CALCIUM METABOLISM IN HELA CELLS AND THE EFFECTS OF PARATHYROID HORMONE

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ABSTRACT

Calcium metabolism was investigated in HeLa cells. 90% of the calcium of the cell monolayer is bound to an extracellular cell coat and can be removed by trypsin-EDTA. The calcium concentration of the naked cell, freed from its coat, is 0.47 mM. The calcium concentration of the medium does not affect the concentration of the naked cell calcium. However, the calcium of the cell coat is proportional to the calcium concentration in the medium. Calcium uptake into the cell coat increases with increasing calcium concentration of the medium, whereas uptake by the naked cell is independent of the calcium of the medium. Anaerobic conditions and metabolic inhibitors do not inhibit calcium uptake by the cell, a fact suggesting that this transfer is a passive phenomenon. The calcium in the extracellular cell coat, was not affected by parathyroid hormone. In contrast, the hormone increased the cellular calcium concentration by stimulating calcium uptake or by enhancing calcium binding to some cell components. These results suggest that, contrary to current thinking, parathyroid hormone influences the cellular calcium balance by mobilizing calcium from the extracellular fluids in order to increase its concentration in some cellular compartment. It is proposed that these effects can enhance calcium transport.

INTRODUCTION

Preliminary studies have suggested that parathyroid hormone (PTH) may influence the in vitro distribution of calcium between cells grown in tissue culture and their medium (1–3). It has also been reported that PTH administered in vivo promotes accumulation of cellular calcium in skeletal muscle, myocardium, pancreas, and aorta (4). Before further investigation of these effects of the hormone could be undertaken, it was deemed necessary to study in greater detail calcium content and calcium uptake in isolated cells. It has been difficult to measure the concentration and exchange of calcium in soft tissues because calcium is distributed among several pools (5). The determination of calcium in small samples of biological material presents a technical problem, and furthermore it is often difficult to estimate the total intracellular calcium concentration since a significant fraction of what is estimated to be intracellular is, in fact, extracellular. This difficulty arises because calcium binds to extracellular ground substance components, collagen, and mucopolysaccharides, and to a pool usually overlooked, the extraneous mucoprotein cell coat. This cell coat is the layer of glycoprotein which is present outside the phospholipid layer of the plasma membrane (6). Cell cultures are ideal for the study of calcium metabolism because their use easily overcomes this difficulty, since the role of the cell coat in over-all calcium metabolism can be identified. It appears from these studies that the major part of the total cell calcium is

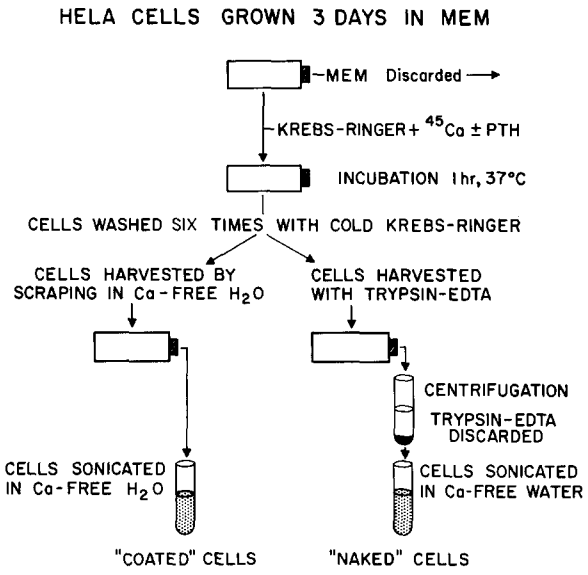

FIGURE 1 Schema showing the difference in harvesting technique between coated and naked cells. The experimental period of incubation is identical in both cases.

bound to the extracellular cell coat and can be removed by treatment of the cells with trypsin-EDTA. The calcium concentration of the cell itself, freed from its glycoprotein coat, was found to be 0.47 mmoles/kg cell water. Calcium uptake by the coat could be accounted for by its binding to the glycoproteins according to the law of mass action. The uptake by the cell seems to be a passive phenomenon since its Q_{10} is low and since it is not inhibited by anaerobic conditions or by metabolic inhibitors. The effects of parathyroid hormone (PTH) on these parameters of calcium metabolism are different from those produced by high or low calcium concentrations in the medium. The calcium in the medium seems to affect the calcium in the cell coat, whereas the hormone increases the calcium uptake and concentration in the cell itself.

MATERIALS AND METHODS

Monolayers of HeLa cells were grown in milk bottles at 37°C in minimum essential medium (MEM) with Earle's salt solution containing 10% calf serum and antibiotics. The cells were grown for 3 days before being used for the experiments which were performed as follows: The growth medium was decanted, and the monolayer was washed twice with a calcium- and magnesium-free saline solution, and then preincubated for 1 hr in 15 ml of Krebs-Henseleit bicarbonate buffer containing 10 mM glucose and varying concentrations of calcium in a gas phase of air with 5% CO_2. After the preincubation period, 20 μc of ^{45}Ca per flask was added and the cells were incubated for 60 min (unless otherwise stated). When the effects of PTH were investigated, 5 units of parathyroid extract (Lilly Co., Indianapolis) per ml of medium were added with the isotope at 0 time. A substitute for the hormone was added to the control groups; it contained 1.6% glycerol, 0.2% phenol, and 0.7% bovine albumin fraction V. At the end of the incubation, the medium was decanted and saved for specific activity determination. The monolayer was washed six times with cold buffer. The flasks were then divided into two groups, and the cells were harvested by two different procedures as shown in Fig. 1: *Procedure 1* (coated cells)—after the last rinse, the flask was inverted and drained for 1 min. 4 ml of distilled water were then added, and the cells were scraped off the glass with a polyethylene policeman, transferred to a plastic test tube, and sonicated. *Procedure 2* (naked cells)—after the last rinse, the cells were harvested by exposing the washed monolayer for 10 min to a trypsin-EDTA solution and by decanting the suspended cells into a centrifuge tube. The cells were then centrifuged for 10 min and washed once with saline. The cell pellet was suspended in 4 ml of distilled water and sonicated. It should be emphasized here that the experimental period is identical in both cases, the cells being incubated as a monolayer with their extracellular coat. The difference between coated and naked cells is an analytical one in the harvesting technique used at the end of the experiment, some of the cells being freed from their extraneous protein coat to yield the naked cells. Aliquots of the sonicates were taken for determination of protein, DNA, ^{40}Ca, and ^{45}Ca. Protein analyses

TABLE I

Protein, Water, and Calcium Content of HeLa Cells

	Coated cells	Naked cells
Cell protein (mg protein/mg DNA)	21.2±0.44 (20)	16.7±0.31 (18)
Cell water (mg water/mg protein)	8.64±0.64 (8)	12.29±0.95 (8)
Cell calcium (mμmoles Ca/mg protein)	43.52±2.60 (25)	5.74±0.30 (44)
(mμmoles Ca/mg DNA)	848.8±49.5 (16)	84.4±2.66 (38)
(mmoles Ca/kg cell water)	5.04±0.48 (8)	0.47±0.04 (8)

Values are mean ± SEM. In parentheses, number of determinations.

were made according to Oyama and Eagle (7), and DNA was determined by the method of Burton (8). Calcium was measured by the automatic fluorometric titration method of Borle and Briggs.[1] ^{45}Ca activity was measured on a Tri-Carb liquid scintillation spectrometer, by adding 0.5 ml of cell sonicate or of medium to a toluene-methyl cellosolve-PPO-POPOP cocktail mixture.

RESULTS

Calcium Content of HeLa Cells

When the calcium content of the coated cells was determined after the cells were scraped from the culture flask, a value of 43.52 mμmoles Ca/mg protein was found (Table I). On the other hand, when the cells were harvested by the classical cell culture method of trypsinization, a value of 5.74 mμmoles Ca/mg protein was obtained. It should be emphasized here that the harvest of cultured cells by trypsin-EDTA is a standard technique for dispersal of cells for reinoculation which does not affect their viability and their growth potential, and hence in all respects the "naked" cell is a normal cell. Since trypsin-EDTA disperses cell monolayers by removing the extracellular coat formed by coacervation of mucopolysaccharides, proteins, and calcium (6, 9–12), and since 87% of the total calcium of the HeLa cells in monolayer is removed by this treatment, we can assume that this fraction of the calcium is present in the coat or on the cell surface. The calcium concentration of the naked cells was measured after various trypsinization times, in order that the optimum time for complete removal of the coat and its calcium could be determined. Each time period was divided equally between exposure to

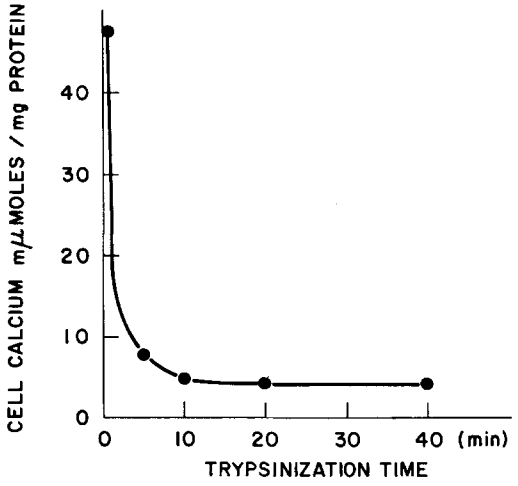

FIGURE 2 Influence of trypsinization time on the calcium content of naked cells. The cells were incubated for 1 hr in a Krebs-Henseleit bicarbonate buffer containing 1.25 mM Ca. After 1 hr, the cells were harvested according to procedure 2, with increasing trypsinization and centrifugation time. The time is divided equally between exposure to trypsin-EDTA and centrifugation. The first point represents the coated cells. Each point represents the mean of three experiments. The SEM, too small to be included in the figure, had an average of ±0.52.

[1] A. B. Borle and F. N. Briggs. 1968. Microdetermination of calcium in biological material by automatic fluorometric titration. *Analyt. Chem.* In press.

TABLE II

Influence of Six Successive Washes with EDTA, Trypsin, or Trypsin-EDTA on ^{45}Calcium Activity, ^{40}Calcium, and Protein Content of Naked Cells

	No. of washes	mg protein/mg DNA			mμmoles Ca/mg DNA			cpm^{45}Ca/mgDNA		
		EDTA	Trypsin	Trypsin EDTA	EDTA	Trypsin	T+E	EDTA	Trypsin	T+E
Coated cell	0	——21.3±0.43——			——848.8±49.5——			18,350	13,300	12,250
Naked cell	1	16.7	13.0	16.2	57	105	101	5,780	5,620	4,140
	2	14.5	14.3	15.6	82	100	89	3,540	2,720	1,680
	3	15.4	14.9	16.6	69	77	69	3,310	2,680	1,520
	4	14.2	11.6	13.0	65	77	113	3,730	1,510	737
	5	14.7	14.5	11.6	83	95	59	2,560	1,280	283
	6	14.7	10.3	11.4	68	115	77	3,140	1,930	248
Slope		−0.303	−0.463	−1.13	−1.785	+4.07	−4.74	−449	−558	−698
Intercept		16.09	14.72	18.02	74.14	83.99	101.26	5,248	4,680	3,870

Each value is the mean of two to four determinations.

trypsin-EDTA and centrifugation, i.e. a time of 20 min represents 10 min of exposure and 10 min of centrifugation. Fig. 2 shows that a steady state was reached between 10 and 20 min. Therefore, a standard trypinization time of 20 min (10 min-exposure + 10 min-centrifugation) was used for routine procedures. In addition, in order for us to study the effects of trypsin and of EDTA on the naked cell itself, the cells were washed and centrifuged several times either with EDTA alone, or trypsin alone, or trypsin-EDTA. Proteins, DNA, calcium and ^{45}Ca were analyzed after each series of washes which consisted of one to six consecutive cycles of washing and centrifugation of 10-min duration. The decrease in each component after each wash (Table II) can be compared with the differences between coated and naked cells. It is obvious that after the first wash with EDTA, trypsin, or trypsin-EDTA, which removes the cell coat, the decrement in protein, calcium, and ^{45}Ca is very slow. The slope of the regression line obtained from the gradual fall of each component was obtained by computer analysis, and the intercept of the regression line was compared with the value obtained after the first wash. The intercept extrapolated to zero wash (theoretically unwashed naked cell) is not significantly different from the value obtained after the first wash, which can then be accepted for that of the naked cell. On the other hand, in each case the difference between the coated cell and the naked cell obtained after the first wash is significantly greater than after subsequent washes, indicating two different processes; first, the removal of the cell coat, and then, progressive washout of cellular components.

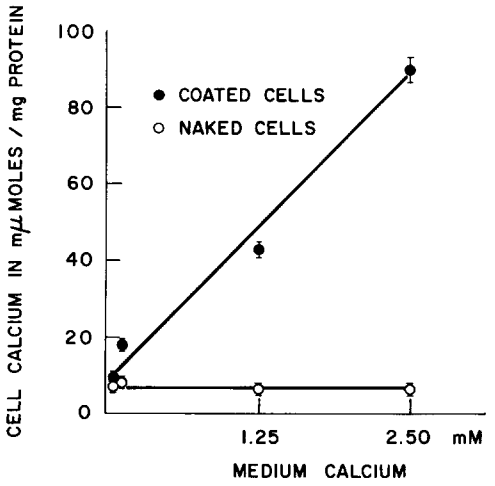

FIGURE 3 Influence of the calcium concentration in the medium on the calcium content of coated and naked HeLa cells. The cells were incubated for 1 hr in a Krebs-Henseleit bicarbonate buffer containing 0–2.5 mM calcium, then harvested by procedure 1 or 2. Each point represents the mean ± SEM of four to six experiments.

Finally, the cell water was calculated from the wet and dry weight of the cells, dried in an oven to constant dry weight. The concentration of cal-

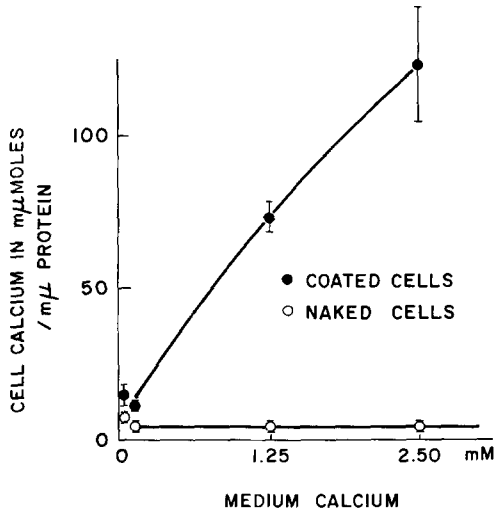

FIGURE 4 Influence of the calcium concentration in the medium on the calcium content of coated and naked cells of monkey kidney. Experimental conditions are identical with those described in Fig. 3. Each point is the mean ± SEM of five to 12 experiments.

cium expressed per kg of cell water was calculated on this basis (Table I).

The concentration of calcium in the medium $(Ca)_0$ was found to be an important factor with regard to the calcium content of the coated cells. Fig. 3 shows that the calcium content of coated cells increases from 9.68 to 90.2 mμmoles/mg protein when the $(Ca)_0$ is increased from 0 mM to 2.5 mM. In contrast, the calcium content of naked cells was unaffected by increasing the $(Ca)_0$. This finding suggests that an interaction occurs between the proteins or the mucopolysaccharides of the extraneous coat and the calcium in the medium and that this interaction does not affect the calcium concentration of the naked cell.

DeLong et al. and Coman (13, 14) have reported that cancer cells lack adhesiveness and seem to be incapable of binding adequate amounts of calcium at their surface. Since HeLa cells are derived from malignant tissue, one may ask whether they are a valid model for cellular calcium metabolism. However, results obtained with monkey kidney cells, which have a slightly higher coat calcium, show that the general relationship between calcium in the medium and calcium in the coated or naked cell is identical to the relationship found with HeLa cells (Fig. 4).

Calcium Uptake

In order for us to determine the uptake of calcium by coated cells and by naked cells, the monolayers were first preincubated with the appropriate medium for 1 hr. At 0 time, 20 μc ^{45}Ca were added, and a sample of the medium was saved for a determination of specific activity. The cells were incubated for 10, 20, 30 and 45 min. At the end of the incubation, the medium was decanted and saved for specific activity determinations. The monolayers were then harvested either by procedure 1 for the determination of calcium uptake in the coated cells, or by procedure 2 for a similar determination for the naked cells. Calcium uptake was calculated according to equation 1.

$$\frac{\text{Cell } ^{45}Ca \text{ radioactivity in cpm/mg cell protein}}{\text{Medium } ^{45}Ca \text{ specific activity in cpm/m}\mu\text{moles}} = \text{uptake in m}\mu\text{moles/mg cell protein} \quad (1)$$

Fig. 5 shows that calcium uptake in both coated and naked cells follows an exponential curve. Although the uptake by the naked cell is only a third of the uptake by the coat, it should be noted that the calcium pool of the naked cell is only a tenth of the total pool. The rate of calcium turnover, therefore, could be greater for the naked cell than for the coated cell. Fig. 6 shows that the relative specific activity and thus the percentage of the pool which has exchanged is greater in the naked cells than in the coated cells, supporting the idea that the naked cells have a faster calcium turnover.

The influence of the calcium concentration of the medium $(Ca)_0$ on calcium uptake was then studied for concentrations of 0 - 2.5 mM (Fig. 7). Uptake by the naked cell is not affected at all by changing the $(Ca)_0$, whereas uptake by the coated cell increases from 0.842 to 2.59 mμmoles/mg protein when the $(Ca)_0$ is increased from 0 to 2.5 mM, suggesting that the calcium exchange between the coat and the medium might obey mass-law considerations and might be expressed in the form of the Michaelis-Menten equation. This suggestion is supported by a Lineweaver-Burke plot (Fig. 8). It is evident that the coated cells follow saturation kinetics, whereas the naked cells do not since their uptake is independent of the $(Ca)_0$. The pattern of calcium uptake in the coated cell is compatible either with (1) a simple

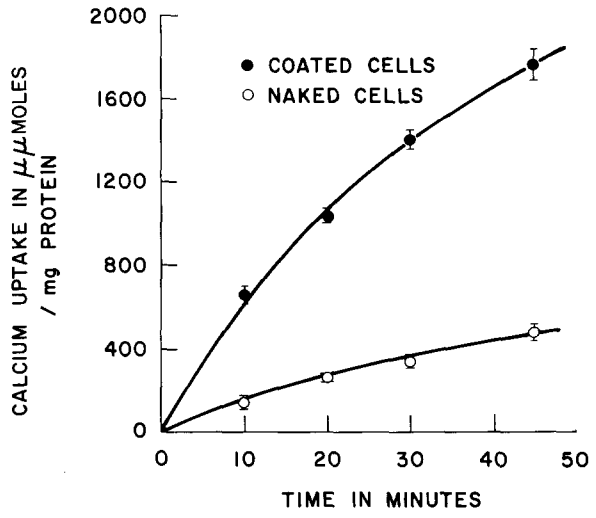

FIGURE 5 Calcium uptake in coated and naked HeLa cells. The cells were preincubated for 10–45 min in a Krebs-Henseleit bicarbonate buffer containing 1.25 mM calcium. At 0 time, 20 μc Ca^{45} were added to each flask. At the time indicated, the cells were harvested by procedure 1 or 2. Each point represents the mean ± SEM of four experiments.

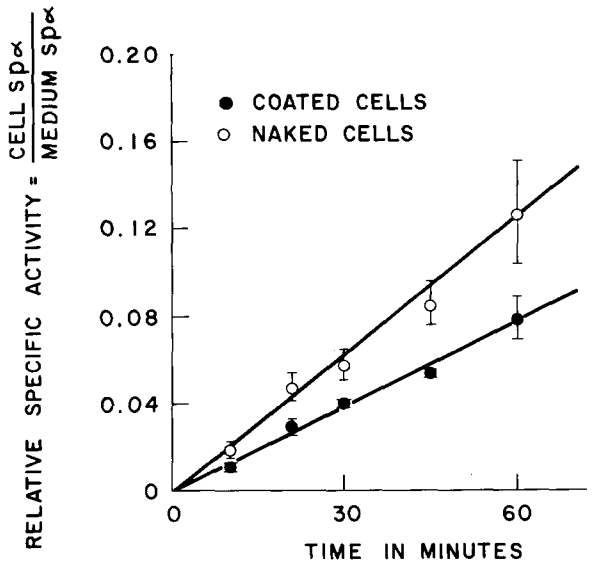

FIGURE 6 Relative specific activity of calcium of coated and naked HeLa cells at different times. Experimental conditions were identical with those described in Fig. 5. Each point represents the mean ± SEM of four experiments. The per cent of each calcium pool which has exchanged at each time point (% exchange) can be obtained by multiplying the values in the ordinate by 100.

binding to the glycoprotein of the coat, or with (2) a carrier-mediated transport, the kinetic pattern reflecting an interaction between extracellular calcium and some carrier molecule. The first possibility is more likely, however, since the greater part of the calcium of the coated cells is in the extracellular glycoprotein coat removable by trypsin-EDTA.

In contrast, calcium uptake into naked cells may represent translocation into the cell interior, although it is recognized that the ultimate proof of the actual translocation is lacking at the present time. The uptake could be interpreted also as a binding to a deeper site of the membrane or to a site unaffected by trypsin-EDTA, but the lack of any saturation kinetics as reflected by the fact that the uptake is independent of changes in external calcium argues strongly against that interpretation, unless all the sites be completely saturated at the lowest calcium concentration obtainable in the medium, i.e., 0.05 mM.

Active versus Passive Uptake

For determining whether calcium uptake into the cell is an active or a passive process, ^{45}Ca uptake was studied under anaerobic conditions, or in the presence of metabolic inhibitors. In addition, the Q_{10} of the process was also determined.

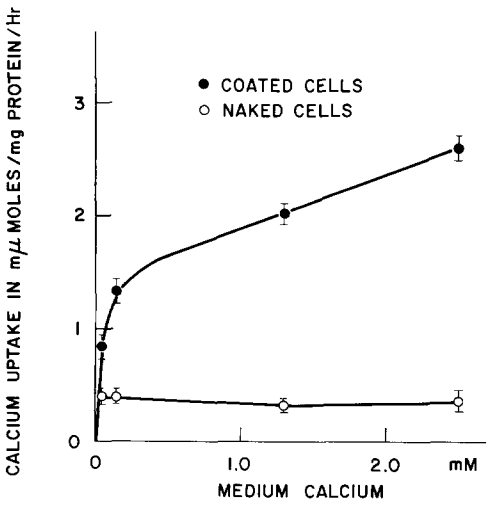

FIGURE 7 Influence of the calcium concentration in the medium on the calcium uptake in coated and naked HeLa cells. The cells were preincubated for 1 hr in a Krebs-Henseleit bicarbonate buffer containing 0–2.5 mM calcium. At 0 time, 20 μc Ca^{45} were added to each flask, and the cells incubated for 1 hr. After 1 hr, the cells were harvested by procedure 1 or 2. Each point represents the mean ± SEM of seven experiments at 0.1 mM $(Ca)_0$ of eight experiments at 1.25 mM $(Ca)_0$ and of four experiments at 0 and 2.5 mM $(Ca)_0$.

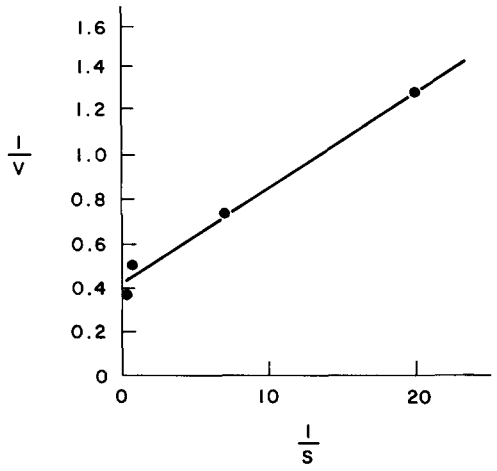

FIGURE 8 Lineweaver-Burke plot derived from the data obtained on coated HeLa cells. Experimental conditions identical to those in Fig. 7.

CALCIUM UPTAKE UNDER ANAEROBIC CONDITIONS AND WITH METABOLIC INHIBITORS: The experimental procedure used for the study of calcium uptake under anaerobic conditions by coated and naked cells when the $(Ca)_0$ was 1.25 mM was identical to that described above, except that at 0 time (after 1 hr of preincubation) the gas phase of the experimental cells was flushed with 95% N_2 and 5% CO_2. The results are shown in Fig. 9. Although the coated cells show a slightly decreased uptake under anaerobic conditions, the naked cells show no significant change. This finding suggests that calcium uptake might be a passive process or, at least that it is independent of aerobic metabolism. Even the very slight decrease in uptake obtained in the coated cells is certainly not typical of an energy-dependent process.

The results of the study of the effect of two metabolic inhibitors, dinitrophenol and iodoacetate, on calcium uptake are also shown in Fig. 9. The inhibitor was introduced during the preincubation period, 10 min before the addition of ^{45}Ca at 0 time. The inhibitors did not significantly inhibit calcium uptake in coated or in naked cells, suggesting that calcium uptake into the coat or into the cell is not dependent upon metabolic energy. The fact that calcium uptake was not affected by anaerobic conditons still left the possibility that some energy could be supplied by the

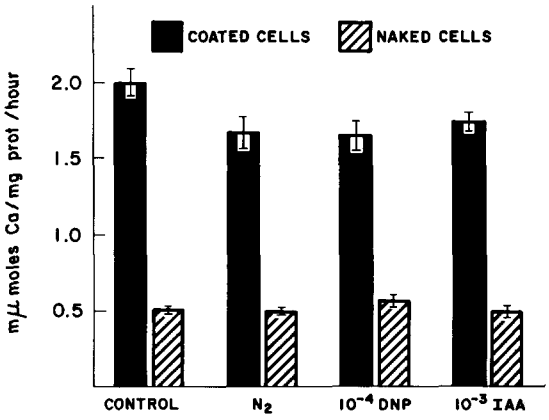

FIGURE 9 Effect of metabolic inhibitors on calcium uptake. The cells were grown for 3 days in MEM, then preincubated for 1 hr in a Krebs-Henseleit bicarbonate buffer containing 1.3 mM Ca. At 0 time, 20 μc of ^{45}Ca were added to the incubation medium and the cells incubated for 60 min. The usual gas phase 95 O_2–5% CO_2 was replaced by 95 N_2–5% CO_2 at 0 time. The metabolic inhibitor was added during the preincubation period, 10 min before 0 time. Values are means ± SEM of seven experiments for the controls, of three experiments for anaerobiosis, of three experiments for DNP, and of six experiments for IAA.

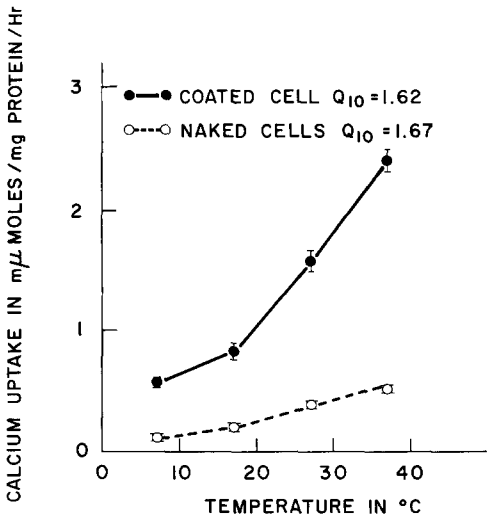

FIGURE 10 Q_{10} of calcium uptake into coated and naked Hela cells. The cells were preincubated for 1 hr in a Krebs-Henseleit bicarbonate buffer containing 1.25 mM calcium, at 7°, 17°, 27°, and 37°C. At 0 time, 20 μc of ^{45}Ca were added to each flask and the cells were incubated for 1 hr, at the same temperature. The cells were then harvested by procedure 1 or 2. Each point represents the mean ± SEM of four experiments.

anaerobic Emden-Meyerhof pathway. However, since iodoacetate which inhibits glycolysis does not affect calcium uptake, this possibility can be discarded. It appears very likely that the uptake is not energy dependent and, therefore, may be passive.

THE TEMPERATURE COEFFICIENT OF CALCIUM UPTAKE: Calcium uptake in coated and naked cells was studied at different temperatures to determine its Q_{10}. Cells were incubated at 7°, 17°, 27°, and 37°C with ^{45}Ca as before and analyzed by the standard procedure. The results are presented in Fig. 10. Although the Q_{10} varies slightly from 1.37 to 1.89 at different temperatures, the averages for coated and naked cells were 1.62 and 1.67, respectively. There is no statistical difference in the Q_{10} between the two groups of cells. These results suggest again that calcium uptake is a metabolically independent process, and they bring further support to the idea that calcium transfer into the cell is a passive phenomenon.

Effect of Parathyroid Hormone on the Calcium Content of HeLa Cells

Fig. 11 shows the difference in calcium content between control coated and naked cells when the monolayer is incubated in media of increasing calcium concentration in the presence of the hormone substitute. The coated cells contain more calcium than those in Fig. 3, but it should be noted that, in this case, the control medium contained glycerol, phenol, and albumin which might shift the calcium equilibrium between the medium and the coat. The calcium concentration of the coated cell is still proportional to the calcium concentration of the medium, whereas the calcium concentration of the naked cells is independent of it. The effects of parathyroid hormone are shown in Figs. 12 and 13. The curve relating the calcium of the coated cells to the calcium concentration of the medium is not affected by the hormone (Fig. 12), while the calcium concentration of the naked cells is increased 200% by PTH treatment (Fig. 13). It is interesting to note that when no source of calcium was available, the hormone did not increase the calcium concentration of the naked cells; on the contrary, the cells appeared to have lost calcium. It is clear that the two calcium pools detected by our method behave quite differently: the cell coat calcium is dependent on the calcium concentra-

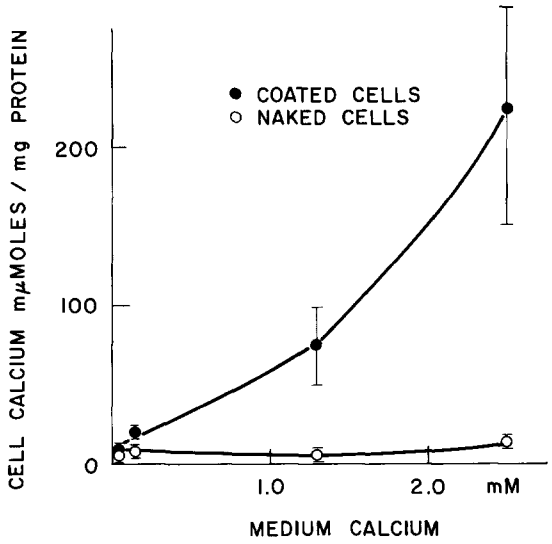

FIGURE 11 Influence of the calcium concentration in the medium on the calcium content of coated and naked HeLa cells. The cells were incubated for 2 hr in a Krebs-Henseleit bicarbonate buffer containing 0–2.5 mM calcium and were then harvested according to procedure 1 or 2. The points represent the mean ± SEM of four experiments at 0 and at 2.5 mM $(Ca)_0$ and the mean ± SEM of six experiments at 0.12 and 1.25 mM $(Ca)_0$.

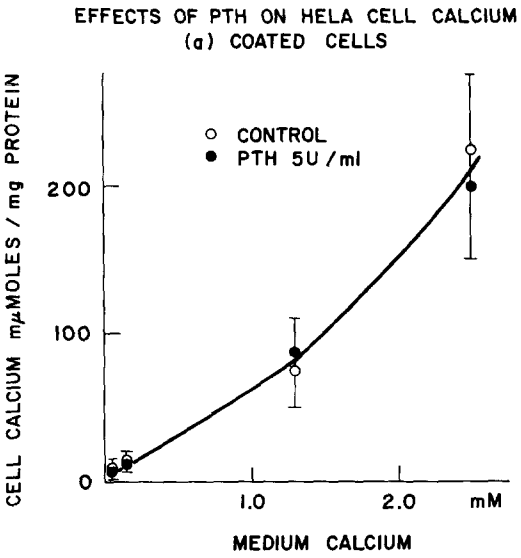

FIGURE 12 Influence of parathyroid hormone on the calcium content of coated cells at different calcium concentrations of medium. The cells were preincubated for 1 hr in a Krebs-Henseleit bicarbonate buffer containing 0–2.5 mM calcium. At 0 time, 5 units of parathyroid hormone/ml of medium were added to the medium, and the cells were incubated for 1 hr and then harvested according to procedure 1. Each point represents the mean ± SEM of four to six experiments.

tion of the medium but it is not affected by PTH, whereas the cellular calcium pool is independent of the extracellular calcium concentration but it is affected by parathyroid hormone.

Effects of Parathyroid Hormone on Calcium Uptake

Calcium uptake was studied by first preincubating the cells for 1 hr in a Krebs-Henseleit bicarbonate buffer containing 0–2.5 mM calcium. At the beginning of the experimental period, 20 μc of ^{45}Ca and 5 units of PTH per ml were added to the medium, and the cells were incubated for 1 hr. At the beginning and the end of the incubation, a sample of the medium was withdrawn so that its specific activity could be measured. The cells were washed six times with cold buffer and harvested by the two procedures described above. Calcium uptake was calculated according to equation 1.

In Fig. 7, it was evident that calcium uptake by coated cells follows a pattern compatible with binding of calcium to some components of the cell coat, and that calcium uptake by naked cells is independent of the calcium concentration of the medium. Parathyroid hormone does not affect calcium uptake by the coated cells (Fig. 14). This

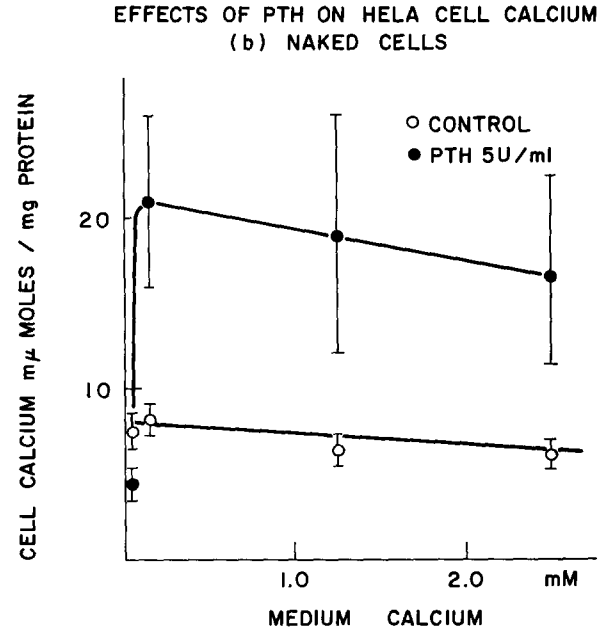

FIGURE 13 Influence of parathyroid hormone on the calcium content of naked cells at different calcium concentrations of medium. The experimental conditions were identical with those described in Fig. 12. After the experimental incubation period, the cells were harvested according to procedure 2. Each point represents the mean ± SEM of six experiments. Ordinate scale 10 × scale of Fig. 11 and Fig. 12.

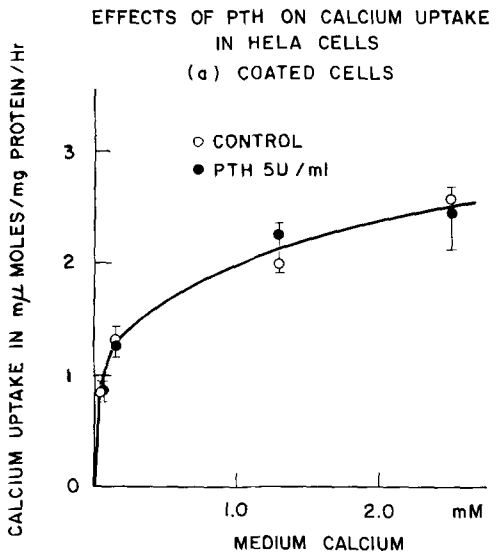

FIGURE 14 Influence of parathyroid hormone on the calcium uptake by coated cells at different calcium concentrations of medium. The cells were preincubated for 1 hr in a Krebs-Henseleit bicarbonate buffer containing 0–2.5 mM calcium. At 0 time, 20 μc of ^{45}Ca and 5 units PTH/ml of medium were added to the experimental flask. The cells were incubated for 1 hr and harvested according to procedure 1. Each point represents the mean ± SEM of four to six experiments.

fact suggests that the hormone has no effect upon the calcium-protein interaction in the cell coat. However, the hormone causes almost a doubling of calcium uptake by the cell itself when the calcium concentration of the medium is 1.25 and 2.5 mM (Fig. 15). When the $(Ca)_0$ is 1.25 mM, the calcium uptake is increased 74% ($p < 0.05$), while at 2.5 mM $(Ca)_0$ the 81% rise in uptake is not statistically significant because of the wide standard deviation ($p < 0.3$).

Effects of Purified Parathyroid Hormone on Kidney Cells

In order to test the specificity of these findings and to see whether another cell strain would respond to PTH, we studied the effects of purified parathyroid hormone, prepared according to Hawker, Glass, and Rasmussen (15), in monkey kidney cells. The Ca uptake and the per cent of Ca exchanged at 1 hr were increased 54 and 84%, respectively, with 1 PTH unit/ml of medium with 1.3 mM Ca (Table III).

DISCUSSION

It is difficult to study calcium metabolism in soft tissues because of the presence of several pools of calcium. Two groups of data can be gathered from the literature concerning the concentration of cellular calcium: in one group, the cellular calcium ranges from 1.0 to 5.0 mmoles/kg of cell water (16–18), and in the other group, it ranges from 0.4 to 0.8 mmoles/kg cell water (19–21). This discrepancy can be compared with the difference we observed between coated cells and naked cells which contained, respectively, 4.95 and 0.47 mmoles of calcium per kg of cell water. The question arises whether our "naked" and "coated" cells reflect real physiological entities or merely technical artifacts introduced by harvesting methods. Several considerations lead to the conclusion that these pools have a physiological basis. First, the calcium concentration of the coated cell, 4.95 mmoles/kg cell water, is in good agreement with a calcium pool of 5.8 mmoles/kg wet tissue reported by Wallach et al. in liver (22). This pool was defined kinetically as a rapidly exchanging

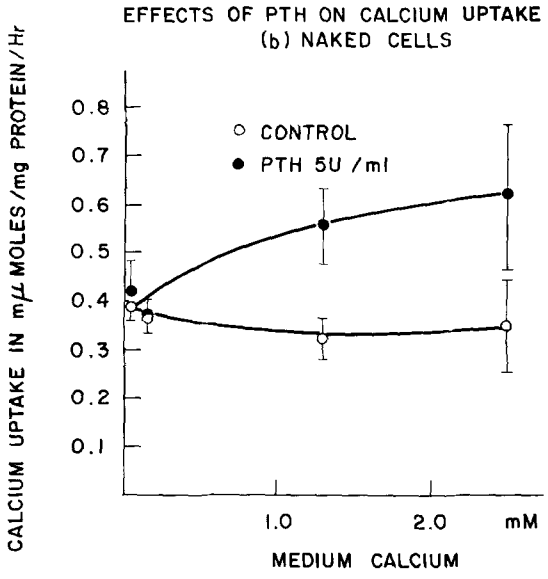

FIGURE 15 Influence of parathyroid hormone on the calcium uptake by naked cells, at different calcium concentrations of medium. The experimental conditions were identical with those described in Fig. 14. After the experimental incubation period, the cells were harvested according to procedure 2. Each point represents the mean ± SEM of four to six experiments. Ordinate scale 4 × scale of Fig. 14.

TABLE III

Effect of 1 Unit of Pure PTH/ml of Medium on Ca Uptake by Kidney Cells in mμmoles/mg Protein/Hour

	Exp.		Increase	p
Control	12	1.188±0.047		
PTH 1 U/ml	8	1.871±0.101	58%	<0.001
% Exchange*				
Control	12	6.87±0.63		
PTH	8	12.63±1.97	84%	<0.01

The experimental procedures were idential to those of Fig. 15. The calcium concentration of the medium was 1.3 mM. Values are mean ± SEM.

* % Exchange = relative specific activity or (Cell sp act/Medium sp act) × 100.

phase. Second, preliminary kinetic studies of calcium influx and efflux in HeLa cells done in this laboratory reveal at least two compartments which have different rate constants and which have the same order of magnitude as our chemically defined compartments. Third, the calcium concentration of the naked cells, 0.47 mmoles/kg cell water, is in excellent agreement with the best measurements of total intracellular calcium in nerve and muscle (19–21). Thus, it seems very likely that the true cellular calcium is of the order of 0.4 mM and that the higher values reported in the literature reflect some contamination from an extracellular compartment. Even in our system, devoid of a vascular space, without binding of calcium to collagen and to the ground substance present in whole tissue, 90% of the calcium is still removable with the extraneous glycoprotein coat of the cell and is, therefore, extracellular. In support of these data, Rambourg and Leblond (6) in a recent paper reported that the presence of a cell coat is a common feature of vertebrate cells, that the material present at the surface of cells is rich in glycoprotein and in acidic groups, and that this coat is external to the plasma membrane and can be removed by trypsin without affecting the membrane itself (6).

One would expect that kinetic studies of calcium exchanges would bring to light several calcium pools, but even when the different compartments are kinetically defined it is difficult to assign to each phase a morphological or physiological identity. Thus, Langer (23) reports in muscle at least five phases, two extracellular, two intracellular, and one undefined. Wallach et al. (22), although describing only two phases in liver slices, estimate the size of the rapidly exchanging cell fraction calcium to be 5.8 mmoles/kg slice. According to them, "this value exceeds the average cell concentration of exchangeable calcium by almost 300% and suggests that the rapidly exchanging cell fraction is restricted to cell loci comprising less than 25% of the total cell mass." Since we have shown that the protein fraction of the cell coat was only 19% of the total cell protein but contained close to 90% of the calcium, it seems probable that the rapidly exchanging cell fraction of calcium reported by Wallach et al. is actually extracellular. This fraction appears to be in equilibrium with external calcium—be it the serum calcium in their case (24) or the calcium of the medium in our system—reflecting some interaction between the divalent ion and the proteins or mucoproteins of the coat. Instances of such relationship have been published, but the fluctuations in calcium concentration of the tissue have often been attributed to the intracellular phase (4, 16, 24, 25). If the distinction between cell and coat is applicable to all cells, which is likely (6), the reported rise in tissue calcium with increasing external calcium would not represent an increase in cellular calcium, but rather increased binding to an extracellular compartment. We have shown, indeed, that the naked cell calcium does not vary with external calcium concentration. It seems, therefore, that the cell is immune to wide fluctuations in extracellular calcium concentration and maintains a constant level of cellular calcium. The importance of recognizing these two different compartments becomes obvious when one studies the effects of hypercalcemia and parathyroid hormone on calcium metabolism at the cellular level.

The data on calcium uptake very strongly suggest that this uptake is a passive process. This concept is in accordance with the results of Hodgkin

and Keynes in nerve (26), of Gilbert and Fenn in muscle (16), and of Wallach et al. in liver (22). In the first two systems, an electrochemical potential gradient exists which favors downhill movement of calcium inside the cell. In HeLa cells, a chemical potential gradient also exists, but its magnitude is uncertain since we have no measurement of that fraction of the intracellular calcium which is free and ionized. As for the electrical potential gradient, measurements done in this laboratory showed that HeLa cells in monolayer at 37°C have a membrane potential of -15.45 ± 0.57 mv, which is in good agreement with the potential of -13.4 ± 0.5 mv found by Redmann et al. (27) in cultures of KB cells. However, the fact that an ion movement goes downhill is not a final proof of its passive nature since calcium could be transported faster than expected from thermodynamic considerations. No single test can conclusively elucidate this point. However, three arguments which favor a passive transfer of calcium are: its low Q_{10}, its independence from anaerobic conditions, and the lack of its inhibition with metabolic inhibitors. These results agree very well with those of Wallach et al. on calcium transport in liver slices (22). The Q_{10} recalculated from their influx data averages 1.37 as compared with 1.62 obtained in HeLa cells. They also report an increased influx and not a decrease with metabolic inhibitors such as cyanide and iodoacetate. In our system, uptake is also increased 12% with DNP, but it is not significantly affected by iodoacetate. These results not only support the idea of a passive calcium influx, but also suggest that there might be an active extrusion of calcium from the cell which is affected by some metabolic inhibitors. A "calcium pump" for calcium extrusion from the cell has already been proposed by Gilbert and Fenn in muscle (16) and by Hodgkin and Keynes in nerve (26). If such a pump exists, one would expect an increased intracellular calcium concentration in the presence of metabolic inhibitors. Such an observation has been reported by Wallach et al. (22), and preliminary experiments on calcium efflux done in this laboratory seem to confirm it.

The passive transfer of calcium probably is not a simple diffusion process but may be a considerably more complex process involving calcium binding to the cell surface or the intervention of some specific carriers. But whatever the mechanism of translocation, the interaction of calcium with the membrane and the changes in the physical properties of this membrane might be important factors, perhaps the regulating factors in calcium transport. It is essential to make a sharp distinction between these modifications of the passive properties of the membrane and the activity of a presumed "calcium pump" regulating calcium efflux, if one is to study the effects of agents affecting calcium transport.

The influence of parathyroid hormone on calcium transport and calcium metabolism has been studied mainly in kidney, bone, and intestine. Only a few reports deal with the effects of this hormone on other soft tissues which are not generally recognized as target organs for the hormone. However, in hypoparathyroid patients, kinetic studies performed with ^{45}Ca reveal that the total exchangeable calcium decreases by more than 50% (28, 29). The pool which is mostly affected is the tissue calcium pool (29). This decrease in cell calcium is detectable even when the serum calcium concentration is normal. In parathyroidectomized dogs, kinetic studies with ^{47}Ca show that calcium concentration is decreased by 15 to 40% in liver, skeletal muscle, myocardium, brain, nerve, and pancreas (4). On the other hand, in dogs treated with PTH the total calcium concentration is increased from 20 to 465% in all soft tissues, except brain (4). Injection of PTH into rats produces metastatic calcification in muscle, and the calcium content of kidney rises more than 100-fold despite the absence of hypercalcemia (30). Deposition of calcium salts after PTH treatment has also been observed in the cells of the proximal convoluted tubules (31). With radiostrontium utilized as tracer, an increased tissue retention has also been reported after high doses of parathyroid extract (32). The data presented in the present paper support all these observations, although the effects of parathyroid hormone were studied in vitro in an entirely different system. The hormone caused the calcium content of HeLa cells to increase 200% at all external calcium concentrations studied, except in calcium-free media. These data also show that the calcium pool of the extracellular coat is not affected by parathyroid hormone, but is dependent upon the calcium concentration of the medium. In contrast, the cellular calcium pool is independent of the calcium concentration in the medium, but is affected by parathyroid hormone. This observation fits well with the reports of Wallach et al. (4, 24) which show that the calcium pool affected by parathyroid

hormone seems to be different from the pool affected by hypercalcemia. Those authors demonstrate that the cellular calcium concentrations are considerably greater in hyperparathyroidism than in acute hypercalcemia of comparable severity, and according to them, "in those tissues of hyperparathyroid dogs which manifest increments in cell calcium concentration, significant fractions of the increments are unexchangeable, whereas in acute hypercalcemia the increments are almost entirely exchangeable" (4).

In studying calcium exchange in the soft tissues of rats, Mulryan et al. (33) found no significant alteration of the normal pattern of exchange after parathyroid hormone treatment. The authors point out, however, that the great bulk of tissue calcium might be extracellular. It is not surprising, therefore, that their experimental procedure could not reveal any significant changes since the data presented here show that the extracellular calcium representing the main fraction of the total tissue calcium is not affected by the hormone. Had those authors been able to detect the very small cellular pool of calcium which is affected by PTH, they might have obtained results similar to those of Wallach et al. (4), and to ours. Another report which seems in contradiction with the present data describes an inhibition of in vitro uptake of calcium by voluntary muscle after parathyroid treatment (34). In this case, however, the calcium concentration in the medium as used by the investigators was extremely low, 0.1 mg/liter, (or 0.0025 mM) a concentration lower than the presumed intracellular, free calcium ion concentration (5). Our results show that hormone action was demonstrable only when the extracellular calcium concentration exceeded 0.050 mM. When the concentration was below this value, we also observed a slight fall in the calcium content of the cell after hormone treatment. It is possible that the hormone is unable to cause an increase in intracellular calcium concentration when the calcium concentration in the extracellular fluids is less than that inside the cell.

Since we have shown that influx of calcium into HeLa cells was, in all probability, a passive phenomenon, we have to assume that, in order to maintain a steady state, the efflux of calcium from the cell is an active process. The action of parathyroid hormone on the calcium exchange between the cell and its environment—increased uptake and rise in the intracellular pool size—could then be explained by two different mechanisms: (1) an inhibition of the active efflux, or (2) an increase in passive uptake. The possibility of an inhibition of an active calcium "pump" by parathyroid hormone is not a very attractive one. After all, numerous investigators have shown that the hormone stimulates calcium transport across both the intestinal epithelium and the renal tubule. Furthermore, the investigations of the mode of action of parathyroid hormone on calcium transport in mitochondria seem to be in contradiction with this possibility (35–38). Since calcium accumulation in mitochondria has been shown to be an energy-dependent process (39–41), calcium release is likely to be passive. It has been shown that parathyroid hormone stimulates the *release* of calcium from mitochondria, but does not affect its uptake (35–38). Such results are, thus, a good indication that parathyroid hormone might stimulate a passive translocation of calcium. Our data also support this alternative, since the hormone enhances the calcium uptake by the cell which we have shown to be a passive process. Of course, since we do not know the molecular mechanism of calcium transport through a biological membrane and the different steps involved, it is impossible to draw any definite conclusion. One can only speculate that if the passive transfer of calcium occurred by facilitated diffusion, parathyroid hormone might produce some modification in the physical properties of the membrane which would increase the diffusion coefficient of the calcium-carrier complex. One piece of evidence in favor of an increased passive membrane permeability to calcium is the fact that when the thermodynamic conditions are reversed, by incubating the cells in calcium-free medium, the effect of parathyroid hormone is also reversed and the cells lose calcium as compared with the control group (Fig. 13). This finding is also supported by the results of Reaven et al. (34) obtained under similar conditions. On the other hand, parathyroid hormone could increase the number of binding sites, or increase the affinity of some membrane constituent for calcium, possibilities which would be reflected in an increased partition coefficient for calcium and ultimately in an increased transport if some preliminary steps are involved in the translocation of calcium, such as binding to a membrane carrier. The observation that PTH increases membrane electrical resistance in toad bladder, simulating the effects of calcium on

membrane electrical properties (2), supports the idea of an increased binding of calcium to some membrane constituents after parathyroid treatment. Whatever the molecular mechanisms, parathyroid hormone seems to affect a passive transfer of calcium and not an energy-dependent calcium "pump" in cellular and subcellular systems. This effect of the hormone on the passive uptake of calcium can ultimately result in an increased transport across an epithelium, without involving primarily the calcium "pump" postulated for the extrusion of calcium from the cell. In a recent review on calcium transfer (5), we have gathered evidence showing that in the two systems in which an active transport of calcium can be demonstrated, sarcoplasmic reticulum vesicles and mitochondria, this transfer is related to the concentration of free calcium ion on the side of the membrane from which calcium is being actively transported. If such a "pump" exists in the plasma membrane, it would be stimulated by the rise in intracellular calcium caused by the increased passive transport induced by para thyroid hormone. In addition, many other effects of the hormone could be secondary to the increased concentration of cellular calcium: the inhibition of collagen synthesis (42) and the stimulation of RNA synthesis by bone cells (43), the inhibition of aconitase which would result in an increased citrate accumulation (44), and the increased calcium accumulation by mitochondria in vivo (45) in contrast to the in vitro experiments (35–38).

In conclusion, our data suggest that parathyroid hormone influences calcium balance in soft tissue by mobilizing calcium from the extracellular fluids in order to increase either intracellular calcium concentration or some binding to a cellular compartment. This concept is in contrast to the hypothesis which assumes that parathyroid hormone mobilizes calcium from soft tissue in order to elevate and support the plasma level of calcium (34, 36). According to the data presented here, the hormone acts by increasing the passive uptake of calcium by the cell. As a result, the intracellular calcium pool is increased. In an epithelium, the increased intracellular calcium may lead to a greater directional extrusion of calcium, resulting in an increased calcium transport.

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