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THE LOCALIZATION OF ZINC-65 IN GERMINATING CORN TISSUES*

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Zinc is known to be involved in several enzyme systems in plants and to play a role in protein metabolism [12]. It its also required for the synthesis of tryptophane, a precursor of indoleacetic acid [22]. It is thought to be of intermediate mobility in plants [4], and since zinc deficiencies in many plants are common a more detailed knowledge of its function and intracellular localization would be useful. Wood and Silby [26] report that up to half of the total zinc in leaves can be located in the chloroplasts, depending on the plant species, and that dialysis of an aqueous brei of oat leaves results in no loss of zinc.

The purpose of the following work was to localize the zinc of various plant tissues.

Materials and methods

Aresan-treated corn seeds were germinated on moist filter paper in petri dishes. In Experiments A and B and for the solubility study, 50 μc of Oak Ridge high specific activity zinc-65 were added per 100 seeds, five days after germination commenced. In Experiment A, after two days, and in B after three days of the zinc-65 addition, the plants were thoroughly rinsed in tap water, separated into roots, grains and leaves, and weighed. In Experiment C, the isotope was added after four days of germination and the plants cropped after an additional five days.

Each tissue was ground in 0.067 *M* sodium phosphate buffer (pH 6.7) in an Omnimixer at full speed. The homogenizer was kept in an ice bath during the

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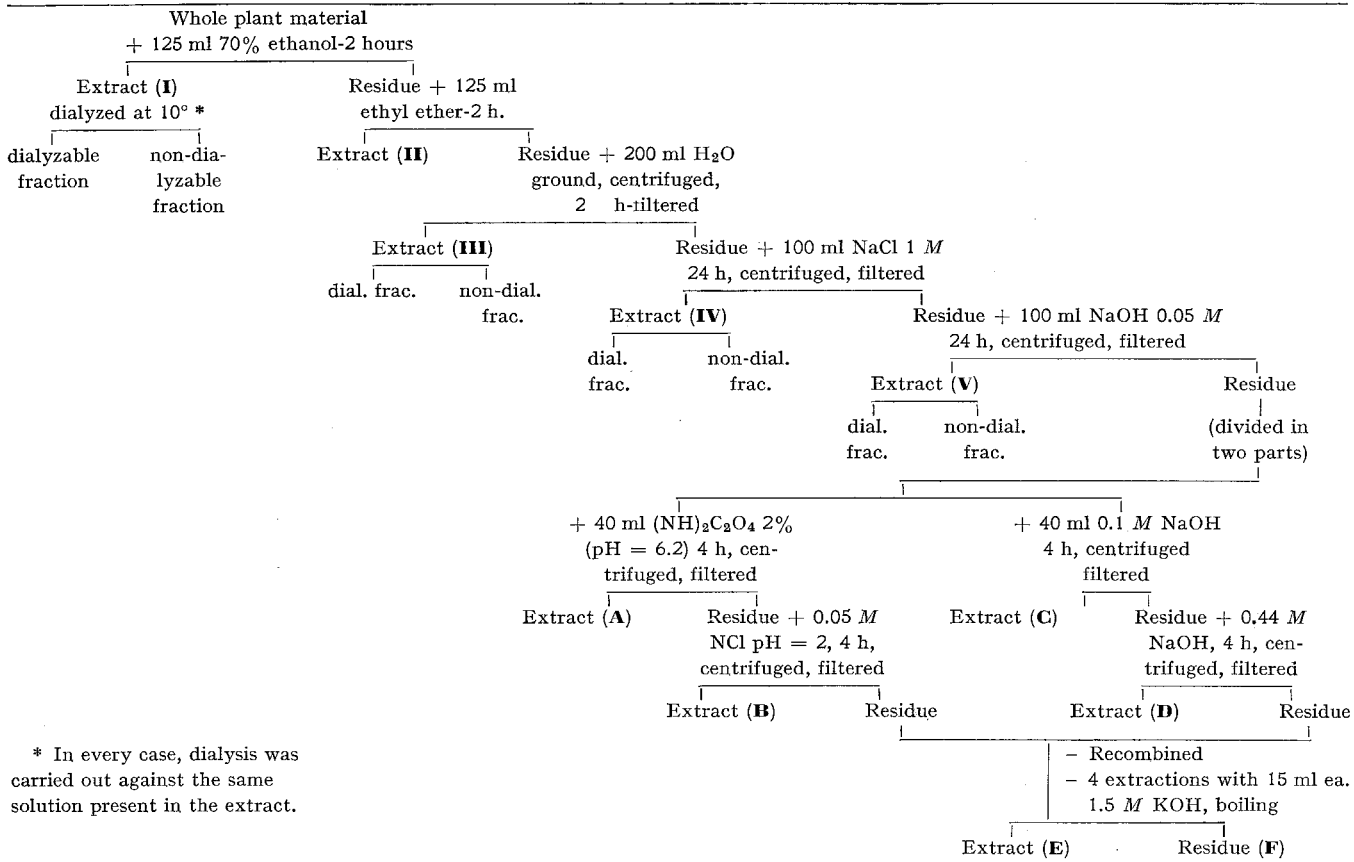
TABLE 1

Extraction scheme for the corn seedlings used in Experiment B



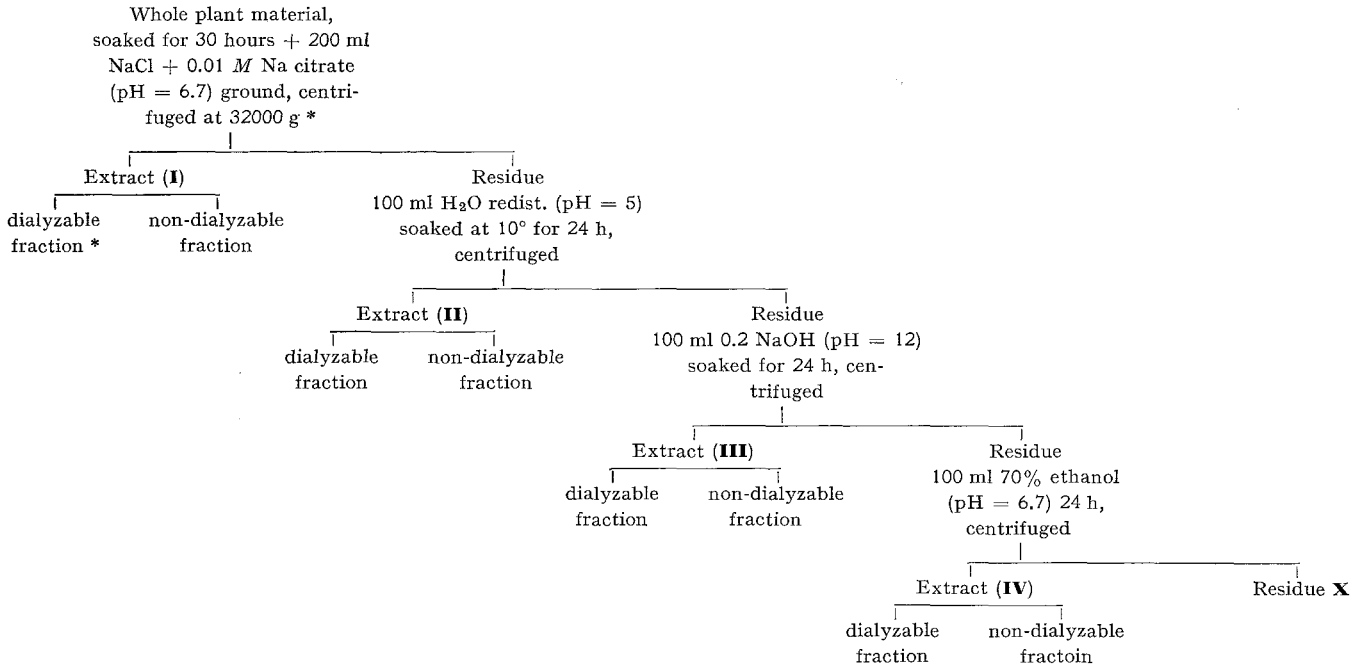
* In every case, dialysis was carried out against the same solution present in the extract.

Substances corresponding to fractions of the above Table 1.

I – prolamines +
II – fats
III – albumines, histones, globines, protamines +
IV – globulines +
V – glutelines +
A – pectates
B – protopectines
C – polyuronide hemicelluloses
D – non-cellulosic polysaccharides
E – part of the protopectines, lignine
F – α-cellulose

TABLE 2

Extraction scheme for the protein fraction of corn seedlings used in Experiment C



* Dialysis at 10°C against 0.07 *M* sodium phosphate buffer, pH 6.7.

grinding. The radioactivity in samples of the resulting brei was counted in a well scintillation detector and the remainder of the brei was centrifuged at 31,000 × *g* for 20 minutes. The supernatant solution was dialysed against the same sodium phosphate buffer at 10°C until the buffer showed background radioactivity. In Experiment B, the residues were fractioned in accordance to the methods of Bonner [1], Hawk *et al.*[9], and Davidson [5], as indicated in Table 1. The cell wall constituents were fractionated by an adaption of the technique of Boroughs and Bonner [3], as given in Table 2.

Radioactivity was determined on triplicate aliquots of each sample in a well scintillation counter. To have comparable data, all samples were counted until at least 4000 counts per sample were registered.

To study the velocity of solubilization of zinc-65 in tissues, another batch of grains was germinated, treated with zinc-65 as before, and harvested after three days, following the application of the radioactive substance. It was then processed ,as described, up to the separation of the different plant fractions. Half of the roots, grains or leaves was covered with water, and the other half with 70 percent (v/v) ethanol. After two hours, the solutions were decanted and the plants washed three times with similar solutions. The washings were combined with the extracting liquids, brought to volume, and aliquots of each counted. This process was repeated after four hours and 24 hours, for both liquids, and in addition, after six and eight hours for ethanol.

To obtain more information concerning the location of zinc in plants, a radioautograph was prepared. Washed and dried seedlings grown for two days in the presence of zinc-65, were put on X-ray film for 35 days. To obtain an adequate exposure, the grains were cut in such a way as to leave only a thin central section exposed.

RESULTS

A general idea of the distribution of zinc in germinating corn tissues is given in Table 3. This table demonstrates the distribution of the element in the different parts of the seedlings as obtained in Experiment A. Comparing the percentages of total activity, it can

TABLE 3

Distribution of Zn-65 in different parts of corn seedlings in Experiment A

Tissue	Fresh Weight (g)	Total cpm	cpm/k	% of total	% of total activity in parts		
					Solid res-idue	Total sol. frac.	Non-dial. part of sol. frac.
Whole plant	120.9	373,946	3,090	100	—	—	—
Roots	33.4	189.694	6,360	50.7	46.7	54.0	10.0
Grains	50.0	135,750	2.710	36.3	57.2	45.5	5.5
Leaves	37.5	48,502	1,290	13.0	38.6	71.3	21.6

be noted that more zinc remains in the solid residue of the seedlings than is extracted, in the cases of roots and grains, while the opposite holds true for leaves. The latter tissue also has a considerably higher non-dialysable zinc fraction.

In Table 4, the data from Experiment B are presented in a form which permits their comparison with the data from Experiment A, presented in Table 3. The portions of zinc-65 detected in the different fractions obtained in accordance to the scheme in Table 1 were added together and presented in groups similar to the ones used to obtain the figures in Table 3. Comparing Tables 3 and 4, it

TABLE 4

Distribution of Zn-65 in different parts of corn seedlings derived by summing the fractions of Experiment B obtained in accordance to the separation of Table 1 in Experiment B.

Tissue	Fresh Weight (g)	Total cpm	cpm/g	cpm frac. as % of total	% of total cpm		
					protein + frac *	cell wall + frac **	Total nondial. Zn-65 as %
Whole plant	91.2	635,198	6,970	100	67.4	32.6	19.7
Roots	24.4	218,881	8,970	34.4	85.3	14.7	22.9
Grains	44.4	345,852	7,780	54.3	56.4	43.6	12.5
Leaves	22.3	70,465	3,150	11.1	65.8	34.2	23.8

* Includes the fractions soluble in 70% ethanol, water, 1 *N* NaCl solution and 0.05 *N* NaOH solution.

** Includes 2% $(NH_4)_2C_2O_4$, 0.05 *N* HCl, 0.1 *N* NaOH, and 1.5 *N* KOH.

could be noted in both experiments that the roots were the most active and the leaves the least active tissue, confirming various reports [18] [26]. The activity of the non-dialyzable fraction was the same for both experiments, being highest for leaves, followed by roots, and finally by grains.

The results of fraction activity are presented in Table 5. These results indicated that the largest part of the 'protein plus' fraction in grains and leaves was water extractable. In roots, the 70-percent ethanol-extractable part was highest, followed by the water-extractable fraction. No ethyl ether-extractable Zn-65 was detected.

The fractionation of the principal constituents of cell walls is presented in Table 6, indicating that most zinc was found in the solubility group of protopectines, and of hemi-celluloses. These two

TABLE 5

Zinc-65 in different protoplasmic fractions of Experiment B						
Zn in 'protein'	Roots		Grains		Leaves	
	Total activity cpm	Non-dializable frac. cpm	Total activity cpm	Non-dializable frac. cpm	Total activity cpm	Non-dializable frac. cpm
'Protein fraction'	186,810	50,025	195,041	43,228	46,368	16,760
Ethanol 70% extraced fraction	74,305	19,030	40,656	12,470	2,585	1,322
H_2O-extractable fraction	59,116	27,710	103,350	20,000	22,633	13,420
NaCl 1 *M* extractable fraction	46,245	1,070	28,585	733	18,376	573
NaOH 0.05 *M* extractable fraction	7,144	2,215	22,450	10,025	2,774	1,445

TABLE 6

Zn-65 distribution in the fractions of cell wall constituents in Experiment B						
Fraction of Zn extracted with:	Roots		Grains		Leaves	
	Total activity cpm	% of total cell wall	Total activity cpm	% of total cell wall	Total activity cpm	% of total cell wall
Total Zn-65	32,071	100	150,811	100	24,097	100
$(NH_4)_2C_2O_4$ 0.02%	717	2.2	7,761	5.2	789	3.3
HCl 0.05 *N*	12,013	37.5	7,763	5.2	10,110	41.9
NaOH 0.1 *N*	12,307	38.4	135,087	89.6	8,609	35.7
NaOH 0.45 *N*	3,976	12.4	—	—	2,628	10.9
KOH 1.5 *M*	3,058	9.5	—	—	—	—
Residue, α-cellulose	0	0	—	—	—	—

solubility fractions included at least 75 percent of the activity of the cell walls examined. From the grains, only three solubility fractions could be separated, since the treatment with 0.45 *N* NaOH resulted in the formation of a jelly from which no residue or supernatant could be separated. The remaining α-cellulose fraction was free of zinc-65.

In Experiment C, the seedlings were allowed to grow five days in the presence of the isotope and a second protein fractionation scheme, given in Table 2, was employed. The results are given in Table 7. Comparing Table 4 with Table 7, it could be observed that a higher and more uniform portion of zinc-65 was found in the 'protein plus' fraction after five days than after three days. There

TABLE 7

Distribution of Zn-65 in different parts of corn seedlings obtained by summing the activity of the fractions in Experiment C according to the separation in Table 2

Tissue	Fresh weight (g)	Total cpm	cpm/g	cpm of fraction as % of total	% total cpm		Non-dializable Zn-65 as % of total
					'protein plus' fraction *	cell wall + fraction **	
Whole plants	60.3	638,333	10,580	100.	91.9	8.1	3.6
Roots	17.0	266,869	15,700	41.8	93.8	6.2	2.6
Grains	17.9	161,128	8,990	25.2	90.3	9.7	5.3
Leaves	25.4	210,336	8,280	33.0	90.7	9.3	2.8

* Includes fractions soluble in 70 percent ethanol, in 0.5 *N* NaOH, in H_2O and in 1 *M* NaCl plus 0.01 *M* Na/citrate (pH = 6.7).

** Includes fractions soluble in two percent $(NH_4)_2C_2O_4$, in 0.05 *N* HCl, in 0.1 *N* NaOH, and in 0.44 *N* NaOH and in 1.5 *N* KOH.

TABLE 8

Zn-65 in different 'protein fractions' in Experiment C

	Roots		Grains		Leaves	
	Total activity	Non-dial. fraction	Total activity	Non-dial. fraction	Total activity	Non-dial. fraction
Total Zn-65 in 'protein' fraction	250,308 cpm	7,032 cpm	145,540 cpm	8,456 cpm	190,871 cpm	6,003 cpm
NaCl 1 *M* + Na citrate soluble	236,040	3,024	126,000	506	181,251	2,631
H_2O redist. soluble	8,824	1,487	2,268	187	6,357	666
NaOH 0.2% soluble	4,357	1,484	4,216	1,380	2,227	1,905
Ethanol 70% soluble	1,087	1,037	13,056	6,383	1,037	801

was also a considerable decrease of the non-dialyzable fraction for all tissues.

Table 8 represents the distribution of zinc in the 'protein' fractions of the seedlings in Experiment C. The 70-percent ethanol- and water-soluble 'protein plus' fractions have decreased in comparison to the figures given in Table 5. The extractants used in Experiment C (1 *M* NaCl and 0.01 *M* sodium citrate) contained over 86 per cent of the total zinc activity. Most of this zinc fraction was lost by dialysis while the remaining fractions had appreciable non-dialyzable portions.

Table 9 gives the zinc distribution of the cell wall components from Experiment C. Here, as before, the formation of a jelly as the

TABLE 9

Zn-65 distribution in the fractions of cell wall constituents in Experiment C

Fraction of Zn extracted with:	Roots		Grains		Leaves	
	Total activity cpm	% of total cell wall	Total activity cpm	% of total cell wall	Total activity cpm	% of total cell wall
Total Zn-65	16,561	100	15,588	100	19,465	100
$(NH_4)_2C_2O_4$ 0.2%	580	3.5	1,250	8.0	326	1.7
HCl 0.05 *N*	6,943	41.9	4,484	28.8	7,477	38.4
NaOH 7%	5,478	33.1	9,854	63.2	5,867	30.2
NaOH 17.5%	2,050	12.4	—	—	3,504	18.0
KOH 1.5 *M*	1,510	9.1	—	—	2,291	11.8
Residue, α-cellulose	0	0	—	—	0	0

result of the 0.45 *N* NaOH treatment of the grains prevented a complete fractionation. Over two-thirds of the total activity was found in the fractions obtained with 0.05 *N* HCl and 0.1 *N* NaOH. This agrees quite closely with the data shown in Table 6. Again, a zinc fraction was observed, so firmly attached to the cell wall components, that only boiling 1.5 *N* KOH treatment could remove it.

In Table 10, the zinc activities in the main fractions obtained in the three experiments are compared. Due to the slightly different extraction techniques, and in order to obtain comparable figures in Experiments B and C, the activity due to the 'prolamine' and 'gluteline' fractions had to be transferred from the protein to the solid residue. It could be noted, generally, that the distributions of Zn-65 detected in the first two experiments were rather similar for all three plant fractions. For the last experiment, a large increase in

TABLE 10

Activity of Zn-65, expressed as percentage of total recovered activity in the main tissue fractions obtained in Experiments A, B and C

Experiment	Time of harvest after Zn-65 application.	Roots % of Zn in:			Grains % of Zn in:			Leaves % of Zn in:		
		Protein +	Solid residue	Non-dial. frac.	Protein +	Solid residue	Non-dial. frac.	Protein +	Solid residue	Non-dial. frac.
Exp. A	3 days	54.0	46.7	22.8	45.5	57.2	5.5	71.2	38.6	21.5
Exp. B	3 days	48.8	52.2	27.9	40.5	59.5	15.7	60.0	40.0	34.0
Exp. C	5 days	91.8	8.2	1.8	80.3	19.7	0.4	89.4	10.6	1.8

TABLE 11

Zn-65 activity extractable from roots and grains of corn seedlings with H_2O and C_2H_5OH expressed cpm extracted per unit period of two hours

Extraction time, hours	Grains cpm		Roots cpm	
	C_2H_5OH	H_2O	C_2H_5OH	H_2O
2	28,700	24,000	51,300	38,500
4	10,450	4,070	8,300	4,050
6	7,140	*	6,140	—
8	4,600	—	3,600	—
24	1,350	695	1,290	335

* No extractions were made with H_2O at 6 or 8 hours.

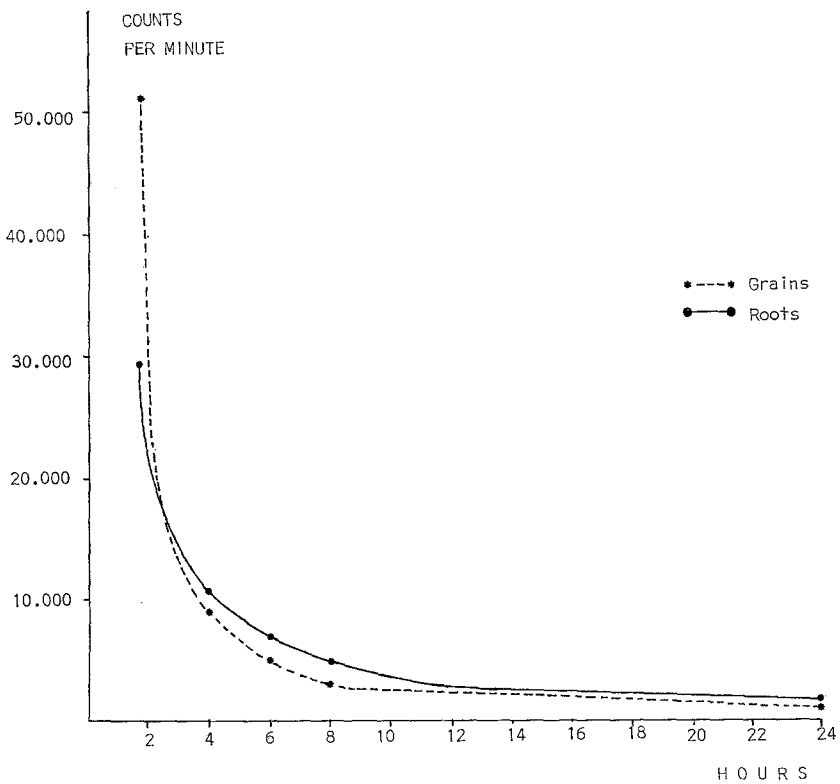


Fig. 1. Cpm *vs* time in the ethanol extraction of corn tissues.

the soluble part was observed. It is suspected that this figure might include some broken-down fractions of a non-proteinaceous nature.

The results of an experiment on the solubilization of the zinc-65 in corn seedlings are presented in Table 11. Only results for grains and roots are presented, since even after 24 hours, no zinc-65 was extracted from the leaves.

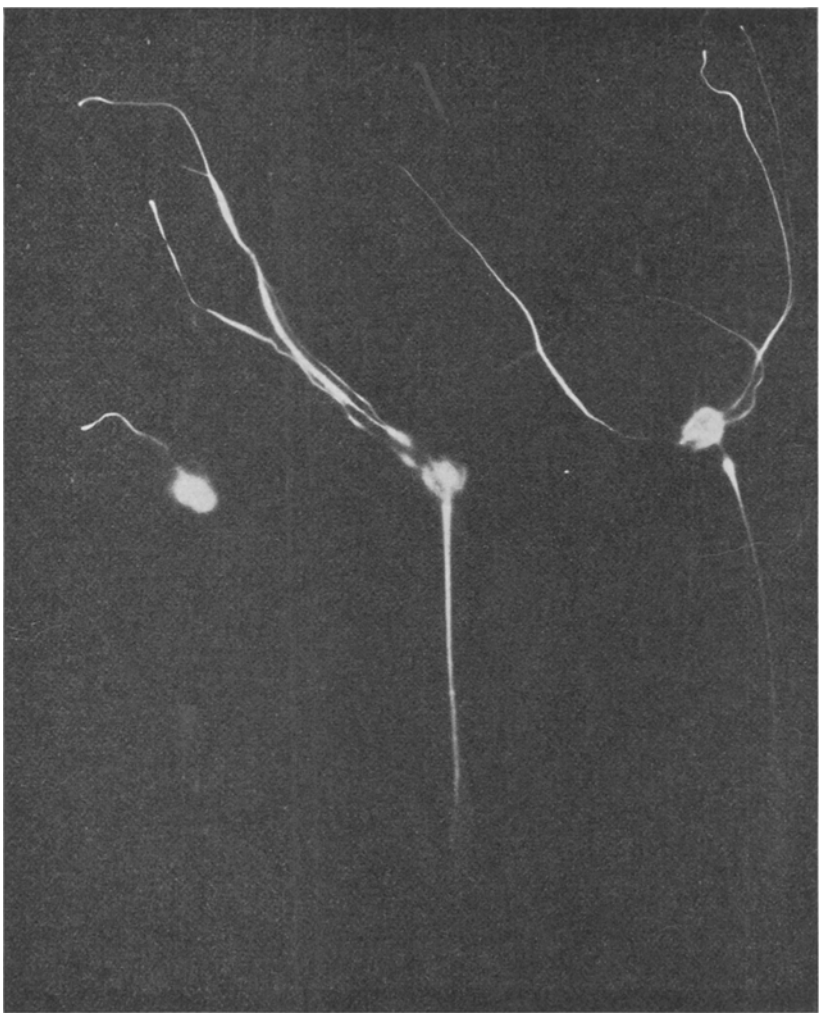


Fig. 2. Radioautograph (80% original size) showing Zn-65 distribution in corn seedlings.

In Fig. 1, the cpm measured are plotted against time for the ethanol extraction, based on unit times of two hours each. The extraction curves are very similar for roots and grains and appear to be of a hyperbolic nature.

The movement of zinc in the seedlings is illustrated by calculating the rations of the 'specific activities' (cpm/g fresh tissue) of roots, versus leaves. The obtained ratios are given in Table 12, for the different periods the seedlings were allowed to grow in the presence of Zn-65. This table clearly shows the movement of zinc-65 from the roots to the leaves, confirming the observations of many previous investigators [11 14 16 18 21 25].

TABLE 12

Ratios of cpm/g tissue of roots *vs* leaves, at different times after adding Zn-65 to germinating corn seeds			
Days between harvest and Zn-65 application	2	3	5
cpm/g tissue of roots *vs* leaves	4.94	2.85	1.90

The radioautograph of seedlings given in Fig. 2 obtained from plants exposed for two days to zinc-65, also shows the largest concentration in the roots and grains and only smaller amounts in the leaves.

DISCUSSION

The data show that a considerable part of the zinc absorbed by plants is closely associated with the proteinaceous components. It can be noted from the results of Experiments B and C, that the extraction techniques separated more than half of the zinc of the plant in the soluble 'protein plus' fraction. For both experiments, most of the zinc was in the roots and leaves, that is, in the actively growing tissues. This zinc was extracted with the basic proteins, albumines and globulines, a fraction including nucleo-proteins and many enzymes. The solubility class of prolamines and glutelines also contained an important fraction of zinc. The presence of non-dialyzable zinc could be observed in all separated fractions. Its concentration was never less than 0.1 per cent of the total absorbed. This agrees with the report of Sibly and Wood [19], indicating at

least 0.56 per cent zinc in their carbonic anhydrase preparations. It appears probable that zinc is bound, in one or more forms, to proteins. This bond, or one of them, is believed to be a rather strong one [12]. The capacity of zinc to form stable complexes is well known. In these complexes, sulphur is considered as the most adequate electron pair donor. Zinc forms stable complexes with organic amines, diamines, and alkyl groups as well [15]. The reports of Kägli and Vallee [13] and of Friden [6], indicating the interference of zinc complexing molecules as 8-hydroxyquinoline-5-sulfamic acid with the functioning of zinc enzymes, make reasonable the assumption that a chelate-type binding exists between zinc and the protein moieties. The lack of exchange with added zinc-65 in short term experiments as reported by Tupper *et al.*, would also support this observation [23].

A variable amount of zinc remained in the residue (6.2–43.6 per cent of the total) after separating the cytoplasmic fraction. The largest proportion of this corresponded to the solubility fractions designated as protopectines and as hemicelluloses in Table 1. It is worth noting the presence of a small but definite amount (0.6–2.9 per cent) of zinc-65 which remains after most of the cell wall constituents are extracted. The importance of zinc in cell walls is evident from the work of Hewitt *et al.* [10], reporting exudations as the result of cell wall deterioration in case of zinc deficient plants. This evidence indicated a role for zinc in the structure and function of cell walls.

The remaining zinc residues could only be extracted by boiling with 1.5 *N* KOH, leaving a rather pure α-cellulose behind.

The most marked effect of zinc deficiency in higher plants is the decrease of stem elongation. Experiments by Skoog [20], have shown the close relationship between the auxin and zinc, possibly due to the importance of the element in the formation of the precursor molecules as indicated by Tsui [22]. While the function of auxin in cell elongations is still not understood, there is some reactive site in the complex group of cell wall materials which is essential for auxin action as shown by Galston and Purves [8]. There is ample evidence proving an attachment of auxin to some receptors in the cell, based on kinetic experiments and on structure-activity considerations [2 24]. However, in the presence of a buffer which forms stable metal complexes, Galston and Kaur [7] did not

find any evidence for the bindings of auxines to cell sites. It might be possible, that zinc is a binding agent forming a bridge between the cell sites and the auxin or auxin-enzyme complexes. This could explain in part, the almost ubiquitous distribution of zinc including its presence in the hemicellulose extracts, and its roll in auxin inhibition noted by Galston and Kaur [7].

SUMMARY

The distribution of zinc in the different solubility groups of proteins and cell wall constituents was studied, using Zn-65 in small maize plants.

Most of the Zn-65 was recovered in the soluble 'protein' fraction. The highest values correspond to the solubility groups for basic proteins, albumines and globulines. The prolamines and glutelines also contained an appreciable proportion. Non-dialyzable zinc percentages varied considerably between 0.1 per cent and 19.0 per cent, of the zinc absorbed by a tissue being the lowest figures characteristic for the saline extracts and especially for the grains.

Between the cell wall constituents, most Zn-65 was present in the solubility groups of protopectines and hemicelluloses. After different extractions, a rather tightly bound zinc fraction remains attached to the cell wall. This can only be separated by boiling with KOH, leaving zinc-free cell wall material. The pure α-cellulose walls contained no Zn-65.

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LITERATURE CITED

1 Bonner, J. S., Plant Biochemistry. Academic Press, N. Y. 537 pp. (1950).
2 Bonner, J. S., The Hormonal Control of Plant Growth. Harvey Lectures, Ser. **48**, 1–34 (1954).
3 Boroughs, H. and Bonner, J., Effects of indoleacetic acid on metabolic pathways. Arch. Biochem. Biophys. **46**, 279–290 (1953).
4 Bukovac, M. F. and Wittwer, S. H., Absorption and mobility of foliar applied nutrients. Plant Phys. **32**, 428– 435 (1957).
5 Davidson, J. N., The Biochemistry of Nucleic Acids. 4th. ed. J. Wiley, N. Y. 288 pp. (1957).
6 Friden, C., The dissociation of glutamic dehydrogenase by reduced diphosphopyridine nucleotide (DNPH). Biochim. et Biophys. Acta **27**, 431–32 (1958).
7 Galston, A. W. and Kaur, R., An effect of auxines on the heat coagulability of the proteins of growing plant cells. Proc. Natl. Acad. Sci. U.S. **45**, 1587–1590 (1959).

8 Galston, A. W. and Purves, W. K., The mechanism of action of auxin. Ann. Rev. Plant Physiol **11**, 239–276 (1960).
9 Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical Physiological Chemistry. 13th. ed. McGraw-Hill, N. Y. 1439 pp. (1954).
10 Hewitt, E. J., Bolle-Jones, E. W., and Miles, P., The production of copper, zinc, and molibdenum deficiencies in crop plants grown in sand culture with special reference to some effects of water supply and seed reserves. Plant and Soil **5**, 205–222 (1954).
11 Hewitt, W. and Gardner, M. E., Some studies on the adsorption of zinc sulfate in Thomson seedless grape canes. Plant Physiol **31**, 393–99 (1956).
12 Hoch, F. L. and Vallee, B. L., The metabolic role of zinc. *In* Trace Elements. eds. C. A. Lamb, O. G. Bentley, and J. M. Beattie. pp. 337–363. Academic Press Inc., N. Y. (1948).
13 Kägli, J. H. R. and Vallee, B. L., The role of zinc in the alcohol dehydrogenase. V. The effect of metal binding agents on the structure of yeast alcohol dehydrogenase molecule. J. Biol. Chem. **238**, 3188–3192 (1960).
14 Langstone, R., Distribution patterns of radioisotopes in plants. Proc. Am. Soc. Hort. Sci. **68**, 370–76 (1956).
15 Moeller, T., Inorganic Chemistry, an Advanced Textbook. J. Wiley, N. Y. 966 pp. (1952).
16 Riceman, D. S. and Jones, G. B., Distribution of zinc in subterranean clover grown to maturity in culture solutions containing zinc labelled with the radioactive isotope Zn 65. Australian J. Agr. Research **9**, 730–744 (1958).
17 Riceman, D .S. and Jones, G. B., Distribution of zinc and copper in subterranean clover (*Trifolium subterraneum* L.) in culture solutions supplied with dugraated amounts of zinc. Australian J. Agr. Research **9**, 73–122 (1958).
18 Sayre, J. D., Accumulation of radioisotopes in corn leaves. Ohio Agr. Exp. Sta. Res. Bull. N° 723. 30 pp. (1952).
19 Sibly, P. M. and Wood, J. G., The nature of carbonic anhydrase from plant sources. Australian J. Sci. Research Ser. B-**4**, 500–510 (1951)
20 Skoog, F., Relationships between zinc and auxin in growth of higher plants. Am. J. Botany **27**, 939–951 (1940).
21 Stout, P. R., Micronutrients in crop vigor. J. Agr. Food Chem. 4, 100– 106. (1956)
22 Tsui, Ch., The role of zinc in auxin synthesis in the tomato plant. Am. J. Botany, **35**, 172–79 (1948) .
23 Tupper, R., Watts, R. W. E., and Wormall, A., Some observations on the zinc in carbonic anhydrase. Biochem. J. **50**, 429–432 (1951).
24 Veldstra, H., The relation of chemical structure to biological activity in growth substance. Ann. Rev. Plant. Physiol. **4**, 151–198 (1953).
25 Woltz, S., Toth, S. J., and Bear, F. E., Zinc status of New Jersey soils. Soil Sci. **76**, 115–122. (1953).
26 Wood, J. G. and Sibly, P. M., The distribution of zinc in oat plants. Australian J. Sci. Research Ser. B-**3**, 14–27 (1950).