25. ROBERTS, R. B., D. B. COWIE, P. H. ABELSON, E. T. BOLTON, AND R. S. BRITTEN. 1955. Studies of biosynthesis in *Escherichia coli*. Carnegie Institute of Washington, Publication No. 607.
26. SPACKMAN, D. H., W. H. STEIN, AND S. MOORE. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30: 1190–1206.
27. SMITH, D. C., J. A. BASSHAM, AND M. KIRK. 1961. Dynamics of the photosynthesis of carbon compounds. II. Amino acid synthesis. Biochim. Biophys. Acta 48: 299–313.
28. TULECKE, W. 1964. A haploid tissue culture from the female gametophyte of *Ginkgo biloba* L. Nature 203: 94–95.
29. TULECKE, W. AND L. G. NICKELL. 1960. Methods, problems, and results of growing plant cells under submerged conditions. Trans. N.Y. Acad. Sci. 22: 196–206.
30. UMBARGER, H. E. 1962. Integration of metabolic pathways. Ann. Rev. Plant Physiol. 14: 19–42.
31. WANG, D. AND R. H. BURRIS. 1963. Carbon metabolism of C^{14}-labeled amino acids in wheat leaves. II. Serine and its role in glycine metabolism. Plant Physiol. 38: 430–39.
32. WEINSTEIN, L. H., W. TULECKE, L. G. NICKELL, AND H. J. LAURENCOT. 1962. Biochemical and physiological studies of tissue cultures and the plant parts from which they are derived. III. Paul's Scarlet Rose. Contrib. Boyce Thompson Inst. 21: 371–86.
33. YEMM, E. W. AND B. F. FOLKES. 1958. The metabolism of amino acids and proteins in plants. Ann. Rev. Plant Physiol. 9: 245–80.
34. YOSHIDA, S. AND G. H. N. TOWERS. 1963. Synthesis of shikimic acid-C^{14} in *Pinus resinosa*. Ait. from some C^{14}-labeled compounds. Can. J. Biochem. Physiol. 41: 579–86.

Calcium Activation of Orthophosphate Absorption by Barley Roots [1]

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Introduction

Calcium, an essential element for plant growth, is involved in a wide spectrum of events including development of the meristematic regions (1) and absorption rates of other ions (3, 7). Other alkaline earth cations do not completely substitute for calcium in fulfilling this growth requirement (1, 8).

The presence of Ca^{++} in the nutrient media of plants has been observed to influence the absorption of other ions. In general, the effect was one of inhibiting the rate of sodium absorption (4), but of enhancing the rate for potassium (4, 10, 11, 14, 21), sulfate (13), chloride (17, 20), and phosphate (18, 19). Thus, Ca^{++} affects the rate of absorption of a specific salt although the absorption rate of the cation or anion is usually considered to be independent of each other.

Although its general influence has been known for several years, the mechanism of action of Ca^{++} on ion absorption is not fully understood. This investigation considers the mode of Ca^{++} activation of phosphate absorption by kinetic analysis of measurements of steady-state absorption.

Materials and Methods

Roots of 2 varieties of *Hordeum vulgare* L., Atlas 46, and Trebi, were obtained from plants grown essentially as described by Epstein and Hagen (5). Seeds (30 g) were soaked for 24 hours in 600 ml of continuously-aerated demineralized water and distributed on cheesecloth supported on a stainless steel screen. The screen was placed on top of a 4-liter beaker containing 2×10^{-4} M $CaSO_4$ solution, so that the seeds were 1 cm above the solution. A

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second stainless steel screen covered with cheesecloth was placed over the seeds. A sintered glass aerator was placed in the solution and the beaker was covered with a watch glass and placed in a constant temperature chamber. Plants were grown 5 days in the continuously-aerated $CaSO_4$ solution maintained at 24° in darkness. Forty-eight hours after planting, the top cheesecloth and steel cover were removed. The following day, the seedlings were removed from the solution, rinsed with demineralized water and placed in a fresh $CaSO_4$ solution. Roots 10 to 15 cm long were obtained 5 days after planting. These were excised about 5 mm below the seeds, rinsed with demineralized water and placed in 2 liters of aerated demineralized water.

The excised roots were removed from the demineralized water, in which they had been suspended, and blotted on cheesecloth to remove adhering water. One-g portions were weighed and each was placed in 50 ml of demineralized water. The roots were rinsed twice with water prior to placement in the phosphate solutions. At zero time, the roots were transferred to 1 liter of phosphate solution at a temperature of 24° ± 2°.

Radioactive, carrier-free phosphate, as orthophosphoric acid in dilute hydrochloric acid obtained from Oak Ridge National Laboratory, was used as a tracer. Removal of the chloride was accomplished by adding the radioactive solution to a nonradioactive phosphate solution containing a known amount of phosphate, and evaporating the solution to dryness under an infrared lamp. The solution volume was adjusted to give a concentration of 1×10^{-6} M ΣP (ΣP = H_3PO_4 equivalents). Isotopic dilution of this stock solution was such that the specific activity was less than 3 mc per g P in the experimental solutions. At this specific activity, radiation damage was not detectable.

Within 5 minutes after the addition of the roots, solutions were readjusted to the desired pH value with 0.1 N KOH or 0.1 N HCl. Solutions having low total phosphate concentration required pH adjustments at intervals of 15 to 20 minutes. Adjustments of pH were less frequent for solutions $> 5 \times 10^{-5}$ M ΣP.

Absorption was terminated by decanting the phosphate solution and rinsing the roots in 4 aliquots of demineralized water. After the roots were transferred to one-fourth ounce metal cups, they were dried under an infrared lamp. Radioactivities in the samples were measured with a Geiger-Mueller tube. From the known specific activity of the particular phosphate solution, the amounts of phosphate absorbed by the roots (moles/g fr wt) were calculated.

Kinetic Interpretation. From an initial combination between an ion and a binding compound, ion absorption is assumed to proceed as in equation I, where R represents a binding compound, M the ion, MR the intermediate, and k the rate constant for each reaction (5). Decomposition of this intermediate complex results in the absorption of the ion into the cell, as described by equation II.

$$R + M_{\text{outside}} \underset{k_2}{\overset{k_1}{\rightleftarrows}} MR \qquad \text{I}$$

$$MR \underset{k_4}{\overset{k_3}{\rightleftarrows}} R' + M_{\text{inside}} \qquad \text{II}$$

The k_3 is the rate-limiting step of absorption.

A steady-state exists as shown by a linear rate of phosphate absorption throughout an absorption period of 5 hours. Under these experimental conditions the absorption of phosphate by barley roots has been shown to be essentially irreversible (12), and hence the k_4 is negligible.

An absorption equation may be derived from equations I and II which is analogous to the steady-state analysis described by Michaelis and Menten (15) for kinetic studies of enzyme reactions. Eadie (2) pointed out that the equation is linear in form upon rearranging, as shown by equation III.

$$v = -\frac{v}{[\Sigma P]} K_m + V_{max} \qquad \text{III}$$

where v is moles of phosphate absorbed, V_{max} represents the maximum absorption at infinite ion concentration, $[\Sigma P]$, the steady-state phosphate concentration, and K_m, the Michaelis constant. When a steady-state absorption of phosphate, v, is plotted against $v/[\Sigma P]$, a curvilinear relationship is obtained. This may be interpreted as 2 or more first-order reactions acting both simultaneously and independently on different substrates which are in a nonrate-limiting equilibrium (9).

Results

Barley roots absorbed more phosphate from a phosphate solution containing Ca^{++} than from a solution lacking it. Absorption increase varied with the concentrations of phosphate, calcium, and, to a lesser extent, hydrogen ion.

The rate of phosphate absorption by roots from solutions containing 10^{-6} or 10^{-4} M ΣP of varying concentrations of $CaCl_2$ is presented in figure 1. The points on the ordinate represent the amounts of phosphate absorbed in 3 hours from 10^{-6} and 10^{-4} M solutions without the addition of calcium (Indigenous Ca^{++} = 4.2×10^{-6} moles/g fr wt of roots). Maximum activation was attained in 10^{-4} M ΣP solutions with $[Ca^{++}]$ approximating 5×10^{-3} M. Absorption from 10^{-6} M ΣP solutions was increased to the same extent at all $[Ca^{++}]$ levels tested. Comparing the phosphate absorption at the end of a 3-hour period, the maximum increase at both phosphate concentrations with added Ca^{++} was approximately 100 %.

Under most conditions, magnesium increased phosphate absorption to a similar extent. Although

absorption at pH 4 was increased less by Mg^{++} than by Ca^{++}, these cations were equally effective at the higher pH values. Increase in absorption in the presence of Ca^{++} or Mg^{++}, relative to their absences, may be observed in table I. These data represent

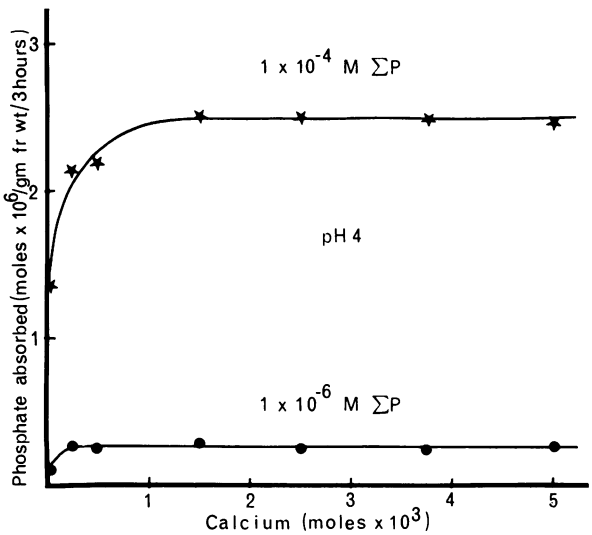

FIG. 1. Absorption of phosphate by excised barley roots as influenced by Ca^{++} concentration in the nutrient solution.

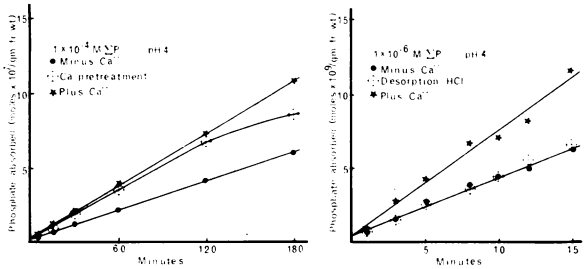

FIG. 2. Phosphate absorption by excised barley roots from 10^{-4} M phosphate at pH 4 as a function of time and Ca^{++} (5×10^{-3} M).

FIG. 3. Phosphate absorption by excised barley roots from 10^{-6} M phosphate at pH 4 as a function of time and Ca^{++} (5×10^{-3} M).

Linearity of absorption with respect to time was maintained for approximately 5 hours, after which the absorption rate declined. Calcium did not appear to alter the time at which the absorption rate began to decrease. Observations of absorption over a 22-hour period in the presence and absence of Ca^{++} are reported in table II. The values indicate absorption by 1 g of roots in a 1×10^{-5} M ΣP solution at pH 4 in the absence or presence of 5×10^{-3} M $CaCl_2$. Roots in the presence of Ca^{++} absorbed more phosphate at all time periods than those in its absence. The initial rates were 3.5×10^{-9} and 5.3×10^{-9} moles g^{-1} min^{-1} in the absence and presence of calcium, respectively. During the 5- to 22-hr period, the rate of absorption decreased both in the presence and absence of calcium. After 20 hours, the absorption rate approached zero. It is of interest to note that calcium increased both the rate of phosphate absorption and the total amount of labeled phosphate in the roots at apparent equilibrium.

Table I. *Phosphate Absorption from 10^{-4} M Solution in the Presence of 5×10^{-3} M Ca^{++} or Mg^{++} as a Percentage of Absorption in the Absence of These Cations in the Solution and as Affected by pH*

pH	% Activation of P in Ca^{++}	% Activation of P in Mg^{++}	P Absorption, (−) divalent cation moles × 10^7
4	134	120	3.5
5	150	145	3.2
6	150	151	3.1
7	142	145	2.8

Table II. *Phosphate Absorption from 10^{-5} M Solution as a Function of Time, Absence, and Presence of 5×10^{-3} M Calcium*

Hr	P, moles × 10^7/g fr wt of roots (−) Ca^{++}	(+) Ca^{++}	(+) Ca^{++} / (−) Ca^{++}
1	2.1	3.2	1.52
2	4.2	6.4	1.52
3	6.1	9.6	1.57
5	10.5	16.0	1.57
22	20.2	32.1	1.57

absorption for a 1-hour time period from 1×10^{-4} M ΣP solutions adjusted to various pH values. Calcium and magnesium, as chlorides, were at a concentration of 5×10^{-3} M.

Other divalent cations, such as Ba^{++} or Sr^{++}, increased phosphate absorption, although neither was as effective as an activator as Ca^{++} or Mg^{++}.

Phosphate absorption by barley roots was a linear function of time from 1 through 300 minutes over the range of 1×10^{-6} to 1×10^{-3} M ΣP. The time course of phosphate absorption by roots from a 1×10^{-4} M ΣP solution of pH 4, in the presence and absence of Ca^{++}, is shown in figure 2. Calcium increased the absorption rate approximately 1.4-fold from 4.1×10^{-9} to 5.8×10^{-9} moles g^{-1} min^{-1}.

Absorption of phosphate can be increased by pretreatment of roots in 5×10^{-3} M $CaCl_2$ before exposing them to phosphate. Roots were placed in Ca^{++} solution for 30 minutes, which was sufficient time to achieve equilibrium of the readily-exchangeable Ca^{++}. At this time, the roots were removed,

washed in three 100-ml volumes of water, and transferred to a 10^{-4} M ΣP solution. Phosphate absorption by roots receiving the Ca^{++} pretreatment is compared to unpretreated roots in the presence and absence of Ca^{++} during absorption in figure 2. Roots which were exposed to Ca^{++} prior to phosphate absorption had an initial rate slightly less than that of roots in the presence of Ca^{++}. At longer time periods, the Ca^{++}-pretreated roots deviated from the steady-state and the rate approached that for roots in the absence of calcium.

Effects of a Ca^{++} pretreatment were found to be reversible by placing the roots immediately in 10^{-4} M HCl or EDTA for 30 minutes prior to the observation of phosphate absorption (fig 3). Thus, the rate of absorption was similar to that for roots which had not received a pretreatment in $CaCl_2$.

Absorption of phosphate by roots, as a function of phosphate concentration, is presented in figures 4 and 5. A curvilinear relationship was obtained upon plotting absorption against absorption/phosphate concentration over the pH range 4 to 7 in the presence or absence of added calcium. Each curve was separated into 2 linear components as suggested by Eadie (2). A common linear line (b) was found for absorption data obtained in the presence and absence of calcium. The other component

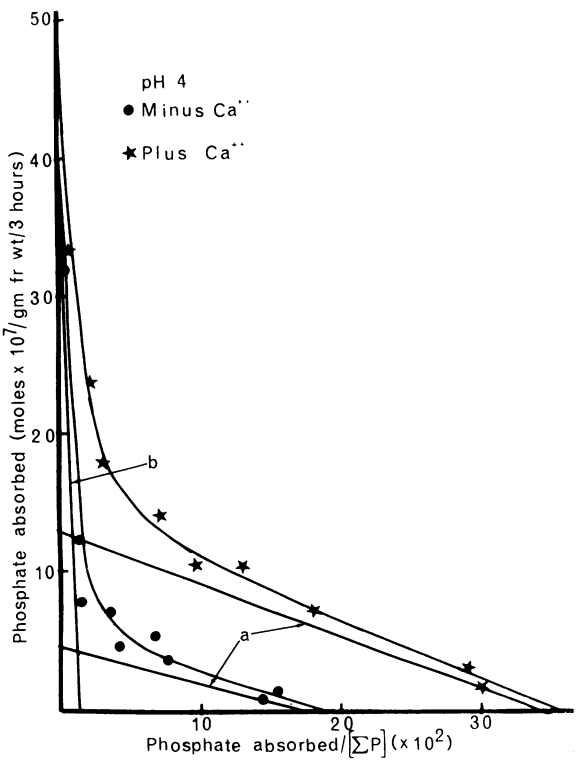

FIG. 4. Eadie plot of phosphate absorption by excised barley roots as influenced by calcium at pH 4. ([Ca^{++}] = 5×10^{-3} M).

for each curve, lines (a), varied primarily in the intercept values with a smaller change in the slopes. Differences between the slopes of lines (a) were greater at pH 7 than at pH 4 (table III). In the

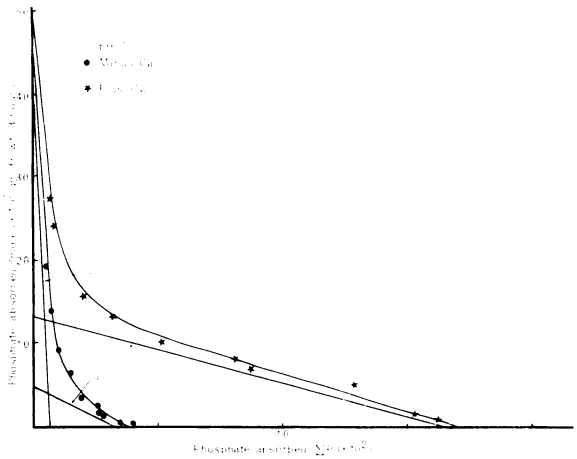

FIG. 5. Eadie plot of phosphate absorption by excised barley roots as influenced by calcium at pH 7. ([Ca^{++}] = 5×10^{-3} M).

Table III. *Apparent K_m Values for Absorption of Phosphate by Barley Roots as Related to pH and 5×10^{-3} M Calcium (see fig 4, 5)*

Condition	K_m value for line a	K_m value for line b
pH 4 (−) Ca^{++}	2.7×10^{-6}	2.5×10^{-4}
(+) Ca^{++}	4.0×10^{-6}	2.5×10^{-4}
pH 7 (−) Ca^{++}	13.9×10^{-6}	5.0×10^{-4}
(+) Ca^{++}	8.3×10^{-6}	5.0×10^{-4}

presence and absence of Ca^{++} the slope of line (a) increased with an increase in pH. A thousand-fold increase in OH^- increased the slopes 5.1- and 2.1-fold in the absence and presence of Ca^{++}, respectively. Therefore, relative to the Ca^{++} treatment, absorption at pH 7 in the absence of Ca^{++} required a 2-fold increase in the phosphate concentration to obtain half maximum absorption.

In other words, the addition of Ca^{++} decreased the apparent inhibition of OH^- relative to the no Ca^{++} treatment (line a). The other linear reaction (line b), while being inhibited by OH^-, was not influenced by Ca^{++}. A quantitative treatment of OH^- inhibition on phosphate absorption by barley roots was presented by Hagen and Hopkins (9).

Discussion

Kinetic analysis of ion absorption by plant cells may be treated in a manner strictly analogous to that applicable to enzyme reactions. Absorption rates

are observed at steady-state conditions where the influx is greater than the efflux of the ion being followed. When these conditions pertain, the Michaelis-Menten equation describes a reaction which has a rate-limiting step of absorption. Kinetic analysis of absorption data describes the rate-limiting step characteristics at an established condition. However, neither the identity nor the sequential location of the rate-limiting step can be established until more specific information is known of reactants and products. Nevertheless, one can characterize the overall absorption process, fully aware that more than one step may be involved in the entry process.

Phosphate absorption by barley roots from 1×10^{-4} M ΣP was increased to a maximum value in the presence of 5×10^{-3} M $CaCl_2$. The effect was one of an increased absorption rate (fig 1). This may have been related to the concentration of an intermediate, the turnover number, or both. The turnover number is implicated since the absorption curves, both in the presence and absence of Ca^{++}, extrapolate to an identical point on the ordinate. Furthermore, a slight change was observed in the K_m values (table III, fig 4, 5) in agreement with an increased net turnover where the value of k_3 is significant compared to k_2, since $K_m = k_2 + k_3 \div k_1$.

Phosphate absorption by roots, in the presence and absence of Ca^{++} as a function of $[\Sigma P]$, delineated a curvilineal relationship when plotted by use of equation III (fig 4, 5). The curves were resolved into 2 linear components by graphic separation (2). Absorption at the higher $[\Sigma P]$ was not affected by Ca^{++} (line b). The influence of Ca^{++} was only effective at the lower phosphate concentrations and is represented by displacement of lines a.

The maximum absorption was increased by Ca^{++} as represented by a different intercept of line a on the ordinate. A change in V_{max} signified that absorption was activated by Ca^{++} at a location different from the loci for phosphate absorption. Interaction of Ca^{++} and phosphate at the rate-limiting step would be represented by a common ordinate intercept.

Calcium increased the amounts of phosphate associated with the roots at apparent equilibrium (table II), as well as the initial absorption rate. A possible explanation of this increase in total phosphate is as follows: if phosphate absorption were to proceed by 2 separate Ca^{++}-activated reactions, one fast (reaction x) and the other slow (reaction y). the apparent equilibrium of the latter would not be reached for some time after that of the former has been achieved. If the rate of the absorption due to reaction y were slow enough, there could be no measurable effect on the absorption rate after the apparent equilibrium for reaction x had been reached.

By prior saturation of the roots with Ca^{++}, an increase in phosphate absorption was mediated (fig 2). This activation can be removed (fig 3) by exchanging Ca^{++} for H^+, as shown by Epstein and Leggett (6). Calcium is absorbed slowly relative to other ions (3), thereby accounting for the long-term effect on phosphate by Ca^{++} pretreatment. In mitochondria, under certain conditions, phosphate has been shown to be accumulated in the presence of Ca^{++} as a Ca-phosphate complex (16). This would not seem to be the case in barley roots as indicated by total analysis of the roots for Ca^{++} and phosphate after a 24-hr absorption period, which showed that Ca^{++} increased absorbed approximately 2-fold, whereas, a change in total Ca^{++} was not measurable.

The mechanism of Ca^{++} action is one of an increased influx, since no efflux of P^{32} is observable in the absence or presence of Ca^{++}. When these conditions held, the amount of phosphate entering the cell per unit time is equal to the (intermediate concentration) $\times$ (k_3). The apparent K_m value will be modified with a change in k_3, whereas a change in the intermediate concentration will not influence K_m. In these studies, an observed change in K_m for phosphate absorption by barley roots is suggestive of an increased k_3 mediated by Ca^{++}.

Summary

Phosphate absorption by barley roots (*Hordeum vulgare* L.) was a linear function of time for a period of at least 5 hours over a range in phosphate concentration from 10^{-6} to 10^{-3} M. With respect to phosphate concentration, absorption by the roots appeared to be mediated by at least 2 rate-limiting steps. These reactions had apparent K_m values of 10^{-4} and 10^{-6}.

Addition of calcium chloride to the phosphate solution resulted in an increase in the rate of phosphate absorption by barley roots. For long absorption periods, where the absorption rate was decreased essentially to zero, the activation of phosphate absorption by calcium persisted similar to that under initial steady-state conditions. Hence, calcium increased the initial rate of phosphate absorption as well as the amount of phosphate at an apparent equilibrium.

Specifically, calcium acted as an activator of phosphate absorption mediated through a reaction which had an apparent K_m of 10^{-6}. Furthermore, the activation was expressed primarily as an increase in the V_{max} of this reaction, with a lesser influence on the K_m value.

It is suggested that the increase in phosphate absorption induced by calcium was associated with an increased turnover number rather than a change in the concentration of a rate-limiting intermediate. In any case, a change in the V_{max} of phosphate absorption implies that the coupling of calcium activation to absorption was removed from the loci of the rate-limiting step of phosphate entry.

Literature Cited

1. BAMFORD, R. 1931. Changes in root tips of wheat and corn grown in nutrient solutions deficient in calcium. Bull. Torrey Botany Club 58: 149–78.
2. EADIE, G. S. 1942. The inhibition of cholinesterase by physostigmine and prostigmine. J. Biol. Chem. 146: 85–93.
3. EPSTEIN, E. 1956. Mineral nutrition of plants: Mechanisms of uptake and transport. Ann. Rev. Plant Physiol. 7: 1–24.
4. EPSTEIN, E. 1961. The essential role of calcium in selective cation transport by plant cells. Plant Physiol. 36: 437–44.
5. EPSTEIN, E. AND C. E. HAGEN. 1952. A kinetic study of the absorption of alkali cations by barley roots. Plant Physiol. 27: 457–74.
6. EPSTEIN, E. AND J. E. LEGGETT. 1954. The absorption of alkaline earth cations by barley roots: Kinetics and mechanism. Am. J. Botany 41: 785–91.
7. GAUCH, H. G. 1957. Mineral nutrition of plants. Ann. Rev. Plant Physiol. 8: 31–64.
8. GILBERT, W. A. AND J. C. O'KELLEY. 1964. The effects of replacement of calcium by strontium on the reproduction of *Chlorococcum echinozygotum*. Am. J. Botany 51: 866–69.
9. HAGEN, C. E. AND H. T. HOPKINS. 1955. Ionic species in orthophosphate absorption by barley roots. Plant Physiol. 30: 193–99.
10. JACOBSON, L., D. P. MOORE, AND J. HANNAPEL. 1960. Role of calcium in absorption of monovalent cations. Plant Physiol. 35: 352–58.
11. KAHN, J. S. AND J. B. HANSON. 1957. The effect of calcium on potassium accumulation in corn and soybean root. Plant Physiol. 32: 312–17.
12. LEGGETT, J. E. 1954. Influence of time, concentration, and pH on phosphate absorption by barley roots. M. S. Thesis, University of Maryland, College Park, Maryland.
13. LEGGETT, J. E. AND E. EPSTEIN. 1956. Kinetics of sulfate absorption by barley roots. Plant Physiol. 31: 222–26.
14. MARCHWORDT, U. 1963. Investigations on cation relationships in enriched barley roots. Landw. Forsch. 16: 6–12.
15. MICHAELIS, L. AND M. L. MENTEN. 1913. Die Kinetik der Invertinwirkung. Biochem. Z. 49: 333–69.
16. MILLARD, D. L., J. T. WISKICH, AND R. N. ROBERTSON. 1964. Ion transport by plant mitochondria. Proc. Natl. Acad. Sci. 52: 996–1004.
17. PITMAN, M. G. 1964. The effect of divalent cations on the uptake of salt by beetroot. J. Exptl. Botany 15: 444–57.
18. SWANBACK, T. R. 1939. Studies on antagonistic phenomena and cation absorption in tobacco in the presence and absence of manganese and boron. Plant Physiol. 14: 423–47.
19. TANADA, T. 1956. Effects of ultraviolet radiation and calcium and their interaction on salt absorption by excised mung bean roots. Plant Physiol. 31: 251–53.
20. VIETS, F. G., JR. 1944. Calcium and other polyvalent cations as accelerators of ion accumulation by excised barley roots. Plant Physiol. 19: 466–80.
21. WELTE, E. AND U. MARCHWORDT. 1963. Relationship between certain cations in the enrichment of barley seedlings. Agriochemica 7: 161–72.