INVESTIGATION OF ZN-BOUND PROTEINS IN ALFALFA USING Zn^{65}

by

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TABLE OF CONTENTS

INTRODUCTION

As early as 1914, the requirement of zinc as a trace element for plant growth in both vegetative and forage crops was noticed (29). It is known that the lack of zinc in the soil produces such outward effects in the tomato plant as chlorosis, rosettes of small leaves, stunted growth and severe necrosis (22). Other scientists have found that the detrimental effects upon plant growth to be universal throughout the many different plants that have been studied (34, 6, 7, 31, 30, 23).

The exact metabolic role that zinc plays is not known. It is generally agreed that the zinc in some way is responsible for the production of indole-3-acetic acid which is an auxin or plant growth regulator (28). One investigator has found that zinc is necessary for the synthesis of tryptophane in the tomato plant (17). Another investigator has shown that the zinc is concerned with the phosphate turnover in the tomato plant (24). A third investigator found that zinc is responsible for the aldolase activity in the clover plant (21). There has been relatively little work reported regarding the function of Zn in the plant and its ultimate location in plant tissue. The purpose of this investigation is to determine the uptake, distribution and final location of zinc in the alfalfa plant (Medicago sativa).

One method of zinc analysis that can be used is the single color dithizone method. This is the method of analysis accepted by the A.O.A.C. (20). Due to the advent of the nuclear reactor,

a suitable zinc radiotracer has been made available. It is Zn^{65} which has a half-life of 250 days and is both a gamma ray and a weak beta emitter (9). This makes possible the "tagging" of zinc-conteining substances and subsequent radiotracer analysis (15).

SURVEY OF LITERATURE

In 1925, Sommer and Lipman studied the effect of the lack of zinc in the sunflower (Dwarf var.) and barley plants (29). Photographs accompanying the text showed the effect of lack of zinc. Both types of plants grown in Zn-deficient solution were stunted in growth and had poor root systems. They stated that the effects were manifest in the early stages of growth showing the need for zinc from the very start. Sommer, in a later paper, showed that the buckwheat plants developed the same effects (30). The Windsor beans and the red kidney beans responded in a slightly different manner. In both of these cases, there was no visible difference between plants grown with and without zinc until the flowering stage was reached. At this stage, there was a sudden and rapid abscission of leaves.

Hoagland, Chandler and Hibbard have shown that the "Little-leaf" disease or rosette of fruit tree leaves was caused by a lack of zinc (13). They gave further evidence on the removal of such effects from the orchard trees and several plants which they had investigated. Chandler, in a later paper, gave strong evidence of the necessity of zinc in higher plants (8). He

discussed many of the 59 references to the work done in detecting zinc deficient symptoms and subsequent removal of the deleterious effects. He also advanced the idea that zinc is a catalytic agent in some essential carbohydrate reaction.

Stout and Arnon found that poor root development and stunted growth in the tomato plant could be attributed to the lack of zinc in the nutrient solution (31). They also found the deficiency symptoms to appear quite early in the growth of the plant.

Reed (23) did a very careful cytological study of vegetative buds of the peach and apricot trees which were zinc-deficient. The principal effects of zinc deficiency were shown by several well-defined biological changes. He also pointed out that there was an increase in the accumulation of a phenolic substance, such as phloroglucinol, in the cell. He made the valid assumption that the trees mobilized their scanty supplies of zinc and transported it to the centers of maximum activity.

Several workers have investigated the kidney bean (7). They found effects produced from lack of zinc similar to those reported earlier in the tomato plants. In a recent investigation Viets, et al,(34) observed the deficiency symptoms in field-grown Red Mexican beans. The symptoms were similar to those found in other plants. The symptoms were general chlorosis, subsequent stages of interveinal chlorosis, formation of brown spots in leaf mesophyll, abscission of flowers, curvature of leaves, and necrosis.

Hewitt and Bolle-Jones (11) studied the legumes for the effects of zinc and copper deficiencies. They found that alfalfa was less affected by lack of zinc than copper deficiency.

Skoog (28) concluded that Zn has an important function in maintaining the normal auxin concentration in the tissues. His experiments carried out on Zn-deficient tomato plants under different environmental conditions gave different auxin concentrations. From his experiments, he stated that Zn is not principally required for the synthesis of auxin. When an auxin, indole-3-acetic acid (IAA), was added to Zn-deficient plants, it was inactivated more rapidly than by control plants. This was shown by the retarding of stem growth. These plants also had a higher oxidation capacity than the control plants. The increased capacities for oxidation and auxin inactivation are possibly correlated. Addition of a small amount of zinc to the plants caused an increase within 24 hours in auxin production and elongation of the stem took place soon after.

Tsui (33) has grown tomato plants in water culture solutions with and without zinc. He found that the free auxin content of the control plants gradually increased with the age of the plants. The auxin decrease in the Zn-deficient plants occurred before there was any decrease in growth or any symptoms were noticeable. When zinc was added to the nutrient solution the free auxin and the enzyme digestible bound auxin increased within two days, whereas the bound auxins released with acid and alkali showed no increase until four days after Zn addition. Synthetic l-tryptophane was converted to an active growth substance to

the same extent by leaf-discs from control and affected plants. Zn-deficient plants contained significantly smaller amounts of tryptophane than the control, even before there were visible symptoms of Zn-deficiency. Three days after the addition of Zn, there was a noticeable increase in tryptophane content. He finally concluded that Zn is required directly for the synthesis of auxins.

Nason (17) found from his experiments with a specie of fungi that a relationship exists between zinc and the enzyme which converts indole and serine to tryptophane. Cell-free enzyme extracts from organisms grown in Zn-deficient media have shown considerable less tryptophene production than controls.

The translocation of auxin might give a lead as to where the auxin was formed. Skoog (27) investigated the auxin translocation in both the tomato and squash plants. He found that when IAA (auxin) was applied externally to the roots that it was transported up through the xylem. It moves laterally from the xylem into surrounding tissues of the stem and leaves and is then re-exported by the normal polar transport. He concluded that the auxin may act in two ways. First, high concentrations may be absorbed and act directly on these tissues. Second, very low concentrations (ca 5×10^{-5} mg per liter) are not absorbed into the aerial portions but may act indirectly through the influence of the roots.

If Zn is bound to some auxin, a method of extraction will have to be found in order to identify this constituent. Thimann and Skoog (32) investigated one specie of fungi. They have found

that the auxin can be removed by extraction with ether for three months. Other solvents, such as chloroform, ethyl alcohol, and water, are less satisfactory. The extraction process cannot be quickened by steaming, boiling at different pH values, extraction in the soxhlet apparatus or grinding. They found that water was essential for the hydrolytic liberation of auxin from tissue. High temperature stops the process which sets free extractable auxin. Low auxin solubility was attributed to slow process at work in tissue which sets auxin free.

Since it was noted earlier that Zn has a direct influence on tryptophane content, it was thought necessary to refer to a reference on tryptophane production. Virtanen and Laine (35) have shown that there is a definite increase in the tryptophane content from early stages of growth to maturity in both the pea and red clover plants. The content reaches a maximum at an early stage of growth, before blooming. At this stage the per cent of tryptophane-N in both species is double that of their respective seeds. The per cent content of tryptophane then falls rapidly until the start of blooming, at which time it is only slightly greater than in the seeds.

Reed (24) through his experiments on the tomato plant, found that Zn-deficient plants contain greater amounts of inorganic phosphate and phenol oxidase than in the controls. He has shown that affected plants contained more reducing sugars, but slightly less sucrose and starch than those of healthy plants. This indicated that one of the essential enzyme systems failed or was blocked, presumably by stoppage of the hexokinase catalyzed

glucose to glucose-6-phosphate reaction. He also stated that affected plant stems were richer in phosphatase and poorer in hydrogenase. He discussed a reference by Warburg and Christian who worked with the yeast enzyme, zymohexase (aldolase). This enzyme is a metal protein containing 1 gram atom of Cu and 1 gram atom of Zn per 3×10^6 grams protein. The enzyme may be blocked by the formation of a complex with cystein but can be reactivated by Zn, Cu, Fe, and Co. Zn reactivates it regardless of the oxygen tension and its presence is of prime importance in the sugar metabolism of the cell. Reed stated that the data now at hand are sufficient to justify the conclusion that the presence of Zn is necessary for the production and consumption of carbohydrates.

Quinlan-Watson (21) has examined Cu- and Zn-deficient oat plants for aldolase activity. While the aldolase activity in Cu-deficient plants did not differ from the controls, the aldolase activity in Zn-deficient plants was markedly decreased. He stated that the breakdown of normal carbohydrate metabolism is due to the decreased aldolase activity, which catalyzes the reversible reaction between hexose phosphate and triosephosphate. This last statement is in agreement with the earlier reference of Warburg and Christian.

Jagannathan and Singh (14) have shown that Zn is necessary for the activation of aldolase in fungus. The enzyme was inhibited by metal chelating agents as EDTA, a, a'-dipyridyl and o-phenanthroline but the inhibition was reversed by addition of

Zn. Nason, et al., (18) worked with cell-free extracts of the apical leaflets of the tomato. They investigated, separately, the effect of Zn, Cu, Mn, Fe, Mo and B deficiencies on the enzymes. They found that Zn-deficient plants contained greater amounts of polyphenol oxidase, ascorbic acid oxidase, peroxidase, lactic dehydrogenase, glycolic dehydrogenase, DPNH diaphorase but a less endogenous oxygen uptake. They have experimentally ruled out the fact that direct participation of the metals as inhibitors of those enzymes which increase under deficiency conditions. Addition of the metals to the corresponding deficient extracts has given no change in enzyme activities. However, addition of missing ions to corresponding nutrient solutions resulted in varied responses of the already modified enzyme patterns.

Arnon, et al., (1) have analyzed the chloroplast fragments and whole ground leaves of the sugar beet and chard plants for B, Mn, Cu, Zn and Mo. They found that Mn, Zn, and Mo were not concentrated in the chloroplast (grana) fragments but that Fe and Cu were.

Foster and Denison (10) studied two acid-producing fungi as to Zn-metabolism. They concluded that Zn is essential for the synthesis of pyruvic carboxylase even though indications are that the metal is not part of the enzyme. They stated that it is possible under conditions of Zn-deficiency an inhibitor is formed.

Some work has been done with Zn^{65} distribution in plants. Bergh (3) sprayed $ZnSO_4$ containing Zn^{65} around the soil of

10 plants of "Pisum sativum var. medullare (pea plant)." Thirty-six days later the plants were harvested and the amounts of Zn^{65} in the plants were determined. 1.04 per cent of Zn^{65} added to soil was recovered as follows: pea 0.19 per cent, shell 0.16 per cent, flower 0.01 per cent, blade 0.27 per cent, stem 0.11 per cent, root 0.30 per cent. Biddulph (4) discussed several references on translocation of radioactive mineral nutrients in bean plants. The accompanying radioautograms of leaves and roots showed that Zn^{65} is deposited mainly in the veins and active growing portions. He attributes this deposition to the precipitation of Zn as Zn-phosphorus complex. He pointed out that as the P concentration increased, the amount of Zn^{65} deposited in the leaves also increased. Addition of 1 ppm of Fe reversed this increase of Zn^{65} deposition due to the formation of the more insoluble ferric phosphate complexes. When Zn^{65} was injected into the veins of the leaflets some Zn^{65} was translocated to the roots. This showed that there is a downward movement to the roots of Zn^{65}. Wallihan, et al. (36) have shown, through their experiments with citrus trees, that absorption and translocation of Zn^{65} occurred more rapidly in young leaves than old leaves. They found no difference in the ultimate distribution of Zn in the plant whether it was absorbed through the roots or through the leaves. Although, Zn is more rapidly absorbed when applied near the center of the leaf than when applied near the margin. Their radioautograms have indicated that Zn^{65} is deposited for the most part in the veins of the leaves. This is in agreement with that found earlier by Biddulph (4).

Bean (2) attacked the problem of Zn metabolism on the basis that lack of Zn blocked one of the steps in the nitrate protein buildup. He found that in Zn-deficient tomato plants the protein nitrogen decreased more than in controls. He went further and stated that this was due to the retarding of nitrate absorption and reduction. He has found an increase in soluble organic nitrogen in Zn-deficient tomato plants. He discusses many of the 104 references listed.

EXPERIMENTAL

Instruments Used

The instrument used in the colorimetric analysis of Zn was the "Evelyn" photoelectric colorimeter. A light filter with maximum transmittance at 540 millimicrons was used in all transmission readings.

The NMC model PC-3 alpha-beta-gamma proportional counter was used in all radioactive sample counting. The gas mixture used was P-10 (10 per cent methane, 90 per cent argon). All operations were performed as directed by the operating manual (19). To obtain the operating voltage for beta-gamma counting, a standard curve of voltage vs. counting rate was made. The operating voltage was found to be 1750 volts. The standard curve is on the following page. Instrument variations with time of each counting were overcome by counting a National Bureau of Standards beta-emission standard (no. 3213, Ra D and E) with each group of samples. All samples counted at subsequent times were corrected

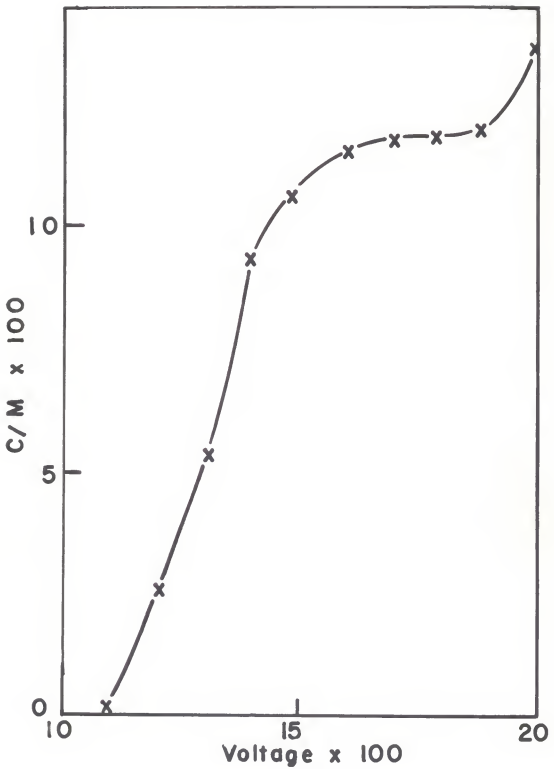

Fig. 1

according to the original standard count rate of the beta standards. Decay of samples was not included in the correction. The minimum detectable amount of Zn^{65} is about 3×10^{-12} grams. This gives a rate approximately three times the background.

Colorimetric Analysis For Zinc

Soxhlet Extraction. Two different samples of alfalfa were used for the colorimetric zinc analysis. Both were grown in the same field but one was commercially dried at the dehydrator (ca 650°C) and the other was dried at 50°C. After drying both samples were ground to pass through a 30 mesh screen.

Approximately 40 grams of each alfalfa meal were placed in individual soxhlet extractors. The first five extractions in order were: diethyl ether, skellysolve B (hexane), 95 per cent ethyl alcohol, redistilled water, and 0.1 N HCl. All solvents were distilled before using. Water was double-distilled in all pyrex glassware. The extractions were made for a period of eight hours since previous work has shown this to be sufficient time (26). The extracts were all evaporated to dryness and weighed. The weighed extracts were then dissolved with a minimum of con. H_2SO_4, oxidized as far as possible with con. HNO_3 and finally with 30 per cent H_2O_2. Volumes of H_2SO_4, HNO_3 and 30 per cent H_2O_2 used were recorded.

The remaining meal was removed from the extraction thimbles and extracted at room temperature with first, five per cent Na_2SO_4 and then 0.1N NaOH. This was done by stirring the meal

and the extractant for three hours, followed by filtering. Fresh extracting solution was added and the extraction repeated twice. Thus, total extraction time was nine hours and about 900 ml. of solution was obtained in each case. The same method was repeated using a 0.1N NaOH solution.

In the first experiment, the five per cent Na_2SO_4 extracts were evaporated to dryness, taken up in concentrated H_2SO_4 and oxidized as before. Weighed samples of Na_2SO_4 and NaOH were analyzed for zinc. These corrections for zinc were subtracted after suitable blank determinations.

In the following two experiments, the five per cent Na_2SO_4 fractions were placed in dialysis tubing and dialyzed against running water for 48 hours. The 0.1N NaOH extracts were neutralized with con. H_2SO_4 and also dialyzed for 48 hours. The dialyzed extracts were evaporated to dryness and oxidized as previously described.

The remaining residues were weighed and ashed overnight in platinum crucibles at 600°C. The entire procedure for both alfalfa meals was repeated for duplication of results.

The oxidized and ashed residues of both dehydrated and low temperature dried alfalfa samples were filtered through quantitative paper and the volume adjusted to 200 ml. 25 ml. aliquots were withdrawn and analyzed for Zn according to the modified A.O.A.C. method (20). The concentration of Zn in parts per million was determined from a calibration curve relating per cent transmission to concentration of Zn. The curve is shown in Fig. 2.

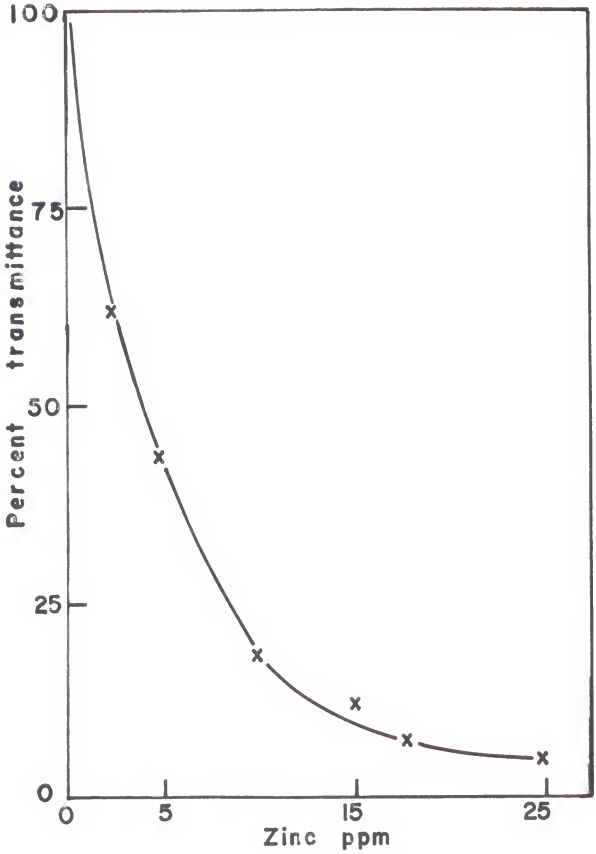

Fig. 2.

All acids, NH_4OH, solvents and water used in the procedure were distilled. Zn determinations were run on the oxidizing reagents and the blanks were subtracted from the value obtained for each extract. The amount of Zn in the extracts are shown in Tables 1 and 2.

Duplicate two gram samples of both types of alfalfa meal were ashed and analyzed for Zn according to the modified A.O.A.C. method (20). The number of ppm of Zn in these samples were multiplied respectively by the total weight extracted to arrive at the total Zn. In the case of dehydrated alfalfa, 87.88 per cent of the Zn was obtained while 84.74 per cent was obtained in the case of low temperature dried alfalfa.

The sequence of extractions were repeated on both types of alfalfa meal at a lower extracting temperature. This was done by reducing the pressure in the extractors so that the meal temperature during any extraction did not rise above 60°C. The ethyl ether boils at a low enough temperature that reducing the pressure during the extraction was not necessary. Both the five per cent Na_2SO_4 and 0.1N NaOH extractions were carried out as before.

The extraction procedure was repeated again on both types of alfalfa meal to provide fractions for Kjeldahl nitrogen determinations. These results and the results of the Zn determinations are included in Tables 1 and 2. The total Kjeldahl nitrogen found in the dehydrated alfalfa was 3.26 per cent while the low temperature dried alfalfa contained 3.34 per cent.

Separation into Cellular Constituents. A different type of extraction procedure was carried out on fresh alfalfa (5). Fresh

Table 1. Zn in alfalfa fractions at normal and reduced temperatures.*
Protein in alfalfa fractions.

Solvent	Normal Temperature: Per cent Protein (%N x 6.25)	Per cent of Total Weight Extracted (av.)	Per cent Zn of Total Zn Extracted (av.)	Reduced Temperature**: Per cent of Total Weight Extracted	Per cent Zn of Total Zn Extracted
Diethyl ether	2.87	2.59	18.49	3.03	16.05
Hexane	1.18	0.32	7.14	0.39	2.76
95% Ethyl Alcohol	15.50	12.78	11.38	5.09	15.84
Zn-free H_2O	20.13	13.92	18.27	7.85	13.38
0.1N HCl	18.63	4.94	13.47	8.35	14.64
5% Na_2SO_4	14.44	21.31	8.71	0.79	4.80
0.1N NaOH	7.75	14.58	10.88	6.61	16.56
Residue	17.94	22.29	11.66	48.64	15.97
Totals	97.94	93.21	100.00	80.75	100.00

* alfalfa dried at temperatures below 50°C
** soxhlet extracting temperature did not rise above 60°C.

Table 2. Zn in dehydrated alfalfa fractions at normal and reduced temperatures. Protein in dehydrated alfalfa fractions.

Solvent	Per cent Protein (%N x 6.25)	Normal Temperature: Per cent of: Total Weight Extracted (av.)	Normal Temperature: Per cent Zn of Total Zn Extracted (av.)	Reduced Temperature*: Per cent of: Total Weight Extracted	Reduced Temperature*: Per cent Zn of Total Zn Extracted
Diethyl Ether	3.44	2.19	8.79	3.11	0.0
Hexane	4.25	0.30	6.09	0.52	0.0
95% Ethyl Alcohol	9.88	10.40	15.80	5.17	1.62
Zn-free H_2O	12.38	12.42	25.34	7.54	20.51
0.1N HCl	10.81	6.19	12.15	10.62	36.24
5% Na_2SO_4	13.31	23.26	13.66	1.36	20.52
0.1N NaOH	7.50	15.34	7.28	8.20	0.72
Residue	22.44	21.25	10.88	50.43	20.39
Total	84.01	91.45	100.00	86.95	100.00

* soxhlet extracting temperature did not rise above 60°C.

frozen alfalfa plants (above ground portion) were allowed to thaw at room temperature and then immersed in a beaker of ether for two hours. The ether was poured off and evaporated to dryness. The alfalfa plants were chopped to a slurry with water in a Waring blender with a minimum of water (ca 100 ml.) for four minutes. More water was added and the chopping continued for eight minutes. The green colored slurry was then filtered through a strong cotton muslin cloth. The cotton cloth was tied in the form of a bag and placed in a Carver laboratory press. It was pressed until the pressure remained constant at 7,000 lbs/sq.in. The liquid that was removed was added to the filtrate from the muslin cloth. The pressed solid material contained unbroken cells and fragments of broken cells.

The green colored liquid was centrifuged in 250 ml. centrifuge bottles at 2,000 rpm for 35 minutes. The straw colored supernatant liquid contained the vacuolar liquids and cytoplasm. The green residue was composed mainly of the chloroplasts.

The three fractions were dried, weighed and dissolved in a minimum amount of concentrated H_2SO_4 and oxidized with concentrated HNO_3 and 30 per cent H_2O_2. The cell wall material was dried, weighed, and ashed overnight in platinum crucibles at 600°C. The ash and residues were taken up in a minimum volume of 1N HCl and hot water and filtered through Whatman no. 2 paper into 200 ml. volumetric flasks. The hot water rinsings were added to the residues on the filter paper. The volumes of the liquids in the flasks were made to 200 ml. and 25 ml. aliquots were withdrawn for Zn analysis. The Zn determinations were done according to the modified A.O.A.C. method (20).

The entire extraction procedure, except for ashing and oxidations, was repeated to provide fractions for Kjeldahl nitrogen determinations. These were performed by the college chemical service laboratory. The results of Zn determinations of the second extraction procedure and the nitrogen determinations are found in Table 3. One gram samples were found to contain an average of 3.04 per cent nitrogen.

Radiotracer Analysis for Zn^{65} in Alfalfa

Alfalfa containing Zn^{65} (beta, gamma emission, half-life of 250 days) was obtained in the following way. About 300 alfalfa seeds were planted in soil in 30 five-inch clay pots. These pots were placed on a sand table in a greenhouse. At the end of three weeks, about 50 of the best plants were chosen and transferred to liquid nutrient solutions. The nutrient solutions were contained in a pair of two-gallon capacity crockery jars with masonite board lids. The covers had 15 three-eighths inch holes drilled in each of them and the lids were covered with paraffin. The alfalfa plants were removed from the clay pots and the roots were washed free of soil. The plants were placed together in pairs; the stems partially wrapped with glass wool and placed through a hole in the masonite lids. The crocks were filled to within an inch of the lids with "Hyponex" solution. This is a commercial powdered plant food for growing plants in liquid nutrients. This was found to be more successful for growing alfalfa plants than Hoagland's solution (12). A Zn analysis was performed on duplicate two-gram samples of "Hyponex" and was

Table 3. Zn and protein in cellular constituents of fresh alfalfa.

Composition:	Per cent Protein: (%N x 6.25)	Per cent of Total: Fresh Weight Extracted (av.)	Per cent Zn of Total Zn Extracted (av.)
Coloring Pigments	5.00	0.67*	2.21*
Cell-wall materials	16.50	13.35	44.02
Vacuolar, cytoplasm, liquids	15.44	3.48	43.25
Chloroplasts	42.94	0.81	10.52
Totals	79.88	18.31	100.00

* average of two, the rest are averages of three determinations.

found to contain an average of 39.2 micrograms of Zn per gram.

After one week of growth in the liquid nutrient, eight of the better plants were chosen for the experiment. Four of the plants were placed together and put through a hole in a rubber stopper and supported by small wooden stakes in the stopper. The stopper, plants and support were placed on top of a two liter bottle. The same was done with the other four plants. The outside of both bottles were painted to exclude light. Both bottles were placed on a 24 x 30 x 1 inch steel tray.

One mg. of Zn^{65} ($ZnCl_2$ in 0.9N HCl) was received from Oak Ridge National Laboratory with an activity of 1.34 mc/ml. Preliminary work had shown that 0.2 ml of Zn^{65} solution which has a total activity of 0.258 mc was sufficient for two liters of nutrient solution during the entire growth period of about 30 days. This corresponds to 6 x 10-3 microgram of Zn^{65} per gram

of solution. This amount was added to both bottles of nutrient solution and one-half hour later, three-10 microliters samples were taken from each bottle and counted. Leaves were clipped from the plants at regular intervals in order to determine the rate of uptake of Zn by alfalfa.

Soxhlet Extraction. After 30 days, the above ground portion of the plants were removed and dried at 50°C. A small Wiley mill was placed inside a dry box to prevent air contamination by radioactive alfalfa. The meal was then ground to pass through a 30 mesh screen. An aliquot sample was weighed from the ground radioactive meal for total activity counting and the remainder was placed in a small soxhlet extractor. The five soxhlet extractions were performed at atmospheric pressure. The inorganic salt and base extractions were carried out as before.

The soxhlet extracts were all evaporated to dryness and oxidized with con. HNO_3 and 30 per cent H_2O_2 under infra red heat lamps. The residues were dissolved in hot H_2O and the volume adjusted to 25 ml. in volumetric flasks. The inorganic salt and base extracts were dialyzed against running water for 48 hours, oxidized and made to the same volume as the soxhlet extractions.

Three-25 lambda aliquots were removed from each flask and placed on copper planchettes. The samples were evaporated to dryness under infra red lamps and counted for 5 minutes in the NMC model PC-3 proportional counter at 1750 volts.

The entire experiment of growing alfalfa on radioactive nutrient, extraction and subsequent counting was repeated. The results of both experiments are in Table 4.

Table 4. Radiotracer analysis of fractions of 50°C dried alfalfa grown on Zn^{65} containing nutrient

Solvent	Per cent Weight of Extracts 1	Per cent Weight of Extracts 2	Total Activity (c/m) 1	Total Activity (c/m) 2	Per cent of Total Zn^{65} added to Nutrient Solution 1	Per cent of Total Zn^{65} added to Nutrient Solution 2	Per cent of Total extract activity 1	Per cent of Total extract activity 2
Ethyl Ether	3.11	4.03	1,480	0	0.002	0	0.139	0
Hexane	0.52	1.07	3,240	0	0.005	0	0.303	0
95% Ethyl Alcohol	10.40	7.60	11,500	111,000	0.017	0.102	1.078	2.106
Zn-free H_2O	21.31	24.49	149,800	560,000	0.22	0.512	14.029	10.626
0.1N HCl	10.62	7.97	331,800	1,265,000	0.49	1.157	31.08	24.004
5% Na_2SO_4	4.36	3.82	358,200	1,917,000	0.53	1.754	33.56	36.376
0.1N NaOH	15.34	20.38	5,100	379,000	0.008	0.347	0.510	7.192
Residue	22.79	20.86	206,000	1,038,000	0.030	0.950	19.293	19.696
Total	88.62	90.22	1,067,120	5,270,000	1.572	4.822	100.00	100.00

Separation into Cellular Constituents. Alfalfa grown on liquid nutrient containing Zn^{65} was extracted in the same way as the non-radioactive alfalfa as previously described.

The radioactive fractions were oxidized in the same manner as before and made to volume in 25 ml. volumetric flasks. Three-25 lambda samples were withdrawn, placed and dried on steel planchettes, and counted for three minutes. The results are in Table 5. In trial one, 30.28 per cent of the fresh weight was recovered in the four fractions. In trial two, 24.14 per cent of the fresh weight was recovered in the four fractions.

Uptake and distribution of Zn^{65} in Alfalfa. Each time a group of alfalfa plants were grown on Zn^{65} containing nutrient solution for extraction purposes, leaves were removed at regular intervals of time. The first leaf was taken about eight to ten inches above the roots about one-half hour after addition of Zn^{65}

Table 5. Radiotracer analysis of cellular constituents of alfalfa grown on Zn^{65} containing nutrient solution.

Composition	Per cent of Total Extract Activity	Weight (grams)	Per cent of Total Extract Activity	Weight (grams)
Coloring Pigments	0.00	0.0282	0.00	0.0587
Cell-wall Material	4.67	1.2841	7.08	1.1436
Vacuolar-Cytoplasm, Liquids	84.11	0.6132	81.91	0.2560
Chloroplasts	11.22	0.1486	11.01	0.2083
Totals	100.00	2.0741	100.00	1.6666

to the nutrient solution. The succeeding removal of leaves were from this height at regular intervals for about 30 days. During the 30 day period of sampling, new growth developed at the base of the plant. Leaves from new growth also were removed.

The leaves were weighed, dried at 75°C and weighed again. They were then ashed overnight in one inch diameter stainless steel cups at 600°C, treated with dilute HCl and the residue dried for counting. Counting was done on NMC model PC-3 windowless proportional counter at 1750 volts. The curves are in Figs. 3 to 6.

Radioautographs of several of the leaflets from two different experiments were made. This was done by placing the radioactive leaflets directly against the emulsion of No-screen X-ray film and held in position with a weight placed on a masonite board. The exposure time was computed from the activity and area of the leaflets. In the first radioautograph, the exposure time was 16 hours while in the second it was 48 hours. The film was developed with standard X-ray film developer. The radioautographs are in Figs. 7 and 8.

DISCUSSION

A difference in the solubility of zinc containing substances has been found between commercial dehydrated (ca 650°C) alfalfa and low-temperature (50°C) dried alfalfa as shown by soxhlet extractions. In the latter, the greatest concentration of bound zinc was in the ethyl ether extract while in the former it occurs in the water extract. This might be explained on the basis that

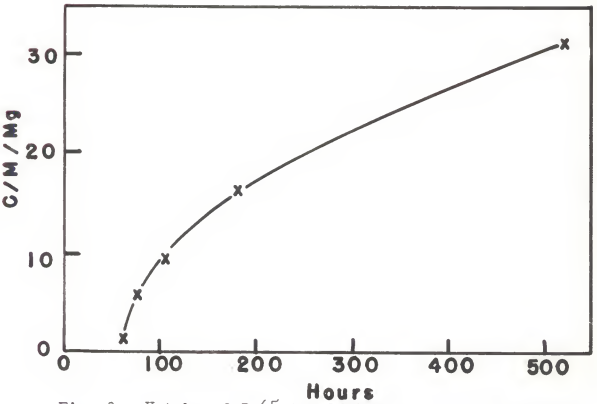

Fig. 3. Uptake of Zn^{65} in alfalfa plant. The nutrient solution contained 0.258 mc Zn^{65} in 2000 ml. of solution.

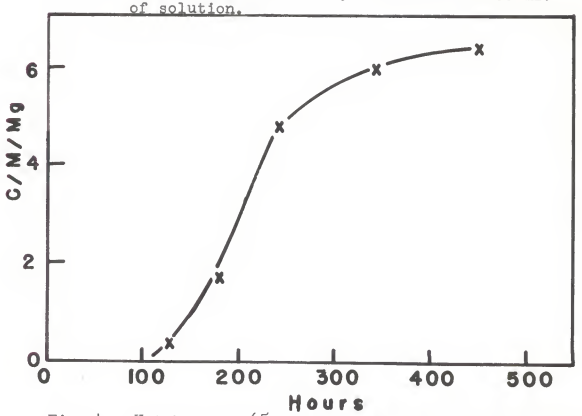

Fig. 4. Uptake of Zn^{65} in alfalfa plant. The nutrient solution contained 0.0258 mc of Zn^{65} in 2000 ml. of nutrient solution.

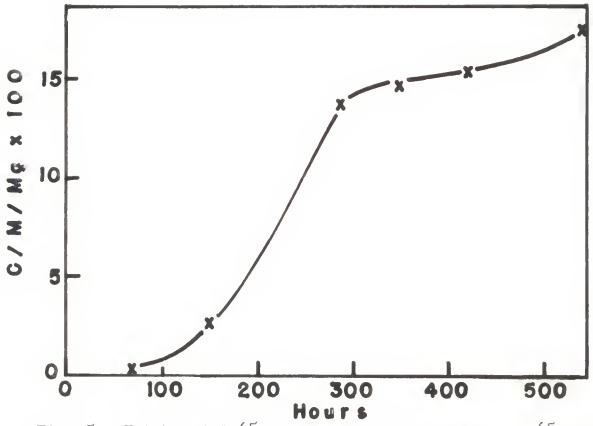

Fig. 5. Uptake of Zn^{65} in alfalfa plant grown on Zn^{65} containing nutrient solution. The nutrient solution contained 0.250 mc in 2000 ml. of solution.

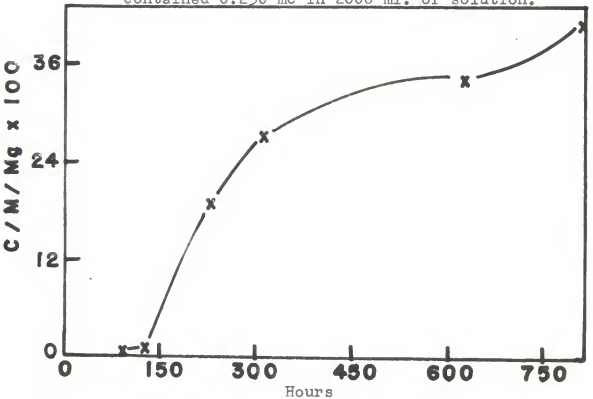

Fig. 6. Uptake of Zn^{65} by new growth from alfalfa plants grown on Zn^{65} containing nutrient solution. The nutrient solution was the same as in Fig. 3.

Fig. 7. Leaves removed from alfalfa plant grown on Zn^{65} (0.258 mc) containing nutrient solution. Leaves were removed 11 to 14 days after addition of Zn^{65} to nutrient solution. The small leaves are new growth. The large leaves with only the veins showing activity were removed 11 days after addition.

Fig. 8. Leaflets removed from alfalfa plant grown on Zn^{65} (0.258 mc) containing nutrient solution. Time of removal from plants after addition of Zn^{65} to nutrient solution are as follows (left to right): 232, 89, 356; 604, 523, 496 hours.

the solubility of the substance to which Zn is bound is altered during commercial dehydration. Proteins are substances whose solubilities are altered when exposed to temperatures of 40°-75°C (5). Several references have stated that Zn is bound to certain proteins (enzymes) (37, 24, 16). It has been found that there is no loss of Zn during the dialysis of Zn-protein complexes (37, 18).

The proteins of the commercially dehydrated alfalfa are already at least partially denatured due to the temperature of the dehydration process. Therefore, keeping the extraction temperature at a minimum should have little effect on the solubility. It has been found that a high per cent of Zn was obtained in the water extracts of the dehydrated alfalfa.

If the Zn is bound to some enzyme in the plant, then there should be a large amount of Zn in the water and dilute acid solutions extracts (5). This was found in all but one of the extractions. In the radiotracer analysis, the greatest concentration of Zn was found in the Na_2SO_4 extract, although, 78-80 per cent of the total Zn was found in the water, dilute acid and neutral salt extracts. In the colorimetric analysis of the fractions from the dehydrated alfalfa, 50-76 per cent of the Zn was found in these three extracts. The corresponding fractions of the low-temperature dried alfalfa are lower.

Bonner (5) stated that the plant-cell enzymes occur in the cytoplasm. Both the colorimetric and radiotracer analysis of the cytoplasm (cell-liquids) fraction show the greatest concentration

of Zn. The per cent Zn in this fraction was more evident in the radiotracer analysis than in the colorimetric analysis. It is possible therefore that Zn may be bound to certain enzymes in the cytoplasmic fluids.

A large amount of Zn has been found in the cell-wall material by the colorimetric analysis. The corresponding fraction by the radiotracer analysis is much lower. If the Zn-bound substance is found principally in the cell-liquid, then any Zn present in cell-wall material may be assumed to be due to incomplete cell-rupture and removal of all cell-liquids.

Arnon, et al., (1) found no Zn in the chloroplast fraction of the chard plant. This is in contrast with results of work reported on alfalfa in this paper. It has been found that about ten per cent of the Zn, as shown by both colorimetric and radiotracer analysis, was contained in the chloroplasts of the alfalfa plant.

From the curves of activity vs. time, it was found that between 65-100 hours after addition of Zn^{65} to the nutrient solution a noticeable increase of Zn^{65} occurred in the mature leaves. This contrasts with that found by Schaff (25) who noted P^{32} in wheat leaves one hour after being added to the nutrient solution. Tsui (33) has found that the bound auxin increased within 96 hours after addition of Zn to the roots of Zn-deficient plants. This is a significant parallel in that Zn has been assumed to be involved in auxin production. The uptake curves do not indicate whether the plant absorbs the Zn late in the metabolism cycle or if the plant takes this much time (65 hrs.) before any additional Zn-bound enzyme is needed.

New leaflets had a phenomenal uptake rate of Zn^{65}. The first detectable uptake of Zn^{65} was 23 c/m/mg at 110 hours after addition of Zn^{65} to nutrient solution. At the end of 230 hours, the activity was 2600 c/m/mg while the final sample contained 3875 c/m/mg at 33 days. The rapid and large uptake by young leaves is consistent with that found by Wallihan, et al. (36).

Inspection of the radioautographs in Fig. 7 show that Zn is contained throughout most of the individual leaflets. The leaves taken earlier in the experiment show a high concentration of Zn in the veins. The veins being the source of liquid movement from the roots would naturally contain a higher concentration of Zn than surrounding tissues. It is possible that the Zn might have been deposited in the leaf veins early in the course of the experiment.

In Fig. 8, the leaflets taken at the various intervals of time show that the Zn^{65} is contained in the leaflets throughout the experiment. It is indicated that once the Zn^{65} is deposited in the leaves it remains as such. The petioles of leaflets in both figures show a heavy concentration (light areas) of Zn^{65}. This might be due to the narrow area through which the Zn^{65} must pass, thus showing a high concentration in a small area.

CONCLUSIONS

From the foregoing statements, it is concluded that the zinc in alfalfa is bound to some non-dialyzable substance which is found mainly in the cell-liquid (cytoplasm) fraction. It is also concluded that the Zn-bound substance is more soluble in water,

dilute acid, and neutral salt solutions than in organic solvents. The solubility in dilute acid is attributed to the partial hydrolysis of the Zn-bound substance.

The exact compound (prosthetic group) to which Zn is bound and the chain of events which produce this compound cannot be explained on the basis of this investigation. It can be postulated that Zn is not required or used immediately in the metabolism system. Zn has been found to be absorbed between 65 and 100 hours after addition to the roots. The final location of Zn is in the leaflets of the alfalfa plant.

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INVESTIGATION OF ZN-BOUND PROTEINS IN ALFALFA USING Zn^{65}

by

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This research was conducted to determine the uptake, distribution and final location of Zinc in the alfalfa plant (Medicago sativa). Colorimetric and radiotracer methods are available for the analysis of Zn.

Two different kinds of dried alfalfa were investigated. One was commercially dried at the dehydrator (ca 650°C) and the other at a low temperature (50°C). Both were subjected to soxhlet extractions using, in order: ethyl ether, hexane, 95 per cent ethyl alcohol, redistilled water, and 0.1N HCl. These extractions were carried out both at room temperature and at lower temperatures. The alfalfa samples were further extracted with first five per cent Na_2SO_4 and then 0.1N NaOH. The extracts from all the extractions were analyzed for Zn by the accepted colorimetric A.O.A.C. method.

Low temperature dried alfalfa was separated into cellular constituents. These constituents included ether soluble material, cell-wall material, cell liquids, and chloroplasts. Each extract was analyzed for Zn by the accepted colorimetric A.O.A.C. method.

Several groups of alfalfa plants, which had first been started on soil, were transferred and grown on liquid nutrient solution containing Zn^{65} (as $ZnCl_2$). The various groups were grown on liquid nutrient for a period of 24 to 33 days. During each growth of plants, leaves were removed from the plants at regular intervals of time from one-half hour to a final time, in one case, of 33 days. These leaves were weighed and ashed and counted for activity. A plot was prepared of c/m/mg vs. time.

Several of the leaves taken from the plants at various intervals of time were radioautrographed by placing them against X-ray film. The Zn^{65} containing alfalfa plants were then harvested and the Zn^{65} extracted by the two previously mentioned procedures.

A difference in the solubility of Zn containing substances has been found between commercial dehydrated alfalfa and low temperature dried alfalfa as shown by the soxhlet extractions. In the latter, the greatest concentration of Zn was in the ethyl ether extract while in the former it occurs in the water extracts. In the radiotracer analysis of low temperature dried alfalfa, 78-80 per cent of the total Zn was found in the water, dilute acid and neutral salt extracts. This is consistent with that found by the colorimetric analysis.

Both radiotracer and colorimetric analysis of the cell liquids (cytoplasm) show the greatest concentration of Zn. It is concluded that the Zn in alfalfa is bound to some non-dialyzable substance which is found mainly in the cell liquid (cytoplasm) fraction. Zn has been found to be absorbed into the leaves between 65 to 100 hours after addition to the nutrient solution. One of the final locations of Zn is in the leaflets of the alfalfa plant.