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PERSONAL REFLECTIONS ON A GALVANIZING TRAIL

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ABSTRACT

This article encompasses my perception of, and experience in, an exciting segment of the trace element era in nutrition research: the role of zinc in the nutrition of animals and humans. Zinc has been a major player on the stage of trace element research, and it has left a trail that has galvanized the attention of many researchers, including myself. It is ubiquitous in biological systems, and it plays a multitude of physiologic and biochemical functions. A brief historical overview is followed by a discussion of the contributions the work done in my laboratory has made toward understanding the physiological and biochemical functions of zinc. The effort of 40 years has led to the belief that one of zinc's major roles, and perhaps its first limiting role, is to preserve plasma-membrane function as regards ion channels and signal transduction. Although substantial knowledge has been gained relating to the importance of zinc in nutrition, much remains to be discovered.

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PROLOGUE

During the middle of the twentieth century, research related to micronutrients shifted rather dramatically from the "vitamin era" to the "trace element era." The essentiality of a few microelements—including copper, zinc, iron, and iodine—had been established, but until crystalline vitamins and amino acids became available for the preparation of highly purified experimental diets, progress in trace element research had been hampered. Concurrent with the availability of crystalline vitamins and amino acids, much more sensitive and convenient tools for quantitative analysis of tissues and food for inorganic elements were developed. With these new tools for trace element research, and with the exhaustion of the number of new vitamins that can be discovered, it was inevitable that research interest would shift toward the trace elements.

Having grown up on a farm in rural Missouri, I learned early in life the importance of good nutrition for animal growth and productivity. No doubt those farm experiences were influential in my choice of a career in nutritional biochemistry. Another early experience, the Great Depression of the 1930s, which was under way during my high school days, no doubt also had a significant effect on my career and lifestyle. There was no money for college when I graduated from high school, and I started teaching in a one-room rural school, qualified by a teachers' certificate based on an examination and one summer at a teachers' college. By attending summer school and taking correspondence courses while teaching, I completed in 4 years the equivalent of 2 years of college. With a little saving from the meager salary of those depression days, I was able, in 1937, to enter the University of Missouri. There I received a bachelor's degree with a major in chemistry. I was attracted to the biological aspects of chemistry and, upon entering graduate school, had the good fortune to come under the tutelage of the late Albert G. Hogan, Chair of the Department of Agricultural Chemistry. Dr. Hogan had an active nutrition research program and had just identified a new vitamin required by chicks, which he named B_c. My assignment was to isolate the vitamin, using anemia in chicks as the assay tool. Although progress was made in purifying the vitamin, I did not see pure crystalline vitamin B_c, now known as folic acid, until I joined the research laboratory of Parke-Davis in Detroit, MI. Nevertheless, I left the University of Missouri with two very important acquisitions, a good education in nutritional biochemistry and the acquaintance of my wife, Vera Stone. Both have provided constant support since that time.

At Parke-Davis, the research team I was on succeeded in isolating crystalline vitamin B_c from both liver and yeast (53). When I returned to the University of Missouri as an assistant professor, my interest in folic acid continued. In cooperation with Professor Hogan, I observed that deficiency of folic acid in pregnant rats caused neural tube defects, notably hydrocephalus, in the pups (50). This was followed by the observation, also in rats, that vitamin B_{12} deficiency produced similar congenital anomalies (51).

In retrospect, the isolation of folic acid and vitamin B_{12} closed the vitamin era and ended a period of research during which we expected the announcement of a new vitamin or growth factor at nearly every Federation (FASEB) meeting. Many of us had been pursuing the search for new growth factors, putative vitamins, using natural products as potential sources. It was during such a search that Jim Savage and I observed that the growth factor in distillers dried solubles was not a vitamin but an inorganic nutrient, zinc (48). That was the beginning of the galvanizing trail.

HISTORICAL OVERVIEW

Although there was earlier evidence that zinc exerted an essential biological function, the first clear demonstration that it was essential in the diet of higher animals derived from the work of Todd et al (61) at the University of Wisconsin. Their observation was followed by the isolation of carbonic anhydrase and evidence that zinc is an essential component of this enzyme (29). These cardinal observations were followed, slowly at first but with accelerating pace, by other observations showing the nutritional significance of zinc. Table 1 lists highlights (not comprehensive but of special significance to me) of zinc research as it relates to nutrition.

Skin lesions, including parakeratosis, were known by 1941 to be a cardinal sign of zinc deficiency (18), but the role of zinc in preventing parakeratotic lesions in pigs raised under practical conditions of swine production was not appreciated until more than a decade later (62). It was even longer before there was evidence that zinc deficiency occurs in normal free-living humans (23, 54), as well as in humans afflicted with the genetic disease acrodermatitis enteropathica (33). An important aspect of the occurrence of zinc deficiency in animals and in humans who consume diets composed of natural foods relates to the low bioavailability of zinc in certain food components. Phytic acid, an organic phosphorus compound associated with seed proteins, decreases the bioavailability of zinc (49) and may induce zinc deficiency in the presence of otherwise adequate dietary zinc levels. Concurrent with the elucidation and description of zinc's physiological functions, biochemical work revealed the extensive occurrence of zinc in metalloenzymes (64) and in gene transcription

Table 1 Highlights of zinc research

Year	Observation	Reference
1934	Zinc is an essential nutrient: deficiency in rats retarded growth	61
1939	Carbonic anhydrase is a zinc metalloenzyme	29
1941	Zinc deficiency in rats caused parakeratosis and hyperkeratosis	18
1955	Zinc prevented swine parakeratosis accentuated by high calcium intake	62
1957	Isolation of metallothionein	31
1960	Phytic acid decreased zinc bioavailability	49
1961	Signs of zinc deficiency in young men living in Iranian villages	54
1966	Zinc deficiency resulted in congenital malformations	26
1968	Zinc deficiency led to dystocia and excessive blood loss at parturition	1
1970	Decreased appetite and cyclic food intake in rats fed a low-zinc diet	13
1972	Zinc deficiency observed in US children	23
1974	Acrodermatitis enteropathica is caused by failure of zinc absorption	33
1977	Zinc deficiency associated with helper T-cell dysfunction	19
1977	Experimental zinc deficiency in adult women	25
1981	Concept that zinc's first limiting function relates to plasma membrane	3
1984	Concept that zinc metalloenzymes are ubiquitous	64
1985	Concept of zinc fingers in gene transcription proteins	24, 32
1990	Zinc deficiency affects locomotion by inducing peripheral neuropathy	41
1991	Zinc deficiency impairs platelet function by decreasing calcium influx	43
1994	Zinc deficiency decreases calcium flux across brain synaptic membranes	10

factors (2). Despite the fact that zinc is essential for the function of these proteins—which provide critical support for metabolism and gene expression—assessment of their activities has not proven useful in the evaluation of zinc status, probably because they bind zinc tenaciously. More recent work points to a first limiting role for zinc in the preservation of cell plasma membrane function (10, 43). Before addressing these biochemical aspects of zinc in nutrition, I wish to discuss the development of the galvanizing trail from the biased and provincial viewpoint of our laboratory.

BIOAVAILABILITY OF ZINC IN FOOD

In my case, transition from the vitamin to the trace element research era resulted from the serendipitous observation that zinc was the unknown growth factor in a natural product used to supplement a purified diet (45, 48). That zinc is essential to mammals was well established, but this somewhat fortuitous observation occurred because my colleagues and I, as well as several other investigators, had recently changed protein in the basal diet from an animal to a plant source. Unknown at the time was the fact that the mineral supplement commonly used in casein-gelatin–based diets did not supply sufficient zinc

when soy was the source of protein. This observation led us to seek the basis for the incongruity.

One obvious difference between proteins of plant seeds and those of animal origin was that plant proteins contain phosphorus in the form of phytic acid whereas animal proteins do not. Furthermore, phytate was known to form stable and insoluble complexes with divalent cations; phytin, for example, a calcium/magnesium phytate complex, was a well-recognized by-product of the corn milling process. Based on these facts, Jim Savage and I decided to determine the effect on zinc bioavailability of adding phytic acid to a casein-gelatin–based diet. The response of animals fed a phytate-supplemented, casein-gelatin diet (Figure 1) was comparable to those fed a soy-based diet with a similar concentration of phytate (49). In an avian species phytate clearly decreased zinc

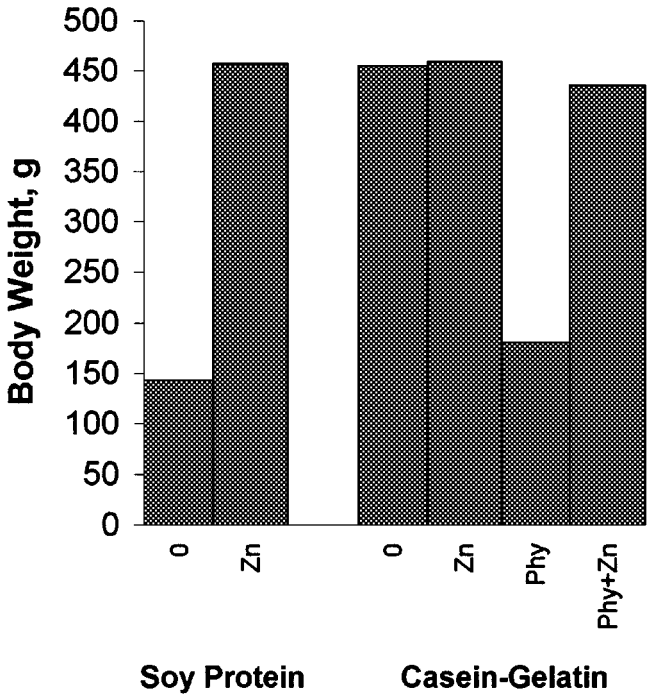

Figure 1 Phytate decreases zinc bioavailability in growing chicks. Chicks were fed basal diets based on soy protein or casein plus gelatin. Zinc (55 mg/kg) improved the growth rate of chicks fed the soy but not the casein-gelatin basal diet. Zinc improved growth when the latter diet was supplemented with phytate at a level comparable to that found in the soy protein diet. (Adapted from Reference 49.)

bioavailability. The results were confirmed in pigs (34) and rats (35). Earlier work in our laboratory (45) had shown that high levels of calcium aggravated signs of zinc deficiency when added to diets in which the components were primarily of plant origin. That this effect resulted from an interaction of calcium, phytate, and zinc was demonstrated in rats (35), pigs (34), and chicks (52) using casein-based diets supplemented with phytate. A high-calcium concentration in the diet increased the requirement for zinc only when phytate was present. The calcium-zinc-phytate complex was even less soluble than was the zinc-phytate complex. In this regard, it is interesting that addition of another zinc chelator, EDTA, to the diet was beneficial when the diet contained phytate, increasing the bioavailability of zinc in rats (35) and chicks (52). EDTA forms a complex with sufficient stability to compete successfully with phytate for zinc, but importantly, the complex is soluble. Presumably, intestinal zinc absorption sites can compete with the soluble EDTA complex for zinc, although not as successfully as with the naturally occurring peptide-zinc complexes commonly found in the intestine. From observation of these calcium-zinc-phytate interactions has come the idea that the bioavailability of zinc in a food correlates inversely with that food's phytate concentration and even better with the product of its calcium $\times$ phytate concentrations.

We next attempted to evaluate quantitatively the bioavailability of zinc in foods and diet ingredients using the growth response of rats and chicks as a measure of zinc absorption and utilization (40). The relative bioavailability of the zinc in cereal grains was substantially lower than that in foods of animal origin. Later, the slope-ratio method was used in a cooperative project involving four laboratories on the University of Missouri at Columbia campus. The relative bioavailability of zinc was measured in beef and soy flour using four different species, human, pig, rat, and chick (36). The zinc in beef had a relative bioavailability of nearly 100% as measured in all species, but that in soy flour was generally low and varied among species. The zinc bioavailability in soy flour, as evaluated in humans and pigs, was actually negative. As measured in chicks, it was low, approximately 20%, and in rats it was approximately 75%. Thus, the pig provided the best experimental model for humans; the commonly used laboratory species, the rat, provided the poorest model. There are relatively few data to provide guidance as to the bioavailability of zinc in human diets, but dietary phytate and calcium concentrations serve as useful indicators.

PHYSIOLOGICAL FUNCTIONS

A common technique for determining the function of a nutrient is to observe the pathology that results from the lack of it. Gross and microscopic pathology provide insight into the physiologic and biochemical systems that are first

limited by deficiency. As in mammalian species, zinc deprivation in growing chicks led to decreased growth rate and food intake, skin and bone lesions, and dysfunction of the neuromuscular system (45). Zinc deprivation during gestation in rats led to reproductive difficulties, including dystocia and excessive blood loss during a prolonged and labored parturition. The females exhibited a type of physiological shock, including low blood pressure, low body temperature, and general depression (47). The bases of these pathologies were pursued experimentally and are discussed below.

Growth and Appetite

Clearly, animals cannot grow without eating, but there is mounting evidence that loss of appetite in zinc-deprived organisms results from their inability to grow, i.e. failure of cell division. The lack of zinc in growing rats quickly induces growth failure accompanied by loss of appetite and cyclic food intake. Cyclic feeding patterns also occur in other species, including the chicken (see 46). To determine the basis of the decreased and cyclic food intake, Phil Reeves and I (55) allowed rats free choice of low- and high-protein diets and measured the respective intakes. Rats that consumed diets inadequate in zinc chose a lower proportion of protein but continued cyclic intake. At the cycle nadir they consumed the same percentage of protein as did the controls, but at the high-intake point they consumed a lower percentage. The tyrosine concentration in brain was elevated in deficient rats, and this may relate to the depressed food intake. Tyrosine is a precursor of the hypothalamic catecholamines that are involved in the control of food intake. During the early stage of zinc deprivation, hypothalmic norepinephrine concentration was above normal, but this anomaly did not endure (56). Feeding zinc-deficient rats a low-zinc diet based on pure amino acids without tyrosine significantly increased food intake over that of zinc-deficient rats given 0.6% tyrosine. The results of these experiments suggest that catecholaminergic neurons are involved in the regulation of food intake in zinc-deprived rats, but undoubtedly other appetite factors are also involved. In recent studies with rats, my colleagues and I (9) have been able to uncouple food intake and growth. Oral administration of megestrol acetate, a progestin, significantly increased the food intake of zinc-deficient rats and largely eliminated its cyclic aspect. After a few days of treatment the food intake of the zinc-deficient rats was not different from than that of rats with an adequate amount of zinc; however, growth rate was not improved by megestrol acetate. The mechanism by which megestrol acetate stimulates appetite is not clear, but it appears to bypass the pathway impaired by zinc deficiency.

While it is obvious that young animals cannot grow unless they eat, they appear to lose appetite if cell division cannot occur. Zinc has long been associated with DNA synthesis, an important component of the cell cycle, but

its role in the process is unknown. Several different growth factors are required to initiate and sustain the cell cycle. Recently, we deprived murine 3T3 fibroblasts of zinc by addition to the culture media of an impermeant chelator, diethylenetrinitrilopentaacetate (DTPA), and measured thymidine uptake (RS MacDonald, LC Wollard-Biddle, JD Browning, WH Thornton, BL O'Dell, submitted for publication). When the cells were stimulated sequentially with platelet-derived growth factor, epidermal growth factor, and insulin-like growth factor-I (IGF-I), the critical need for zinc occurred during the IGF-I–stimulation step, suggesting that IGF-I signal transduction is impaired by lack of zinc. It is significant that DTPA, which binds zinc tenaciously and reduces free zinc to a negligible concentration, did not decrease the measurable concentration of zinc in 3T3 cells. Extrapolating these results to the whole animal, one might predict that failure of IGF-I signal transduction is associated with growth failure in zinc deficiency. The plasma concentration of IGF-I is low in zinc deficiency, but its restoration did not restore growth in zinc-deprived rats (9). Growth failure and loss of appetite may result from impaired transduction of the same, or closely related, biochemical signals.

Bone and Integument

Zinc deficiency in growing chicks led to shortened and thickened long bones and enlarged tibial-metatarsal joints (45). The epiphyseal cartilage was reduced in width and showed depressed cell division. Consumption of a low-zinc diet led not only to bone pathology, but also to a markedly lower bone zinc concentration (59). While decreased bone zinc concentration may not seem remarkable, zinc deprivation had little or no effect on the zinc content of most soft tissues. Similar observations have been made in guinea pigs (37). Bones of pigs lost zinc during zinc deprivation, so bone zinc concentration served as a good index of long-term zinc status (8).

Pathology of the skin and its appendages, first described in rats (61), is one of the most obvious signs of zinc deficiency. In early studies with chicks, skin lesions and poor feather development were observed (45). Most prominent was hyperkeratinization of the skin, particularly on the legs and feet, but there was also parakeratosis of the esophagus similar to that observed in the skin of pigs. Later work uncovered a connection between dietary fatty acids and zinc, manifesting in skin lesions (5). The consumption of diets low in polyunsaturated fatty acids protected chicks against dermal lesions when zinc was limiting. While the basis of this protective action is not entirely clear, later observations (6) suggested that consumption of polyunsaturated fatty acids aggravated oxidative damage to tissues when zinc was limiting. Addition of antioxidants, particularly vitamin E, to the diet exerted a protective effect against skin lesions.

Parturition and Smooth Muscle

Zinc deficiency causes reproductive failure in both male and female rats. In the female, there is failure of conception, failure to suckle, death of fetuses and neonates, and congenital malformation of pups. Another reproductive problem is the prolonged and labored parturition associated with zinc deprivation during the gestation period. At or near term, pregnant rats exhibited signs of low blood pressure, low body temperature, and excessive blood loss. These signs were analogous to those of aspirin toxicity (47) and led to the exploration of a possible relationship between zinc status and impaired prostaglandin (PG) production and function. Aspirin inhibits the production of PGs, modulators of parturition, blood pressure, and hemostasis. It was predicted that low PG concentrations would be observed in zinc deficiency. However, measurement of PG metabolites in plasma showed that they were not lower but actually higher in the plasma of deficient than in that of control female rats (38). The possibility remains that PG function is impaired because of failure of signal transduction. In any case, the difficult parturition and low blood pressure associated with zinc deficiency are undoubtedly the result of smooth muscle malfunction in the uterus and vascular system.

Blood Pressure and Body-Water Distribution

Low blood pressure and a dehydrated appearance are prominent signs of zinc deficiency in rats near the end of gestation (47). Smooth muscle function, whether in the uterus or the vascular system, is regulated by nerve and hormone signals that must be transduced at the plasma membrane. Impairment of the transduction process would explain many of the observed physiological signs of deficiency. To measure response of the vascular system during zinc deficiency, rats were treated with two vasodilators, bradykinin and prostacyclin (12). The blood pressure response was impaired, suggesting that the signals were not properly conveyed to smooth muscle cells. Angiotensin II, a product of the reaction catalyzed by angiotensin-converting enzyme (ACE) and a vasopressor, is another regulator of blood pressure. Activity of this enzyme was found to decrease by 30% in rats deprived of zinc for 4 days and ACE activity was restored within 12 hr by zinc repletion or by addition of zinc to the assay medium (57). Thus, ACE activity reflected primarily plasma zinc status. Furthermore, depressed plasma ACE activity in zinc-deficient rats had no effect on the angiotensin II concentration (58). The plasma concentration of angiotensin II is not limiting in zinc deficiency, but angiotensin II function may be impaired.

Dehydrated appearance and chronic diarrhea have been observed in many species deficient in zinc. Associated with these conditions are higher-than-normal hematocrit values (45) and an increased preference for salt in drinking

water. In spite of these signs of dehydration, my colleagues and I found no evidence in either chicks or rats that total body water was decreased. However, the distribution of body water in zinc-deficient chicks shifted from the extra- to the intracellular compartment (7). The high hematocrit values of zinc-deficient chicks were restored to normal within a few hours after administration of zinc. While the zinc-dependent mechanism involved in abnormal tissue-water distribution is unknown, it is significant that the pathology is readily reversible by restoration of the extracellular zinc concentration.

Erythrocyte Stability, Hemostasis, and Platelets

Red blood cells from zinc-deficient rats exhibit decreased osmotic stability (3), and the response is affected by other dietary factors, including concentration and source of amino acids and level and type of dietary fat. Abnormal osmotic fragility due to zinc deficiency is readily reversed; zinc repletion for 1 day restored fragility to normal, but addition of zinc in vitro was ineffective (39). The ready reversibility of the decreased red cell stability and the abnormal tissue-water distribution may involve the same or similar mechanisms.

Associated with abnormal osmotic fragility of erythrocytes in zinc deficiency is a tendency to increased bleeding, a sign observed originally in rats at parturition. To investigate the basis of this pathology, Phil Gordon and I (20) studied hemostasis in young male rats. Both bleeding time and blood loss were significantly higher in rats that consumed a low-zinc diet for only 4 days. Blood coagulation was not affected by zinc status, so our attention turned to platelet function (21). Short-term zinc deprivation impaired the response of platelet-rich plasma to such aggregatory agents as ADP, collagen, and arachidonate. This work was extended and confirmed using thrombin (68) as well as ADP (14) in a more quantitative assay. Similar observations were made in humans, and the effect was promptly reversed by zinc repletion (22). Platelet malfunction offers an explanation for the increased bleeding tendency observed in zinc deficiency.

Locomotion, Posture, and Peripheral Nerves

Abnormal posture and locomotion are among the common signs of zinc deficiency. Particularly dramatic were the abnormal stance and gait described in zinc-deficient chicks (45). Associated with these signs was a propensity of the chicks to assume a squatting posture, thereby avoiding support of body weight. Similar behavior was not observed in mammals, with the exception of guinea pigs. This species developed not only abnormal posture but also hypersensitivity to touch, manifested by vocalization during handling (37). A single dose of zinc caused remission of these neuromuscular signs within 5 days, but without further treatment they regressed to the severe stage within 7 days.

These observations accompanied by lack of muscle histopathology suggested peripheral nerve dysfunction. To explore this avenue, my colleagues and I (41) made electrophysiological measurements of sciatic nerves. Of prime significance was the low nerve conduction velocity observed in the zinc-deficient guinea pigs, a parameter that correlated with the neuromuscular signs. Zinc repletion reversed both the neuromuscular signs and the low nerve conduction velocity within 10 days. A later longitudinal study (60) confirmed and extended these observations. After 5.5 weeks of depletion, the conduction velocity of sensory, as well as excitatory, impulses was slower than normal. Significantly, the nerves exhibited no histological lesions. Low nerve conduction velocity in zinc-deficient chicks provided similar evidence of readily reversible peripheral neuropathy (42). The results of these experiments support the idea that locomotion and posture abnormalities of zinc deficiency relate to a biochemical defect in the nervous system rather than in the bones and joints, as many of us had thought earlier.

BIOCHEMICAL FUNCTIONS

So far as is known, zinc exerts all its biochemical functions in association with proteins. The stability of the zinc-protein complexes is highly variable, giving rise to what are termed zinc metalloproteins, highly stable complexes that do not lose zinc during their isolation, and zinc-protein complexes, which dissociate more readily. It follows that the latter would be the first to lose zinc during dietary zinc deprivation. Any zinc-protein complex that performs an essential function would be among the first limiting zinc-dependent systems. However, it is difficult to detect and study such complexes.

Zinc in the form of metalloproteins performs two major biological functions: enzymatic catalysis and maintenance of protein conformation. Zinc appears to perform another function, the preservation of cell plasma membrane function, and it is the latter function that becomes first limiting, presumably because it is dependent on a low-affinity zinc protein complex.

Metalloenzymes

Zinc is an integral part of at least 300 known metalloproteins that exhibit catalytic function. These metalloenzymes are found in all six classes of enzymes and have been isolated from many different species of animals, plants, and microorganisms; they catalyze more than 50 different biochemical reactions. By use of X-ray crystallography, zinc ligands have been identified in 12 of the metalloenzymes (63). When zinc functions at the catalytic site—as in carbonic anhydrase, carboxypeptidase, phospholipase C, and alkaline phosphatase—two histidine residues serve as ligands. The third amino acid ligand varies but is

commonly a carboxy amino acid; the fourth ligand of the tetrahedral complex is a water molecule. In contrast, zinc plays a strictly structural or conformational role in aspartate transcarbamoylase, in which four cysteine residues serve as the binding ligands. While these enzymes and others such as DNA and RNA polymerases and Cu,Zn-superoxide dismutase play critical roles in metabolism, there is no evidence that the activity of these or other zinc metalloenzymes becomes physiologically limiting during the development of dietary zinc deficiency.

DNA Binding Proteins

Since the discovery (24) that zinc is essential for the binding of transcription factor IIIA (TFIIIA) to the 5S RNA gene, it has been determined that this protein contains a series of loops held together at the base by zinc (2). These zinc-containing domains were widely termed zinc fingers, a term inadequate to describe the many structural configurations involving zinc in DNA binding proteins. The zinc ligands in TFIIIA include two cysteine and two histidine residues that form a stable complex with zinc and thereby stabilize a conformation that binds to DNA. However, relatively few transcription factors—only TFIIIA, GAL4, G32P, and glucocorticoid receptor—have been demonstrated experimentally to contain zinc. More than 10 different classes of zinc binding domains have been identified, involving different ligands and giving rise to such names as zinc twist, zinc cluster, and zinc ring. While gene transcription is a highly important function in biology, and zinc plays a critical role in the process, there is no evidence that transcription factor function is impaired by dietary zinc deficiency.

Plasma Membrane: Ion Channels and Signal Transduction

A major long-term goal of my research in zinc nutrition has been to establish a valid index of zinc status in man and animals, an assessment that would augment another essential criterion, low plasma zinc. Such an index should have a biochemical basis and reflect the first limiting biochemical function that occurs during the early stages of zinc deprivation. As might have been predicted because of their extreme stability, zinc metalloenzymes and transcription factors have not proven to be useful indices. For this reason my colleagues and I directed our research effort toward another function of zinc, its role in protecting plasma membrane function.

The first indication that zinc plays a role in preserving plasma membrane function was the observation of increased osmotic fragility in erythrocytes from zinc-deficient rats (3). This observation was a surprise in that the decreased tissue concentration of a nutrient would be expected to be associated with deficiency pathology. There was no measurable change in the zinc concentration

of the deficient red blood cells. Further probing showed that the increased erythrocyte fragility was associated with decreased plasma membrane zinc in rats (28) and pigs (27); the change in this small compartment of the cell was not detectable by total cell analysis.

Since erythrocyte function is largely passive in nature, the blood platelet was used to determine biochemical function. Platelets are cell-like structures that exhibit a sensitively controlled physiological function, aggregation. Platelets must take up calcium from the external medium in order for aggregation to occur. The external calcium concentration required for rat platelets is higher than that for human platelets, so "citrated" rat plasma does not permit aggregation (15). Calcium uptake by rat platelets provides a useful model for the study of membrane function. Platelets from rats fed a low-zinc diet exhibited decreased calcium uptake when stimulated with ADP (43), thrombin (68), or fluoride (16). The decreased uptake correlates with impaired aggregation and offers a biochemical explanation for the observed pathology.

When we began to search for the biochemical defect in the nervous system of zinc-deficient guinea pigs, it seemed reasonable to pursue calcium uptake in this tissue, considering the similarities of excitability in platelets and neurons. Both have agonist-gated calcium channels that are activated by organic stimulants, thrombin and glutamate, respectively. Glutamate is the primary neurotransmitter in the brain. Calcium uptake due to potassium depolarization was decreased in synaptic membranes prepared from both the cortex (10) and hippocampus (11) of zinc-deficient guinea pigs. Glutamate-stimulated calcium uptake was also impaired. Extrapolation of these results to the neuromuscular junction could explain the abnormal posture and gait observed in zinc-deficient guinea pigs.

Calcium serves as a second messenger for many cell signals. Figure 2 summarizes our concept of how zinc deficiency impairs the function of calcium in platelet aggregation. Protein kinase C (PKC) is a calcium-dependent enzyme; it is also a zinc metalloenzyme, but its concentration in platelets is not affected by zinc status. Thrombin stimulation of platelets results in a transient increase in calcium concentration, thereby activating PKC, an enzyme whose activity is essential for platelet aggregation (68). While in vitro addition of zinc increased phorbol ester binding to platelets and platelet membranes (65), low zinc status had no effect on the aggregation response to phorbol ester stimulation when calcium in the medium was low (66). Increasing the calcium concentration of the external medium improved the response of control platelets but had no effect on platelets from zinc-deficient rats. The location of PKC within the platelet (66) is regulated by calcium concentrations in cell compartments, a condition that in turn is affected by zinc status. In spite of PKC's critical role in platelet function, its concentration and cellular location are not limiting in zinc deficiency; failure

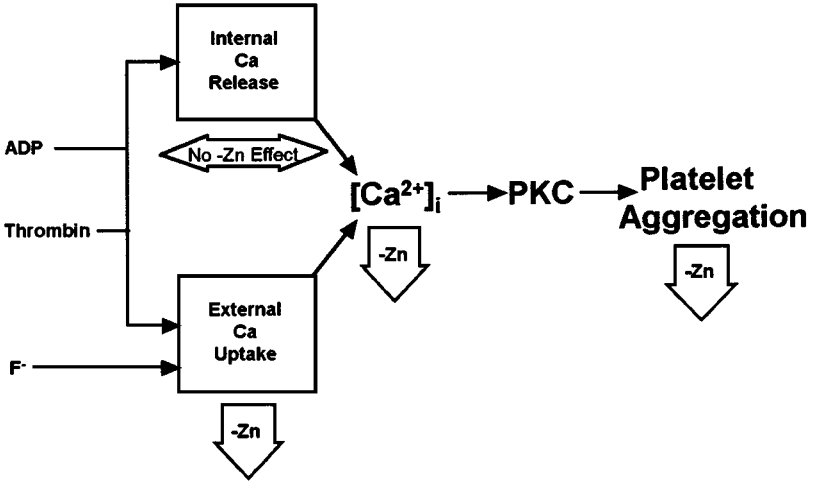

Figure 2 Zinc deficiency in the rat (-Zn) impairs platelet aggregation and calcium uptake when platelets are stimulated by ADP, thrombin, or fluoride. Protein kinase C (PKC) activity is calcium dependent and is essential for platelet aggregation. (Adapted from Reference 68.)

of the transient increase in cytosolic calcium that is required for PKC activation appears to be the limiting factor.

As pointed out above, zinc exerts a distinct role in the maintenance of plasma membrane function, particularly calcium-channel function. Zinc appears to exert a protective role. Supporting this idea is the fact that the plasma membrane of red cells from zinc-deficient rats contains less zinc than normal. This decrease correlates with low plasma-zinc concentration; in contrast, cytosolic zinc concentration does not change significantly. The protective mechanism is unclear, but Jinming Xia and I (67) observed that the thiol concentration in plasma membrane proteins is decreased during zinc deprivation, and that the thiol concentration is inversely correlated with osmotic fragility. Both conditions were reversed within 2 days by oral zinc repletion. Clearly, zinc protected against a post-translational modification of plasma membrane proteins that was readily reversible by zinc in vivo. Addition of zinc in vitro was ineffective (39). Measurement of thiol concentration was then extended to platelet membranes (44). Platelet membrane proteins from zinc-deficient rats also contained lower-than-normal sulfhydryl group concentration. Importantly, addition of glutathione to whole blood in vitro restored both platelet aggregation and calcium uptake to normal levels. Erythrocytes played an essential role in restoration of platelet sensitivity. The platelet defects in zinc deficiency may involve glutathione concentration in erythrocytes, but earlier results (17)

showed no significant effect of zinc status on plasma glutathione concentration. Regardless of the mechanism(s) involved, zinc exerts a protective role in the plasma membrane by way of preventing reversible oxidation of protein sulfhydryl groups. Sulfhydryl groups are essential to the function of calcium channels.

Because of the predominant role of calcium as a second messenger in cell-signal transduction, impairment of calcium-channel function could explain most, if not all, of the pathology associated with zinc deficiency. Calcium transients are associated with nerve transmission, cell division, cell differentiation, and many other physiological functions impaired by zinc deficiency. Maintenance of calcium-channel function in plasma membranes offers a unifying hypothesis for the first limiting role of zinc.

EPILOGUE

Besides my interest in zinc, another major research appeal has been the biochemical role of copper. As important as I consider copper to be in nutrition and as great as my fascination with this nutrient is, I do not have the brass to combine that story with the zinc trail. Suffice it to say, we found that copper plays a key role in development of the cardiovascular system through its function in collagen and elastin cross-linking. Another system we found to be particularly vulnerable to copper deficiency is the striatal area of the brain. The dopaminergic neurons of the striatum are particularly at risk, and their loss results in ataxia. Before developing an interest in copper and zinc, we explored the nutritional role of magnesium, particularly its function in the prevention of soft-tissue calcification. Another early research interest was the arginine-lysine interaction and the related role of acid-base balance.

Most of us in academe sit on a three-leg stool—teaching, research, and service. The three components of academic life are highly interactive and each one strengthens the other. My teaching of biochemistry encompassed undergraduate and graduate students alike, as well as students in varying fields—medicine, veterinary medicine, and agriculture. In these experiences, I found nutrition a useful theme for teaching the concepts of metabolism. One of the strengths—some might say weaknesses—of the university system is the committee structure. I certainly paid my dues in many hours of committee work concerned with all sorts of problems, ranging from building planning to disciplinary action. At the national level, I found service on the National Institutes of Health Nutrition Study Section and on several editorial boards both demanding and rewarding. Service on committees and as president of the American Institute of Nutrition, as well as on numerous FASEB committees, provided rich opportunity for professional advancement and development of priceless friendships. I found

sabbatical leaves to be valuable experiences, not only for personal renewal but also for the expanded vision that I believe proved to be a good investment for the University. Particularly valuable were the leaves in Cambridge, England, and Adelaide, Australia. These international experiences and the friendships acquired provided precious memories.

Little did I realize when I started down this galvanizing trail where it would lead or how exciting it would be. Of course, there were many bumps in the road, but fortunately one remembers primarily the highlights and euphoria of new findings and insights. Even more memorable than the science are the people—the students, post-docs, technicians, and colleagues—with whom one has interacted along the way. I will not attempt to acknowledge all the people who have contributed to—truly made possible—the work discussed in this discourse. Many of their names appear in the bibliography. One colleague, James E. Savage, with whom I worked closely in the early phase of the trail, must receive special recognition for his important contributions. Besides the many local, national, and international colleagues who have contributed to my interesting career, I thank the University administrators who supported our research efforts and provided space and facilities, particularly after my retirement. Most of all I want to thank my wife Vera, my daughter Ann, and my son David for their patience when I had to go back to the lab or to a meeting.

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