PLANT PHYSIOLOGY

VOLUME 30 MAY, 1955 NUMBER 3

IONIC SPECIES IN ORTHOPHOSPHATE ABSORPTION BY BARLEY ROOTS[1]

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Absorption of phosphate by higher plants has been considered to involve the $H_2PO_4^-$ ion (7, 10). This conclusion has been based on simple correlations of phosphate sorption with $H_2PO_4^-$ concentration.

Results obtained from this study indicate that both $H_2PO_4^-$ and $HPO_4^=$ are absorbed under aerobic conditions by excised barley roots, and that the absorption of both ion species is competitively inhibited by hydroxyl ion. Expressions are derived that are in quantitative agreement with the observed absorption of phosphate. Radioactive phosphate was used as a tracer as well as a very low root mass per unit volume, and a sorption period of short duration.

MATERIALS AND METHODS

Excised roots of *Hordeum vulgare*, variety Atlas 46, were grown essentially as described by Epstein and Hagen (3). Twenty grams of seed were soaked for 24 hours in one liter of continuously aerated demineralized water at 24° C in a dark chamber. They were then rinsed in demineralized water and distributed on boiled cheesecloth supported on a stainless steel screen having a diameter of 12 cm. Each screen was made with four flaps which were turned down over the top of a four-liter beaker containing 1×10^{-4} M $CaSO_4$ solution, so that the seeds rested one centimeter above the solution. Another steel screen was placed over the seeds and this in turn was covered with boiled cheesecloth. Sintered glass aerators were placed in the solution and the beaker assembly was then covered with a watch glass and placed in a dark chamber. Seedling roots were grown five days in the continuously aerated $CaSO_4$ solution maintained at 24° C in darkness. Forty-four hours after planting, the glass and cheesecloth covers were removed; and three days after planting, the seedlings were removed from the solution, rinsed twice with demineralized water and placed in renewed $CaSO_4$ solution. During each of the latter two operations, the seedlings were exposed to low intensity incandescent light for about three minutes. Healthy roots 10 to 15 cm long were obtained by this procedure. These were excised with clean shears about 5 mm below the seeds. Excised roots were rinsed and placed in 3 liters of demineralized water.

In preliminary experiments, one liter of solution was found necessary for each one-gram portion of roots to maintain the concentration of phosphate essentially constant over an absorption period of three hours. Thus, one-liter solutions having the appropriate total phosphate concentration and specific activity were made up in one-liter Erlenmeyer flasks. The pH of the solutions was adjusted to the desired value with 0.1 *N* KOH or 0.1 *N* HCl. Flasks were placed in a water bath at 30° C and the solutions, under constant aeration, came to the temperature of the water bath.

A calculated amount of non-radioactive phosphate was added to the P^{32} in 0.04 *N* HCl, obtained from the Oak Ridge National Laboratory, with a specific activity of 40,000 mc/gm P. The solution was then evaporated to dryness under an infrared lamp to remove the acid and the residue was dissolved in demineralized water to prepare the phosphate stock solution. Phosphate absorption in three hours was not affected by levels of specific activity up to 3200 mc P^{32}/gm P. In all other experiments the specific activity was between 0.32 and 3.2 mc/gm P.

Excised roots were removed from the demineralized water in which they had been suspended for a period of 30 minutes and were blotted on cheesecloth to remove adhering water. One-gram portions were weighed out and placed in 50 ml demineralized water at 24° C in a test tube. When the solutions contained in flasks previously placed in a water bath reached a temperature of 30° C, the water in each test tube containing one gram of excised roots was decanted and 50 ml demineralized water added. At zero time roots were transferred, en masse, to phosphate solutions in one liter flasks. From 60 to 120 minutes elapsed from excision of roots to placement in the absorption flasks.

The flasks were returned to the water bath and aerated continuously during the absorption period with CO_2-free air at a rate of approximately 12 l/hr × l solution. In preliminary work, bicarbonate ion at a concentration of 4×10^{-4} M did not interfere with absorption of phosphate from solutions at 5×10^{-6} M phosphate, and pH levels of 5.0 and 7.7. However, removal of CO_2 was adopted as a standard procedure in view of the possibility that bicarbonate might conceivably interfere with absorption of other ions.

Flasks were removed from the bath within three minutes after addition of roots and the solutions readjusted to the desired pH value. Solutions having high total phosphate concentration required infre-

[1] Received July 28, 1954.

quent adjustment during absorption periods, once each half-hour being sufficient. At low total phosphate concentrations and all pH values, however, adjustments had to be made at intervals of 15 minutes. In solutions having total phosphate concentrations between 10^{-6} to 10^{-5} M, and with initial pH values ranging from 4.0 to 5.0, the pH tended to increase up to one-half unit over the first 30 minutes of the three-hour absorption period. Under similar conditions of phosphate concentrations, and in solutions having initial pH values ranging from 6.0 to 8.0, the pH tended to decrease as much as one-half unit.

At the end of the absorption periods, 50-ml portions of the solutions were saved for assay of activity and the roots were rinsed with demineralized water to remove adhering solution. The lots of roots were dried under an infrared lamp preparatory to measurement of radioactivity. Activity in samples of the stock solutions, with the same geometry as the root samples, were measured with a Geiger-Mueller tube. No correction for self-absorption was necessary because of the high energy beta emission of P^{32} and the range of counting was so high that the effect of the presence of K^{40} was negligible. The activity remaining in the solutions was measured. The extent of recovery of activity ranged from 95 to 100 % of that initially present.

Each point expressing the absorption under conditions of known phosphate and hydrogen ion concentrations, figures 1 to 3, is a mean value obtained from five experiments.

Basic Equations and Results

Experimental design was based on the assumption that absorption of ions involves formation of reversibly dissociable intermediates. Further, the rate of association and dissociation of the active intermediates is very rapid when compared with the irreversible reaction rate of the breakdown of the intermediate. These assumptions are formally expressed in the following equations:

$$(1) \qquad R + M_{outside} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MR$$

$$(2) \qquad MR \underset{k_4}{\overset{k_3}{\rightleftharpoons}} R' + M_{inside}$$

where R represents a metabolically produced site, M the ion, MR the active intermediate, and k the rate constant for each reaction. The k_3 is the rate-limiting step of absorption. Such assumptions have been shown to be valid for the absorption of alkali cations (3), as well as the halides (2).

Elementary application of these assumptions to experimental results requires a steady-state approximation. Within the limits of the experimental conditions a steady-state was shown to exist by a linear rate of phosphate absorption throughout an absorption period of three hours. Further, the absorption of phosphate by barley roots has been shown to be essentially irreversible (8), the k_4 is negligible.

A velocity equation may be derived from equations *1* and *2* which is entirely analogous to the steady-state analysis described by Michaelis and Menten (11) for kinetic studies of enzyme reactions. Lineweaver and Burk (9) pointed out the velocity equation is linear in form upon taking the reciprocal of both sides, as shown by equation *3*;

$$(3) \qquad 1/v = \frac{K_M}{V_{max}(S)} + 1/V_{max}$$

In the analogous expression describing absorption, substrate concentration, (S), will be expressed as $[\Sigma P]$ for total phosphate concentrations or in certain cases as the concentration of a specific ion species of orthophosphate. The observed absorption of phosphate is denoted by v, and the maximum absorption at infinite substrate concentration as V_{max}. The K_M is equal to the apparent dissociation constant of an activated intermediate of phosphate, RM.

If the process of phosphate absorption at steady-state involves a single first order reaction, described by the velocity equation, a double reciprocal plot of the variables, v and $[\Sigma P]$, would result in a straight line. That the experimental measurements cannot be described by a single first order reaction is evident from figure 1. To test whether more than one independent reaction is acting on a single substrate, or on different substrates which are in a non rate-limiting equilibrium, a plot of absorption, v, against $v/[\Sigma P]$ was made to permit separation of the component reactions by graphical methods (6), and figure 2. The observed absorption at pH 4.0 and 7.0, curve c, is composed apparently of two first order reactions, curves a and b, with both reactions acting simultaneously and independently on orthophosphate, per se. The two reactions may be resolved from the observed curve by the use of polar coordinates. In the case where the dissociations constant, K_M, of one reaction differs widely from the other, an extrapolation to the intercept of both coordinates may be made for the first order reaction that is approximated at low values of $v/[\Sigma P]$ as well as that reaction approximated at relatively high values of $v/[\Sigma P]$. The highest extrapolated

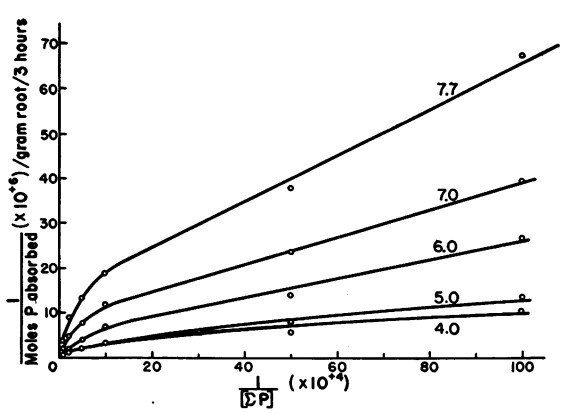


Fig. 1. Double reciprocal plot of phosphate absorption at varying pH.

TABLE I

ABSORPTION OF PHOSPHATE AT VARYING pH VALUES AS % OF ABSORPTION AT pH 4.0

[ΣP]	pH			
	5.0	6.0	7.0	7.7
	%	%	%	%
1×10^{-6}	89.2	37.8	25.8	15.0
2×10^{-6}	85.7	39.4	23.4	14.4
5×10^{-6}	117.4	48.9	29.7	19.4
1×10^{-5}	125.0	61.3	27.5	16.8
2×10^{-5}	134.0	81.4	34.5	16.9
1×10^{-4}	114.7	115.3	64.6	18.9

value on both the ordinate and the abscissa is a summation of the values contributed by both reactions. Thus, a subtraction of the extrapolated values on the ordinate, as well as the values on the abscissa, establishes the points which are determinate to the two curves. The latter procedure was considered to be applicable in the present instance.

The maximum absorption, V_{max}, is given directly by the intercept with the ordinate, figure 2. At pH 4.0 the individual reactions a and b comprising the observed absorption differ in their V_{max} by one factor of ten. Further, at pH 7.0 both first order reactions are inhibited, but at infinite phosphate concentrations the maximum absorption, in each case, is the same as that at pH 4.0. Since $V_{max} = Rk_3t$, it is evident that neither hydrogen ions nor hydroxyl ions are involved in irreversible changes in the binding sites, R_a^+ or R_b^+, or the rate constants. Thus, a strictly competitive interference of phosphate absorption is shown implicitly by a factor associated with increasing pH values.

Two different sites are involved evidently in phosphate absorption. The ion species of orthophosphate actively involved, and the factor associated with increasing pH which exhibits a competitive inhibition of phosphate absorption, may be established by qualitative inspection of the results.

Qualitative inspection of figure 2 shows the absorption curves can be best represented as two processes, as previously stated. Further, a number of mechanisms have been eliminated as a description of the system by not being consistent with the experimental results. A system of two species of orthophosphate absorbed through separate types of sites, with differing pH-activity relationships, however, is qualitatively consistent with the results in the following manner. At higher [ΣP] values, the site with the greatest V_{max}, curve a, contributes the largest percent of the total phosphate absorbed, figure 2. At the high value of [ΣP] of 1×10^{-4} M, absorption approximately parallels the percent of the singly charged species, $H_2PO_4^-$, with varying pH, tables I and II. Thus, $H_2PO_4^-$ is a logical substrate for the process of absorption denoted by curve a, figure 2. At the low concentrations of total phosphate the decrease in absorption is greater than the decrease in percent $H_2PO_4^-$ at the intermediate pH values. Since this decrease in absorption occurs despite an increase of $[HPO_4^=]$ with increasing pH values, table II, a pH effect, per se, must be invoked. However, the rate of decrease of absorption becomes less than the rate of decrease of $[H_2PO_4^-]$ with unit increases of pH values from 5.0 to 7.7, table I. With these increasing pH values, the concentration of $HPO_4^=$ increases to a significant value, and a large percent of the total concentration of phosphate. Absorption of $HPO_4^=$ through its respective site is consistent with these results. The process for which $HPO_4^=$ is the substrate is denoted by curve b, figure 2. The strictly competitive nature of the pH-activity effect on the absorption of an anion, phosphate, figure 2, suggests that hydroxyl ion, OH^-, is the competitive ion.

The above considerations result in a system described by an effect of pH on the species of phosphate ion in solution:

$$(4)\quad H_3PO_4 \rightleftharpoons H^+ + H_2PO_4^- \rightleftharpoons H^+ + HPO_4^= \rightleftharpoons H^+ + PO_4^{\equiv}$$

the combination of two active ion species with their respective binding sites;

$$(5)\quad R_a^+ + H_2PO_4^- \rightleftharpoons R_aH_2PO_4$$

$$(6)\quad R_b^+ + HPO_4^= \rightleftharpoons R_bHPO_4^-$$

and an additional effect of pH expressed by a competitive interference by the hydroxyl ion, OH^-, for both binding sites;

$$(7)\quad R_a^+ + OH^- \rightleftharpoons R_aOH$$

$$(8)\quad R_b^+ + OH^- \rightleftharpoons R_bOH$$

EXPRESSION OF EXPERIMENTAL OBSERVATIONS

The effective concentration of the species of orthophosphate ions in solution is limited by the ionization equilibria, as shown by equations *9*, *10*, and *11*.

$$(9)\quad K_{1h} = \frac{[H_2PO_4^-][H^+]}{[H_3PO_4]}$$

$$(10)\quad K_{2h} = \frac{[HPO_4^=][H^+]}{[H_2PO_4^-]}$$

$$(11)\quad K_{3h} = \frac{[PO_4^{\equiv}][H^+]}{[HPO_4^=]}$$

Different binding sites, or carriers, are involved in the absorption of $H_2PO_4^-$ and $HPO_4^=$. Further OH^-

TABLE II

SPECIES OF PHOSPHATE IONS AT VARYING pH VALUES AS % OF ΣP. THE RATIO OF SPECIES IS INDEPENDENT OF [ΣP]

ION SPECIES	pH					
	4.0	5.0	6.0	7.0	8.0	9.0
$H_2PO_4^-$	98.6	99.2	93.8	60.3	13.3	1.5
$HPO_4^=$	0.06	0.65	6.1	39.2	86.7	98.5

competitively inhibits each species at its respective binding site as expressed by the following equations:

$$(12) \qquad K_{1M} = \frac{[R_a^+][H_2PO_4^-]}{[R_aH_2PO_4]}$$

$$(13) \qquad K_{1i} = \frac{[R_a^+][OH^-]}{[R_aOH]}$$

$$(14) \qquad K_{2M} = \frac{[R_b^+][HPO_4^=]}{[R_bHPO_4^-]}$$

$$(15) \qquad K_{2i} = \frac{[R_b^+][OH^-]}{[R_bOH]}$$

The conservation equations for total phosphate, $[\Sigma P]$, and the two binding sites, $[\Sigma R_a^+]$ and $[\Sigma R_b^+]$, are as follows:

$$(16) \quad [\Sigma P] = [H_3PO_4] + [H_2PO_4^-] + [HPO_4^=] + [PO_4^{\equiv}] + [R_aH_2PO_4] + [R_bHPO_4^-]$$

$$(17) \quad [\Sigma R_a^+] = [r_a^+] + [R_aOH] + [R_aH_2PO_4]$$

$$(18) \quad [\Sigma R_b^+] = [r_b^+] + [R_bOH] + [R_bHPO_4^-]$$

A cubic equation results when equations *9* through *18* are combined with the elimination of all variables except either $[R_aH_2PO_4]$ or $[R_bHPO_4^-]$, the intermediate complexes. Solution was obtained by mapping from the following partially combined equations:

$$(19) \quad [R_aH_2PO_4] = \frac{[R_bHPO_4^-][H^+](K_{2M})(K_{1i})[R_a^+]\{K_{2i} + [OH^-]\}}{(K_{1M})(K_{2i})(K_{2h})\{[R_b^+] - [R_bHPO_4^-]\}\{(K_{1i}) + [OH^-]\} + [R_bHPO_4^-][H^+](K_{2M})(K_{1i})\{(K_{2i}) + [OH^-]\}}$$

and

$$[\Sigma P] = \frac{[R_bHPO_4^-][H^+]^2(K_{2M})\{[H^+] + (K_{1h})\}\{(K_{2i}) + [OH^-]\} + [R_bHPO_4^-](K_{2M})(K_{1h})(K_{2h})\{[H^+] + (K_{3h})\}\{[OH^-] + (K_{2i})\} + [H^+](K_{2i})(K_{1h})(K_{2h})\{[R_bHPO_4^-] + [R_aH_2PO_4]\}\{[R_b^+] - [R_bHPO_4^-]\}}{[H^+](K_{2i})(K_{1h})(K_{2h})\{[R_b^+] - [R_bHPO_4^-]\}}$$

Since $[R_aH_2PO_4]$ and $[R_bHPO_4^-]$ are functions solely of the parameters $[H^+]$, $[OH^-]$, $[\Sigma P]$, $[\Sigma R_a^+]$, $[\Sigma R_b^+]$ and the several equilibrium constants, it is apparent that absorption must be described as follows:

$$(20) \quad \text{Absorption} = \{[R_aH_2PO_4](k_{a3}) + [R_bHPO_4^-](k_{b3})\}t_{sec}$$

To test the expression, values for R_a^+ and R_b^+ as well as for k_{a3} and k_{b3} must be obtained. Consideration of the results shows that only limits and approximations of these values are necessary.

Kinetic studies of enzyme reactions are usually expressed as:

$$(21) \qquad E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P$$

where k_4 is negligible, and the rate of the observed velocity of the reaction is directly proportional to the concentration of the enzyme-substrate compound, ES, at all values of the concentration of the substrate, ΣS. Thus, $v = (ES)k_3t$ and at infinite substrate concentration the numerical constant representing the maximum velocity, V_{max}, obtained when E exists completely in the form ES, may be expressed as $V_{max} = (E)k_3t$. These considerations are valid to most enzymatic measurements, as either the appearance of product, P, the disappearance of substrate, S, or the intermediate, (ES), is determined.

Formally analogous considerations are involved in ion absorption, equations *1* and *2*. However, total ion associated with the root is measured in absorption studies. Thus, the absorption involves MR as well as M_{inside}. Absorption must then be expressed as shown in equation *22*.

$$(22) \qquad \text{Absorption} = (MR)k_3t + (MR),$$

and the maximum absorption as:

$$(23) \qquad V_{max} = (R)k_3t + (R).$$

Further, if non-specific adsorption is involved, the expression is complicated in the following manner:

$$(24) \qquad V_{max} = (R)k_3t + \{(R) + (\text{adsorption})\}.$$

A small, but measureable, amount of the sorbed phosphate is exchangeable in 30 minutes (8). It cannot be ascertained whether such exchange involves adsorbed phosphate, phosphate in combination with the carrier, or both. Inspection of equation *24* shows that if a measureable amount of adsorption contributed to the results, it would only suffice to make (R) smaller. Therefore, the highest possible value for the amount of carrier, R, may be determined by assuming a negligible non-specific adsorption according to equation *23*.

The maximum absorption, V_{max}, was determined by graphical treatment of results from three different absorption periods at $[\Sigma P]$ of 10^{-4} M to 10^{-6} M at pH 7.0, table III. Since the V_{max} for both absorption processes remain constant at varying pH values, figure 2, no restriction of the results is invoked by the selection of pH 7.0 for determining the V_{max}.

$$5.96 \times 10^{-7} = (R^+_{a+b})k_{3_{a+b}}\,3600 + (R^+_{a+b})$$

$$8.34 \times 10^{-7} = (R^+_{a+b})k_{3_{a+b}}\,5400 + (R^+_{a+b})$$

$$13.56 \times 10^{-7} = (R^+_{a+b})k_{3_{a+b}}\,9000 + (R^+_{a+b})$$

Solution of the three combinations of paired equations gave $(k_{3_{a+b}})$ values of 1.1×10^{-3}; 1.6×10^{-3}, and

TABLE III

MAXIMUM ABSORPTION, $(V_{a_{max}} + V_{b_{max}})$, DETERMINED FROM THREE DIFFERENT ABSORPTION PERIODS AT $[\Sigma P]$ OF 10^{-4} M TO 10^{-6} M AT pH 7.0

ABSORPTION PERIOD SEC	$(V_{a_{max}} + V_{b_{max}})$ MOLES P/GM ROOT $\times t_{sec}$
3600	5.96×10^{-7}
5400	8.34×10^{-7}
9000	13.56×10^{-7}

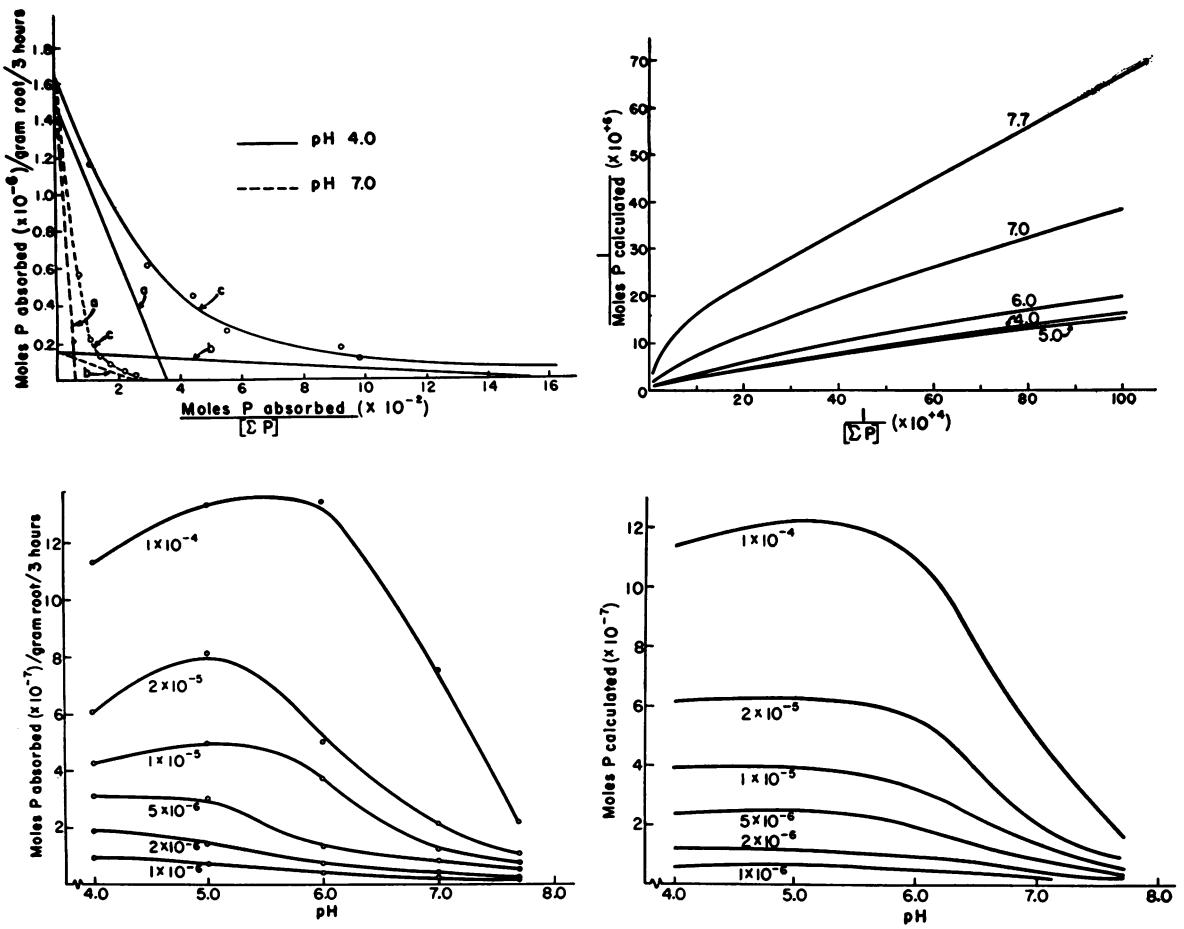


FIG. 2 to 5. Phosphate absorption by excised barley roots.

FIG. 2 (*top left*). Graphical separation of reactions, curves a and b, comprising phosphate absorption at pH 4.0 and 7.0.

FIG. 3 (*bottom left*). Absorption as a function of pH and total phosphate concentration.

FIG. 4 (*top right*). Double reciprocal plot of calculated values of phosphate absorption at varying pH.

FIG. 5 (*bottom right*). Calculated absorption as a function of pH and total phosphate concentration.

2.8×10^{-3} moles P/mole (R^+_{a+b})/gm root × sec, and from these values the mean value of (R^+_{a+b}) was calculated to be 8.72×10^{-8} moles. The average value for (R^+_{a+b}) is the greatest total amount of carrier that could be involved in absorption of both $H_2PO_4^-$ and $HPO_4^=$ from the external solution.

Absorption has been shown to be composed of two first order reactions with V_{max} for $HPO_4^=$ and $H_2PO_4^-$ absorption as 1.5×10^{-7} and 1.5×10^{-6} moles P/gm root × 3 hrs, respectively, figure 2. Thus, the $(R^+_{a+b})(k_{3_{a+b}})$ values vary by one factor of ten. To ascribe this to a difference of (R^+_{a+b}), $(k_{3_{a+b}})$, or both is not possible. Therefore, the assumption was made that equal amounts of both carriers were present, having a high value of 1×10^{-8} moles. With this value, the rate constants k_{3_a} and k_{3_b} for $HPO_4^=$ and $H_2PO_4^-$ absorption are calculated to be 1.39×10^{-3}, and 1.39×10^{-2} moles P/moles R^+/gm root × sec, respectively. A simplifying assumption, discussed in detail later in this paper, is made that the amount of carrier associated with the roots is effective throughout the volume of one liter and as such may be expressed in units of concentration. The amount of R^+ in moles is then assumed to be equivalent to moles/l of R^+. It will be shown later that the assumption of 10^{-8} M (R^+) is not critical insomuch as only $[R^+]$ greater than 10^{-6} M significantly affects the results.

The three steps in the ionization equilibria of solutions of orthophosphoric acid are: $K_{1h} = 7 \times 10^{-3}$; $K_{2h} = 6.5 \times 10^{-8}$; and $K_{3h} = 5 \times 10^{-13}$ (4).

The steady-state analysis of Michaelis and Menten is based on the assumption that the uncoupled substrate can be approximately represented by the total concentration of the substrate. This assumption is not inherent in the mathematical expression of the system, *19*, since the substrate conservation equation has been considered by *16*. However, in order to obtain the numerical values of the related constants involved in the expression, it was necessary to experi-

mentally approximate the conditions of steady-state throughout the absorption periods. Within the limits of the experimental conditions, a linear rate of phosphate absorption was shown to exist throughout an absorption period of three hours. Thus, a sufficient total amount of substrate, M, in respect to the coupled intermediate, MR, was present to maintain the concentration of M essentially constant throughout the absorption periods. This allowed determinations of constants from expressions based on the assumption of steady-state (9, 11).

The dissociation constants of the carrier phosphate intermediates, $R_aH_2PO_4$ and $R_bHPO_4^-$, can be determined only in the absence of interfering ions. Inspection of figure 1 shows that with a ten fold increase in the concentration of the competitive hydroxyl ion, in the range of pH 4.0 to 5.0, a relatively small inhibition of phosphate absorption occurs. From this, it is inferred that the effect of the competing hydroxyl ion is negligible at pH 4.0, and at this pH the dissociation constants of the phosphate intermediates may be determined. In these experiments, pH values below 4.0 were avoided because of the possibility of irreversible damage to the roots. The validity of the above reasoning will be shown in a later portion of this paper when the determination of the dissociation constants of the carrier hydroxyl ion complex is discussed.

Both processes involved in phosphate absorption at pH 4.0 are expressed by a velocity equation, *3*, derived for reactions in absence of interfering ions or competing substrates. The intercepts with the ordinate give the V_{max} directly for each reaction, when a plot is made of the variables, v against $v/[\Sigma P]$, figure 2. The V_{max} for the reactions are 1.5×10^{-6} M and 1.5×10^{-7} M, respectively. The values of the slopes give dissociation constants, K_M, of the two first order reactions in respect to the total phosphate concentration, $[\Sigma P]$. Since the dissociation constants are expressed as the equilibria between the specific ion species and its respective carrier, equations *12* and *14*, the constants must be calculated in respect to the concentration of the particular ion species under consideration. The constants describing the three ionization equilibria of orthophosphoric acid are known, and the concentration of the various species of phosphate ions can be calculated at any pH value. At pH 4.0, the $H_2PO_4^-$ and $HPO_4^=$ are approximately 98.6 % and 0.064 % of the total phosphate, respectively. Thus, the slope, K_M, of each line may be recalculated on the basis of the total concentration of the specific ion species. The numerical values for the apparent constants K_{1M} and K_{2M} were calculated to be 4.0×10^{-5} M and 5.5×10^{-10} M, respectively.

At pH 7.0 the hydroxyl ion, OH^-, interferes in a competitive manner with the absorption of both $HPO_4^=$ and $H_2PO_4^-$, figure 2. The absorption contributed by each of the two first order reactions to the total absorption may be calculated at any total substrate concentration, $[\Sigma P]$, at this pH value. The recalculation of the concentration of the specific ion involved under these conditions, pH 7.0, for each respective curve shows that $[H_2PO_4^-]$ and $[HPO_4^=]$ comprise 60.3 % and 39.2 % of the total phosphate concentration, respectively. The dissociation constant of a strictly competitive inhibitor is expressed by the following expression:

$$(25) \qquad K_i = \frac{(K_M)(I)(v)}{(V_{max})(S) - (K_M)(v) - (S)(v)}$$

As previously shown, the respective K_M and V_{max} have been determined for each of the ion species involved. The K_{1i} and K_{2i} are calculated to be 2.5×10^{-8} M and 2.0×10^{-11} M, respectively, by substitution of the respective K_M and V_{max} and the calculated concentration of the specific ion, $H_2PO_4^-$ or $HPO_4^=$ involved in its respective contribution, v, to the total absorption at a total inhibitor concentration, $[OH^-]$, of 1×10^{-7} M.

The appearance of K_M in equation *25* permits testing the validity of the observation of an insignificant inhibition of hydroxyl ion at pH 4.0. Using the value of K_M based on this observation, the values of K_{1i} and K_{2i} were calculated from pH values 6.0, 7.0 or 7.7, respectively, and shown to remain essentially constant. A significant error in the numerical value of K_M due to hydroxyl ion competition at pH 4.0 would be reflected by varying values of K_{1i} and K_{2i} when calculated from the results obtained at the different pH values.

Agreement with Experimental Observations

Substitution of the apparent constants into equation *19* permitted values for the intermediates, $[R_aH_2PO_4]$ and $[R_bHPO_4^-]$, at varying $[\Sigma P]$ to be calculated for each pH level. Since the concentration of each intermediate complex is assumed to be equivalent to the amount in moles, the values were multiplied by the respective k_3t according to equation *20*, where t is equal to 10,800 seconds. A double reciprocal plot of the calculated total absorption against $[\Sigma P]$ is shown in figure 4. Calculated total absorption at different pH values is shown in figure 5. Essential agreement with experimental results, figures 1 and 3, indicates that the expression describes the system for phosphate absorption by excised barley roots.

Discussion

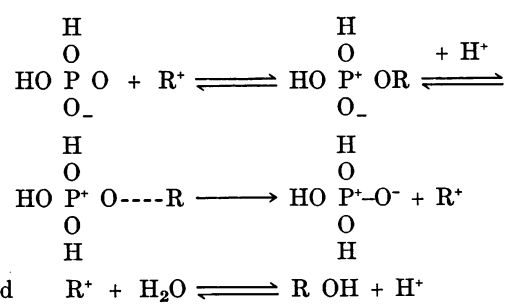
Association of the binding sites involved in phosphate absorption with roots describes a heterogeneous system. Although the general kinetic treatment employed in this paper is applicable to both homogeneous and heterogeneous reactions, certain restrictions are imposed on the results obtained from heterogeneous systems.

Consideration of equilibrium in intermediate complex formation necessitates assignment of concentration values for the intermediate complexes, $R_aH_2PO_4$ and $R_bHPO_4^-$, and the binding sites, R_a^+ and R_b^+. The units of concentration are expressed in respect to the external system and do not indicate the concentration of the sites in the roots, per se. Since the absolute concentrations of the binding sites are not known, it is evident that constants describing reactions with

these sites must be recognized as apparent or relative constants. Under the experimental conditions, it has been shown that the greatest amount of $(R_a^+ + R_b^+)$ effective in phosphate absorption from the external solution is approximately 1×10^{-8} moles. The measured maximum absorption for each reaction is expressed by an $(Rk_3t + R)$ which dictates that varying amounts of R^+ are compensated by changing values of k_3. Since the moles of R^+ have been assumed to be equivalent to the concentration of R^+ in respect to the external system, it can be shown that only $[R^+]$ of 1×10^{-6} M or greater significantly affect the equilibrium as described by equation *19*. The assignment of the highest value to the concentration of the binding sites is not critical to this analysis since $[R^+]$ is 1×10^{-8} M, or less.

Phosphate absorption by barley roots emphasizes the danger in assigning importance to an ion solely because of its predominant concentration in the system. The first step in the ionization equilibria of orthophosphoric acid differs from the second step by approximately five factors of ten, being 7×10^{-3} M and 6.5×10^{-8} M for K_{1h} and K_{2h}, respectively. Consideration of concentration of the ions, per se, would dictate that $HPO_4^=$ is unimportant in the absorption of phosphate at acid pH values, since the ionization of $H_2PO_4^-$ to $HPO_4^=$ is small. However, the apparent dissociation constants of the two phosphate intermediates differ by five factors of ten; the K_{1M} and K_{2M} being 4×10^{-5} M and 5.5×10^{-10} M, respectively. Since total phosphate has been shown to be greatly in excess to that of the binding groups, $(R_a^+ + R_b^+)$, and the ionization of orthophosphate is not rate limiting, it is apparent that the $HPO_4^=$ ion, in less prominent concentration but with greater affinity, attains importance in absorption equal in magnitude with that of the $H_2PO_4^-$.

The competitive nature of the hydroxyl ion, OH^-, indicates the formation and breakdown of the labile, intermediate complexes involves the cleavage of an R–O bond, as described by the following reactions:



and $R^+ + H_2O \rightleftharpoons R\,OH + H^+$

If the P–O bond were involved, a hydrogen ion competition would be expected, which is not the case, figure 2. Cleavage of such R–OPO_3 bonds is known to be involved in such reactions as the acid hydrolysis of glucose-1-phosphate (1) and numerous examples of phosphorylase action on glucosyl–OPO_3 (1, 5).

Summary

Kinetics of phosphate absorption by excised roots of *Hordeum vulgare* var. Atlas 46 were studied in constantly aerated solutions at 30° C with phosphate containing P^{32}. In order that concentrations might remain essentially constant during three hour sorption periods one liter quantities of solutions were used with one gram of roots. Principle variables were phosphate (10^{-4} to 10^{-6} M) and hydrogen ion (pH 4 to 8) concentration. A quantitative interpretation of the results was found. This interpretation is based on the formation and breakdown of phosphate complexes with metabolically derived reactants (sites).

(1) In excised barley roots $H_2PO_4^-$ is absorbed through one site and $HPO_4^=$ through another.

(2) Hydroxyl ion competitively inhibits absorption of both $H_2PO_4^-$ and $HPO_4^=$.

(3) Neither hydrogen nor hydroxyl ions in the external medium affects the concentrations of either absorption site effective in phosphate absorption from the external solution.

(4) The rate limiting breakdown of both kinds of intermediate phosphate compounds involves cleavage of an R–O bond.

This investigation was supported by the U. S. Atomic Energy Comission. The critical interest of S. B. Hendricks, P. C. Jackson and J. E. Leggett is gratefully acknowledged.

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