

Kinetic Analyses of Calcium Movements in Cell Cultures

III. *Effects of calcium and parathyroid hormone in kidney cells*

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ABSTRACT Calcium compartments and fluxes were measured by kinetic analyses in kidney cell suspensions in a three-compartment closed system. The fast phase influx and compartment size increase linearly with the medium calcium and the half-time of exchange is only 1.3 min which suggests that the fast component is extracellular. The slow phase compartment rises linearly from 0.1 to 0.5 mmole calcium/kg cell water when the medium calcium is raised from 0.02 to 2.5 mM. The slow phase calcium influx exhibits the pattern of saturation kinetics with a V_{\max} of $0.065 \mu\text{mole cm}^{-2} \text{sec}^{-1}$ and a K_m of 0.3 mM indicating that it is a carrier-mediated transport process. PTH has no effect on the fast phase of calcium influx, but increases both calcium influx and the calcium pool size of the slow component. The maximum effect is obtained at medium calcium concentration of 1.3 mM. Below 0.3 mM extracellular calcium, the effects of the hormone cannot be demonstrated. PTH increases the V_{\max} of calcium influx from 0.065 to $0.128 \mu\text{mole cm}^{-2} \text{sec}^{-1}$ while the K_m rises from 0.3 to 1.15 mM. These findings suggest that PTH increases the translocation of the calcium-carrier complex across the membrane and not the carrier concentration or its binding affinity for calcium.

We have demonstrated that it was possible to detect two different calcium pools in cell culture monolayers (1-3). First, with rather crude methods, involving treatment of the cells with trypsin and EDTA, we have shown in HeLa and kidney cells that the calcium of the extracellular cell coat was proportional to the calcium concentration in the medium (Ca_o) whereas the naked cell calcium remained unchanged (1). Then, by kinetic analyses we were able to confirm the existence of two distinct calcium pools in HeLa cell monolayers and we showed that the parameters of the slow phase of calcium exchange—influx, efflux, and compartment size—were consistent

with an exchangeable intracellular calcium pool (2, 3). We also brought evidence in support of the idea that calcium influx is a passive process while calcium efflux is a metabolically dependent or active transport (2, 3).

The purpose of the present investigations was several fold. First, we wanted to improve the sampling technique of our kinetic studies to be able to determine accurately the parameters of different phases from a single uptake curve instead of using as before the mean of four or five pooled experiments. To do so, we used cells in suspensions and a very fast sampling technique instead of working with the more cumbersome cell monolayers. Second, we wanted to compare the results obtained in HeLa cells with the unidirectional fluxes and the compartment sizes¹ of kidney cells, a nonneoplastic strain from a recognized target organ for parathyroid hormone. Third, we wanted to study with this improved technique the effects of extracellular calcium on the kinetic parameters of calcium transport, and bring forth more information concerning the mechanisms of the transport processes. Finally, we decided to investigate the effects of parathyroid hormone (PTH) on the different parameters of calcium transport by the same kinetic analyses. We were interested in determining which phase was influenced by the hormone, and whether calcium influx, the rate constant, or the compartment size was primarily affected. Furthermore, by studying the effects of the hormone at different extracellular calcium concentrations (Ca_o) we were curious to see whether we could gain some insight on the mode of action of PTH. Specifically, we tried to find out whether the hormone stimulates the binding of calcium to a postulated carrier, whether it increases the amount of carrier, or whether it stimulates translocation of the calcium-carrier complex through the plasma membrane.

We found that the fluxes and pool sizes of kidney cells and HeLa cells are practically identical. The exchange rate and the compartment size of the fast phase vary linearly with the medium calcium. The size of the slow compartment also increases linearly with Ca_o , but the influx of the slow phase exhibits the pattern of saturation kinetics suggesting the involvement of a carrier mechanism. Furthermore, we found that parathyroid hormone stimulates calcium influx and increases both the rate constant and the pool size of the slow compartment. The hormone is most effective when the concentration of calcium in the medium is 1.3 mM. Both the V_{max} and the K_m of calcium influx in the slow phase are increased by PTH suggesting that translocation and not binding is primarily affected.

METHODS

1. *Cell Preparation* Monkey kidney cells (LLC MK₂) were grown as monolayers in plastic T-flasks by standard cell culture techniques in minimum essential medium

¹ From hereon, pool or compartment will mean exchangeable calcium compartment.

and Earle's salt solution supplemented with 10% calf serum and antibiotics. After 1 wk, during which the medium was changed once, the cells were harvested for the experiments. The medium was decanted, the monolayer washed once with isotonic salt solution, and gently detached in Krebs-Ringer bicarbonate buffer with a soft rubber policeman. A total of nine T-60 flasks was pooled in a 50 ml centrifuge tube, the cells centrifuged for 5 min at 700 rpm in an International centrifuge, and re-suspended in fresh buffer.

2. Calcium Uptake Experiments Approximately 1 ml of packed cells representing 60–80 mg cell protein was resuspended in 36 ml of buffer in a 50 ml Erlenmeyer flask and placed on a submersible magnetic stirrer in a 37°C water bath. The buffer contained from 0 to 2.5 mM Ca, 200 mg% glucose, and 200 mg% albumin, and the gas phase consisted of 5% CO₂ in air. The cells were preincubated for 1 hr to assure steady-state conditions. At 0 time, 50 μCi of ⁴⁵Ca were added to the medium and the cells incubated for 2 more hours. Samples of the cell suspension were taken at 1, 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, and 120 min. The sampling procedure was as follows: for each time point two samples of 1 ml each were taken with a 1000 μl Eppendorf pipet and squirted into two 50 ml centrifuge tubes containing 40 ml of ice-cold isotonic choline chloride at pH 7.4. The two tubes, previously balanced, were immediately centrifuged at 2,500 rpm for 45 sec and stopped with the electric brake. The choline chloride was rapidly decanted and the tubes inverted for further drainage. The whole procedure, from the time of sampling to the separation of the cell pellet from the choline chloride, lasted approximately 1 min. After the tubes were thoroughly drained, 4 ml of deionized water were added and the cells were homogenized with a high intensity ultrasonic probe. Aliquots of the homogenate were analyzed for ⁴⁰Ca by the method of Borle and Briggs (4) and for cell protein by the modified Lowry method of Oyama and Eagle (5). ⁴⁵Ca was counted on a Beckman ambient temperature scintillation spectrometer. In our studies of PTH, the hormone was added at the beginning of the preincubation period, 60 min before the addition of the isotope ⁴⁵Ca, in order to maintain the steady-state conditions required for kinetic analyses and which are implied in our model. The hormone was purified by the method of Hawker, Glass, and Rasmussen (6) and had a specific activity in the range of 600 to 1000 units/mg protein. It was present in the medium at a concentration of 5 μg peptide/ml.

The methods for the measurement of the cell surface and of the cell water content have been previously published (1, 2).

3. Calculations The mathematical model of the three-compartment closed system, and the calculations of the different kinetic parameters have been published (2). From the experimental points of calcium uptake, expressed as (⁴⁵Ca activity/mg protein) divided by the medium specific activity, the uptake curve was drawn by hand. The time derivative of each point was derived from the experimental curve, plotted on semilog paper, and the parameters of the two exponentials were obtained by graphical analysis. The slope and intercept of both phases were obtained by computer. A theoretical curve was reconstructed and compared with the original data by feeding the derived constants to a computer (2). Figs. 1 and 2 show the uptake curve, the semilogarithmic plot, and the reconstructed curve of one typical experiment.

RESULTS

1. *Determination of Exchange Rate and Compartment Size* Five experiments were performed in standard Krebs-Ringer bicarbonate containing 1.3 mM calcium. One representative curve and the graphical analyses of its two phases are shown in Figs. 1 and 2. The different parameters of calcium exchange and their derived values are shown in Table I. The values obtained

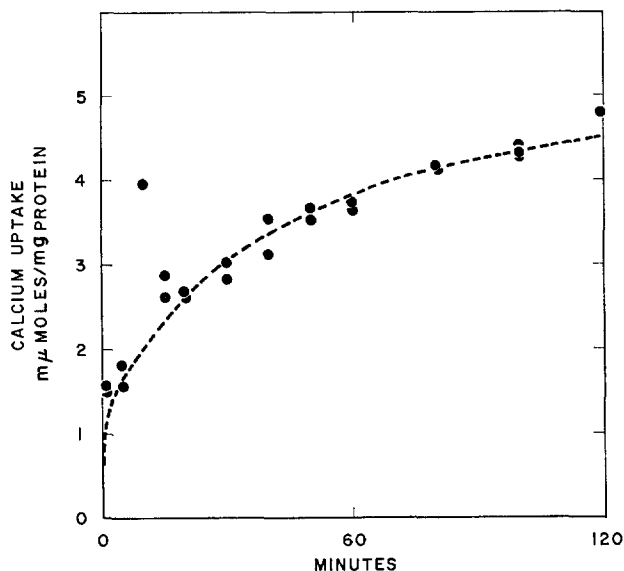


FIGURE 1. Calcium uptake in kidney cells, in a medium containing 1.3 mM calcium 1.2 mM phosphate, and 1.2 mM magnesium. The points are the actual data from one typical experiment. The dotted line is the curve reconstructed by computer from the constants derived in Fig. 2 which shows the graphical analysis of the two phases of the same experiment.

in HeLa cells (2) are included in this table for comparison between the two cell strains. The measurements of the cell surface, the cell water, and of the cell protein used for these calculations are shown in Table II. It is immediately apparent that none of these parameters is very different from those reported in HeLa cells (2). Exchange rates and compartment sizes obtained from human intestinal cells are also practically identical.² Although the calcium uptake of the cells in suspension is greater than the uptake of a cell monolayer, the calculated flux rate per surface area is of the same order of magnitude. This confirms the validity of the assumption made in our previous paper with regard to the surface area available for exchange in a cell

² A. B. Borle. Unpublished data.

monolayer (2). Although it is difficult to assign to each phase a morphological or physiological identity, we shall consider the fast compartment to be extracellular and the slow compartment to be the intracellular exchangeable calcium pool. The reasons for this choice have already been offered in pre-

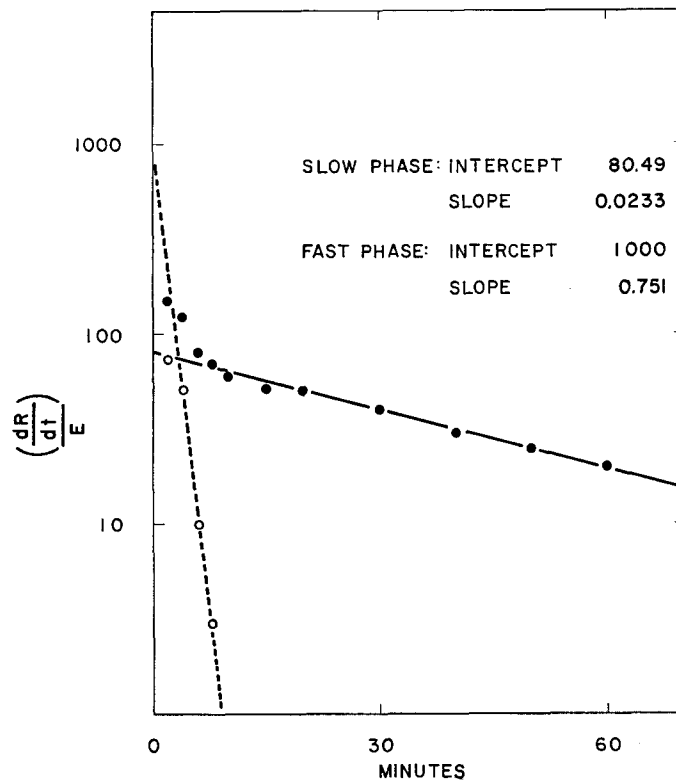


FIGURE 2. Graphical analysis of the two exponential phases derived from the uptake curve of Fig. 1. The slope and intercept of both phases were obtained by computer. From these constants a curve was reconstructed according to the formula

$$\left(\frac{dR}{dt}\right) \frac{1}{E} = -1000 e^{-(0.751)t} - 80.49 e^{-(0.0233)t}$$

and is shown in Fig. 1 as the dotted line. t is expressed in minutes.

ceding papers (2, 3): (a) The magnitude of the slow compartment size in our kidney, intestinal, and HeLa cell cultures matches the exchangeable intracellular calcium concentration reported in other cells. (b) Influx and efflux are of the same magnitude as those reported for the exchange rate between intra- and extracellular compartments in nerve and muscle. (c) The passive nature of calcium influx in the slow compartment and the fact

that efflux from the same compartment is a metabolically dependent process are in agreement with the thermodynamic conditions prevailing in the cell; i.e., with the activity gradient and the electrical potential difference.

2. *Effect of Extracellular Calcium* In this series of experiments, the cells were prepared and incubated in buffers containing concentrations of cal-

TABLE I
EXCHANGE RATES (ϕ) AND COMPARTMENT
SIZES (S) IN KIDNEY AND HeLa CELLS

	Kidney	HeLa*
A. Fast phase		
ϕ_{21} , $\mu\text{moles}/(\text{mg protein}\cdot\text{min})$	1161 \pm 223	474
Derived ϕ_{21} , $\mu\text{mole}\cdot\text{cm}^{-2}\cdot\text{sec}^{-1}$	0.580 \pm 0.011 ‡	0.430 §
S_1 , $\mu\text{moles}/\text{mg protein}$	1.90 \pm 0.43	1.06
B. Slow phase		
ϕ_{23} , $\mu\text{moles}/(\text{mg protein}\cdot\text{min})$	96.0 \pm 12.0	60.0
Derived ϕ_{23} , $\mu\text{mole}\cdot\text{cm}^{-2}\cdot\text{sec}^{-1}$	0.048 \pm 0.006 ‡	0.055 §
S_3 , $\mu\text{moles}/\text{mg protein}$	3.21 \pm 0.17	2.69
Derived S_3 , $\text{mmole}/\text{kg cell water}$	0.292 \pm 0.016	0.224

The cells were incubated in a medium containing 1.3 mM calcium.

Values are mean \pm SE.

* From reference (2).

‡ Obtained from the suspended cell surface of 33.5 cm²/mg protein.

§ Obtained from a cell surface available for exchange in a monolayer of 18.3 cm²/mg protein (2).

|| Calculated on the basis of the cell water content of 10.9 mg/mg protein.

TABLE II
PARAMETERS FOR THE MEASUREMENT OF KIDNEY
CELL SURFACE AND WATER CONTENT

Cell protein, $\mu\text{g}/10^6$ cells	347	\pm 22.3
Cell diameter, μm	19.25	\pm 0.46
Cell surface, $\mu\text{m}^2/\text{cell}$	1165	
Cell surface, $\text{cm}^2/10^6$ cells	1.165	
Cell surface, $\text{cm}^2/\text{mg protein}$	33.5	
Water content, $\text{mg}/\text{mg protein}$	10.9	\pm 0.39

Values are mean \pm SE.

cium varying from 0.02 to 2.5 mM. Aliquots of the incubating medium were taken throughout the experiment and their analysis showed that the medium calcium concentration remained constant. Fig. 3 presents the reconstructed curves of representative experiments made at several calcium concentrations. It shows that the over-all calcium uptake is markedly increased with increasing medium calcium. The effects of Ca_o on the fast component of influx are

shown in Fig. 4. Influx increases linearly 15-fold from 0.17 $\mu\text{mole}/(\text{mg protein} \cdot \text{min})$ at 0.02 mM Ca_o to 2.56 at 2.5 mM. Concurrently, the pool size increases eightfold from 0.44 to 3.71 $\mu\text{moles}/\text{mg protein}$ as shown in Fig. 5. The half-time of the reaction is very short, 1.3 min, supporting again our contention that this fast phase represents calcium exchange with an extra-

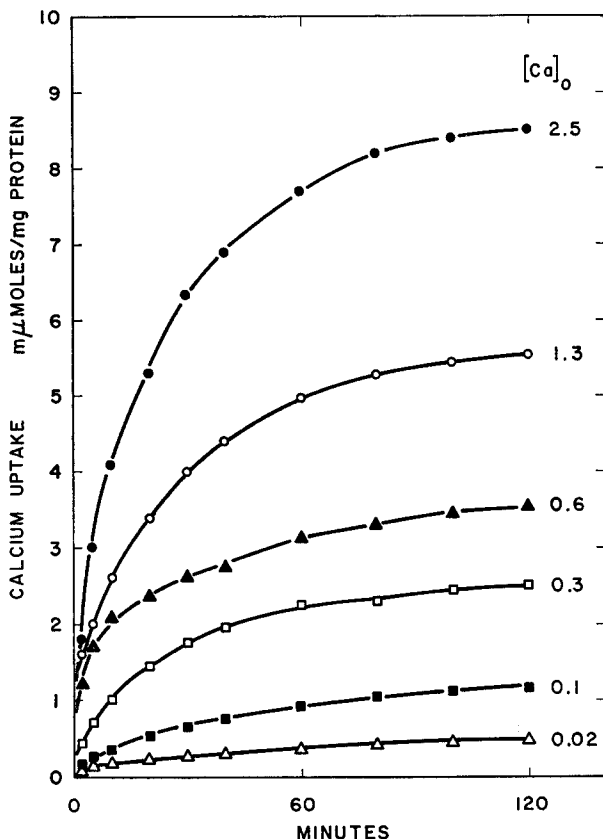


FIGURE 3. Calcium uptake curves of six representative experiments conducted in medium calcium concentrations ranging from 0.02 to 2.5 mM.

cellular calcium compartment. It could represent for instance calcium binding to some prosthetic group on the cell membrane.

The effect of the medium calcium concentration on the compartment size of the slow phase is shown in Fig. 6. Contrary to what we reported earlier (1), the exchangeable calcium pool increases linearly with increasing medium calcium. It varies from 0.08 mmole/kg cell water in calcium-free medium to 0.52 at 2.5 mM Ca_o . It is likely that the crude chemical measurements of total cellular calcium made in naked cells treated with trypsin-EDTA (1) could not detect the small changes in the exchangeable calcium fraction

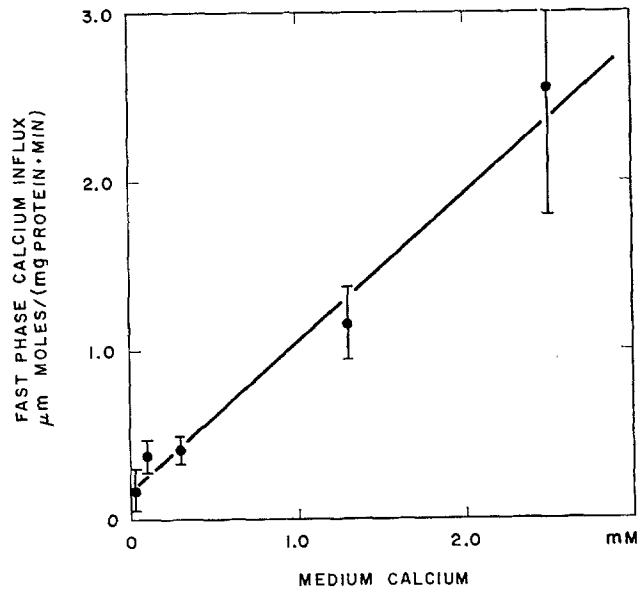


FIGURE 4. Relationship between the calcium influx of the fast phase and the medium calcium concentration. The points are the mean \pm SE of two to five experiments for each concentration.

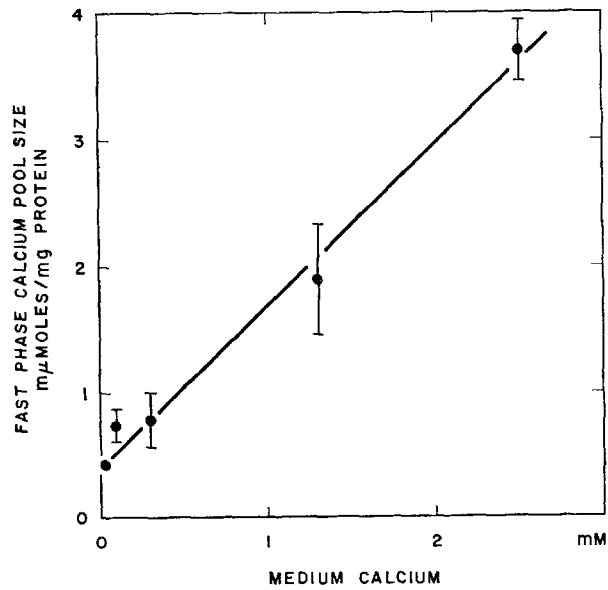


FIGURE 5. Relationship between the calcium compartment size of the fast phase and the medium calcium concentration. The points are the mean \pm SE of two to five experiments for each concentration.

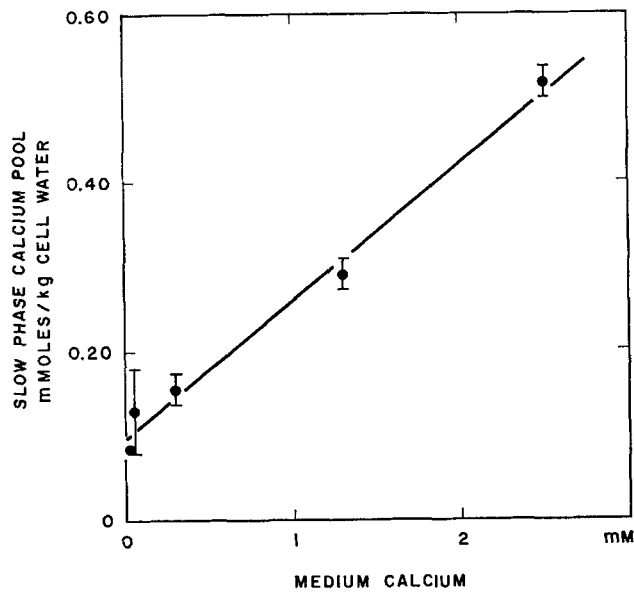


FIGURE 6. Relationship between the calcium compartment size of the slow phase and the medium calcium concentration. The points are the mean \pm SE of two to five experiments for each concentration.

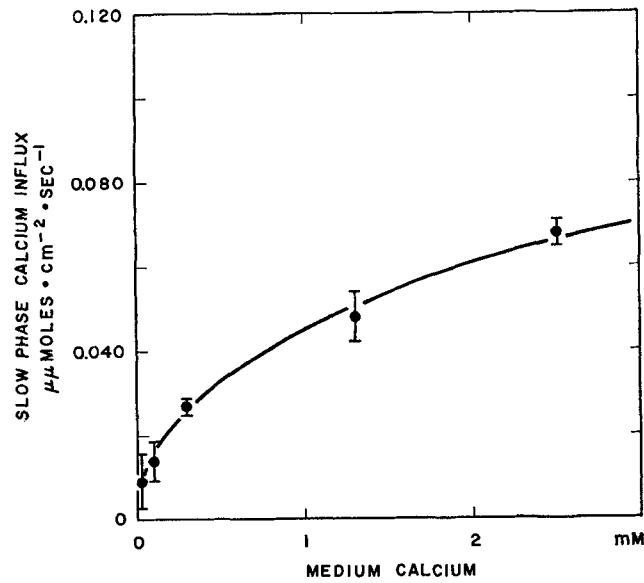


FIGURE 7. Relationship between the calcium influx of the slow phase and the medium calcium concentration. The points are the mean \pm SE of two to five experiments for each concentration.

obtained by kinetic analyses. It is also possible that the percentage of exchangeable calcium of the slow compartment increases with Ca_o at the expense of the unexchangeable fraction without changing significantly the total cellular calcium.

The effects of the extracellular calcium concentration on the slow phase of influx are quite different. Fig. 7 shows that the relationship between influx and Ca_o follows the pattern of saturation kinetics suggesting the involvement

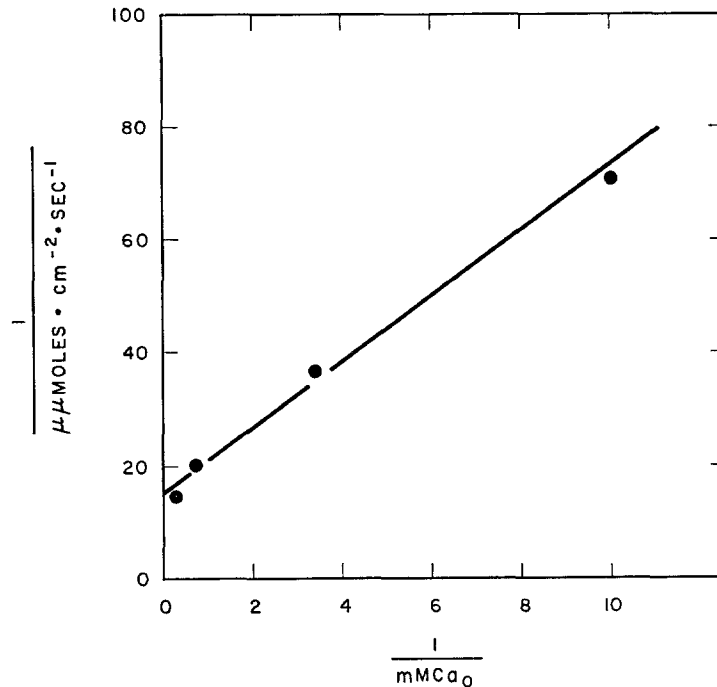


FIGURE 8. Lineweaver-Burk plot of the calcium influx into the slow compartment of exchange. The V_{max} obtained from the plot is $0.065 \mu\mu\text{mole cm}^{-2} \text{sec}^{-1}$ and the K_m is 0.37 mM , as shown in Table V.

of a carrier mechanism. This is consistent with our hypothesis that calcium transport between the ECF and the intracellular compartment is a carrier-mediated transport. A Lineweaver Burk plot of calcium influx in the slow compartment is shown in Fig. 8. Since the exact concentration of calcium in the medium cannot be known with sufficient accuracy below 0.1 mM , the point obtained at 0.02 mM was omitted. From this plot the V_{max} and the K_m of the calcium transport process were calculated and found to be $0.065 \mu\mu\text{mole cm}^{-2} \cdot \text{sec}^{-1}$ and 0.37 mM , respectively.

3. *Effects of PTH at 1.3 mM Ca* Five control experiments and five with $5.0 \mu\text{g PTH/ml}$ were performed in Krebs-Ringer bicarbonate buffer con-

taining 1.3 mM calcium, 200 mg% of glucose, and 200 mg% albumin. The data were calculated by two different methods which gave qualitatively identical but quantitatively slightly different results. With the first method, each experiment was analyzed individually and the fluxes and compartment size of each phase were computed for that particular experiment. The mean and standard error of each parameter of five controls and five PTH experiments were calculated and are shown in Table III. Although the parameters of the fast phase are slightly larger in the PTH group than in the controls,

TABLE III
EFFECT OF 5 $\mu\text{g/ml}$ PTH ON CALCIUM FLUXES
AND CALCIUM COMPARTMENT SIZE IN KIDNEY CELLS
CALCULATED FROM SINGLE EXPERIMENTS

	Control		PTH calculated individually	
A. Fast phase				
ϕ_{21} , $\mu\text{moles}/(\text{mg protein}\cdot\text{min})$	1161	± 223	1336	± 60
		(5)		(5)
S_1 , $\mu\text{moles}/\text{mg protein}$	$1.90 \pm$	0.43	$2.43 \pm$	0.27
		(5)		(5)
$t/2$, min	$1.30 \pm$	0.12	$1.22 \pm$	0.11
		(5)		(5)
B. Slow phase				
ϕ_{22} , $\mu\text{mole}\cdot\text{cm}^{-2}\cdot\text{cm}^{-1}$	$0.043 \pm$	0.004	$0.077 \pm$	0.009*
		(5)		(5)
S_2 , mmole/kg cell water	$0.302 \pm$	0.016	$0.431 \pm$	0.041†
		(5)		(5)
$t/2$, min	$26.2 \pm$	1.9	$22.3 \pm$	3.4
		(5)		(5)

Experiments performed at a medium calcium concentration of 1.3 mM.

Values are mean \pm SE.

Number in parentheses indicates number of experiments.

† $p < 0.02$.

* $p < 0.01$.

the difference is not significant. In the slow phase, however, PTH increases influx 80% from 0.043 to 0.077 $\mu\text{mole cm}^{-2} \text{sec}^{-1}$ and the calcium pool size 43% from 0.302 to 0.431 mmole/kg cell water, both of these effects being statistically significant.

With the second method of calculation, the time points of the uptake curves of all five experiments in both groups were averaged and a mean calcium uptake curve was drawn and analyzed. The mean and standard error of each time point are shown in Fig. 9. From these curves, the parameters of calcium uptake were calculated as usual and the results are shown in Table IV. With this method, the effects of parathyroid hormone are more

pronounced. Calcium influx is increased 195%, the pool size 61%, and the rate constant 86%.

4. *Effects of PTH at Different Extracellular Calcium Concentrations* The effects of 5 $\mu\text{g}/\text{ml}$ of parathyroid hormone were investigated at 0.02, 0.1, 0.3, 1.3, and 2.5 mM. Each experiment was analyzed separately to obtain the

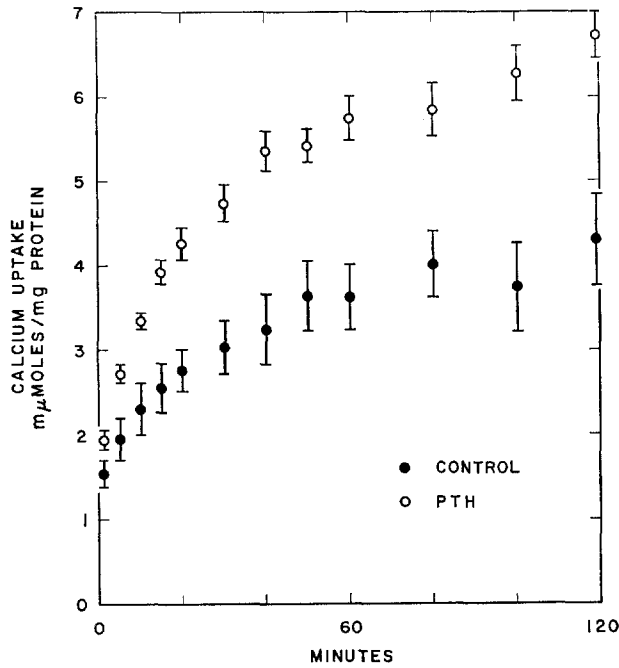


FIGURE 9. Calcium uptake in kidney cell suspensions in a Krebs-Ringer bicarbonate buffer containing 1.3 mM calcium. Pure parathyroid hormone was added in the experimental group at a concentration of 5.0 $\mu\text{g}/\text{ml}$ medium. The values are the mean \pm SE of five experiments in each group.

TABLE IV
PARAMETERS OF THE SLOW PHASE OF CALCIUM UPTAKE
CALCULATED FROM THE CURVES OF FIG. 9

	Control	PTH*	Change
			%
ϕ_{23} , $\mu\text{mole cm}^{-2} \cdot \text{sec}^{-1}$	0.021	0.062	+195
S_3 , mM/kg cell water	0.255	0.410	+61
k , min^{-1}	0.0147	0.0273	+86
$t/2$, min	46.8	25.4	

* Five experiments were performed in each group in a medium containing 1.3 mM Ca. PTH was added at a concentration of 5 $\mu\text{g}/\text{ml}$.

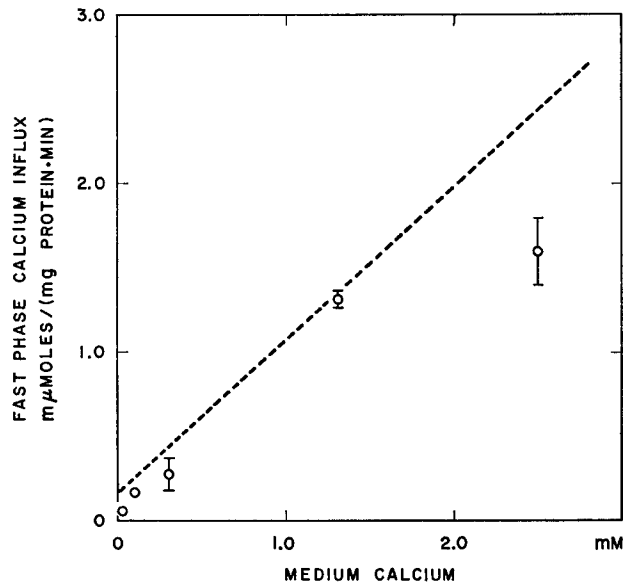


FIGURE 10. Effect of 5.0 $\mu\text{g/ml}$ of PTH on the calcium influx of the fast compartment in media ranging from 0.02 to 2.5 mM calcium. The values are the mean \pm SE of two to five experiments at 0.3, 1.3, and 2.5 mM Ca_o . Single experiments were performed at 0.02 and 0.1 mM Ca_o . The dotted line represents the relationship obtained in control experiments.

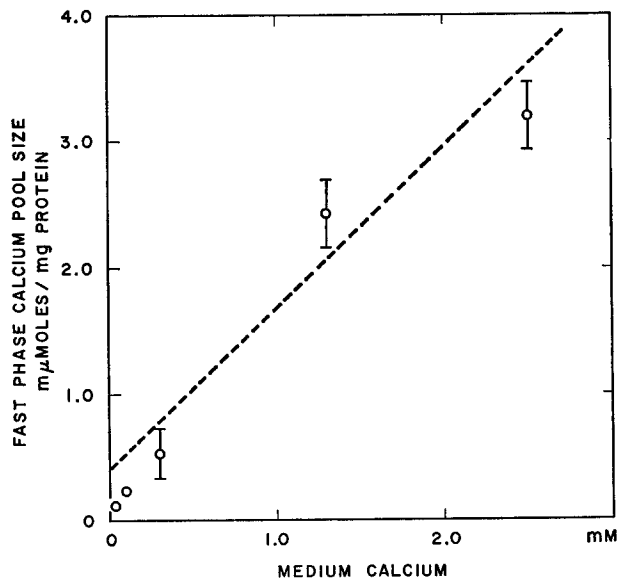


FIGURE 11. Effect of 5.0 $\mu\text{g/ml}$ PTH on the calcium pool of the fast phase. The dotted line represents the relationship obtained in control experiments. The experimental conditions are the same as those described in the legend for Fig. 10.

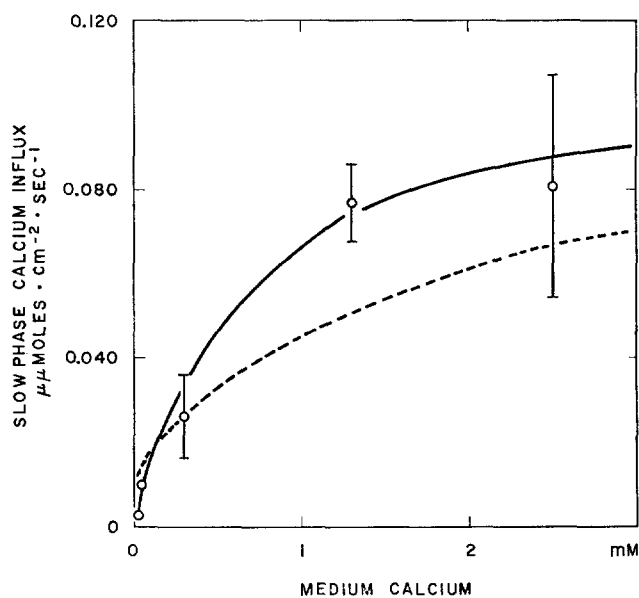


FIGURE 12. Effect of 5.0 $\mu\text{g}/\text{ml}$ PTH on the calcium influx in the slow compartment. The dotted line is the curve obtained in control experiments. The values are the mean \pm SE of two to five experiments. At 0.02 mM Ca_o a single experiment was performed.

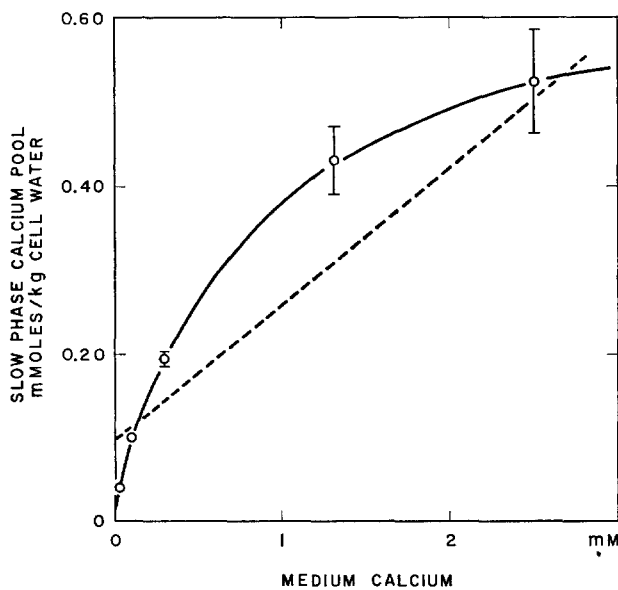


FIGURE 13. Effect of 5.0 $\mu\text{g}/\text{ml}$ PTH on the calcium compartment size of the slow phase. The dotted line represents the relationship obtained in control experiments. The experimental conditions are the same as those described in the legend for Fig. 12.

different parameters of calcium uptake in the two compartments. Fig. 10 shows that PTH does not affect the calcium exchange rate of the fast phase at any medium calcium concentration. The same is true for the calcium pool size of the fast phase as shown in Fig. 11. The small deviations from the dotted lines, which are drawn from the control data, are not statistically significant.

The effects of PTH on the calcium influx and pool size of the slow phase are shown in Figs. 12 and 13. It is interesting to see that the maximum effect

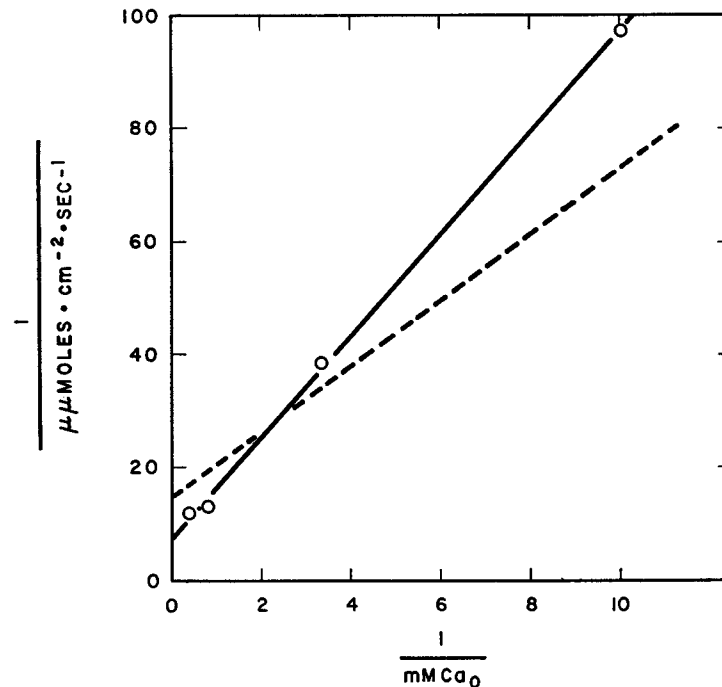


FIGURE 14. Lineweaver-Burk plot of calcium influx in the slow compartment in the presence of 5.0 $\mu\text{g}/\text{ml}$ of PTH. The dotted line represents the Lineweaver-Burk plot of control experiments. The V_{max} and K_m derived from the plot are presented in Table V.

of the hormone is obtained at 1.3 mM Ca_0 . Below 0.3 mM the values for both influx and pool size are, if anything, less than control. At a medium calcium concentration of 2.5 mM influx is slightly greater than control but the compartment size is identical. The relationship between medium calcium and compartment size is linear in the control while it is curvilinear in PTH-treated cells. It would be interesting to study this relationship at medium calcium concentrations higher than 2.5 mM. Unfortunately, precipitation of calcium phosphate usually occurs at higher concentrations of calcium and it is impossible to ascertain whether the difference in the linearity of the curves between control and PTH is a real one. It is interesting to note, however,

that the same difference in pattern has been observed between control and PTH-treated kidney slices with regard to the cellular activity of calcium-dependent enzyme systems (H. Rasmussen, personal communication).

A Lineweaver-Burk plot of the calcium influx in the slow phase after PTH treatment is shown in Fig. 14, and can be compared with the control plot. The V_{\max} and K_m obtained in both control and PTH-treated groups are shown in Table V. Parathyroid hormone doubles the V_{\max} of calcium influx

TABLE V
EFFECTS OF 5 $\mu\text{g/ml}$ PTH ON THE V_{\max} AND K_m
OF CALCIUM INFLUX IN THE SLOW COMPARTMENT

	Control	PTH
V_{\max} , $\mu\text{mole cm}^{-2}\cdot\text{sec}^{-1}$	0.065	0.128
K_m , mM Ca_o	0.37	1.15

TABLE VI
COMPARISON BETWEEN THE OBSERVED CALCIUM FLUXES
AND THE VELOCITIES CALCULATED FROM EQUATION 7

Medium calcium	Control		PTH	
	Observed ϕ_{23}	Calculated * V	Observed ϕ_{23}	Calculated † V
	$\mu\text{mole cm}^{-2}\cdot\text{sec}^{-1}$			
0.1	0.014 ± 0.005	0.014	0.010 —	0.010
0.3	0.027 ± 0.002	0.029	0.026 ± 0.011	0.026
1.3	0.048 ± 0.006	0.050	0.077 ± 0.009	0.068
2.5	0.068 ± 0.003	0.057	0.081 ± 0.027	0.088

Experimental values are mean \pm SE.

* Calculated from a V_{\max} of 0.065 and a K_m of 0.37.

† Calculated from a V_{\max} of 0.128 and a K_m of 1.15.

from a control value of 0.065 to 0.128 $\mu\text{mole cm}^{-2}\text{ sec}^{-1}$. In addition, the K_m is increased threefold from 0.37 to 1.15 mM Ca_o . The comparison between the theoretical fluxes calculated from the V_{\max} and K_m obtained and the original data is shown in Table VI.

DISCUSSION

It is immediately apparent from these experiments that the flux rate and the compartment size of the two phases in kidney cells are practically identical to those obtained in HeLa cells, provided that one bears in mind that HeLa cell monolayers have only one surface exposed, while in kidney cell

suspensions the whole surface is available for exchange. This means that only the fluxes expressed on the basis of surface area are literally comparable while fluxes expressed on the basis of milligrams of cell protein would be expected to show a roughly twofold difference as can be seen in Table I. The calcium pool of the slow component of exchange, ranging from 0.1 to 0.5 mmole/kg cell water, agrees well with the exchangeable intracellular calcium of other tissues, and the calcium fluxes observed are also in the same order of magnitude (7-15). It appears, however, that the average calcium influx reported here is a basal rate which can fluctuate significantly with the external medium composition and in the presence of agents such as parathyroid hormone. In kidney cells, the mean calcium influx with a medium containing 1.3 mM calcium and normal concentrations of phosphate and magnesium ranges between 0.03 and 0.05 $\mu\mu\text{mole cm}^{-2} \text{sec}^{-1}$. In the absence of both phosphate and magnesium, and a medium calcium of 5.0 mM, calcium influx increases 10-fold to 0.3 $\mu\mu\text{mole cm}^{-2} \text{sec}^{-1}$.³ These results agree very well with those obtained in squid axon in artificial seawater containing 11 mM calcium and no phosphate in which calcium influx averages 0.15 $\mu\mu\text{mole cm}^{-2} \text{sec}^{-1}$, but varies between 0.04 and 0.6 $\mu\mu\text{mole cm}^{-2} \text{sec}^{-1}$ (14). The medium composition is not the only factor which influences calcium influx: parathyroid hormone, for instance, can double calcium influx in the slow phase, increasing the V_{max} of the transfer from 0.065 to 0.128 $\mu\mu\text{mole cm}^{-2} \text{sec}^{-1}$. But despite these variations due to the composition of the extracellular fluids and the metabolic activity of the cells, calcium metabolism does not seem to markedly differ from cell to cell.

The slow time constant and the pattern of saturation kinetics obtained in the slow component of influx are good arguments in support of our contention that it represents translocation of calcium down an electrochemical potential gradient from an extracellular compartment to an intracellular phase and that it involves a carrier mechanism (2, 3).

Since calcium influx into the cell is a limiting factor in the over-all directional transport, one can wonder whether these fluxes are of sufficient magnitude to allow, for instance, tubular reabsorption of calcium. Assuming that 99% of the calcium filtered in the glomeruli is reabsorbed and that calcium reabsorption occurs in the proximal tubule only, the calcium influx per day for the total surface area available should be able to reach the value of the filtered load. With an average glomerular filtration rate of 120 ml/min and a normal filterable calcium of 1.6 mmoles/liter, the total calcium filtered per day in man is 276 mmoles or 11 g. The proximal tubule is reported to have a diameter of 60 μm and a length of 14 mm (16). In addition, the microvilli increase the cellular surface area of the proximal convoluted tubule some 40 times (17). Since up to 4.5 million nephrons per kidney have been

³ A. B. Borle, in preparation.

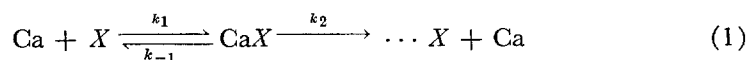
reported in man (16), the total surface area available in the proximal tubules alone is $9.5 \cdot 10^6 \text{cm}^2$. With calcium influx rates ranging from $0.055 \mu\text{mmole cm}^{-2}\text{sec}$ (at 1.3 mM Ca_o and in the presence of phosphate) to 0.3 (at 2.5 mM Ca_o without phosphate), calcium reabsorption can fluctuate between 1.8 and 9.9 g/day . If one takes into account the physiological effects of parathyroid hormone normally present, it is evident that our calculations of calcium influx, in the proximal tubule alone, are in the same order of magnitude as the filtered load.

The fact that the compartment size of the slow phase increases with rising medium calcium concentrations is also of interest. If one accepts our conclusion that the slow compartment represents the intracellular exchangeable calcium pool, these results suggest that extracellular calcium does influence the intracellular calcium concentration. This would explain why increasing extracellular calcium concentrations can mimic the effects of parathyroid hormone which has been shown to increase the intracellular calcium concentration (1, 18, 20, 21). We have to assume, however, that the ionized calcium which is only a small fraction of the intracellular calcium pool is proportionally affected, although the actual proof that this is so is presently lacking.

The linear increase of the calcium exchange rate and of the pool size of the fast phase with increasing medium calcium, and the very short half-time of the process further support our previous assumption that this fast phase is extracellular. The dramatic increase in pool size which rises eightfold with medium concentrations of calcium ranging from 0.02 to 2.5 mM may very well be the experimental equivalent of the extracellular calcification observed *in vivo* with hypercalcemia (21, 22).

It is clear that our previous conclusions concerning the effects of parathyroid hormone on calcium transport are abundantly substantiated in this series of experiments (1, 18, 19). Although the experimental techniques were entirely different, the results are practically identical. The fact that PTH does not affect the exchange rate and the pool size of the fast component of influx which we consider to be extracellular is in complete agreement with the lack of effect of PTH on the calcium concentration of the extracellular coat of HeLa cells (1). Similarly, the stimulation by PTH of calcium influx and the increase in pool size of the slow compartment which represents the intracellular exchangeable calcium pool are comparable with the larger calcium uptake and the increased cell calcium concentration obtained in HeLa and kidney cells (1, 18, 19).

The effects of parathyroid hormone on the V_{max} and the K_m of the slow phase of calcium influx bring forth an interesting clue regarding the mode of action of the hormone. According to the classic formulation of carrier-mediated transport kinetics, the process can be described as follows:—



where Ca is the extracellular calcium and X, the postulated carrier located in the membrane. Following the Briggs-Haldane derivation, the velocity of the reaction, v , will be

$$v = k_2[\text{CaX}] \quad (2)$$

Since

$$[\text{CaX}] = \frac{[X_t] \cdot [\text{Ca}]}{[\text{Ca}] + \frac{k_2 + k_{-1}}{k_1}} \quad (3)$$

where X_t is the total concentration of carrier, we can write

$$v = \frac{k_2[X_t][\text{Ca}]}{[\text{Ca}] + \frac{k_2 + k_{-1}}{k_1}} \quad (4)$$

By definition

$$K_m = \frac{k_2 + k_{-1}}{k_1} \quad (5)$$

and

$$V_{\max} = k_2[X_t] \quad (6)$$

therefore

$$v = \frac{V_{\max} [\text{Ca}]}{K_m + [\text{Ca}]} \quad (7)$$

In our case, parathyroid hormone increases both the V_{\max} and the K_m . Moreover, we observed that calcium influx is stimulated above 0.3 mM Ca_o and depressed below that concentration. The only explanation involving the modification of a single factor is that PTH increases k_2 . An increased rate of transfer k_2 would perfectly account for (a) the increased K_m according to equation 5, (b) an increased V_{\max} according to equation 6, and (c) an over-all stimulated transfer through the membrane. In addition, the decreased velocity of transfer v , below 0.3 mM Ca_o , can also be accounted for, since an increased k_2 affects the numerator and the denominator of equation 4 differently depending on the medium calcium concentration. Table VI shows the theoretical velocities of calcium influx calculated from the respective V_{\max} and K_m at given medium calcium concentrations as compared with the experimental data. The table shows a very good agreement between the observed and calculated values. According to the same calculations the

crossover point where PTH will fail to produce a stimulation of calcium influx is in the vicinity of 0.4 mM Ca_o. The implication which can be derived from these considerations is that parathyroid hormone stimulates calcium transport by increasing the rate of transfer of the calcium-carrier complex across the membrane and not by increasing the carrier concentration or raising its binding affinity for calcium. It is clear that the formulation of the transport process shown in equation 1 may be an oversimplified representation of the course of events. Nevertheless, regardless of the number of steps represented by the rate constant k_2 and despite our ignorance of the molecular mechanism involved, we can conclude that parathyroid hormone increases the carrier-mediated influx of calcium.

APPENDIX

The present method of calculation, which was outlined in a previous paper (2), offers several advantages but also has a number of limitations which should be clearly

TABLE VII
HALF-TIME OF THE FAST PHASE AT
DIFFERENT CALCIUM CONCENTRATIONS

Medium calcium	Half-time
<i>mM</i>	<i>min</i>
0.01	1.83 ± 0.20
0.10	1.26 ± 0.16
0.30	1.25 ± 0.13
0.60	0.96 ± 0.10
1.30	1.39 ± 0.11
2.50	1.23 ± 0.15

underlined. Because the fast phase exchanges so rapidly that it becomes almost immediately part of the medium compartment, the parameters of the slow component can be calculated as in a two-compartment system or as in the parallel case of a three-compartment system. It should be emphasized that the equations used will not satisfactorily describe a series model in general but only the special case in which the flux of the fast phase is fast, and many times faster than the flux of the slow phase. In our case the half-time of the fast phase remains within the 1 min range at all calcium concentrations studied (Table VII) and the rate constants of the two phases differ by a factor of 20. The main advantage of the method is that it only requires the determination of the cell ⁴⁵Ca activity and the cell protein. Also the change in specific activity of the medium is so small that it can be neglected. Thus the medium specific activity becomes a constant. Although the parameters of the fast phase can contain a considerable error, the accuracy of the slow phase values is very good and totally independent of the inherent error of the fast phase. This might be the best asset of the method providing that one is mostly interested in the slow compartment.

The method of Robertson et al. (23) can also be used to calculate our data and in

addition it can discriminate between a parallel and a series model. However, there are several disadvantages in applying it to our system. For instance, the very small difference between the medium specific activity at 0 time (X_20) and the specific activity at infinite time (E) has to be known with great accuracy and the smallest imprecision will result in very great errors. In addition, errors of a few per cent in the estimate of the cell specific activity at infinite time may produce differences of up to 25% in the estimate of the fast flux and of the fast compartment size with little effect, however, on the parameters of the slow phase. Such errors are most likely to occur in experiments performed at low medium calcium concentrations because several more hours of incubation may be required to reach steady-state values for the specific activity of the cells.

TABLE VIII
EXPERIMENTAL VALUES AND COMPUTER-GENERATED
CURVES OF AN EXPERIMENT PERFORMED AT A MEDIUM
CALCIUM CONCENTRATION OF 0.013 mM

Time	Calcium uptake		Cell specific activity	
	Data	Computer*	Data	Computer†
<i>min</i>	<i>pmoles/mg protein</i>		<i>cpm/pmoles</i>	
1	116	101	1.76	1.59
5	212	213	3.22	3.27
10	245	243	3.74	3.73
15	268	266	4.09	4.08
20	286	287	4.36	4.41
30	330	324	5.02	5.00
45	382	388	5.83	5.75
60	425	409	6.48	6.34
90	476	464	7.25	7.20
120	498	500	7.59	7.77
150	489	523	7.45	8.13
180	571	538	8.70	8.37

* Computer-generated curve from equation 8.

† Computer-generated curve from equation 9.

In order to compare our method of calculation with that of Robertson et al. (23) an experiment performed at a medium calcium concentration of 0.013 mM was selected and computed according to both methods. The second column of Table VIII presents the experimental values of the calcium uptake curve calculated according to our method: calcium uptake = (cell radioactivity/cell protein) divided by the medium specific activity. The derivation by graphical analysis of the two exponential functions gives the following equation for the curve:

$$\frac{dR_{1+3}}{dt} = 190 e^{-0.688t} + 5.41 e^{-0.0144t} \quad (8)$$

The computer-generated curve obtained from this equation gives the values shown in the third column of Table VIII and they compare favorably with the actual data. Equating the coefficients of the fast and slow exponential terms with the fluxes (ϕ) and the exponential constants with the rate constants (k), we can calculate the compart-

TABLE IX
BASIC DATA FROM THE EXPERIMENT
DESCRIBED IN TABLE VIII

Medium	
Calcium concentration, <i>mM</i>	0.013
Medium calcium, <i>pmoles/ml</i>	13,000
⁴⁵ Ca radioactivity, <i>cpm/ml</i>	1,980,000
Specific activity at 0 time, <i>cpm/pmole</i>	152.3
Specific activity at ∞ time, <i>cpm/pmole</i>	145
Cells	
Cell calcium, <i>pmoles/ml</i>	10,800
Cell protein, <i>mg protein/ml</i>	1
Cell ⁴⁵ Ca activity at ∞ time, <i>cpm/mg protein</i>	88,500
Cell specific activity at ∞ time, <i>cpm/pmole</i>	8.2
Relative specific activity $\left(\frac{\text{cell sp. } \alpha}{\text{medium sp. } \alpha}\right)$ at ∞ time	0.0566
Exchangeable cell calcium, <i>pmole/mg protein</i>	610

TABLE X
DATA FOR THE DETERMINATION OF THE PARAMETERS
OF A THREE-COMPARTMENT CLOSED SYSTEM ACCORDING TO
ROBERTSON et al. (23) FROM SPECIFIC
ACTIVITY CURVE DESCRIBED BY EQUATION 9

C_{11} , <i>cpm/pmoles</i>	3.10
C_{12} , <i>cpm/pmoles</i>	0.085
λ_1 , <i>min⁻¹</i>	0.718
λ_2 , <i>min⁻¹</i>	0.0144
X_20 , <i>cpm/pmoles</i>	152.3
E , <i>cpm/pmoles</i>	145.0
R_20 , <i>cpm/ml</i>	1,980,000

C_{11} , C_{12} are the coefficients of the fast and slow exponential terms and λ_1 , λ_2 , the exponential constants derived from equation 9 describing the cell specific activity curve. X_20 is the medium specific activity at 0 time, E the specific activity at ∞ time, and R_20 , the amount of tracer in the medium at 0 time.

ment size (S) from the equation: $S = \phi/k$. The fluxes and the compartment size calculated by this method are shown in Table XII and can be compared with the values obtained by the Robertson et al. method (23). The cell specific activity (S.A.) used to compute the same data according to Robertson et al. (23) is shown in the fourth column of Table VIII. The curve obtained has the following equation

$$\frac{dS.A.}{dt} = 3.10 e^{-0.718t} + 0.085 e^{-0.0144t} \quad (9)$$

and the computer-generated curve from this equation is given in the fifth column of

Table VIII. Other basic information about the experimental conditions is given in Tables IX and X and the results of the computation are shown in Table XI. Table XII compares the fluxes and compartment sizes obtained by the two methods. It is clear that there is no significant difference among the three sets of results and that

TABLE XI
PARAMETERS OF THE THREE-COMPARTMENT
CLOSED SYSTEM OBTAINED FROM THE DATA OF
TABLE X ACCORDING TO ROBERTSON et al. (23)

Rate constant	Compartment size	Fluxes
min^{-1}	$\mu\text{moles/mg protein}^*$	$\mu\text{moles}/(\text{mg protein}\cdot\text{minute})^*$
Parallel case		
k_{21} 0.0142	S_1 262	ϕ_{12} 184.61
k_{12} 0.7037	S_2 13,000	ϕ_{23} 5.48
k_{23} 0.0004	S_3 392	
k_{32} 0.0139		
Series case		
k_{12} 0.0146	S_1 13,000	ϕ_{12} 190.10
k_{21} 0.6838	S_2 277	ϕ_{23} 5.41
k_{23} 0.0194	S_3 376	
k_{32} 0.0143		

* The units in the solution are actually picomoles per milliliter of cell suspension; but since there is 1 mg cell protein/ml of cell suspension, the results can be directly expressed on a cell protein basis.

TABLE XII
COMPARISON BETWEEN THE RESULTS OBTAINED FOR
THE SERIES AND PARALLEL CASES ACCORDING TO ROBERTSON
et al. (23) AND THOSE OBTAINED BY THE
CALCULATIONS OUTLINED IN THE PRESENT METHOD

	Robertson et al.		Present method*
	Series case	Parallel case	
Compartment sizes $\mu\text{moles/ml}\ddagger$			
Medium	13,000	13,000	13,000
Fast compartment	277	262	279
Slow compartment	376	392	377
Fluxes, $\mu\text{moles/mg protein}\cdot\text{min}$			
Fast component	190	184	190
Slow component	5.41	5.48	5.41

* Values obtained from equation 8 describing the uptake curve of Table VIII.

‡ Values can be expressed either as picomoles per milliliter medium or picomoles per milligram cell protein since there is 1 mg cell protein/ml medium.

the distinction between the series and the parallel case is quite academic in our cell system. But it should be emphasized again that this is so only because of the rapid rate of exchange of the fast compartment and the fit does not preclude a possible error in the estimate of the parameters of the fast phase with either method.

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