Nature of Zinc-Containing Substances in the Alfalfa Plant Cell

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Alfalfa plants grown in nutrient solution containing Zn^{65} as a tracer were blended with water or a buffer and centrifuged. The supernatant liquid containing the cytoplasm was used for studies concerning the nature of zinc and zinc-containing substances in alfalfa plant cells. Presence of free zinc ions was shown by H_2S precipitation and by removal of Zn^{65} by dialysis of the plant extract. Binding of zinc was observed in equilibrium dialysis experiments. Trichloroacetic acid precipitation of protein in a plant extract removed 25% of the Zn^{65}. Paper electrophoresis of buffered plant extract indicated zinc-protein binding. Apparently zinc is both free and bound, and an equilibrium exists such as: Zn^{+2} + protein $\rightleftarrows$ zinc-protein complex + $2H^+$. The equilibrium is pH sensitive. As the pH of a buffer decreases, there is greater removal of Zn^{65} by dialysis and also by precipitation with H_2S. Equilibrium dialysis also indicated less binding in more acidic media.

Results of a study of some characteristics of zinc and zinc-containing substances as they occur in the alfalfa plant cell are presented. The work was undertaken to acquire some of the data needed to help describe the role of zinc in plants. Principal procedures used include dialysis (equilibrium and nonequilibrium) and paper electrophoresis, with radioactive Zn as a tracer.

Dialysis data reported in the literature concerning the behavior of zinc in plant extracts are conflicting. Lewitt and Todd (*4*) observed a loss of zinc on dialysis of extracts from the potato tuber, while Wood and Sibly (*5*) reported that zinc could not be removed by dialysis of leaf extracts from tomato and oat plants. Day and Franklin (*1*) found that dialysis did not remove zinc from extracts of green leaves of the common elderberry bush.

Preliminary work in the authors' laboratories indicated that zinc could be removed from plant extracts of alfalfa by extended dialysis. To obtain additional information concerning these properties of zinc in plant tissue extracts, the following studies were undertaken.

Experimental Methods and Results

Growth of Plants. Alfalfa plants were started in the greenhouse and allowed to grow in soil for about 2 months. They were then transferred to an aerated nutrient solution prepared with the recommended amount of Hyponex, a commercial powdered plant food. The solution of Hyponex as used, contained 0.001*M* KNO_3, 0.001*M* $(NH_4)_2SO_4$, and 0.001*M* $Ca(H_2PO_4)_2$. An iron chelate (EDTA) was added to the solution as needed.

After alfalfa plants had grown about 10 days in the nutrient solution, Zn^{65} was added. In a typical experiment, 50 μc. of Zn^{65} ($ZnCl_2$ in 0.9*N* HCl, carrier-free) were added to each liter of nutrient solution. The alfalfa was harvested at approximately 30-day intervals. Total zinc content of the alfalfa as used in these experiments averaged 75 p.p.m. on a dry weight basis. The plants were washed with distilled water, frozen, and preserved at −20° C. until used.

Extraction Technique. Twenty-five grams of alfalfa (stems and leaves) were blended 10 minutes with 50 to 100 ml. of water or buffer with a Servall Omni-Mixer with the cup placed in an ice bath. The resultant slurry was filtered through muslin. The filtrate was centrifuged in the cold (below 10° C.) to separate the chloroplast fragments from the supernatant liquid, which served as the plant extract for further study.

Radiozinc Assay. For the assay of radioactive zinc-65, a well-type, gamma-ray scintillation spectrometer was used. The radioactivity of the samples, either in solution or solid, was counted by placing them in 20-ml. lusteroid test tubes, which were inserted into the well of the scintillation detector.

Free Zinc Ions. Two experimental techniques were used to show the presence of free zinc ions—dialysis and hydrogen sulfide precipitation.

Dialysis. Plant extracts containing radiozinc were prepared in the manner described above, except that the chloroplasts were not removed by centrifugation before dialysis. For dialysis, extracts were placed in cellulose dialysis tubing, with an inflated diameter of $^3/_4$ inch. The dialysis bags were placed in bottles of distilled water and allowed to dialyze in a refrigerated room (below 10° C.) for 10 days with daily changes of water. The dialyzed material was centrifuged to separate it into two fractions. Both fractions and the dialyzate were assayed for radiozinc.

In five trials, an average of 86.8% of the zinc was removed by dialysis, while 6.5% remained in the supernatant liquid and 6.7% in the residue consisting of chloroplast fragments and other insoluble material.

Buffered Dialysis. Extracts containing the radiozinc were prepared by blending alfalfa with a series of phosphate buffers of various pH values, followed by centrifuging to remove the chloroplasts. The phosphate buffers were prepared using Na_2HPO_4 and H_3PO_4. In no case did the total concentration of phosphate exceed 0.1*M*. The supernatant liquid was dialyzed against a buffer of the same pH as the extract. The results of the radiozinc assay of the dialyzate and the dialyzed material are presented in Table I.

Removal of a large percentage of the zinc by dialysis was observed; more zinc was removed from extracts in buffers at

Table I. Results of Dialysis of a Buffered Extract against Buffer

	pH of Buffer					
	4	5	6	7	8	8.5
pH at end of dialysis	3.9	5.0	6.0	7.2	8.0	8.5
Dialyzed material	0.1[a]	0.7	1.3	3.1	7.8	14.7
Dialyzate	99.9	99.3	98.7	96.9	92.2	85.3

[a] Data are in percentages of the total Zn^{65} activity counted.

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Table II. Results of H_2S Precipitation of Zn^{65} in Plant Extract

All data are in percentanges of the radiozinc in the original and are the averages of five trials

	After Adding H_2S Only	After Adding $ZnSO_4$ and H_2S
In precipitate	82.0	90.6
In supernatant liquid	3.3	2.4
Loss	14.7	7.0

Table III. H_2S Precipitation of Plant Extract and Zn^{65} Solution Buffered to Different pH Values

All data are in percentages of total Zn^{65} activity counted

	0.1M HCl	pH of Buffered Extract or Solution 3	5	7	9	11
Extract plus H_2S only						
In residue	2.1	72.0	57.6	64.1	32.1	18.0
In supernatant liquid	97.9	28.0	42.4	35.9	67.9	82.0
Extract plus $ZnSO_4$ and H_2S						
In residue	4.6	100.0	99.6	99.8	98.3	93.3
In supernatant liquid	95.4	0.0	0.4	0.2	1.7	6.7
Zn^{65} solution plus H_2S only						
In residue	...	51.7	66.9	96.9	95.8	96.3
In supernatant liquid	...	48.3	33.1	3.1	4.2	3.7
Zn^{65} solution, $ZnSO_4$ and H_2S						
In residue	...	94.3	100.0	99.4	100.0	100.0
In supernatant liquid	...	5.7	0.0	0.6	0.0	0.0

Table IV. Results of Equilibrium Dialysis Experiments

Trial	Temp., ° C.	Time, Hours	Zn^{65} Activity[a] Percentages at Equilibrium after Dialysis B	O	I	R
1	30	130	0.98	10.2	89.8	8.8
	0	130	0.94	16.2	83.8	5.2
2	30	105	0.99	19.2	80.8	4.2
	0	105	0.99	37.7	62.3	1.7
3	32	126	0.98	31.0	69.0	2.2
	5	160	0.99	37.0	63.0	1.7
4	32	85	0.98	18.8	81.2	4.3
5	32	55	0.99	35.3	64.7	1.8

[a] B = degree by which the control moved toward equilibrium is the ratio of I to O for the control tube; O = radiozinc activity of the solution outside the dialysis bag at equilibrium, in percentage of the zinc-65 activity on O and I; I = Radiozinc activity of the solution inside the dialysis bag at equilibrium, in percentage of the zinc-65 activity in O and I; and R = ratio of I to O, indicating the degree of binding.

pH 6 and below than in buffers of higher pH. The ion product of hydroxyl, zinc, and phosphate, except the value at pH 8, never exceeded the solubility product of zinc phosphate published by Jurinak (*3*). Also, the work was paralleled using acetate buffers, and the same results were obtained as with phosphate buffers.

Dialysis of filtrates prepared by extraction with 0.1*N* HCl removed all of the zinc radioactivity. Apparently acidic conditions favor breakdown of a zinc complex in the plant extract.

Hydrogen Sulfide Precipitation. To analyze plant extracts further for the presence of free zinc ions, several hydrogen sulfide precipitation experiments were undertaken. Two 5-ml. portions of plant extract were used. To one portion, 1 ml. of 0.1*M* $ZnSO_4$ solution was added as a carrier. Hydrogen sulfide was bubbled through each tube for 10 minutes. After being centrifuged, both the residue and the supernatant liquid were counted for radiozinc. The results, presented in Table II, indicate that hydrogen sulfide precipitated nearly all of the radioactive zinc from the extract. Some loss of Zn^{65} occurred. Since total activity is very low, losses may be attributed to manipulative processes, counting efficiency, and adsorption on walls of vessels used.

The effect of pH on the hydrogen sulfide precipitation also was studied. Hydrogen sulfide was bubbled through extracts prepared in the usual manner but with a series of buffers of different pH values as extractants. To one portion, 0.1*M* $ZnSO_4$ again was added as a carrier. These results, presented in Table III, reveal the tendency of less zinc precipitation as pH values increase, even in the presence of $ZnSO_4$ carrier.

A Zn^{65} solution as a control, buffered at the same pH values, also was treated with H_2S as above, centrifuged, and the level of radioactivity determined. Complete precipitation of the zinc from the control solution (containing the $ZnSO_4$ carrier) occurred at the higher pH values (pH 7 to 11). In the tubes of Zn^{65} only (no $ZnSO_4$ carrier), 3.0 to 4.2% of the radiozinc remained in the supernatant liquid. This contrasts with the results from H_2S precipitation from plant extract presented in Table III.

The results of the sulfide precipitation of the radiozinc agree with those obtained from the dialysis data and substantiate the presence of free zinc ions, or an equilibrium of zinc ions with a loosely bound complex in the extract.

Bound Zinc. From the results of dialysis and hydrogen sulfide precipitation experiments on the effect of pH (Tables I, II, and III), a tendency for binding of zinc was apparent at higher pH values. To study further this property of zinc (II) ions in plant extracts, a number of equilibrium dialysis experiments were performed.

For the equilibrium dialysis studies, the technique described by Hughes and Klotz (*2*) was used. The plant extract was prepared by blending fresh alfalfa (containing no radiozinc) with water and filtering through muslin. The extract was concentrated by circulating air with a fan and then centrifuged at 1000 × G to remove the broken cell fragments and chloroplasts. Concentration time averaged 1 hour. Extract temperature was always below 20° C. The extract was then dialyzed 75 to 100 hours at 0° C. against moving water.

The predialyzed plant extract (containing no radioactive zinc-65) was centrifuged and subjected to equilibrium dialysis by placing 10 ml. of the prepared extract into a previously prepared dialysis bag, which was inserted into a test tube to which a 10-ml. solution containing Zn^{65} was added outside the bag. Dialysis was then allowed to proceed until equilibrium was reached. The temperature of one group of tubes was maintained at 30° or 32° C. by a constant temperature water bath and another group at 0° or 5° C. in a water bath in a refrigerated room. After dialysis, the solutions were analyzed for Zn^{65}. No microbial growth was observed in these solutions. Typical results are presented in Table IV.

Equilibrium dialysis experiments consistently showed an increased concentration of the radiozinc inside the dialysis bags (which contained the plant extract) over that in the solution outside the bag. Since the R values in Table IV are greater than one, zinc binding was indicated.

In a second series of equilibrium dialysis experiments, the concentration of the plant extract inside the dialysis bag was varied. The total volume was made up to 10 ml. by adding a solution of a supporting electrolyte (0.15*M* sodium acetate). The same amount (10 ml.) of Zn^{65} solution was placed outside the bag for each tube. The temperature was maintained at 5° C.; other conditions were kept constant. Typical results are presented in Figure

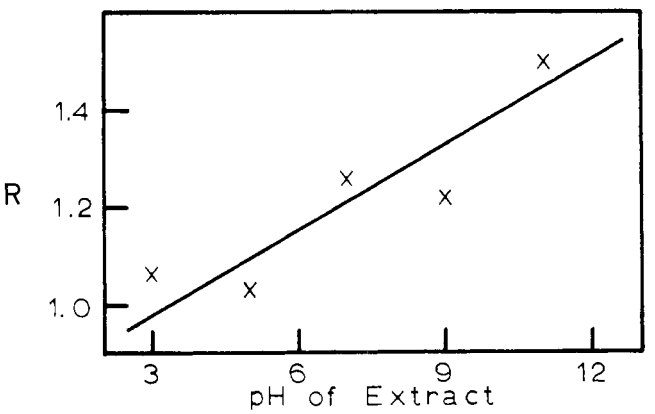

Figure 1. Degree of binding of zinc (*R*) as affected by volume of plant extract added to the solution inside the dialysis bag

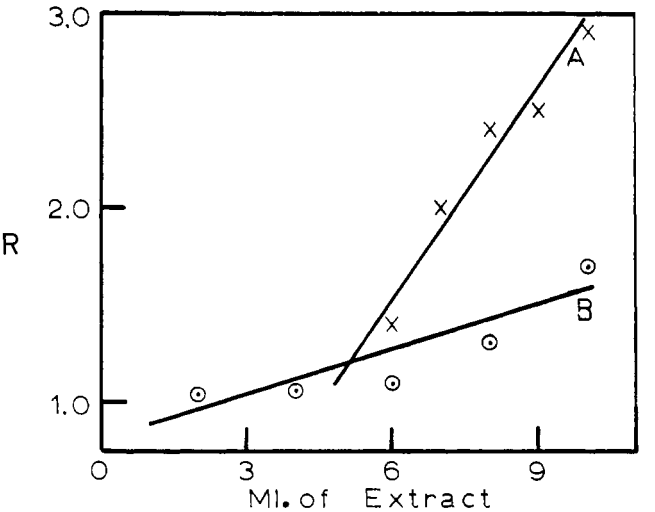

Figure 2. Degree of binding of zinc (*R*) related to pH of the plant extract from results of equilibrium dialysis

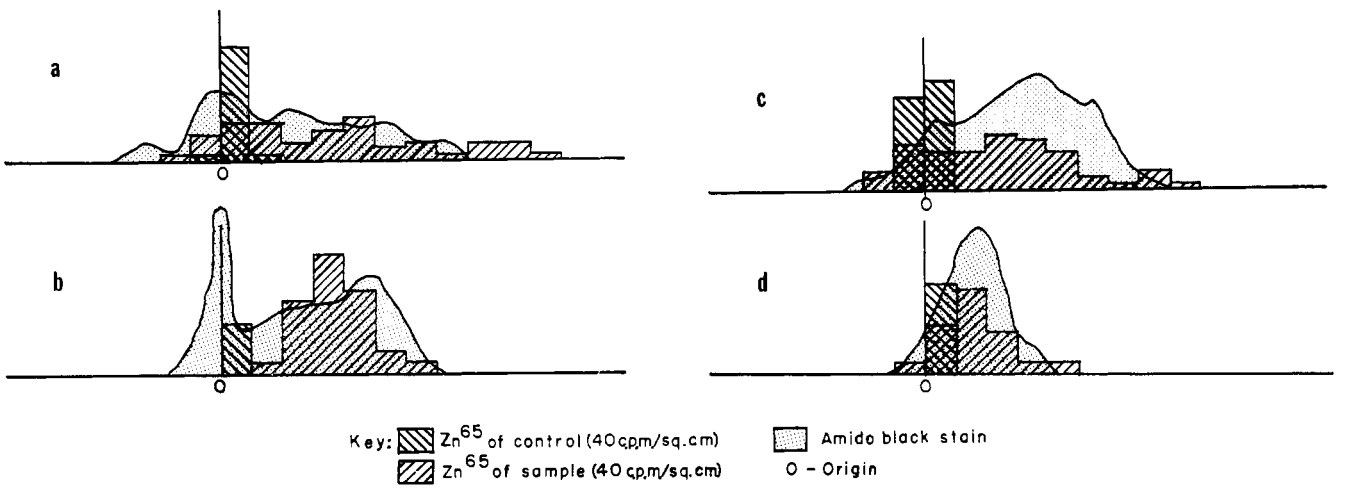

Figure 3. Graphs of paper electrophoresis strips of plant extract showing association of the radiozinc with the fraction stained with amido black

(a) at pH 8, barbiturate buffer as electrolyte; (b) at pH 9, barbiturate buffer as electrolyte; (c) at pH 10, phosphate buffer as electrolyte; (d) at pH 11, phosphate buffer as electrolyte

1 for the equilibrium dialysis of two different extracts (A and B). The *R* values, determined in the same manner as above, were plotted against the volume of extract inside the dialysis bag. The data indicate the extent of binding in relation to the amount of plant extract.

The effect of extract pH on the extent of binding was studied in a third series of equilibrium dialysis experiments. Plant extracts were prepared by blending alfalfa with a series of phosphate buffers of various pH values and treating it as previously described, except that it is not subjected to predialysis. The extract was allowed to dialyze (for 116 hours at 30° C.) against a Zn^{65} solution until equilibrium was reached. A residue separated during dialysis which was removed by centrifugation, and the level of radioactivity of the liquid fractions inside and outside the dialysis bag were determined. From these results, the *R* values were plotted against the pH of the extract (Figure 2). An increased binding of zinc with increased pH is indicated.

Nature of Zinc Binding. Investigations were undertaken to ascertain the constituent to which the zinc was bound that employed trichloroacetic acid precipitation and paper electrophoresis.

Trichloroacetic Acid Precipitation. To precipitate the protein, trichloroacetic acid was added to 10 ml. of plant extract obtained by pressing alfalfa in a hydraulic press. The mixture was centrifuged and the fractions assayed for radiozinc. The precipitate contained 24.9% of the original activity. This agrees with Lewitt and Todd's (*4*) conclusion that 25% of the zinc in potato is protein-bound.

Paper Electrophoresis. For the paper electrophoresis studies, 25 μl. of plant extract, prepared by the method described previously, were placed at the midpoint of a paper strip. A 1- × 11-inch strip of Whatman No. 1 filter paper was used. Six such strips were placed into an electrophoresis apparatus at one time, with the ends dipping into buffer solutions which served as electrolytes. Usually 12-ma. current from a power supply was applied for 4 to 12 hours. Thus 2 ma. were applied to each strip.

To detect the protein on the paper strips after electrophoresis, an amido black stain was used. The paper strips were first heated in an oven to 107° to 110° C. for 15 minutes to harden the proteins. They were then immersed for 5 minutes in the amido black stain made up by saturating amido black solvent with Amido Black 10B. The amido black solvent was a 50:50:10 mixture of ethanol, water, and glacial acetic acid. The paper strips were then washed in the solvent until the paper was almost white; a dark blue protein stain remained. A

densitometer (Photovolt Corp., N. Y., Model 52-C) was used to determine the concentration of the amido black on the electrophoresis strips.

The location of the zinc-65 on the paper strips was detected by cutting the paper into 1-cm. segments, placing them into plastic test tubes, and counting the radioactivity of each segment by means of the scintillation detector.

Typical results, presented in Figure 3, show that the zinc in the plant extract subjected to electrophoresis migrated with the amido black stain of the protein fraction. The free zinc in the control strips did not migrate appreciably. A definite association of the radiozinc with some constituent in the plant extracts was observed. Since zinc-65 correlated with the amido black stain for proteins, a zinc-protein binding was evident.

The results of this investigation led to the conclusion that zinc in plant extract is both free and bound, and that zinc is associated with a protein. The following equilibrium may help to explain the experimental results:

$$Zn^{+2} + \text{protein} \rightleftarrows \text{zinc-protein} + 2H^{+}$$

If this equilibrium exists, an increase in the hydrogen ion concentration would dissociate the zinc-protein complex. This was observed in the buffered dialysis experiments (Table I) and in the hydrogen sulfide precipitation (Table III), in which the removal of zinc increased as pH of the extract was lowered. Acidic conditions caused the complex to dissociate more readily, shifting the above equilibrium to the left to form free zinc(II) ions. Equilibrium dialysis experiments (Figure 2) also presented evidence of greater zinc binding at higher pH values. Evidently the zinc-protein complex is more stable at higher pH.

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TOMATO COMPOSITION

Varietal and Location Influence on Acid Composition of Tomato Fruit

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In tomato fruits from farms located in New Jersey, Pennsylvania, and Ontario, Canada, acidity levels and acid composition varied among varieties and locations. Results from replicated trials indicated major differences between varieties in percentage concentration of citric acid and quantity of acid anion in a titratable form or as a neutral salt. A high acid variety contained a higher percentage of potassium than a low acid variety. No differences were found in the root cation exchange capacities of varieties with low, medium, and high titratable acidity levels. Rootstocks of tomato plants had no significant influence on acidity of fruit from the scion.

THE PURPOSE of this investigation was to determine the acid composition of tomato fruit from varieties having low, medium, and high titratable acidities; the cation content of the vines and fruit; and the influence of rootstock and root cation exchange capacities on acid metabolism.

Reynard (*10*) reported that the titratable acidity of fruit from four tomato varieties maintained the same relative rank when grown at Chicago, Ill.; Davis, Calif.; Riverton, N.J.; and New Toronto, Ontario, Canada. However, levels of titratable acidity varied considerably between locations.

Anderson and Thompson (*1*) found threefold differences in acid content between a number of varieties and strains. Citric acid was reported by Manunta and Lafon (*7*) to be the principal acid in tomatoes. A high correlation was found between total acidity and citric acid content, but no correlation was noted between total acidity and either malic or succinic acid. Manunta and Lafon (*8*) found that F_1 hybrids of crosses between high and low acid varieties had citric acid levels intermediate between the parents but with a tendency toward the low acid parent.

Several investigators (*5, 6, 9*) have observed that potassium fertilization increases tomato acidity. Using soils with exchange capacities up to 13 meq. per 100 grams, Bradley (*3*) found that K fertilizers increased titratable acidity, total acidity, citric acid, and K content of vines and fruit. Potassium comprised up to 85% of the total cations associated with the acid anions as salts in tomato puree.

Experimental

Tomato Acidity as Related to Variety and Location. Seventeen nonreplicated tomato fruit samples, each weighing approximately 25 pounds, were collected in August at a uniform stage of ripeness from fields in New Jersey, Pennsylvania, and eastern Canada. Fruit samples represented varieties Ace, Improved Garden State (IGS), Rutgers, John Baer, and strain Kc54. The samples were pureed and canned, and the acid composition was determined by ion exchange and silica gel partition chromatography (*2*). Inorganic anions eluted from the silica gel partition column were titrated and reported as a group. Titratable and total acidity were determined by titrating a 10-ml. aliquot of filtered puree before and after resin treatment, respectively, with NaOH to a phenol red end point. Results are reported as milliequivalents per liter.

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