

ORGANIC ACID METABOLISM AND ION ABSORPTION IN ROOTS^{1, 2}

LOUIS JACOBSON AND LAWRENCE ORDIN
DEPARTMENT OF PLANT NUTRITION, BERKELEY, CALIFORNIA

It is axiomatic that the total charge of ions inside plant cells must be electrically equal to zero. Nevertheless, preferential absorption or uptake of ions by plants from external environments can and does occur. Since roots may absorb cations and anions at unequal rates from single salt solutions, they must therefore have at their disposal mechanisms for maintaining cation-anion equivalency. In recent years, the role of organic acid metabolism in relation to the maintenance of cation-anion balance have been investigated by Pucher, Vickery and Wakeman (15), Ulrich (17), Overstreet, Broyer, Isaacs and Delwiche (11), Pierce and Appleman (13), Burström (2), Cooil (5), and others.

Ulrich (17), working with excised barley roots, concluded that when excess cations were absorbed, "organic acids were formed as a response to the tendency toward an increase in pH of the root sap. Conversely, when excess anions were absorbed, organic acids tended to disappear leaving the base to balance the increase of inorganic anions." He further concluded that the organic acids were associated with respiration directly or indirectly.

On the basis of buffer curves of plant saps and fractional precipitation with barium salts in alcoholic solution, Burström (2) tentatively concluded that the organic acid fraction from wheat sap consisted largely of malic acid with small amounts of other acids, mostly citric. The citric acid in no case constituted more than 10% of the total acid. Burström states that the buffer system of the wheat plant consists of the formation or destruction of malic acid so its content approximately equals the existing cation excess, and the regulation of the pH of the cell sap by the buffer action of CO₂. In a later paper, Burström (3) relates the role of organic acid in ion balance to the Lundegårdh scheme of anion respiration.

A valid theory of ion absorption should be consistent with all observed changes occurring in the ionic composition of the absorbing tissues. Thus, to study the mechanisms of ion absorption, it becomes highly desirable to examine the nature of the changes in ionic balance during absorption.

Since organic acids play an important role in the cation-anion balance, it likewise becomes imperative to conduct a thorough survey of the organic acids which occur in the absorbing tissue and to follow their changes during the course of absorption. This has now become readily feasible with the recent application of quantitative chromatographic techniques to organic acid analysis:

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MATERIALS AND METHODS

All experiments were carried out with excised roots, usually from 6-day-old barley seedlings (Sacramento variety). Seedlings were grown in a very dilute nutrient solution in the dark as earlier described (7). Also, low-salt roots were obtained from plants grown in the greenhouse in 1/8 strength Hoagland nutrient solution.

The excised roots were rinsed thoroughly in distilled water and centrifuged for five minutes at approximately 65 × g. Weighed portions of roots were then placed into the various solutions. All absorption experiments were carried out at 26°C. In most experiments, 15 gm of excised root material were allowed to absorb from 15 liters of solution. In no case were more than 26 gm of roots used in 15 liters of solution. A large ratio of solution to root weight was used to minimize concentration changes in the culture solution. All solutions were aerated during the experiment. After the absorption period, the treated roots were carefully washed and stored at -18°C until lyophilized. Roots to be used for organic acid fractionation were lyophilized immediately after treatment.

Potassium was determined in ashed aliquots of the dried and ground root material by precipitation as the cobaltinitrite, and subsequently titrated with ceric sulfate. Calcium was precipitated as the oxalate and titrated with ceric sulfate. Bromide was determined by the iodometric procedure of White and Kilpatrick (18) after fusing a suitable aliquot of the dried sample with NaOH in a nickel crucible. Calcium plus magnesium were estimated together by a method essentially similar to the ethylenediamine tetraacetate titration procedure of Cheng and Bray (4); nitrate as described by Johnson and Ulrich (9); inorganic sulfate according to Johnson and Nishita (8); ammonia by the method of Pucher, Vickery and Leavenworth (14); and total organic acid, malic, citric and oxalic acids by the methods of Pucher, Wakeman, and Vickery (16). Fractionation of the organic acids was accomplished through the use of silica gel chromatography as described by Bulen, Varner, and Burrell (1). Inorganic phosphate in the lyophilized root material was determined colorimetrically by the molybdenum blue reaction.

RESULTS

BARLEY SEEDLINGS ROOTS: When ions are absorbed by plant roots, a) equivalent amounts of cations and anions are absorbed, or b) excess cation, or c) excess anion absorption occurs from the external solution. The first condition was examined by allowing excised roots from 6 day old barley seedlings to absorb from 0.005 N KBr solutions for periods up to 3 hours. The

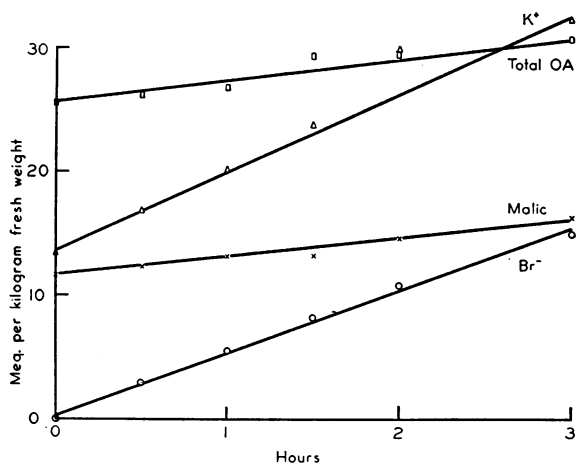


FIG. 1. Potassium, bromide, total organic acid and malic acid contents of excised barley seedling roots placed in 0.005 *N* KBr, as a function of time.

absorption rates of potassium and bromide from KBr are not very different for this root material. The treated roots were analyzed for K, Br, total organic acid and malic acid, as shown graphically in figure 1. In 3 hours, the roots had absorbed 18.9 meq potassium and 15.4 meq Br per kg fresh weight indicating 3.5 meq excess cation absorption. In the same period the total organic acids increased by 5.2 meq per kg fresh weight. The malic acid increased by 4.6 meq. In this case, the increase in organic acid anion more than compensated the relatively small cation excess and therefore other changes in the root ions were involved to a small extent. The pH of the expressed sap of the barley seedling roots (i.e., 5.7 to 6.0) in this, as well as in the following experiments, is such that the organic acids are almost completely dissociated.

As an example of the second case, the experiments of Overstreet, Ruben and Broyer (12) with radioactive carbon suggested that the absorption of potassium from KHCO_3 was largely excess cation ab-

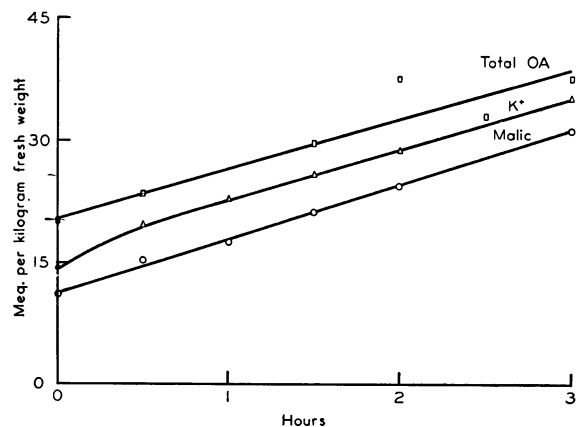


FIG. 2. Potassium, total organic acid and malic acid contents of excised barley seedling roots placed in 0.005 *N* KHCO_3 , as a function of time.

sorption and this view is supported in the following experiment. An experiment carried out as described above, but in 0.005 *N* KHCO_3 , gave the K, organic acid and malic acid results plotted in figure 2. The excess cation absorption (K) was 20.6 meq per kg fresh weight. The increase in total organic acids and malic acid were 18.9 and 19.8 meq respectively. In this instance, the increase in total organic acid anion very nearly approached the excess cation absorbed. The apparently greater production of malic acid than of total organic acid may or may not be significant. Some data reflecting on this point will be described later.

The third case of differential absorption of ions is shown with CaBr_2 . Roots were placed in 0.005 *N* CaBr_2 for various periods up to 3 hours. The Ca, Br, total organic acid and malic acid data are plotted in figure 3; 3.5 meq calcium per kilogram fresh weight and 8.6 meq bromide were absorbed in 3 hours, producing an excess anion absorption of 5.1 meq. Dur-

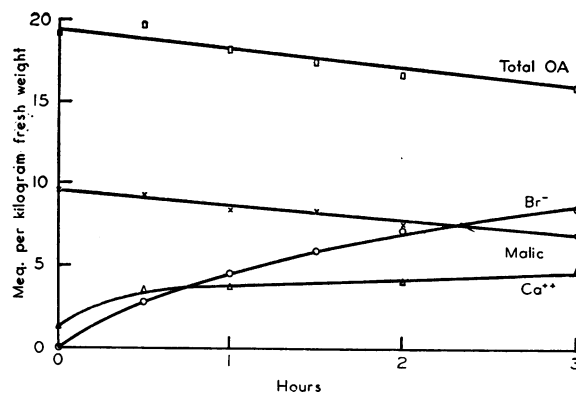


FIG. 3. Calcium, bromide, total organic acid and malic acid contents of excised barley seedling roots placed in 0.005 *N* CaBr_2 , as a function of time.

ing this period, the roots lost 3.7 meq total organic acid and 2.8 meq malic acid. The decrease in total organic acid anion was not sufficient to compensate entirely for the excess absorption of bromide over calcium, the discrepancy amounting to 1.4 meq. However, similar discrepancies amounting to as much as 7.0 meq have been obtained in other experiments.

In a modified experiment, excised barley roots were allowed to absorb from 0.005 *N* CaBr_2 for 3 hours and the treated roots then transferred to distilled water for 3 hours. This was done in order to observe any changes in the organic acid content following the initial treatment. The Ca, Br, total organic acid and malic acid levels were followed over the entire period of the experiment. The values are presented in figure 4. At the end of the CaBr_2 treatment, the roots had absorbed a bromide excess of 6.2 meq per kg fresh weight over the Ca absorbed. The total organic acid anion at this point had decreased by 3.7 meq, leaving an unaccounted excess of 2.5 meq of Br. Upon placing the treated roots into distilled water, some Ca and Br were lost from the roots and at the same time, the

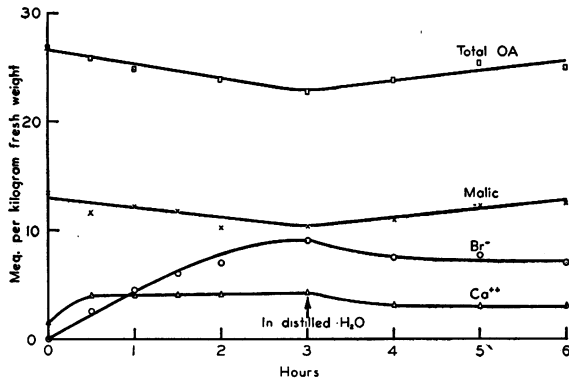


FIG. 4. Calcium, bromide, total organic acid and malic acid contents of excised barley seedling roots as a function of time. Roots placed in 0.005 *N* CaBr₂ for periods up to 3 hours. 3 hour treated roots then transferred to distilled water for periods up to an additional 3 hours.

total organic acid and malic acid increased somewhat. By comparing the initial and final values, it will be observed that for the entire experiment, the roots had gained 5.5 meq more bromide than calcium. The total change in organic acid anion was a decrease of 1.2 meq leaving 4.3 meq of Br to be compensated by other means.

MATURE PLANT ROOTS: The plants listed in table I were grown in dilute nutrient media in the greenhouse. All plants, with the exception of Atlas barley, were cultured for a period of 4 or 5 weeks before harvesting. For Atlas barley, the period was about 2 weeks. Excised roots from these plants were allowed to absorb from solutions of 0.005 *N* KHCO₃ for 3 hours. Analyses were made on the roots, before and after treatment, for K, Ca plus Mg, total organic, malic, citric, and oxalic acids. These data are given in table I. For every root sample reported in the table, Ca

plus Mg was lost from the roots at the same time as K was being absorbed. Roots from Romaine lettuce lost nearly as much Ca plus Mg as K absorbed. In this case, the total organic acid increased but little. With the exception of the above material, poor agreement was obtained between the change in total organic acid content and the changes in the K and Ca plus Mg contents. It would appear that other ions were involved to a marked extent or that changes within the so-called total organic acid fraction were occurring. Some evidence of the latter is seen in the peculiar situation of having malic acid increasing in all the root material by an amount larger than that of the total organic acid.

ACETIC ACID IN BARLEY SEEDLING ROOTS: Initially, in order to fractionate the organic acids, they were extracted by a modification of the method of Isherwood (6). The ground material was acidified with H₂SO₄, mixed with silica gel and eluted with 50% (v./v.) butyl alcohol-chloroform. The eluted acids were re-extracted from silica gel with 50% (v./v.) tertiary amyl alcohol-chloroform. However, the total organic acids extracted by this procedure were invariably lower than when extracted by the method of Pucher, Wakeman and Vickery (16). For untreated barley seedling roots, the Isherwood procedure resulted in values which were only about 60% of those obtained by the Pucher *et al.* method. For roots, treated with 0.005 *N* KHCO₃ for 3 hours, the comparison was better. About 80% was recovered by the Isherwood procedure. Since the total organic acid (Pucher *et al.* method) had doubled as a result of the KHCO₃ treatment, it appeared that there might be an approximately constant amount of some acid present which was extracted by the Pucher *et al.* method, but not by the Isherwood procedure. Such was indeed found to be the case and the acid in question was identified as acetic acid. Initial identification was established through fractionation on silica gel columns and by

TABLE I

THE EFFECT OF 0.005 *N* KHCO₃ FOR 3 HOURS ON THE CONTENT OF POTASSIUM, CALCIUM PLUS MAGNESIUM, TOTAL ORGANIC, MALIC, CITRIC AND OXALIC ACIDS OF THE EXCISED ROOTS FROM MATURE PLANTS

		MEQ PER KILOGRAM FRESH WEIGHT					
		K	Ca + Mg	TOTAL OA	MALIC	CITRIC	OXALIC
<i>Hordeum vulgare</i> (Atlas 46 barley)	Initial	34.1	21.2	53.7	33.4	0.77	0.98
	Final	62.3	16.5	64.4	50.5	2.44	0.98
	Change	+28.2	-4.7	+10.7	+17.1	+1.67	0.00
<i>Soja max</i> (Hawkeye soybean)	Initial	38.8	28.5	50.3	6.7	1.21	0.68
	Final	56.8	23.4	60.7	17.8	1.41	0.56
	Change	+18.0	-5.1	+10.4	+11.1	+0.20	-0.12
<i>Cucurbita maxima</i> (Early white squash)	Initial	16.7	34.7	28.0	4.3	2.17	2.47
	Final	30.7	29.2	30.1	8.9	2.38	0.81
	Change	+14.0	-5.5	+2.1	+4.6	+0.21	-1.66
<i>Medicago sativa</i> (Chilean alfalfa)	Initial	17.6	61.1	61.2	22.8	13.6	0.51
	Final	29.2	52.2	58.8	22.8	12.6	0.00
	Change	+11.6	-8.9	-2.4	0.0	-1.0	-0.51
<i>Lactuca sativa</i> (Romaine lettuce)	Initial	15.3	76.2	85.7	19.8	7.85	48.2
	Final	26.1	67.1	86.8	21.4	8.34	49.0
	Change	+10.8	-9.1	+1.1	+1.6	+0.49	+0.8

paper chromatography. Final identification was accomplished by a Du Claux distillation. In all cases, the correspondence between the unknown acid and acetic acid was exact.

Some preliminary studies of the acetic acid fraction indicate that this acid is not present in the free form in the barley seedling roots. The free acetate is present only in small amounts. The "bound" acetic acid is insoluble since it remains in the residue after extraction of ground root material with water. It is, however, readily hydrolyzed by treatment with 0.1 *N* H₂SO₄ on the steam bath for several hours. After such treatment, all of the acetic acid can be recovered in the free form. The expressed sap from frozen roots also contain but little acetate, free or "bound," the bulk of the acetate remaining in the press cake.

ORGANIC ACID FRACTIONATION: Samples of excised roots from barley seedlings were permitted to absorb for 3 hours from 0.005 *N* solutions of KBr, CaBr₂, and KHCO₃, and a control sample was placed in distilled water. The pH of the KBr solution was adjusted to 4.95, since at this pH and with this material, the rates of uptake of K and Br from KBr are almost exactly equal. After the absorption period, the samples were washed, lyophilized and ground. The percentage dry weight of the samples varied from 5.85 to 6.1%. The total organic acids were extracted from suitable aliquots of the ground material by the ether extraction procedure as described by Pucher, Wakeman and Vickery. The individual organic acids were isolated from this fraction by the silica gel column fractionation procedure of Bulen, Varner and Burrell (1). The distribution of the acids in the effluent fractions were mapped out by running various combinations of known acids. When sufficient material was available, the identity of the isolated root acids was further confirmed through the use of paper chromatography.

The analyses of the initial and treated root samples are given in table II. In addition to the organic acid analyses, data are presented for the major inorganic ions. Acetic acid appears in the organic acid fraction since it is liberated by the extraction procedure. However, it is probably not available for cation compensation and so is not included in the cation-anion balance calculations. Other organic acids than those listed were either absent or present in too small a quantity to detect. The list of acids appears to be fairly complete since their sums check quite well with the corresponding total organic acid values as obtained by the titration procedure for total acids as described by Pucher *et al.* From 93.2 to 94.8% of the total acid values were recovered as isolated acids. This is good agreement with the average recovery figure for the pure acids adsorbed on the silica gel column. Two unidentified acids were present, acids A and B in table II. Both were present in small quantity, the former in all samples and the latter primarily in the KHCO₃ treated roots. Only the trans form of aconitic acid was detected. However, the treatment used to isolate the acids would have been sufficiently strong to have converted any cis-aconitic acid to the trans form.

Although most of the acids increased during excess

cation absorption, malic acid and to a considerably lesser extent succinic acid accounted for most of the increase. The major decrease in organic acid content during excess anion absorption was accounted for by a decline in the malic acid content. Changes in the organic acids of the roots during the distilled water treatment were relatively small. This was also true when conditions of equivalent cation and anion uptake prevailed.

TABLE II

THE EFFECT OF WATER AND 0.005 *N* SOLUTIONS OF KBr, CaBr₂ AND KHCO₃ FOR 3 HOURS ON THE ORGANIC ACID COMPOSITION AND ION-BALANCE IN EXCISED BARLEY SEEDLING ROOTS

	TREATMENT				
	VALUES IN MEQ PER KILOGRAM FRESHWEIGHT				
	INITIAL	H ₂ O	KBr	CaBr ₂	KHCO ₃
<i>Organic acids</i>					
Acetic	8.13	8.00	9.32	8.83	9.40
Pyruvic *	0.079	0.280	0.053	0.265	0.441
Fumaric	0.588	0.511	0.518	0.485	0.626
Succinic	0.423	0.407	0.474	0.428	2.42
Trans-aconitic *	0.391	0.396	0.301	0.458	0.653
Oxalic	0.352	0.228	0.260	0.327	0.322
Malic	11.80	13.80	13.20	8.31	29.31
A †	0.276	0.315	0.243	0.209	0.181
B †	0.000	0.030	0.039	0.000	0.179
Citric	0.216	0.359	0.368	0.258	0.632
Total	22.25	24.33	24.78	19.57	44.16
Total OA	23.8	25.7	26.5	20.7	47.3
(Pucher <i>et al.</i> method)					
<i>Anions</i>					
Total OA-acetic	15.7	17.7	17.2	11.9	37.9
Br ⁻	0.0	0.0	17.5	7.8	0.0
NO ₃ ⁻	1.0	1.4	0.2	0.1	1.1
SO ₄ ⁻	3.6	3.4	3.1	3.0	3.6
H ₂ PO ₄ ⁻	4.8	4.2	4.1	4.8	4.4
Total anion ..	25.1	26.7	42.1	27.6	47.0
<i>Cations</i>					
K ⁺	14.6	14.9	32.0	14.7	38.5
Ca ⁺⁺ + Mg ⁺⁺ ...	9.8	8.6	7.7	11.8	8.2
NH ₄ ⁺	1.5	2.4	2.2	2.9	1.2
Total cation ..	25.9	25.9	41.9	29.4	47.9

* Identification tentative.

† Unidentified.

Measurements of pH made on the expressed sap of frozen roots, showed the changes for the various treatments to be small, varying from a low of 5.73 for the water treated sample to a high of 6.02 for the KHCO₃ treated sample.

DISCUSSION

Cation and anion equivalency is accomplished by absorption, and exchange of mineral ions, and by metabolic gains or losses of ions within the root. When absorption takes place from salts in which

cation and anion absorption rates are equal, no problem exists with respect to cation-anion balance.

If the rate of cation absorption exceeds that of anion absorption, the roots either lose cations of different species to the external solution or through synthesis make available anions within the roots. In roots of older plants, the exchange of cations may be of considerable magnitude as illustrated by Romaine lettuce, where as much as 84% of the K taken up from KHCO_3 exchanged for Ca plus Mg (table I). On the other hand, only a small amount of exchange occurred in barley seedling roots (table II). This difference in behavior may be related to the age of the plant or to the much greater initial calcium plus magnesium content of the more mature plant roots (21.2 to 76.2 meq per kg fresh weight) as compared to the barley seedling roots (9.8 meq). It appears that in the older plant material, the amounts of Ca plus Mg exchanging out are proportional to their initial concentrations. Presumably other cations could participate in this exchange reaction in a similar manner.

It has been pointed out by Jacobson, Overstreet, King and Handley (7) that the overall result of the postulated absorption reaction, $\text{HR} + \text{M}^+ = \text{MR} + \text{H}^+$, is the apparent exchange of a hydrogen ion from the root for a cation present in the external solution. However, this is not an exchange reaction in the same sense as that described above. If there were a simple exchange of pre-existing H^+ within the roots for external cations, the pH of the root sap should rise markedly during excess cation absorption in view of the weak buffering capacity of roots at the higher pH's (2). This does not occur. Evidently the hydrogen becomes available in ionic form only during the actual absorption reaction.

The other important mechanism for compensating excess cation absorption is the formation of organic acid anions within the cell. This is particularly marked in the case of the barley seedling roots. In this material, and probably in the roots of the more mature plants, malate is the most important acid radical participating in cation balancing. It is likely that the increases in the other organic acid radicals are caused by the accumulation of malate. An anomalous situation appears to exist for the samples reported in table I, since the increase in malate is greater than that of the total organic acid (particularly so in the case of Atlas barley) and for most samples, the increase in total organic acid is insufficient to compensate the excess of cations after deducting the calcium plus magnesium exchanged. It is possible that some anion other than an organic acid radical is involved. For example, Coil (5) has shown that bicarbonate in guayule leaves can function in cation balancing. In any event, this would not explain the disproportionate increase of malate over total organic acid. If these root samples contain "bound" acetic acid, either a disappearance or conversion to a free acid, during the course of K absorption from KHCO_3 solutions could account for both the apparent anion deficiency and the apparent excess malate increase over total organic acid increase. How-

ever, it must be pointed out that such is not the case in barley seedling roots where the "bound" acetic acid increases somewhat as a result of the KHCO_3 treatment.

For excess anion absorption to occur, excised roots must either produce cations within the root or lose pre-existing anions. The data of table II indicate that NH_4^+ increases during the CaBr_2 treatment. However, major compensation of the excess anion absorbed is afforded by the loss of anions in the roots. A small amount of inorganic anion is lost, most probably through direct exchange, although possibly by metabolic conversion to a non-ionic form. Much of the decline in initial root anions occurs in the malate content. It is conceivable that malate could exchange for the external anion, but the increase in R.Q. values obtained by Ulrich (17) during excess anion absorption under similar conditions suggest that malate is more likely being lost via metabolism.

The close parallelism between malate synthesis and excess cation absorption as well as the absence of any significant delay in its production strongly suggest that in barley seedling roots, malate is the first organic anion of appreciable stability to be formed during the absorption of excess cations. Burström (3) has suggested that the malate is accumulated as an intermediate of the acid cycle of respiration. He further suggests that an equilibrium exists between malic acid and malate whose value is dependent upon the prevailing pH. When excess cations are present, the dissociation shifts in favor of the malate and "an amount of malate equivalent to the cation excess is withdrawn from the respiration cycle, and is apparently excreted together with the cations into the vacuole."

In connection with this suggestion, it should be noted that malate could not accumulate appreciably as a result of the normal functioning of the tricarboxylic acid cycle since it is a member of the cycle. However, if any member of the tricarboxylic acid cycle (other than acetic acid) were produced by some reaction external to the cycle, it could then use a portion of the cycle as a pathway for the synthesis of the malic acid and thus lead to the accumulation of malate. Among other possibilities are the synthesis of malic acid by reductive carboxylation of pyruvate via malic enzyme and the deamination of aspartic acid.

As yet, there is no evidence indicating the significance of malic acid in the actual absorption mechanism. The synthesis of malic acid may be an additional mechanism which assumes an active role only when excess cation absorption occurs or it may be produced even when equivalent absorption of cation and anion occurs. In the latter case, it would necessarily be metabolized as rapidly as formed. That is, the formation of the malic acid could be a normal consequence of cation absorption and the disappearance a normal consequence of anion absorption. According to the views of Lundegårdh (10) and Burström (3), the accumulation of organic acid radicals is caused by the deficiency of anion absorption rather than by the absorption of excess cations.

By suitable treatment with dilute acid, the rather impressive amount of 0.8 to 0.95% acetic acid, on a dry weight basis, can be obtained from barley seedling roots. Acid treatment such as used in the Isherwood procedure for total organic acid extraction (i.e., pH 2 for 2 to 3 hours at room temperature) is not sufficient to liberate the acetic acid. On the other hand, treatment at pH 0.5 for 16 to 18 hours at temperatures somewhat above room temperature (Pucher, Wakeman and Vickery method) appears to completely liberate the "bound" acetic acid from its insoluble form.

At present, the role of the "bound" acetic acid in the ion absorption process or in metabolism in general is obscure. As yet, little can be said concerning the form in which the acetic acid is bound or even if it exists as acetic acid. The acid could arise as a decomposition product although the relatively mild treatment required to liberate it makes this unlikely. Preliminary analyses for organic phosphorus and organic sulfur indicate that these elements are not involved in the acetic acid binding since they are present in smaller molar quantities than the acetic acid.

CONCLUSIONS

During the absorption process, ionic balance is maintained in excised roots by absorption, by exchange for previously absorbed ions or by metabolic changes within the root, the latter being confined largely to the malic acid fraction. When excess cation absorption occurs, exchange for previously absorbed cations and the production of malate are the most important means of ion compensation. The exchange reaction is particularly significant in older roots, whereas the synthesis of malate predominates in barley seedling roots. When excess anion absorption prevails, much but not necessarily all of the compensation is due to a decrease in malate content.

The accumulation, and presumably the loss, of malate which may occur during the ion balancing process cannot arise via the normal functioning of the tricarboxylic acid cycle since malate is a member of the cycle. To account for the accumulation of malate, it is necessary to assume that either larger amounts of malate are synthesized only when excess cations are absorbed or that malate is utilized at a slower rate under conditions of decreased anion absorption.

Acid treatment of barley seedling roots results in the liberation of nearly 1% acetic acid on a dry weight basis. In untreated barley seedling roots, this acid constitutes about 35% of the total organic acid fraction as determined by the ether extraction procedure.

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