

Competition between Oxalate and Phosphate during Active Calcium Accumulation by Sarcoplasmic Vesicles *

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Sarcoplasmic Calcium Uptake, Oxalate and Phosphate Uptake, Oxalate-Phosphate Competition, Calcium Turnover

1. During ATP supported active calcium uptake oxalate as well as phosphate are accumulated with calcium. The uptake of calcium exceeds that of both anions by a small quantity — accounting for calcium binding to vesicular proteins and lipids.

2. From assay media containing phosphate and oxalate — nearly exclusively either oxalate or phosphate are taken up together with calcium by the sarcoplasmic reticulum vesicles. The mutual exclusion occurs in a very narrow concentration range of the anions.

3. In solutions containing phosphate and oxalate, calcium phosphate or calcium oxalate precipitates are formed according to their solubility properties.

4. When phosphate prevents oxalate from being taken up, calcium transport is inhibited. Inhibition occurs, because the concentration of ionized calcium inside the vesicles rises approximately 100-fold when oxalate is replaced by phosphate. The activity of the calcium dependent ATPase parallels the calcium uptake activity.

5. It is excluded that the inhibition of calcium uptake produced by phosphate is caused by an enhanced permeability of the sarcoplasmic reticulum membranes for calcium in the presence of phosphate.

Introduction

In the presence of calcium precipitating anions like oxalate, phosphate or pyrophosphate the calcium storing capacity of the calcium transporting vesicular fragments of the sarcoplasmic reticulum (SR) is increased 50- to 100-fold. The respective calcium salts are precipitated inside the vesicles^{1, 2}. The enhanced calcium storing capacity has most plausibly been explained by the assumption that the anions follow passively the active inward movement of calcium ions, since the anions are neither activity accumulated by the SR nor do they affect the calcium dependent ATPase^{2–4}. If the product of the activities of calcium and the respective anions inside the vesicles exceeds the solubility product, precipitation occurs and the influx of anions continues as long as calcium ions are pumped inwards. Since monovalent anions for which the SR mem-

branes seem to be quite permeable do not compete effectively for oxalate or phosphate uptake, it is unlikely that an electric potential resulting from calcium translocation may support the movement of the precipitating anions. The relatively simple explanation for the effect of the calcium precipitating agents proved to be of great heuristic value for the analysis of the sarcoplasmic calcium transport. However, in the course of experiments performed to analyze passive and ADP-activated calcium release from calcium loaded vesicles a puzzling competition between the calcium precipitating anions was observed⁵, indicating an intriguing interaction of these anions with the SR membranes. The results presented in this paper show a complicated competition between the different anions during active calcium accumulation. Oxalate and phosphate prevent each other from moving into the SR vesicles. These results further exclude unspecific permeation of the calcium precipitating anions through membraneous pores but rather indicate the involvement of highly specialized membrane structures in anion translocation.

Materials and Methods

The vesicular fragments of the sarcoplasmic reticulum were prepared from rabbit skeletal muscle

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* This work was started in cooperation with the late H. H. Weber. A preliminary report of this work was presented at the International Symposium on Membrane Proteins and Transport Phosphorylation, 1974, Bressanone, Italy.

Abbreviation: EGTA, Ethyleneglycol-bis-(β -aminoethylether)-N-N-tetra-acetic acid.



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as described by Hasselbach and Makinose², modified according to de Meis and Hasselbach⁶. [γ - ^{32}P]-ATP was prepared by the method of Glynn and Chapell⁷.

The standard assay for measuring Ca-uptake, anion-uptake and ATP hydrolysis contained 5 mM ATP, 5 mM MgCl_2 , 40 mM KCl, 20 mM histidine pH 7.0, 0.05–0.1 mg vesicular protein per ml assay, 0.1–2.0 mM CaCl_2 and 0.1–2.4 mM EGTA; pH 7.0, T 23 °C, the incubation time was 15 min. The concentration of ionized calcium has been calculated using the stability constant of Schwarzenbach⁸.

^{45}Ca -, $^{32}\text{P}_i$ -, [^{14}C]oxalate uptake were followed by filtration of the vesicular suspension through Sartorius filters (0.45 μm pore size). The filters were washed with 0.1 M KCl, dried, dissolved in dioxane and the radioactivities, corrected for unspecific adsorption, were determined by liquid scintillation counting.

ATP hydrolysis was monitored by measuring ^{32}P -release from [γ - ^{32}P]ATP. An acid-quenched aliquot of the assay was mixed with a Norit-A charcoal suspension and unlabeled phosphate, shaken intensively for 10 min and filtrated. The radioactivity in the filtrate was measured by liquid scintillation counting. A correlation had to be made for the radioactivity in the filtrate found in control experiments without protein. This background activity was 0.5–1% for freshly prepared [γ - ^{32}P]ATP.

^{45}Ca -, [^{14}C]oxalate and $^{32}\text{P}_i$ were obtained from Amersham Buchler (Braunschweig, Germany). Norit A was purchased from Serva-Feinbiochemica (Heidelberg, Germany).

All reagents were A-grade.

Results

The stoichiometry of calcium and anion uptake

The data presented in Table I illustrate that the uptake of calcium supported by oxalate as well as by phosphate is accompanied by an uptake of nearly

equal amounts of the respective anion. Evidently, calcium is precipitated either as calcium oxalate or as calcium phosphate. The uptake of calcium exceeds that of both anions by as small quantity, being just significant for phosphate and not significant for oxalate. The excess of calcium taken up corresponds approximately to the amount of calcium stored in the absence of oxalate or phosphate. The storing capacity found in the presence of 1 mM oxalate reaches only 30–40% of its maximal value of 8–10 $\mu\text{mol}/\text{mg}$ observed when the assay media contained 5 mM oxalate^{3,4}. In agreement with previous observations, phosphate supports calcium uptake less effectively than oxalate. A concentration of 10 mM secondary phosphate is required to achieve the same storing capacity as found in the presence of 1 mM oxalate.

Competition between oxalate, phosphate and pyrophosphate uptake during calcium storage

When calcium storage is performed in assay media containing mixtures of the precipitating anions, the uptake of the anions exhibits a number of unexpected features. When at a concentration of 1 mM oxalate in the assay the phosphate concentration is raised, the uptake of oxalate starts to decline at a phosphate concentration of 0.6 mM and is completely abolished at 2 mM. At phosphate concentrations above 1 mM, phosphate uptake becomes observable and approaches a plateau at 10 mM (Fig. 1). Proportionally higher concentrations of phosphate are required to suppress oxalate uptake when the oxalate concentration in the medium is raised (Fig. 2). The figure further illustrates that pyrophosphate prevents oxalate from being taken up more efficiently than phosphate. Higher total phosphate concentrations are needed to suppress oxalate uptake at lower pH values. The different curves coalescent if oxalate uptake is plotted versus the

Table I. Stoichiometric relationship between the storage of calcium and oxalate or phosphate in SR vesicles.

Stored ions	No precipitating anions present			1 mM oxalate present			10 mM secondary phosphate present		
	(n)	$\mu\text{mol}/\text{mg}$ prot.	S.E.	(n)	$\mu\text{mol}/\text{mg}$ prot.	S.E.	(n)	$\mu\text{mol}/\text{mg}$ prot.	S.E.
Ca^{2+}	(6)	0.102	± 0.008	(13)	2.66	± 0.14	(5)	2.014	± 0.062
Anion	—	—	—	(8)	2.46	± 0.18	(4)	1.743	± 0.17

The assay medium contained: CaCl_2 0.5 mM, EGTA 0.7 mM, Mg ATP 2 or 5 mM, pH 7.0; other conditions as described in Methods.

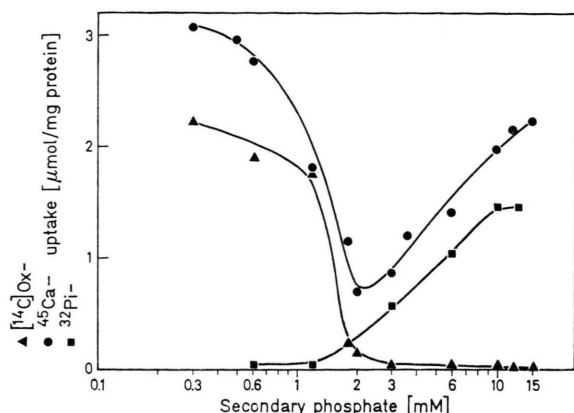


Fig. 1. Storage of oxalate or phosphate accompanying active calcium accumulation. Ordinate: Accumulation of the respective ions ($\mu\text{mol/mg protein}$). Abscissa: Concentration of secondary phosphate (mM). (●) calcium, (▲) oxalate, (■) phosphate. All assays contained 1 mM oxalate. Other conditions as described in Methods.

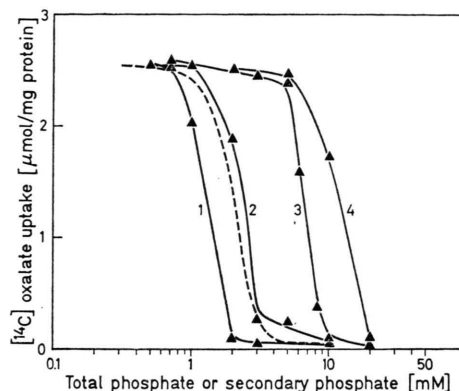


Fig. 3. Inhibition of oxalate uptake (▲) by phosphate at different pH values. Ordinate: Oxalate accumulation ($\mu\text{mol/mg protein}$). Abscissa: Total phosphate or secondary phosphate (mM). The assay contained 2 mM oxalate. pH 7.2 (1); pH 7.0 (2); pH 6.7 (3); pH 6.5 (4); graphs 1, 2, 3, 4 plotted against the concentration of secondary phosphate in the assays (— —).

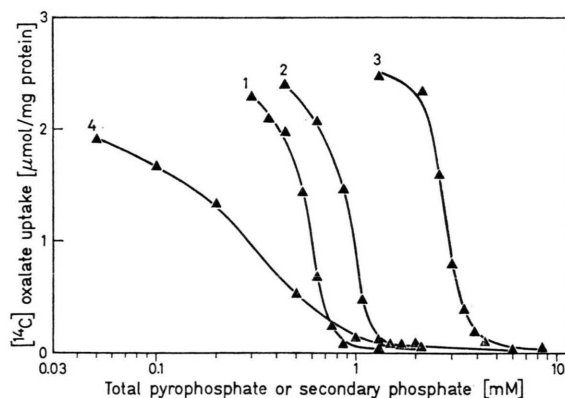


Fig. 2. Inhibition of oxalate uptake by phosphate or pyrophosphate. The inhibition of oxalate uptake by phosphate was measured at 0.75 mM oxalate (1), 1 mM oxalate (2) and 2 mM oxalate (3). The inhibiting effect of pyrophosphate (at pH 6.7) was measured in the presence of 1 mM oxalate (4). Ordinate: Oxalate accumulation. Abscissa: Concentration of secondary phosphate or total pyrophosphate. Other conditions as described in Methods.

concentration of secondary phosphate in the solution, indicating that it is a secondary phosphate ion which seems to compete with oxalate (Fig. 3).

When the concentration of phosphate in the uptake medium is kept constant and the oxalate concentration is raised (experiments not shown), the uptake of phosphate stops completely when the oxalate concentration exceeds the phosphate concentration.

Calcium precipitation from solutions containing oxalate and phosphate together

The formation of calcium precipitates in solutions containing more than one precipitable anion must

be considered to be a rather complex process. It may not only be governed by the solubility product of the respective salts but may also depend on the rates with which the precipitates are formed and with which they are mutually interconverted. Therefore, it is necessary to exclude that the described findings are physico-chemical peculiarities of the precipitation kinetics of calcium phosphate or calcium oxalate. Control experiments were performed imitating the conditions prevailing in the vesicles during precipitation. 2 mM calcium and 2 mM oxalate were added to solutions containing increasing concentrations of phosphate. The formed precipitates were removed by filtration. As shown in Fig. 4, only calcium oxalate is precipitated, even if the solution contained 50 mM secondary phosphate. Therefore, it must be assumed that in the studied concentration range only calcium oxalate precipitates are formed in the internal vesicular space if both anions are present. Even when calcium phosphate initially had been formed inside the vesicles, because it is precipitated more rapidly than calcium oxalate, the formation of calcium oxalate would finally prevail. As illustrated by Fig. 4, calcium phosphate is largely converted to calcium oxalate when, at first, calcium and phosphate are mixed together and 15 min later oxalate is added. Approximately 40 mM secondary phosphate must be present to suppress the conversion of calcium phosphate to calcium oxalate during 15 min.

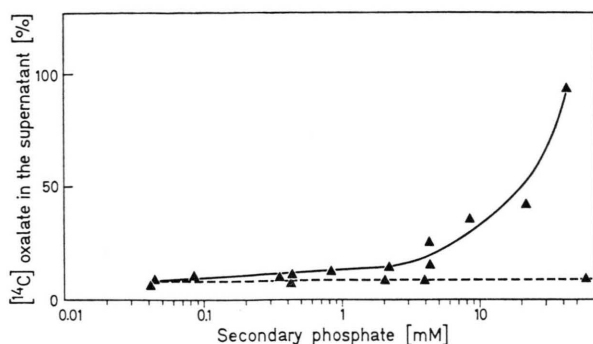


Fig. 4. Precipitation of calcium oxalate in the presence of phosphate. Ordinate: Oxalate remaining in the supernatant as percentage of the added quantities. Abscissa: Concentration of secondary phosphate (mM). 2 mM calcium and phosphate as indicated on the abscissa were mixed. After 15 min 2 mM [^{14}C]oxalate were added, and after another 15 min the precipitated calcium salts were removed by filtration and the activity remaining in the filtrate was measured (\blacktriangle — \blacktriangle). ^{45}Ca , oxalate and phosphate or calcium, [^{14}C]oxalate and phosphate were mixed together. After 5 hours the precipitates were removed and the respective radioactivities in the supernatants were measured (\blacktriangle — \blacktriangle). KCl was added to obtain ionic strength of 0.1 μ .

The occurrence of such high concentrations of phosphate inside the vesicles, however, seems very unlikely under the experimental conditions described before (Table I, Fig. 1). Increasing the phosphate concentration from approximately 4 mM outside to 40 mM inside the vesicles would require an active accumulation of phosphate. This accumulation should lead to an excess of phosphate over calcium inside the vesicles which never has been observed. Therefore, the formation of calcium phosphate in the presence of oxalate cannot be explained by physico-chemical peculiarities of the involved calcium salts. Besides, the experiments confirmed the assumed solubility product of calcium oxalate to be $L_0 = 2 \times 10^{-8} \text{ M}^2$ in 0.1 M KCl. As shown by Fig. 4 (dashed line), the molar solubility of calcium oxalate was about $1.4 \times 10^{-4} \text{ mol/l}$ leading to a solubility product of $L_0 = 2.25 \times 10^{-8} \text{ M}^2$. This finding is consistent with the results of McComas and Rieman¹¹ who found the molar solubility of calcium oxalate to be $1.18 - 1.76 \times 10^{-4} \text{ mol/l}$ in salt solutions of ionic strength 0.1–0.3 $I/2$.

Calcium uptake in the presence of oxalate and phosphate

A more complex mechanism of the observed anion competition becomes evident when the dependence of calcium uptake on the anion composition

of the uptake media is studied. Fig. 1 demonstrates that calcium storage declines from 3 $\mu\text{mol/mg}$ when oxalate is displaced by phosphate. In the presence of 1 mM oxalate, 2 mM phosphate suppress calcium uptake to levels as they are found in the presence of phosphate alone. Calcium uptake raises again when higher concentrations of phosphate are applied. The system behaves as if at phosphate-oxalate ratios higher than 2, no oxalate could reach the interior of the vesicles. The nadir in the calcium uptake curve does not disappear even when the uptake is followed for 40 min (experiments not shown). It slowly rises from 0.6 $\mu\text{mol/mg}$ to 0.9 $\mu\text{mol/mg}$ without any change in the profile of the uptake curve.

The behaviour of the storage of calcium observed in the preceding experiments is in good agreement with the kinetics of calcium uptake. If the activation of calcium uptake produced by phosphate or oxalate is compared at equal concentrations of the anions (2 mM), the rate of calcium uptake in the presence of oxalate (0.7 $\mu\text{mol/mg} \cdot \text{min}$) is 5 times faster than that in the presence of phosphate (0.13 $\mu\text{mol/mg} \cdot \text{min}$). However, as shown above, 2 mM phosphate are sufficient to abolish the activating effect of 1 mM oxalate completely.

The calcium dependent ATPase parallels closely calcium uptake activity (Fig. 5). The ATPase activity is high in the presence of 1 mM oxalate and low in the presence of 3 mM phosphate. The same low activity is observed in systems containing 1 mM oxalate together with 3 mM phosphate. The suppression of the ATPase activity by phosphate is observed only as long as calcium uptake occurs. When the vesicles are destroyed by sonication ATP is split with the same high rate in the presence of either 5 mM oxalate or 5–10 mM phosphate (experiments not shown). A plausible explanation for the reduction of the rate of calcium uptake and of calcium dependent ATP-splitting by phosphate will be given in the Discussion.

The permanent suppression of the calcium storing capacity by phosphate at a constant oxalate level is more difficult to rationalize.

Since calcium uptake ceases before the maximum of the storing capacity is reached, the reduction of the calcium storage may be explained by a diminished ability of the vesicles to concentrate calcium. As shown in Table II, the final calcium level of the medium increases by a factor of 3 when instead of

Table II. Final calcium concentrations and calcium turnover after cessation of net calcium uptake. The uptake of calcium was performed in media containing 5 mM ATP, 5 mM MgCl₂, 20 mM histidine, 40 mM KCl, 0.2 mg vesicular protein per ml assay, 0.1 mM ⁴⁵Ca EGTA for the smaller calcium load and 0.2 mM ⁴⁵Ca EGTA for the larger calcium load. The final calcium concentration in the solution was measured by filtration through Sartorius filters (0.45 μm) after 15 min. Subsequently, carrier-free ⁴⁵Ca was added and its disappearance from the solution was measured for the determination of the rate of calcium exchange. For the calculations of the final activity ratio $L=2 \times 10^{-8} \text{ M}^2$, ^{9, 11} and $L=5 \times 10^{-6} \text{ M}^2$, ¹⁰ were used as solubility products of calcium oxalate (+) and calcium phosphate (++), respectively.

Conditions	Ca load $\mu\text{mol} \cdot \text{mg}^{-1}$	Final calcium concentrations		Final activity ratio ($L/\text{Ca}^{2+} \cdot \text{A}^{2-}$)	Rate of calcium exchange (V) $\text{nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$	Rate coefficients	
		total mM	ionized nM			V/Ca^{2+} $\text{l} \cdot \text{mg}^{-1} \cdot \text{min}^{-1} \cdot 10^{-1}$	$V/(\text{Ca}^{2+})^2$ $\text{l}^2 \cdot \text{nmol}^{-1} \cdot \text{mg}^{-1} \cdot \text{min}^{-1} \cdot 10^{-2}$
Ox 1 mM	0.9	0.02	22	909 (+)	17	7.73	3.51
	0.42	0.015	35	570	17	5.0	1.47
Ox 1 mM } Pi 4 mM }	0.54	0.09	160	12800	88	5.5	0.34
	0.27	0.044	160	12800	76	4.75	0.3
Pi 4 mM	0.57	0.076	120	6830 (++)	44	3.67	0.31
	0.30	0.038	120	6830	50	2.78	0.35

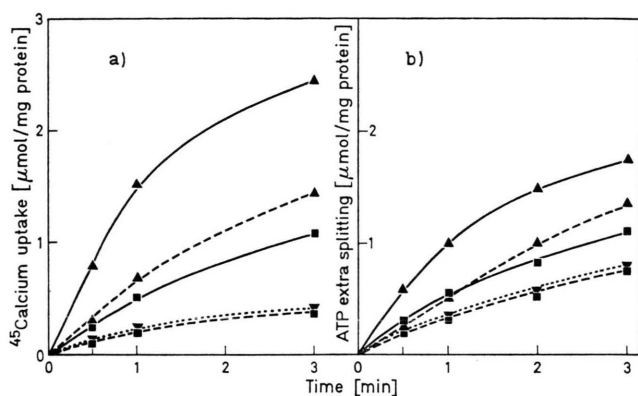


Fig. 5. Time course of calcium uptake and ATP extra splitting. The assay contained 0.05–0.1 mg vesicular protein/ml, 5 mM AT³²P, 5 mM MgCl₂, 20 mM histidine, 40 mM KCl, 2 mM CaCl₂, 2.4 mM EGTA, 5 mM oxalate (▲—▲), 1 mM oxalate (▲—▲—▲), 10 mM secondary phosphate (■—■), 3 mM secondary phosphate (■—■—■), 1 mM oxalate+3 mM secondary phosphate (▼—▼—▼); pH 7.0, $T=20^\circ\text{C}$. a) Calcium was added as ⁴⁵Ca to measure calcium uptake. b) ATP hydrolysis was monitored by measuring ³²P_i release. The basal activity measured in the absence of calcium was subtracted from the total amount of released P_i. Calcium uptake and ATP splitting were measured with different preparations.

1 mM oxalate 10 mM phosphate are used for calcium precipitation or when 4 mM phosphate are added to the assay containing 1 mM oxalate. However, since the solubility product (L_p) of calcium phosphate is 100 times higher than that of calcium oxalate (L_o), the ratio of the respective activity products inside and outside the vesicles $L_o/\text{Ca}^{2+} \cdot \text{Ox}^{2-}$ and $L_p/\text{Ca}^{2+} \cdot \text{P}^{2-}$ which are a measure of the concentrating ability, is higher in the phosphate

than in the oxalate containing medium. Therefore, phosphate per se does not reduce the ability of the vesicles to concentrate calcium. The elevated calcium level in the phosphate containing assays reflects that the calcium concentrations inside the vesicles are considerably higher in the presence of phosphate than in the presence of oxalate. At a given maximal capacity of the vesicles to concentrate calcium, an increase of the internal calcium concentration must result in the elevated external calcium level. This elevated calcium concentration in the medium fully accounts for the increased calcium turnover in the presence of phosphate observed after calcium uptake has levelled off. To transform the observed turnover rates to rate coefficients which reflect concentration independent permeability properties of the membranes, the turnover rates are divided by the external calcium concentrations or by its square values. The division of the turnover rates by the square values of the external calcium concentrations would account for the translocation of two calcium ions during one turnover cycle. The obtained rate coefficients are smaller in the phosphate than in the oxalate containing medium. This fact indicates that the calcium permeability of the membranes is not increased by phosphate and that the enhanced turnover rate rather follows the external calcium level.

Discussion

Under all conditions applied in this study the storage of the calcium precipitating anions, oxalate

and phosphate never exceeds the storage of calcium. This limitation is in agreement with the previous observations demonstrating that calcium and oxalate were stored by the sarcoplasmic vesicles in equimolar quantities². The fact that the calcium/oxalate ratio remained under all conditions approximately 1 was used as an argument to exclude an active uptake of anions. If such a mechanism would exist, the accumulation of the anions should proceed until the solubility product of the respective calcium salts inside the vesicles is reached. At low concentrations of the anions in the assay an excess of the anions over calcium inside the vesicles should easily be detectable. Such an excess of anions has neither been observed for oxalate² nor for phosphate in this study. In the presented experiments the ratio of calcium to phosphate has a mean value of 1.15 (Table I) indicating that most of the calcium is present as CaHPO_4 . The binding of calcium to membrane protein and lipids may account for most of the excess calcium. The fraction bound to the calcium binding proteins is presumably small, since at the prevailing free calcium concentrations inside the vesicles (~ 1 mM) the binding sites are only partially occupied. Part of the excess calcium may be present as $\text{Ca}_3(\text{HPO}_4)_2\text{OH}_2$ which is the predominant calcium salt formed in the mitochondrial matrix¹².

When two calcium precipitating anions are present in the assay media, the nature of the formed precipitates should depend on the solubility products of the respective calcium salts and the concentrations of the anions. As shown, the formation of calcium oxalate and calcium phosphate on addition of calcium to solutions containing oxalate and phosphate corresponds to the expected solubility properties of the calcium salts. Complications such as the tendency of the less soluble salt to form supersaturated solutions so that the more soluble salt would precipitate at first, are of minor significance, if at all. Under all conditions applied in the calcium uptake experiments the total concentrations of calcium, oxalate and phosphate had such values that the inequality $L_0/\text{Ox} < L_p/\text{P}_i$ holds and therefore, calcium should have been precipitated only as calcium oxalate. If, nevertheless, calcium phosphate precipitates are formed in the intravesicular space of the vesicles, the local ratio L_p/P_i must have become smaller than L_0/Ox . Since an active accumulation of phosphate has never been observed, this

change can only be brought about by the exclusion of oxalate so that its intravesicular concentration remains below $20 \mu\text{M}$.

This exclusion of oxalate by phosphate strongly indicates that the membranes can sharply discriminate between the different precipitating anions. The narrow concentration range in which phosphate prevents oxalate from entering the vesicles is an astonishing phenomenon indicating the involvement of multiple binding sites in the anion channel or gate. The same kind of competition explains the finding that rising oxalate concentrations inhibit calcium phosphate uptake. Oxalate replaces phosphate as calcium precipitating anion inside the vesicles quite abruptly when phosphate cannot any longer prevent oxalate from entering the vesicles. This is the case when the oxalate concentrations exceed the phosphate concentrations by a factor of 2. Together with the inflow of oxalate calcium uptake increases considerably.

The sharp transition from oxalate to phosphate or phosphate to oxalate uptake affects calcium transport. That is because the different precipitating anions buffer the internal calcium concentrations at different levels. According to the solubility product of calcium oxalate $L = 2 \times 10^{-8} \text{ M}^2$ 0.2 mM oxalate in the solution allows the presence of 0.1 mM ionized calcium. Since an oxalate concentration of 0.2–0.4 mM produces a just detectable activation of calcium uptake^{4,13}, we must assume that in the absence of oxalate an internal free calcium concentration of 0.1 mM must have existed inside the vesicles before calcium uptake starts to increase by the formation of calcium oxalate. According to the higher solubility product of calcium phosphate higher concentrations of calcium can be expected to exist inside the vesicles. Consequently, when phosphate excludes oxalate from being taken up, the internal calcium level must rise. This rise fully accounts for the observed suppression of calcium uptake and calcium dependent ATPase activity (Fig. 5). A complete exclusion of oxalate by phosphate is in agreement with the sharp decline of the storing capacity of the vesicles for calcium. The high storing capacity of $3 \mu\text{mol/mg}$ in the presence of 1 mM oxalate drops to a considerably lower value as found in assay media supplemented with 2 mM phosphate alone. When the phosphate concentration is increased, calcium storage rises again as if no oxalate were present in the assay.

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