98	3.38	9.663	3.75
86	10.3	2.92	8.567	3.25
96	9.048	2.49	7.637	2.76
106	7.51	2.00	7.003	2.22
116	6.518	1.73	6.052	1.93
126	5.84	1.47	5.692	1.64
136	5.193	1.30	5.692	1.44
146	4.513	1.12	5.057	1.26
156	3.948	0.99	3.262	1.15
166	3.348	0.90	3.112	1.07
176	2.84	0.85	2.863	1.04
186	2.553	0.78	2.805	0.96
196	1.96	0.46	3.435	0.56

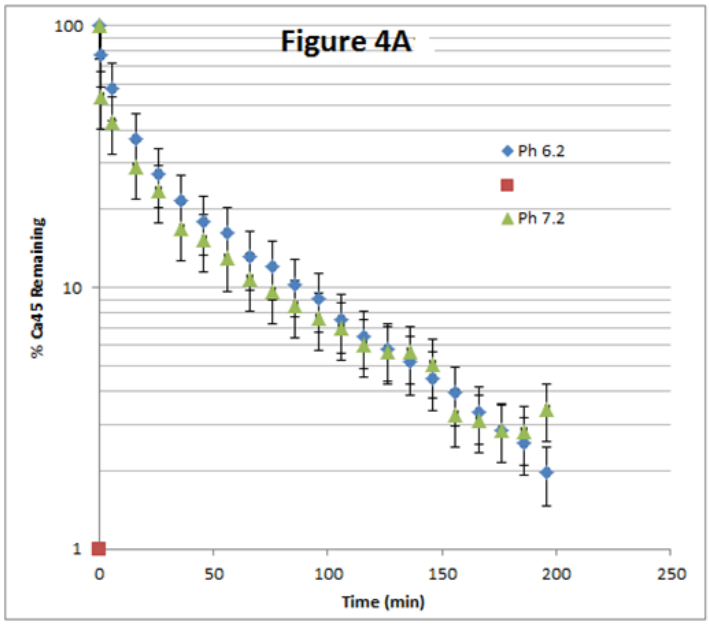


Figure 4A.

Average desaturation curves are plotted using the average of Calcium-45 left in tubules after each successive washing with media having pH 6.1 or pH 7.2 at times indicated (min). The averages were obtained from desaturation curves presented in Apendix I Table 4A/3 (Brazier BW (2016c))

Table 4 B/1. Paired Results from Analysis of Ca^{45} Desaturation Curves When Renal Tubules are incubated in Media with High or Low Sulphate

Experiment Number	Media Sulphate H or L	Ca++ Pool Size (n.mol/mg cell prot.)		Ca++ Exchange Rate (p.mol /rng prot./min)	
		S(2)	S(1)	P(20)	P(30)
1	H	5.66	2.76	0.129	0.012
	L	5.67	1.33	0.114	0.012
2	H	5.58	1.69	0.114	0.012
	L	5.68	1.8	0.117	0.007
3	H	4.79	1.88	0.077	0.0011
	L	4.7	2.36	0.145	0.004
4	H	7.19	0.0007	0.233	0.007
	L	373	2.09	0.114	0.003
5	H	4.03	2.14	0.074	0.006
	L	4.64	1.62	0.079	0.006
6	H	6.04	1.28	0.287	0.005
	L	4.63	2.16	0.125	0.0008

Results of graphical analysis of the six desaturation curves from Appendix 1 Table 4B/3 [22] Brazier (2016c) have been used to calculate the six sets of kinetic data shown. S1 and S2 are taken as intracellular Ca ++ pool sizes for cytosol and mitochondria respectively and the P20 and P30 are taken as the exchange rates between the incubation medium and the cytosol or the medium and the mitochondria respectively.

Table 4B/2. Differences in Paired Calcium Exchange Rates (P20) Between Tubules Incubated in High or Low Sulphate Media

Experiment No.	Exchange Rates			t-test (Paid Differences)	
	High Sulphate	Low Sulphate	Differences		
1	0.129	0.114	0.015		
2	0.144	0.117	0.027	t	1.141
3	0.077	0.145	-0.068	P (1tail)	0. 153
4	0.233	0.144	0.089	Critical t	2.015
5	0.076	0.079	-0.003	P (2tail)	0.305
6	0.287	0.125	0.162	Critical t	2.571
Average	0.16	0.12	0.370		
% change		76.53 of 4A		Total Ave4A+4B	89.12%

Student t-test of paired results are shown comparing the Ca^{45} exchange rate measured in tubules incubated in media with high or low sulphate levels.The P20 exchange rates compared were assumed to be between the cytosol and the medium.

Figure 4B

Time	High Sulphate	SE	Low Sulphate	SE
0	100	SE	100	SE
1	69.64	7.07	53.18	3.26
6	52.27	6.21	37.64	2.20
16	36.84	4.44	29.19	2.72
26	27.2	2.35	22.8	2.78
36	22.5	1.87	21.72	2.37
46	20.44	1.96	18.85	2.08
56	19.1	1.68	16.71	1.98
66	17.66	1.58	15.02	2.20
86	13.34	1.46	12.28	2.14
96	12.27	1.32	10.71	2.16
106	11.01	1.29	9.4	1.94
116	10.26	1.25	8.47	1.91
126	9.54	1.18	7.94	1.78
136	8.97	1.07	7.55	1.64
146	8.19	1.10	7.08	1.58
156	7.57	1.24	6.38	1.50
166	6.92	1.20	6.08	1.51
176	6.26	1.22	5.29	1.32
186	5.76	1.21	4.98	1.34
196	5.53	1.27	4.12	1.38

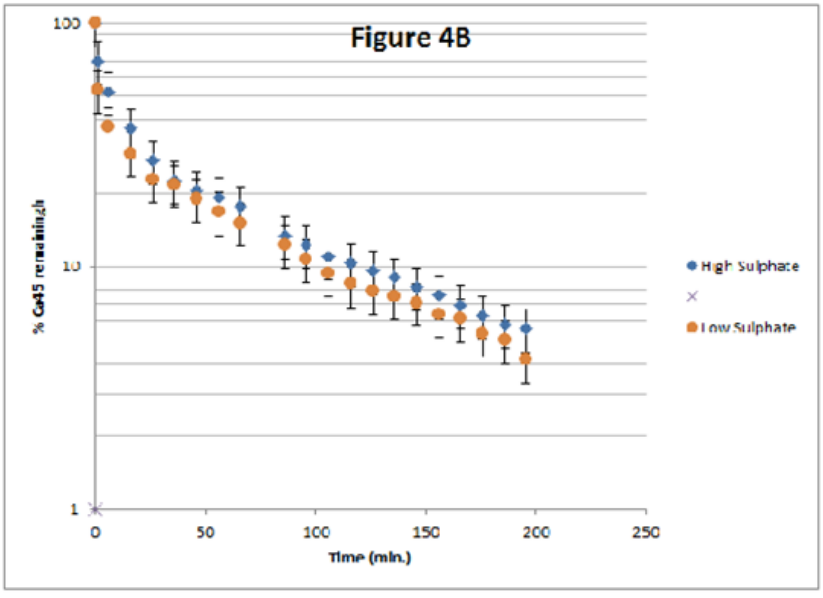


Figure 4B. Average desaturation curves for media with high sulpahrte and low sulphate

Average desaturation curves are plotted using the average percentage of Calcium-45 left in tubules after each successive washing with media with high sulpahrte and low sulphate at times in dicated (min). The averages were obtained from desaturation curves presented in Apendix I Table 4B/3 (Brazier BW (2016c))

Table 4C/l. Paired Results from Analysis of Ca^{45} Desaturation Curves When Renal Tubules are incubated in Media With or Without Glutamine

Experiment Number	Media Glutamine	Ca++ Pool Size (N.mol/mg cell prot.)		Ca++ Exchange Rate (pmol /mg prot./min)	
	(+/-)	S(2)	S(1)	P(20)	P(30)
1	+	1.57	0.52	0.020	0.0018
	-	1.79	188	0.026	0.0016
2	+	1.20	4.53	0.013	0.0069
	-	139	1O.49	0.020	00073
3	+	4.72	1.72	0.097	0.0073
	-	5.36	2.40	0.108	0.0080
4	+	1.07	2.74	0.085	0.0071
	-	1.08	3.203	0.088	0.0070
5	+	1.00	1.89	0.150	0.0020
	-	1.79	1.87	0.160	0.0016
6	+	3.72	1.75	0.050	0.0025
	-	4.60	3.92	.0050	0.0007

Results of graphical analysis of the six desaturation curves from Table 4C/3 Appendix 1 [22] Brazier (2016c) have been used to calculate the six sets of kinetic data shown. S1 and S2 are taken as intracellular Ca ++ pool sizes for cytosol and mitochondria respectively and the P20 and P30 are taken as the exchange rates between the incubation medium and the cytosol or the medium and the mitochondria

Table 4C/2. Differences in Paired Calcium Exchange Rates (P20) Between Tubules Incubated in Media With or Without Glutamine

Experiment No.	Exchange Rates			t-test(Paid Differences)	
	With glutamine	No glutamine	Differences		
1	0.020	0.026	0.006		
2	0.0013	0.020	0.007	t	2.475
3	0.097	0.108	0.011	P (1 tail)	0.028
4	0.085	0.083	-0.002	Critical t	2.015
5	0.150	0.160	0.010	P (2 tail)	0.056
6	0.050	0.050	0.000	Critical t	2.571
Average	0.07	0.07	0.007	Total Ave	89.12%
% Change		90.22% of 4B		4A+4B+4C	80.4%

Student t-test of paired results are shown comparing the Ca^{45} exchange rate measured in tubules incubated in media with high or low sulphate levels. The P20 exchange rates compared were assumed to be between the cytosol and the medium.

Figure 4C

Time	High Sulphate		Low Sulphate	
0	100	SE	100	SE
1	58.75	8.91	38.12	12.20
6	42.88	8.94	42.2	9.80
16	27.48	7.23	26.62	7.55
26	20.1	5.81	15.47	3.40
36	15.15	5.00	10.89	2.73
46	13.27	4.71	8.87	2.22
56	12	4.58	7.8	2.17
66	9.66	4.18	5.67	2.31
76	8.79	3.53	5.69	1.59
86	7.43	3.09	4.8	1.30
96	6.37	2.71	4.26	1.20
106	5.2	2.22	3.78	1.16
116	4.55	1.96	3.42	1.07
126	3.92	1.72	3.18	0.96
136	3.35	1.56	2.98	0.87
146	2.93	1.35	2.73	0.75
156	2.54	1.20	2.26	0.67
166	2.11	1.08	2.18	0.67
176	1.76	0.99	1.85	0.53
186	1.58	0.90	1.56	0.42
196	1.16	0.56	1.42	0.40

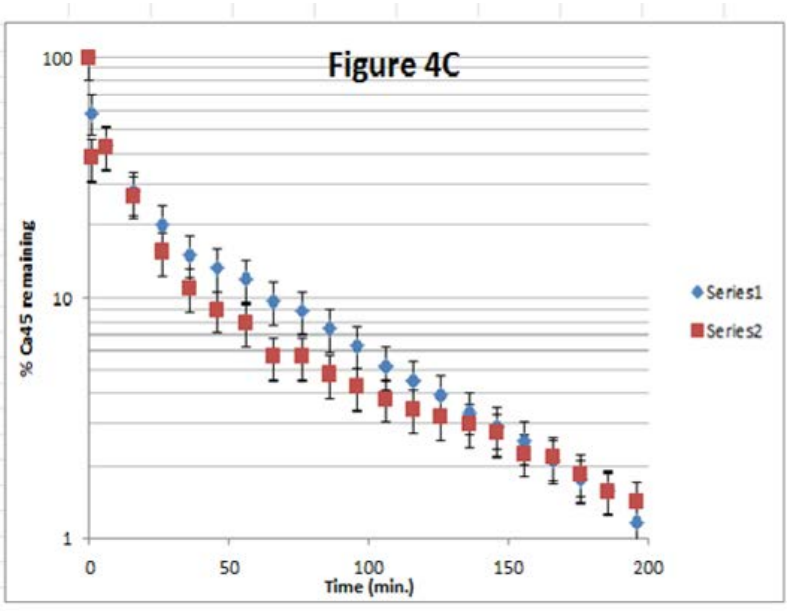


Figure 4C. Average Desaturation Curves for media with or without Glutamine

Average desaturation curves are plotted using the average percentage of Calcium-45 left in tubules after each successive washing with media with or without Glutamine at times indicated (min). The averages were obtained from desaturation curves presented in Apendix I Table 4C/3 (Brazier BW (2016c))

Table 4D/1. Paired Results from Analysis of Ca^{45} Desaturation Curves When Renal Tubules are incubated in Media With or Without Insulin

Experiment Number	Media Insulin	Cart Pool Size (N.mol/mg cell prot.)		Cart Exchange Rate (pmol/mg prot./min)	
	(+/-)	S(2)	S(1)	P(20)	P(30)
1	+	4.67	2.36	0.094	0.0164
	-	0.97	5.07	0.023	0.0070
2	+	5.19	1.76	0.120	0.0014
	-	4.80	2.21	0.069	0.0057
3	+	8.30	2.32	0.090	0.0015
	-	3.79	2.81	0.079	0.0015
4	+	1.79	1.84	0.026	0.0016
	-	0.94	3.68	0.018	0.0049
5	+	2.05	7.16	0.027	0.0044
	-	1.39	10.49	0.020	0.0073
6	+	10.53	1.05	0.136	0.0164
	-	1.50	1.31	0.117	0.0087

Results of graphical analysis of the six desaturation curves from Table 4D/3 Appendix 1 [22] Brazier (2016c)) have been used to calculate the six sets of kinetic data shoum.S1 and S2are taken as intracellular Ca ++ pool sizes for cytosol and mitochondria respectively and the P20 and P30 are taken as the exchange rates between the incubation medium and the cytosol or the medium and the mitochondria respectively

Table 4D/2. Differences in Paired Calcium Exchange Rates (P20)Between Tubules Incubated in Media With or Without Insulin

Experiment No.	Exchange rates			t-test (paid Differences	
	With Insulin	No Insulin	Difference		
1	0.094	0.023	0.071		
2	0.120	0.069	0.051	t	2.768
3	0.090	0.079	0.011	P (1Tail)	0.020
4	0.026	0.018	0.008	Critical t	2.015
5	0.127	0.020	0.107	P (2 Tail)	0.039
6	0.136	0.117	0.019	Critical t	2.570
Total	0.326	0.593	-0.267		
Average	0.05	0.10	-0.045	Total Ave *4A+4B+4C+4D*	146.25%
% change	181.90% of 4C				

Student t-test of paired results is shown comparing the Ca^{45} exchange rate measured in tubules incubated in media with or without insulin. The P20 exchange rates compared were assumed to be between the cytosol and the medium.

Figure 4D

Time	No Insulin		With Insulin	
0	100	SE	100	SE
1	63.82	8.74	52.75	9.35
6	50.44	11.39	35.34	10.46
16	39.36	8.08	22.14	8.23
26	27.24	5.14	14.5	5.11
36	22.01	3.55	11.46	4.48
46	19.88	3.18	11.15	3.89
56	17.01	2.86	9.27	3.96
67	15.51	2.86	8.07	3.87
77	14.16	2.04	7.16	3.43
88	12.04	2.37	6.22	3.29
98	11.66	1.96	5.35	3.18
109	10.82	1.76	4.93	2.99
140	8.2	1.22	4.24	2.57
161	7.58	2.45	3.55	2.43
187	7.1	1.10	3.17	2.31

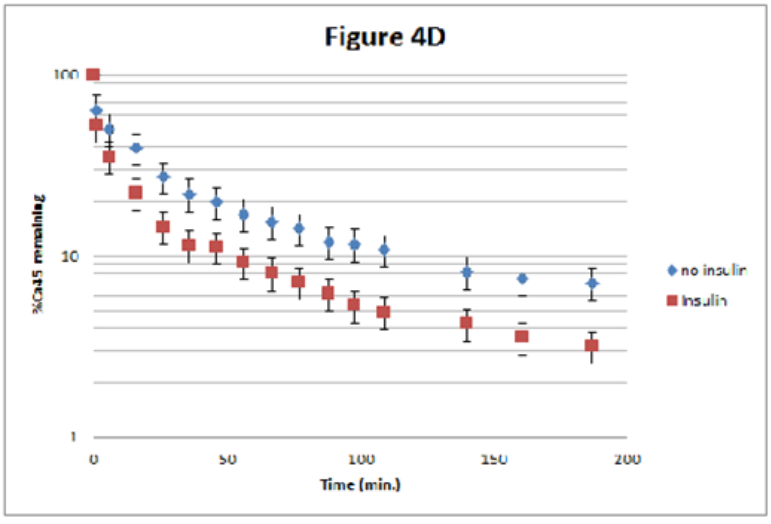


Figure 4D. Average Desaturation Curves for media with or without Insulin

Average desaturation curves are plotted using the average percentage of Calcium-45 left in tubules after each successive washing with media with or without Insulin at times indicated (min). The averages were obtained from desaturation curves presented in Apendix I Table 4D/3 (Brazier BW (2016c))

Table 4E/1. Intracellular Calcium Pool Sizes and Exchange Rates in Renal Tubules Incubated in Media With or Without Acetate

Experiment Number	Media Additions	Ca++ Pool Size (N.mol/mg cell prot.)		Ca++ Exchange Rate (p.mol/mg prot./min)	
	Ac+l-	S(2)	5(1)	P(20)	P(30)
1	+	2.65	1.52	0.095	0.008
	–	1.75	1.47	0.035	0.0073
2	+	631	1.48	0.082	0.0053
	-	5.77	2.37	0.021	0.0064
3	+	7.65	2.33	0.147	0.0005
	-	8.14	1.44	0.112	0.0007
4	+	4.21	3.72	0.113	0.0017
	~	?'.77	4.01	0.08?'	0.0018
5	+	324	3.71	0181	0.0101
	–	2.81	3.57	0.175	0.0093
6	+	4.09	2.43	0.091	0.0059
		4.11	1.47	0.047	0.0039

Table 4E/1. Pair Results from Analysis of Ca^{45} Desaturation Curves when Renal Tubules are incubated in Media with or without Acetate
Results of graphical analysis of six desaturation curves from Table 4E/3 Appendix 1 [22] Brazier BW (2016c) have been used to calculate the six sets of kinetic data. S1 and S2 are taken as intracellular Ca++ pool sizes for cytosol and mitochondria respectively and the P20 and P30 are taken as the exchange rates between the incubation media and the cytosol or the medium and the mitochondria respectively.

Table 4E/2. Differences in Paired Calcium Exchange Rates (P20) Between Tubules Incubated in Media With or Without Acetate

Experiment no	Exchange rates			t-test Paid Means	
	With Acetate	No Acetate	Difference		
1	0.095	0.035	0.074		
2	0.0845	0.021	0.063	t	4.5066
3	0.147	0.112	0.035	P (1 tail)	0.0032
4	0.113	0.082	0.030	Critical t	2.0015
5	0.180	0.175	0.005	P (2 Tail)	0.0064
6	0.090	0.047	0.043	Critical t	2.5740
Totals	0.472	0.709	0.116		
Average	0.08	0.12	0.019	Total Ave *4A+4B+4C+4D +4E*	273.43%
% change	150% of 4D				

Student t test of paired results are shown comparing the Ca^{45} exchange rate measured in tubules incubated in media with high or low sulphate levels. The P20 exchange rates compared were assumed to be between the cytosol and the medium.

Figure 4E

Time	No Acetae		With Acetate	
0	100	SE	100	SE
1	64.06	7.94	55.84	10.94
6	48.11	5.17	35.97	12.68
16	34.44	3.82	21.67	8.34
26	24.08	3.22	16.82	6.16
36	19.34	1.71	13.51	4.74
46	16.16	3.25	10.96	3.78
56	14.49	3.06	9.27	3.41
66	12.69	2.71	7.897	3.16
76	11.05	2.57	7.008	2.71
86	9.998	2.35	6.398	2.32
96	8.873	2.28	6.003	2.17
106	8.125	2.05	5.353	2.03
116	7.397	1.77	4.673	1.77
126	6.923	1.68	4.363	1.67
136	6.621	1.65	4.12	1.63
145	6.352	1.50	3.773	1.54
156	4.663	1.46	3.583	1.60
166	4.533	1.43	3.495	1.58
176	4.433	1.42	3.403	1.58
186	4.238	1.31	4.052	1.58
196	4.112	1.40	3.353	1.58

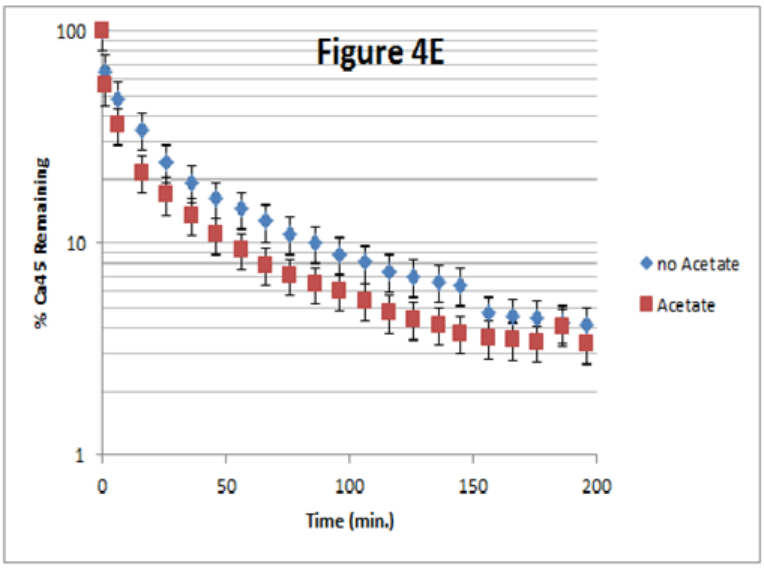


Figure 4E. Average Desaturation Curves for media with or without Acetate

Average desaturation curves are plotted using the average percentage of Calcium-45 left in tubules after each successive washing with media with or without Acetate at times indicated (min). The averages were obtained from desaturation curves presented in Apendix I Table 4D/3 (Brazier BW (2016c)).

The changes in Ca^{45} exchange rate indicate either increase or decrease over results for the previous results of each set of media conditions. These changes are indicated in Table 5 and Figure 5.

Table 5. Percentage change of Ca^{45} exchange rates

Media	pH7.2	pH6.2	+SO4	+Glutamine	+insulin	+ acetate
% change of previous	0	-50.48	+76.53	90.22	181.9	150.32
Total %			89.12	80.40	146.24	273.43

This table shows the percentage change in Ca^{45} over the exchange rates in the previous set of media conditions.

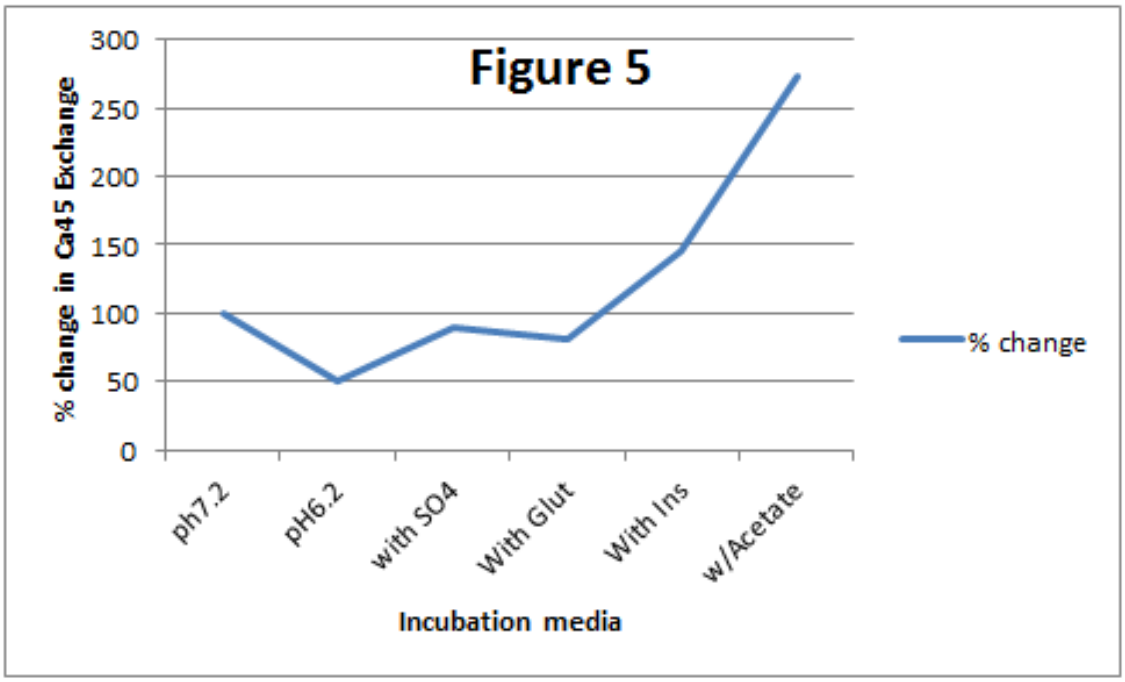


Figure 5. Percentage change of Ca^{45} exchange rates

This table shows the percentage change in Ca^{45} over the exchange rates in the previous set of media conditions.

5. Discussion

The results of experiment 4A comparing high pH with low pH (Table 4A) indicate that reduced pH has a pronounced effect on the plasma membrane transport of calcium. Experiment **B**showed that the presence of sulphate had no significant effect (Table 4B). It has been suggested that metabolic acid affects calcium transport by changing the transferase stereochemistry, [12] Studer and Borle (1979) and it has also been suggested that sulphate may inhibit Calcium uptake by complexing with the Ca^{2+} ions [8] Wlaser and Browden (1958), the latter doesnot seem to occur in this study. 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. However the changes caused by inclusion of glutamine (Table 4C) and acetate (Table 5E) in the incubation media indicates that the reduction in calcium absorption is caused by a metabolic change in thetubule cells rather than physical changes caused by sulphate or H+. Inclusion of glutamine probably causes increased ammoniogenesis, reduced renal pH also produces increased ammoniogenesis as referred to by [23] Brosnan (1978). However the mechanism by which ammonia production causes the change in Ca exchange is not yet established. Although [24] Nissin *et. al* (1991) and [25] Nissim *et. al.* (1990) have suggested that intracellular acidity stimulates ammoniogenesis by inhibiting citrate synthetase and stimulation of glutamase. The indications in this experiment are that glutamine reduced Ca absorption and that insulin increases calcium absorption the effect of glutamine may have been because of more ammoniogenesis connected to gluconeogenesis thereby reducing the availability of ATP for calcium uptake. The observation that insulin greatly increased Ca exchange can be explained by insulin inhibition gluconeogenesis and making ATP more ready available. But both effects connected to ammoniogenesis. These possibilities are considered in a future experiment using larger quantities of material so that ammonia production can be measured in single time point analysis of cellular calcium efflux.

The observation that acetate with insulin increased calcium exchange rate even more that insulin alone could be due to either more energy being made availability through the citric acid cycle or due to an inhibition of ammoniogenesis as described by [26] Tannen (1978) or both. Measurements of intra cellular potassium indicate that the cells remained viable throughout the experiment. The greatly reduced exchange rate of calcium by the tubules incubated at 30Cindicate that the process is an active biological process and not just diffusion.

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