CHELATION OF TRACE METALS IN NUTRIENT SOLUTIONS[1]

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(WITH ONE FIGURE)

Received June 5, 1950

Plant nutrition studies frequently require nearly complete elimination of trace metals from the nutrient media in which the plants are to be grown. Conventional methods of purification by recrystallization and precipitation have been used for this purpose in some studies. These have proved more difficult and less effective than the adsorption technique developed by STEINBERG (**11**) and improved by ARNON and STOUT (**4**), in which heavy metals are coprecipitated with calcium phosphate. In recent years attention has been called to the reaction of heavy metals with certain organic molecules to form chelate complexes which can be removed from nutrient solutions by extraction (**7, 8, 12**). This means of eliminating trace metals from nutrient solutions suggested the possibility that such chelating agents might be used in nutrient solutions in direct contact with the roots of growing plants to provide a continuing control of trace metal contamination in the nutrient media. The present study was undertaken to determine the effect of chelating agents added to nutrient solutions on plants growing in these solutions. Tests reported describe the responses of seedlings of several species and of older tomato plants to six such chelating compounds present in nutrient solutions.

A chelating agent (from the Greek Chele, meaning claw) usually is a complex organic molecule containing two electron-donating groups which, acting similar to the two jaws of a claw, may remove simple metal ions from solution. These chelating reactions are slightly reversible, the degree of reversibility, characterized as the "stability constant," depending on the agent used and the metal ion involved. The mechanism of chelation is illustrated with the following example. Oxine (fig. 1, A), 8-hydroxyquinoline ($HOC_6H_3N:CHCH:CH$), is a double ring compound formed by the fusion (in the ortho position) of a pyridine ring to a benzene ring with the hydroxyl group on the eighth position of the double ring compound. Oxine ionizes to form oxinate and hydrogen ions, the hydrogen coming from the hydroxyl group. The chelating effect of oxine results when the oxygen and nitrogen, each acting as a claw, join to a metal ion (Zn^{++}) thus forming a ring, called a chelate ring. The oxygen, with a free negative valence, forms an electrovalent linkage with the zinc ion while nitrogen forms a co-ordinate covalent bond. Thus the zinc ion is bound securely to the oxine molecule. However, the zinc ion has two free valences and for this reason two molecules of the oxine are required to

[1] Contribution from the Botanical Laboratory, The University of Tennessee, New Ser. no. 119.

bind one zinc ion. The complete structural linkage is diagrammed in figure 1, B. Generally speaking, the above type of complex is formed with any union of heavy metal and chelating agent. Trivalent metals require three molecules of the chelating agent to bind them but the general mechanism is the same. The strength of this association is dependent on several factors (**1, 2**). Although no definite specificity for any one metal can be ascribed to a particular chelate compound, these compounds do form more stable complexes with some metals than with others.

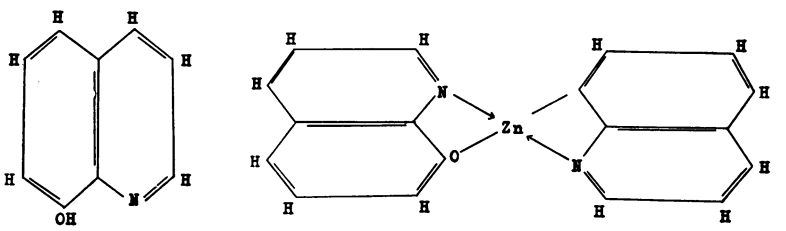

FIG. 1. A, oxine molecule (8-hydroxyquinoline). B, chelate complex of oxine and zinc.

Chelation has been used to some extent as a means of eliminating trace metals from culture solutions. PIPER (**8**) supplemented calcium carbonate coprecipitation of heavy metals from stock solutions with dithizone and chloroform extractions. WARING and WERKMAN (**12**) removed iron from bacterial nutrient solutions by extraction with oxine and chloroform. HEWITT and JONES (**7**) effectively removed traces of molybdenum from stock nutrient solutions by adsorption on a chelate complex. In their procedure oxine was chelated with iron and the molybdenum was removed by coprecipitation with the oxine-iron complex. Several chelating agents used in chlorella cultures have been shown to inhibit photosynthesis and respiration, the inhibitions being reversible upon removal of the chelating agents (**6**). ZENTMYER (**13**) found oxine to be an effective fungistatic agent, but the inhibition of fungal growth could be prevented by adding zinc in amounts sufficient to overcome the chelating action of oxine.

General methods

ALBERT and GLEDSHILL (**1**) investigated 30 chelating agents showing their effects on certain metallic ions at temperatures from 20 to 30° C in the pH range 6.5 to 7.3. Each solution tested was composed of .0001 M metal ions, .05 M borate buffer (pH 7.3) and .001 M chelating agent. Since two molecules of chelating agent combine with one ion of divalent metal the molar concentration of the chelating agent in these solutions was actually five-fold in excess over the divalent metals.

Chemicals used in this study and their reactions with certain metal ions, as determined by Albert and Gledshill, are listed in table I. Salicylic

acid was not characterized by these investigators. Concentration of chelating agents for all tests was based on total metal impurities, estimated at not more than five parts per million or .000078 M metal, computed as copper. Chelating agents, .00078 M, were then supplied to give a five-fold excess in the solutions. This molarity of chelating agents is referred to in all tests as "unit" concentration and all higher molarities used were integral multiples of this unit concentration.

Glass containers used throughout this study were thoroughly washed, then rinsed with tap water, distilled water, concentrated hydrochloric acid, distilled water, and finally redistilled water.

TABLE I

CHELATING AGENTS USED IN THIS STUDY AND THEIR CHELATING REACTION WITH FIVE METAL IONS. AFTER ALBERT AND GLEDSHILL (1)

CHELATING AGENT	MOLECULAR WEIGHT	Mn*	Zn**	Fe**	Fe†	Cu**
Oxine (8-Hydroxyquinoline)	145	**	**	*	*	**
Carbamate (Sodium diethyl-dithiocarbamate)	171	*	†	*	**	†
Chromatropic salt (Sodium 1: 8 dihydroxy-naphthalene 3:6 disul-phonate)	364	†				†
Nitroso-R-salt (Sodium 1-nitroso-2-naphthol 3: 6 disul-phonate)	377			†	**	**
Quinalizarin (Tetrahydroxyanthra-quinone)	272	**	*	*	**	*
Salicylic acid (Hydroxy-benzoic acid)††	138					

* Showed trace chelation with .0001 M metal using .001 M chelating agent
** Showed marked chelation with .0001 M metal using .001 M chelating agent
† Showed trace chelation with .00001 M metal using .001 M chelating agent
†† Not characterized by Albert and Gledshill.

Germination and seedling studies

Germination and seedling tests were designed to study the effects of different chelating agents on root growth in several species of plants. Seeds of tomato, pea, bean, corn, and radish were used in the germination tests. Eight seeds of each species were placed in each of six different solutions: (1) distilled water, (2) carbamate, (3) chromatropic salt, (4) oxine, (5) nitroso-R-salt, (6) quinalizarin. Germination in all solutions, except oxine, was good. Tomato and radish seeds did not germinate in the oxine while the other three species showed no growth after the initial rupture of the seeds.

Seedling studies were made with tomato and corn using nine test solutions (table II). From 200 to 300 seeds of each species were germinated

TABLE II

EFFECT OF CHELATING AGENTS ON GROWTH IN LENGTH OF SEEDLING ROOTS, MEASURED IN CENTIMETERS

PLANTS	REFERENCE SOLUTION*	CARBAMATE 2-FOLD	CARBAMATE 4-FOLD	CARBAMATE 8-FOLD	CHROMATROPIC SALT 2-FOLD
Tomato	4.26	4.21	0.24	0.00	4.27
Corn	16.01	15.12	10.35	4.85	12.18

PLANTS	OXINE 2-FOLD	NITROSO-R-SALT 2-FOLD	QUINALIZARIN 2-FOLD	SALICYLIC ACID 2-FOLD
Tomato	0.00	4.23	4.21	2.99
Corn	2.54	14.68	17.13	14.20

* The reference solution served as a standard of comparison for all other test solutions which contained the chelating agents as well as the basic nutrients.

in Petri dishes using distilled water. Eight corn and 15 tomato seedlings, selected for uniform growth, were transferred to large culture dishes containing the test solutions. After three days for corn, and seven days for tomato, the length of the primary roots was determined as a measure of the effect of the chelating agent on growth. The results (table II) were in agreement with those obtained with germinating seeds and showed that oxine was injurious to root growth of both species, the roots turning brown in less than twelve hours. Both species showed root growth to be inversely related to the carbamate concentration; however, the roots were not discolored and remained turgid in the two lower carbamate concentrations and only half of the roots in the eight-fold concentration were dead. Roots in chromatropic salt, nitroso-R-salt, quinalizarin, and salicylic acid showed growth comparable to that in the reference solution, as did the carbamate at two-fold concentration.

Water culture studies

Tomato, variety Marglobe, was the major test plant in these studies. Plants were germinated in white sand and transferred to 125 ml. flasks containing basic nutrients plus boron, manganese, zinc, and copper. Roots were washed with distilled water and rinsed with double distilled water before being placed in test solutions. Early tests were made in 500 ml. flasks without aeration, while quart Mason jars, covered with brown paper and aerated continuously, were used in later tests. Solutions were renewed every three weeks, and distilled water was added regularly to avoid any major change in the concentration of chelating agents. Stock solu-

tions of each chelating agent, with the basic nutrient salts (**3**) and 0.286% H_3BO_3, were stored in two-liter Pyrex flasks. A stock iron solution of 0.5% $FeCl_3$ and 0.5% tartaric acid and a stock supplementary solution, containing 0.181% $MnCl_2 \cdot 4H_2O$, 0.0222% $ZnCO_4 \cdot 7H_2O$, and 0.0079% $CuSO_4 \cdot 5H_2O$, were prepared so that one ml. of each could be added to a liter of test solution, where desired, to make a complete nutrient solution.

Since chelating agents were used in the nutrient solutions, heavy metals, such as iron, manganese, zinc, and copper, had to be supplied to the plants by a method other than direct addition to the nutrient solution. Spraying with a 0.3% iron solution resulted in poor chlorophyll distribution, the leaves having mottled appearance. This condition was not improved materially by the addition of a little Vel to the spray solution. Since a uniform green color is essential for recognition of various other deficiencies, the spray technique was discarded in favor of the injection method. This procedure, developed by ROACH (**9**) and ROBERTS (**10**) and modified by FELBER (**5**), permitted injection for a period of time. Eight strands of cotton darning thread were soaked in the injection solution and threaded through the stem. One end of the thread dipped into a reservoir of this solution in a small vial tied to the stem. Two injection solutions were used: (1) 0.1% iron, as ferrous ammonium sulphate, acidified with HCl to pH 1.5; (2) 0.1% iron and the supplementary solution, containing manganese, zinc, and copper, at one fifth the concentration of the nutrient stock solution or 200 times the concentration actually used in the nutrient solution. In most cases a single injection of one ml. was sufficient for six to eight weeks' growth. Best results were obtained by injecting immediately after threading under conditions favorable for high transpiration. With these conditions one ml. could be injected and distributed throughout the plant within four hours. Slow injection for eight to 10 hours resulted in considerable injury at the point of injection of the stem. If more than one ml. was injected, toxicity was evident in some leaves and along the stem directly above the point of injection.

Chelating agents used in the water culture tests were the same as in previous studies with seeds and seedlings (table III). However, in these tests with older plants a number of concentrations of some of the agents were used. Oxine, which was used successfully in bacterial studies by WARING and WERKMAN (**12**), was included in these studies, even though seeds and seedlings failed to survive in oxine solutions. Carbamate was studied more extensively than any of the other chelating agents because of the number of metals chelated and the tenacity with which some are held.

Tomato, bean, corn, and Coleus were used in the oxine studies. No growth was evident in any of these plants (table III). The roots died quickly and no adventitious roots formed. The lower stems of all plants, except corn, became constricted to a thin black band. This constriction gradually extended up the stems until, in some cases, even the lower petioles showed constriction. Tops remained turgid throughout the tests

TABLE III

GROWTH AND DEFICIENCY RESPONSES OF PLANTS GROWN IN NUTRIENT SOLUTIONS WITH VARIOUS CHELATING AGENTS ADDED

TEST SOLUTIONS	NO TRACE METALS ADDED	FE, MN, ZN, CU ADDED TO TEST SOLUTION	FE, MN, ZN, CU INJECTED INTO PLANTS	FE INJECTED INTO PLANTS
Reference solution	Fe deficiency	Good growth*	Good growth	Mn deficiency
Oxine	No growth	No growth		
Oxine, two-fold	No growth	No growth		
Carbamate	Fe deficiency	Fe deficiency		
Carbamate, two-fold	Fe deficiency	Fe deficiency	Good growth	Mn deficiency
Carbamate, four-fold	Little growth	Little growth	Little growth	Little growth
Carbamate, eight-fold	Little growth			
Carbamate, twelve-fold	No growth			
Carbamate, sixteen-fold	No growth			
Chromatropic salt	Fe deficiency	Good growth		
Chromatropic salt, two-fold	Fe deficiency	Good growth	Good growth	Fair growth
Chromatropic salt, three-fold	Little growth			
Chromatropic salt, four-fold	Little growth			
Nitroso-R-salt	Fe deficiency	Fe deficiency		
Nitroso-R-salt, two-fold			Fair growth	Little growth
Quinalizarin	Fe deficiency	Fe deficiency		
Quinalizarin, two-fold			Good growth	Mn deficiency
Salicylic acid, two-fold	Fe deficiency	Good growth	Fair growth	Fair growth

* Plants grown in complete nutrient solution without chelating agents were regarded as growth standards.

but showed no growth in six weeks. The leaves showed marginal necrosis which gradually included the whole leaf.

The other five chelating agents (table III) showed no deleterious effects on growth of tomato plants up to two-fold concentration of the agents. Iron deficiencies were characteristic in those tests which received no trace metal additions either through the test solution or by injection. When iron and supplementary solutions were added to the test solution an iron deficiency was still produced in all cases except with chromatropic salt and salicylic acid, which did not chelate iron effectively. Injection of iron and supplementary solutions corrected iron deficiencies in all cases. When the iron deficiency was corrected by injection of iron alone a manganese deficiency appeared in carbamate and quinalizarin tests within four weeks, and reduced growth was noted in the plants in chromatropic salt. Since the manganese deficiency was also apparent in the reference solution one ml. of the supplementary solution was added to those solutions containing manganese-deficient plants. Within four days partial recovery was noted in all cases, regardless of the presence of chelating agents. Within a week all plants in these tests had recovered completely.

Increasing the concentration of chromatropic salt and carbamate in the nutrient solutions (table III) failed to show injury effects which could be identified as specific nutrient disorders. Roots died, lower parts of the stems became constricted, eventually collapsing, and the leaves showed marginal necrotic areas. These injury effects were quite similar to those evident in oxine tests. The rate of injury, at concentrations over two-fold, was directly related to the molarity of the chelating agent up to concentrations of four-fold chromatropic salt and 16-fold carbamate.

Studies were made to determine the nature of the injury caused by oxine and high concentrations of carbamate. The root system of a tomato plant was split lengthwise, half being placed in an oxine solution and the other half in a complete nutrient solution. The roots in oxine died while a good root system was developed in the complete nutrient solution. The plant itself showed good growth in the six weeks it was under test. These results indicate that oxine effects are due to root injury and oxine is not translocated to upper parts of the plant.

ZENTMYER (**13**) obtained good fungus growth in oxine after adding sufficient zinc to complex all the oxine. This was tried in these studies using iron to complex oxine and zinc to complex the 16-fold carbamate. The plants in oxine showed good growth while the plants in 16-fold carbamate showed fair growth, thus agreeing with Zentmyer's results and indicating that the root injury from oxine and carbamate was caused by the chelating action of these two agents.

Discussion

The use of chelation for removal of trace metals in nutrient studies with higher plants has not been explored extensively. Extractions with dithi-

zone and oxine have been used in a few instances as a means of removing heavy metals from stock solutions (**7, 8, 12**). The present study was undertaken to determine if plants could be grown directly in solutions containing chelating agents, thus providing continuing control of heavy metal contamination.

The results of this study indicate that the effectiveness of chelation determines whether plants can grow in solutions containing chelating agents. At low concentrations of all the agents used, other than oxine, chelation was ineffective except for iron in some cases. When iron was added by injection to plants growing in solutions where iron was effectively chelated, plant growth was good. Chelation is a reversible process, the degree of dissociation (stability constant) depending on the agent used and the metal ion involved. Since the unit molarity of chelating agent gave a five-fold excess over the divalent metal ions, these agents, other than oxine, must dissociate appreciably. Consequently, sufficient metal was left in solution in these lower concentrations of chelating agent to provide for good plant growth, except for iron in some cases.

Oxine at two-fold concentration, and carbamate and chromatropic salt at higher concentrations, produce general and severe plant injury. ZENTMYER (**13**) attributed the fungistatic mechanism of oxine to its ability to remove zinc from the culture medium. Quite likely root injury involves this mechanism also. Apparently these chelating agents complex the metals so effectively that their concentration in the solution is infinitely small, resulting in outward diffusion of the metals from the roots into the solution and consequent death of the root cells. Entrance of the chelating agents into the root cells is not likely since the tomato plant with the divided root system thrived even though half of the roots were immersed in the oxine solution.

Results of this study indicate that chelation of heavy metal ions in the nutrient solution of growing plants is not feasible. There seems to be a fairly critical point below which chelation is not effective and above which chelation is effective and plants are injured. This point will vary according to the stability constant for a given reagent and the concentration of metal, as impurities, in any one solution used. In fact concentrations of agents in the solutions vary greatly as water is lost from and replaced to the solutions. Presumably all chelating agents would react similarly, since the chelating mechanism is the same in all cases.

Removal of heavy metal ions in nutrient solutions by extraction with chelating agents (**7, 12**) appears to be a more promising method of purification. Oxine at low concentrations and carbamate at high concentrations should give very good results. Since calcium and magnesium are not chelated at pH 6.5 to 7.3 (**1**), the four stock solutions composing the basic nutrient solution (**3**) could be treated separately with either oxine or carbamate and the chelate-metal complexes then could be removed by extraction with chloroform. Additional studies are planned to test these and

other suggestions concerning the use of procedures involving extraction of chelate-metal complexes as a means of removing trace metals from nutrient solutions.

Summary

1. The effect of chelating agents added to nutrient solutions on plants growing in these solutions was investigated. Chelating chemicals used were: 8-hydroxyquinoline (oxine), sodium diethyldithiocarbamate (carbamate), sodium 1:8 dihydroxynaphthalene 3:6 disulphonate (chromatropic salt), sodium 1-nitroso-2-naphthol 3:6 disulphonate (nitroso-R-salt), tetrahydroxyanthraquinone (quinalizarin), and hydroxy-benzoic acid (salicylic acid).

2. Germination and seedling studies with tomato, pea, bean, corn, and radish, and water culture tests with tomatoes, using various concentrations of the chelating agents, are described, and plant responses to these reagents examined.

3. Iron was the only metal ion effectively removed from solution at low concentrations of oxine, carbamate, nitroso-R-salt, and quinalizarin. Iron was not effectively chelated by either chromatropic salt or salicylic acid.

4. Concentrations of chelating agents in which plants would grow did not chelate metal ions effectively, while concentrations of chelating agents which chelated effectively produced severe plant injury. This injury resulted from heavy metals being extracted from the root cells.

5. Since effective chelation in the nutrient solution results in severe plant injury, the use of such agents in nutrient solutions in direct contact with the roots of growing plants is not a feasible method of controlling trace metal contamination in such solutions.

The authors are pleased to acknowledge technical assistance of Dr. G. K. Schweitzer on chelation aspects of this study.

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