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Review

Zinc metabolism and homeostasis: The application of tracer techniques to human zinc physiology

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Abstract

Tracer kinetic techniques based on zinc stable isotopes have a vital role in advancing knowledge of human zinc physiology and homeostasis. These techniques have demonstrated the complexity of zinc metabolism, and have been critical to estimating the size and interrelationships of those pools of zinc that exchange rapidly with zinc in plasma and which are likely to be especially important for zinc dependent biology. This paper presents findings from recent research linking a steady state compartmental model with non-steady state post-prandial sampling from the intestine, utilizing a combination of intestinal intubation/perfusion and stable isotope tracer kinetic techniques. The gastrointestinal tract has a central role in maintaining whole body zinc homeostasis. While the fractional absorption of zinc from a meal depends on the quantity of exogenous zinc and on such dietary factors as phytic acid, the fractional absorption does not appear to be dependent on the size of the rapidly exchanging pool of the host. In contrast, the quantity of endogenous zinc excreted via the intestine is positively correlated with both the amount of absorbed zinc and the zinc 'status' of the host, and thus this process has an equally critical role in maintaining zinc homeostasis. The observed alterations in zinc metabolism in some disease states can be understood in the context of known homeostatic processes. In other conditions, however, such alterations as inflammation-associated hyperzincuria and zinc redistribution, the links between homeostatic perturbation and cellular biology are yet to be explained. Thus the challenge remains for research at the whole body level to carefully characterize zinc distribution and exchange under diverse circumstances, while research at the cellular level must elucidate the regulatory processes and the factors to which they respond.

Abbreviations: EZP – exchangeable zinc pool; DS – Down Syndrome.

Introduction

Parallel with the exceptional recent advances in our appreciation of the diversity, versatility and extraordinary importance of the cellular biology of zinc (Cousins 1998; McMahon & Cousins 1998), there has been notable progress in understanding human zinc physiology and homeostasis (Hambidge *et al.* 1998; King *et al.* 2000). Furthermore there has been a great expansion in recognition of the clinical and public health importance of this essential micronutrient (Bhutta *et al.* 1999). The purpose of this paper is to synthesize current concepts of human zinc physiology and homeostasis, based largely on isotopic tracer techniques. To complete the process of integration as far as is possible, the paper will conclude with a review of some clinical conditions and the observed changes in zinc homeostasis, for in these circumstances there may well be clues to both normal and abnormal physiology. This also serves as a challenging reminder that there remains a great deal of essential research ahead before knowledge of the cellular biology of this metal can

be adequately integrated with the clinical and public health sequelae of zinc deficiency.

Tracer techniques

Both radioisotopes and stable isotopes of zinc have been utilized in investigations of human zinc physiology and homeostasis. In experienced hands and with sensitive equipment, radio-tracer techniques have made important contributions to knowledge of human zinc physiology over a period of more than half a century. Prior to the 1980s, tracer studies of human zinc physiology depended on radioisotope techniques and these have been employed effectively to develop detailed compartmental models of zinc metabolism (Wastney *et al.* 1986) and to study zinc absorption and bioavailability (Sandstrom & Lonnerdal 1989).

Over approximately the past twenty years, there has been a steadily growing body of experience and expertise in the application of zinc stable isotope techniques to investigate whole body human zinc homeostasis and physiology. This has been facilitated by advances in analytical instrumentation, especially in the development and application of inductively coupled plasma mass spectrometry (ICPMS). State of the art ICPMS instrumentation is capable of relatively rapid, precise, and accurate measurements of zinc stable isotope ratios.

Of greater fundamental importance, zinc has three stable isotopes for which the natural abundance is sufficiently low to allow their utilization as 'tracers'. These are ^{67}Zn (natural abundance 4.1%), ^{68}Zn (18.8%) and ^{70}Zn (0.6%). The availability of these 3 stable isotopes of zinc in low natural abundance concentration makes it possible to administer all three tracers essentially simultaneously via different routes. One example of the application of multi-tracer techniques is in the development of our compartmental model of zinc metabolism to be described later. The development of this model utilized kinetic data derived from administration of different zinc stable isotopes intravenously, orally in the post-absorptive state and orally with all meals on the same day. A second example is the facilitation of the comparison of the effects of different diets and different chemical forms of zinc on zinc bioavailability and homeostasis. Even with improved analytical sensitivity, however, caution is required to ensure that quantities of isotope administered are not themselves of sufficient magnitude to perturb the very physiology that is being examined.

The safety of the stable isotopes is unquestioned, and it is thus possible to utilize them in studies in women during the reproductive cycle and in the growing child, population groups for which zinc nutriture is of special interest. These techniques can be applied in studies of populations far removed from major research centers, especially in the developing world. This provides unique opportunities to learn from populations whose habitual diets are low in zinc (Lei *et al.* 1996) or are high in factors, especially phytic acid, that impair the bioavailability of this micronutrient (Manary *et al.* 2000). The intricacies of appropriate study design and reliable analyses, however, ideally dictate the involvement of experienced investigators and laboratories.

Tracer techniques open the door to probing key variables of zinc homeostasis. The refinement and application of this methodology represent a major advance in our ability to explore human zinc physiology, to understand the complexities of human zinc homeostasis and to gain new insights into why and when zinc deficiency occurs. Although the ability to perform complex whole body kinetic studies is clearly of importance, simpler applications are also frequently of special value. For example, arguably the most important information that has been derived from their application relates to the central role of the gastrointestinal tract in maintaining whole body zinc homeostasis. This can be achieved quantitatively and is dependent on only simple algebraic equations for data processing. In contrast to traditional metabolic balance methodology, application of these tracer techniques allows separation of individual variables of zinc homeostasis while providing greater accuracy and precision of measurements.

Whole body zinc physiology: Kinetic studies and compartmental analysis

Kinetic and other data derived from a combination of human zinc tracer and metabolic studies quickly become very complex. In these circumstances, model-based compartmental analysis is not only of immediate practical value in data analysis but also of heuristic value in exploring and better understanding the complexities of mammalian zinc metabolism. These models can be extended effectively to assist in linking the cellular biology of zinc to whole body zinc metabolism (Dunn & Cousins 1989).

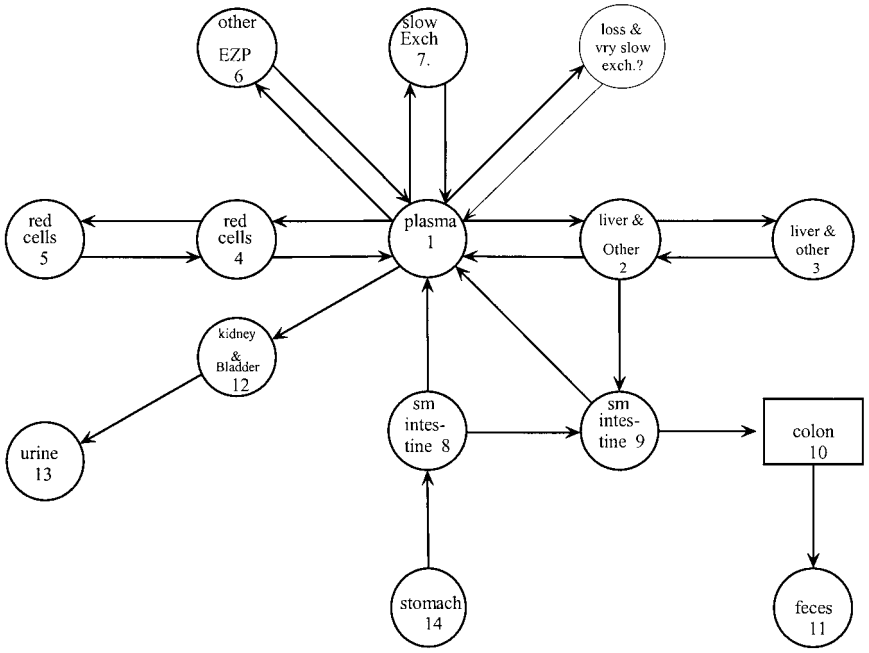

Figure 1. Structure of compartmental model developed to fit zinc stable isotope tracer kinetic data from 5 subjects. The circles represent compartments and are labeled with physiologic/anatomic or kinetic designation. The rectangle indicates the non-mixing delay compartment. (Figure adapted from compartmental model as described in reference Miller *et al.* 2000).

The complexity of human zinc physiology is apparent with the administration of a zinc tracer intravenously even when sampling is limited to blood (plasma and erythrocytes) and excreta. Adequate analysis of such data requires more than a sum of exponential analysis and investigators have turned increasingly to model-based compartmental analyses. Typically, these models have also incorporated additional data derived from the oral administration of tracers, and, in some instances, from regional scanning (radio-tracers only) and a range of steady state data including that derived from simple algebraic equations. Several such models have been reported (Lowe *et al.* 1997; Miller *et al.* 1998, 2000; Wastney, 1989; Wastney *et al.* 1986, 1996, 1991), the complexity of which varies according to the amount of data that is required to fit and other factors. We have recently published a model-based compartmental analysis of the steady state kinetic data obtained from studies in normal adults who received oral (fasting and with meals) and intravenous stable zinc isotopes. The extended multiple studies analysis (EMSA) program of SAAM/CONSAM (Miller *et al.* 1998, 2000) was applied to the individuals' data to derive a composite model (Figure 1). This is in contrast to other reports for which population parameter values were derived from the arithmetic means of individual parameters (Lowe *et al.* 1997; Wastney *et al.* 1986). In our experience, this is the simplest compartmental model that provides an adequate fit for data derived primarily from measurements of enrichment in plasma, erythrocytes, urine and feces over approximately a two week period following administration of zinc stable isotopes orally and intravenously. Both the structural identifiability and validity of this model were thoroughly documented. It includes fourteen compartments and twenty-five kinetic parameters that were not measured directly. Although more slowly exchanging pools were not completely identifiable with the limited duration of these sample collections, this model serves as one illustration of the complexity of human zinc physiology (Miller *et al.* 2000).

Both a strength and a weakness of these compartmental analyses is that, while they help to link kinetic data to some specific organs, the compartments in these models do not, in general, correspond precisely to a specific organ, except for those tissues in which tracer is measured directly. Regional scanning after administration of ^{65}Zn demonstrated, for example, the central role of the liver in zinc metabolism (Wastney *et al.* 1986). It is the principal organ that accounts for the second and third plasma exponential decay curves

for a zinc tracer administered intravenously, and more detailed investigations with an animal model have identified a third rapidly exchanging liver compartment, attributable to hepatic metallothionein (Dunn & Cousins 1989; Lowe *et al.* 1991).

Other rapidly exchanging compartments are less well defined anatomically, but are known to represent zinc in multiple organs. These have been identified to some extent by the use of animal models, in which tracer and tracee have been analyzed in selected individual tissues. For example, zinc in kidney and spleen has been specifically shown to be part of the rapidly exchanging system (House & Wastney 1997). These authors speculated that other components of the immune system contribute to this compartment, and other investigators have demonstrated that the zinc tracer in bone marrow is rapidly exchanging (Dunn & Cousins 1989). Such animal studies have been useful in several ways. For example, the demonstration that even those tissues which account for the great part of the slowly exchanging zinc (e.g., bone) also contain more rapidly exchanging tracer (House & Wastney 1997), suggesting different carriers and/or transporters in tissue subtypes. Even with these animal models, however, there is a notable lack of analytical data for some organs that are of special interest with respect to zinc metabolism, including the central nervous system (Frederickson *et al.* 2000) and the pancreas (Andrews *et al.* 1990; Dalton *et al.* 1996; De Lisle *et al.* 1996; Kelly *et al.* 1996; Onosaka *et al.* 1988; Rofe *et al.* 1999). The importance of these rapidly exchanging pools, defined by their kinetic parameters, will be discussed later in relation to key processes of zinc homeostasis.

Despite the limitations of these models, their full potential has likely not yet been tapped. They have been used only to a limited extent to compare zinc physiology in different populations, for example the elderly (Wastney *et al.* 1992), in conditions of varying zinc intake (Wastney *et al.* 1986), or in disease states in which zinc homeostasis is likely to be perturbed (Lowe *et al.* 1995; Wastney *et al.* 1996, 1999).

The next several paragraphs are devoted to a description of unpublished data by Krebs *et al.* which is included to illustrate the compartmental modeling of a more complex zinc kinetic study that yielded both steady state and non-steady state data and required more detailed modeling of the gastrointestinal tract and intestinal – systemic interchange (Krebs *et al.* 1999). The development of the recently published 'composite' steady state model (Miller *et al.* 2000) (Figure 1) provided the framework to which intestinal perfusion and aspiration data for a four hour non-steady state period after a test meal have been incorporated. The study design included passage of a multilumen intestinal tube into the proximal jejunum, which had duodenal and jejunal perfusion ports for perfusion of nonabsorbable marker to be used to calculate flow rates, and aspiration ports in the duodenum and jejunum for aspiration of intestinal contents after the test meal. The particular subject to whom reference will be made in this text ingested a liquid test meal containing ^{67}Zn as an extrinsic label. Intravenous infusion of ^{70}Zn preceded the test meal by one hour, and was followed by frequent blood sampling to provide kinetic data. To evenly label all of the exchangeable pools, including sources of intestinal endogenous zinc, by the time of the intestinal intubation, ^{68}Zn was infused intravenously 10 days prior. The use of 3 tracers thus allowed us to model separately the movement of exogenous and endogenous zinc.

The non-steady state model of the intestinal aspiration data, including 3 sampling ports in the proximal small bowel, 3 isotopes (tracers) and natural zinc (tracee) (Krebs *et al.* 1999; Krebs *et al.* 1998b), is presented for this subject in Figures 2 and 3. To simplify presentation of these complex data, the models illustrating flow of exogenous zinc and endogenous zinc are shown separately. Additionally, although the steady state model (Figure 1) is not shown in these figures because of space limitations, it is critical to note that the data and models shown below are 'linked' to the steady state system, so the models fit both systems (steady state and non-steady state), and reflect exchange of all 3 tracers.

Figure 2 indicates the total flow of exogenous zinc (labelled with ^{67}Zn) over the ~4 h after the test meal, which contained a total of 5.48 mg Zn. The numbers beside arrows going from the intestinal compartments into the plasma indicate zinc absorbed into the system. The figures between the intestinal compartments indicate amounts (mg) of zinc flowing 'down' the intestine. The amount of exogenous zinc exiting the system via each of the aspiration ports is also shown (circles at bottom of figure). The maximal absorption (0.69 mg) is seen from the most proximal compartment (duodenum), which represents ~12.5% of intake from meal and dose. At the most distal port, 4 mg of exogenous zinc has flowed past, either to be absorbed more distally or be excreted in the feces. The fractional absorption (FAZ) calculated by the model, based on addition of amounts transferred

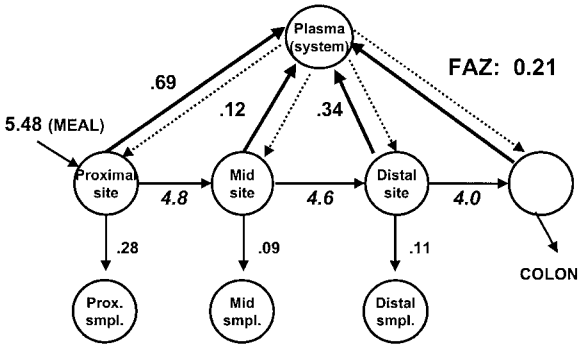

Figure 2. Total flow of exogenous zinc (mg) after test meal (0–4.3 h), based on flow of oral isotope (^{67}Zn). Full non-steady state model is based on intestinal aspiration data, including 3 sampling ports, 3 isotopes (tracers) and natural zinc (tracee), and is linked to steady state model with same tracers. The flow of endogenous zinc for same subject and over same time period is shown in Figure 3. The outflow compartments indicate exogenous zinc exiting the system via sampling from aspiration ports. FAZ = fractional absorption of zinc.

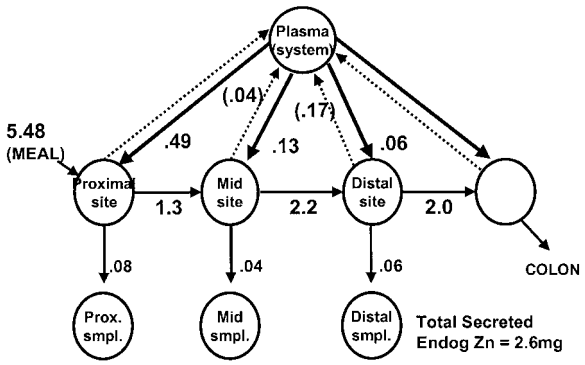

Figure 3. Total flow of endogenous zinc (mg) after test meal (0–4.3 h), based on flow of two intravenously administered isotopes (^{68}Zn and ^{70}Zn). The amounts of zinc flowing between the aspiration sites (proximal site, mid site, and distal site) reflect endogenous zinc present at each of the sites prior to sampling plus amount secreted into the gut from the system after the test meal. Full non-steady state model is based on intestinal aspiration data, including 3 sampling ports, 3 isotopes (tracers) and natural zinc (tracee), and is linked to steady state model with same tracers. The flow of exogenous zinc for same subject and over same time period is shown in Figure 2. The outflow compartments indicate endogenous zinc exiting the system via sampling from aspiration ports.

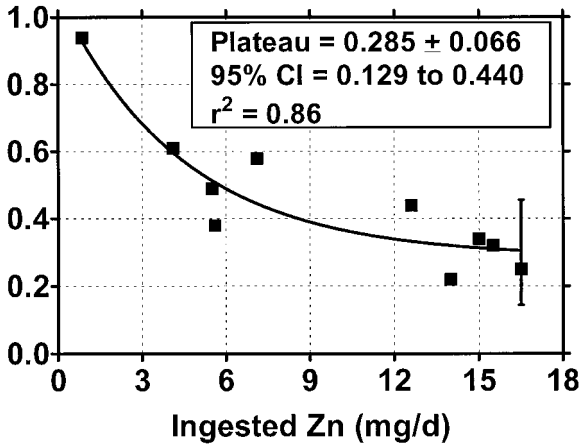

Figure 4. Inverse relationship between amount of ingested zinc and fractional absorption of zinc. Data points represent mean fractional absorption measurements based on stable isotope methods from several studies in healthy adult men. References to specific studies are provided in the text.

into the system = 0.21, is in good agreement with algebraic flow data from the aspirations, and with calculations of fractional absorption from urine isotope ratios (= 0.23). The plasma appearance of the oral tracer remained high through ∼9 h after ingestion of the test meal, whereas the data from intestinal aspirates indicated no further disappearance from the intestinal lumen after ∼ 3 h (Data not shown). Our tentative interpretation of this is that release of zinc from the enterocytes into the portal circulation or from hepatocytes into the peripheral circulation occurred over the longer time frame.

Figure 3 indicates flow of endogenous zinc after the test meal, based on flow of both of the intravenously administered isotopes (^{68}Zn & ^{70}Zn). The model projects not only the amount of each tracer, but the amount/flow of natural zinc, based on isotope 'dilution.' In this portion of the model, the exchange between the plasma and small intestine is bidirectional, indicating some apparent reabsorption of endogenously secreted zinc. The reabsorption figures are in parentheses between intestinal compartments and the plasma/system. It should be noted that the 'plasma' compartment as the source of the endogenous zinc is viewed as preliminary. Subsequent modeling from additional subjects suggests that the model may be better fitted to the data by having endogenous zinc originate from the liver/other compartment (see Figure 1).

The model predicts that the maximum secretion of endogenous zinc after a meal occurs in the proximal compartments, consistent with the hypothesis that a major part of the endogenous zinc secretion comes from the pancreaticobiliary secretions. The total amount secreted with the meal is projected to equal ∼ 2.6 mg. The majority of reabsorption occurs from the distal compartment (arrow indicating 0.17 mg into plasma). Relating this to the anatomic location of the aspiration ports, this would be mid-jejunum and possibly extending into ileum. The model predicts 2.0 mg

endogenous zinc passing between the distal site (jejunum) and the colon during the post-prandial period alone, which can either be reabsorbed more distally or excreted in the feces. Mean daily endogenous fecal zinc over 4 days for this subject was 4.5 mg. We thus predict that some of the endogenous zinc secreted in conjunction with this single test meal is likely to have been reabsorbed in the distal small bowel.

In summary, these data from the intestinal aspiration studies, using multiple tracers, and combining steady state with non-steady state kinetic data, represent an example of very complex application of compartmental modeling. In fact, the compartmental analysis is essentially the only way that all of the data can be analyzed simultaneously to characterize the exchange of tracer and tracee between the gut and the rest of the body. The model supports and extends calculations from data obtained by direct aspiration from the intestinal lumen: absorption of exogenous zinc likely primarily occurs in the proximal small bowel, i.e., between mid-distal duodenum and proximal jejunum, and disappearance from the intestinal lumen is apparently complete within 3 h of intake. If this is correct, there are implications for the anatomic distribution of the cellular absorptive transport mechanism. Secondly, the model suggests that the majority of endogenously secreted zinc enters in the proximal small bowel, consistent with a major source being the pancreaticobiliary secretions, although not necessarily the exclusive source. The model also supports the concepts that substantial amounts of zinc are secreted with meals (Matseshe *et al.* 1980), that maintenance of normal zinc homeostasis will be dependent on some reabsorption of the endogenous zinc, and that this most likely occurs in more distal small bowel, e.g., jejunum and possibly ileum. The role of the gastrointestinal tract in maintaining whole body zinc homeostasis will be considered further in the next major section.

Apart from their contribution to better understanding of the physiology of zinc and of its homeostasis, tracer techniques have potential to provide useful information about zinc nutritional 'status'. This has appeal for at least two reasons. First, despite intensive efforts, no sensitive biomarker of zinc status has yet been identified. Second, a number of studies have demonstrated that even modest depletion of critical pools of zinc result in functional compromise in zinc dependent processes, such as growth and immune function. There is thus considerable attractiveness to measurement of changes in these critical pools, especially rapidly exchanging pools, which may provide useful insights into zinc status (Lei *et al.* 1996; Lowe *et al.* 1995; Miller *et al.* 1994).

To give one example, the compartmental model has allowed us to evaluate the accuracy of estimates of the quantity of rapidly exchanging zinc, the exchangeable pool (EZP), which we define as zinc that exchanges/intermixes with zinc in plasma within three days and which accounts for only approximately 10% of the total body zinc content. In Figure 1, the EZP is comprised of the sum of the masses of compartments 1, 2, 3, 4 and 6.

Sum of exponential analyses after administration of a zinc tracer into the plasma of the systemic circulation indicates that four exponential decay terms are required to fit the tracer disappearance data from the plasma over the first 24 h. Extrapolation of the linear regression line fitting the log-transformed intravenous tracer enrichment data between 3 and 10 days after tracer administration to the y-axis (time 0) provides an estimate of the exchangeable zinc pool (EZP) (Miller *et al.* 1994; Miller *et al.* 1997). This is an estimate of the EZP that can be derived from urine as an alternative to plasma kinetic data and can be obtained under field conditions for adults (Lei *et al.* 1996), children (Manary *et al.* 2000), and infants (Krebs *et al.* 2000a). As will be discussed in the section addressing interrelationships between variables of zinc homeostasis, our experience is that EZP determinations can provide useful insights into zinc homeostasis and to differences in zinc status of individuals.

Zinc homeostasis and the gastrointestinal system

The gastrointestinal tract has the principal role in maintaining whole body zinc homeostasis. This is accomplished by modulation of the quantity of exogenous dietary zinc absorbed and of the quantity of endogenous zinc excreted. In no other organ system is it more necessary to amalgamate advances in knowledge of the cellular biology of zinc with parallel advances in our understanding of zinc physiology.

Fractional absorption of zinc

The fraction of dietary zinc absorbed is affected first by other dietary factors, especially those that reduce the fraction of zinc that is available for absorption by the intestinal mucosa. In general, the efficiency of absorption of zinc ingested with meals of any composition is less than that of zinc ingested as a simple salt

in solution. Human milk has commonly been regarded as promoting the absorption of zinc. Fractional absorption of zinc in some breastfed infants is as high as 0.80, although average fractional absorption is ~0.6 (Krebs *et al.* 1996); (Krebs, unpublished data). Comparison of these figures for absorption of zinc from human milk with that from an aqueous solution (Lei *et al.* 1993), suggests that rather than promoting absorption, zinc absorption is not inhibited to a discernible extent by the sum of other factors in human milk.

The dietary factor that has received most recognition as a major inhibitor of zinc bioavailability is inositol hexaphosphate, or phytic acid (Sandstrom 1997; Sandstrom & Lonnerdal 1989). Phytic acid is present in all seeds, especially grains and legumes and is considered to be a major etiologic factor in human zinc deficiency globally (Gibson 1994). It is present in especially high concentration in cereal grains and legumes which provide the major food staples for many populations in the developing world. The inhibitory effects of phytic acid may be especially noteworthy at times of high requirement (Manary *et al.* 2000). Lumenal factors affecting zinc bioavailability, while important in determining dietary zinc requirements (WHO 1996), are not particularly relevant to bridging the whole body physiology and the cellular biology of zinc. Accordingly, bioavailability per se will not be a focus of this paper.

With the consumption of diets of relatively high zinc bioavailability, there is an inverse relationship between the quantity of zinc ingested and the fractional absorption of that zinc. This is illustrated in Figure 4, which is derived from the mean data for stable isotope studies of young, healthy adult men (Hunt *et al.* 1992; Jackson *et al.* 1984; Lee *et al.* 1993; Taylor *et al.* 1991; Turnlund *et al.* 1986, 1984; Wada *et al.* 1985). This relationship has a major impact on the absolute quantity of zinc absorbed. The decline in fractional absorption with increasing dietary zinc is an outstanding factor in maintaining zinc homeostasis when intake is excessive (Lowe & Jackson 2000; Wastney *et al.* 1986; Weigand 1983). Although fractional absorption increases with dietary zinc restriction (King *et al.* 2000; Lee *et al.* 1993; Taylor *et al.* 1991; Wada *et al.* 1985), there is uncertainty about how effectively this increase is maintained over periods of many months (Lee *et al.* 1993). Despite the inverse relationship between fractional absorption and ingested zinc, the quantity of zinc absorbed each day varies directly with the quantity of ingested zinc over a wide range of intake (Food and Nutrition Board 2001, pre-print; Lei *et al.* 1996). This implies that the changes in fractional absorption in response to changes in the quantity of ingested zinc are alone inadequate to maintain zinc homeostasis, especially with restricted levels of intake.

Whether fractional absorption of zinc is regulated in response to changes in zinc 'status' is not entirely clear. Typically, in zinc depletion studies, the quantity of zinc in tracer-labeled test meals has corresponded to that in the experimental low zinc diet rather than to the quantity of zinc in the baseline 'normal zinc' test meals. It is not possible to determine from such studies if observed increases in fractional absorption of zinc are related to changes in the zinc status of the host rather than attributable to the smaller quantity of absorbable zinc present at the brush border of enterocytes involved in zinc absorption.

There are, to date, only a few observations that are consistent with and most readily explained by regulation of fractional absorption of zinc in response to changes in the physiologic state of the host. The best documented of these is lactation, itself a very special physiologic state. In a rural population in northeast China that has an habitually low dietary zinc intake (Lei *et al.* 1996), fractional absorption of zinc was strikingly higher at six weeks' lactation: 0.53, compared to 0.33 in nonlactating women from the same area on similar diets (Lei *et al.* 2000). This and other reports of increased fractional absorption during lactation (Fung *et al.* 1997; Jackson *et al.* 1988; Moser-Veillon *et al.* 1996) strongly suggest that fractional absorption is indeed responsive to changes in host physiologic condition. The basis of the enhanced absorption during human lactation, which has not been consistently observed during pregnancy (Fung *et al.* 1997), is not known. The hormonal milieu of lactation may affect the intestinal absorptive surface, the transporters involved in zinc absorption, gastrointestinal motility, or other factors (Davies & Williams 1977).

The impact of other physiologic conditions in the host on fractional absorption is less clear. For example, when the typical high phytic acid content of the Malawian diet is reduced during recovery from both malnutrition and infection in young children, fractional absorption of zinc increased significantly (Manary *et al.* 2000). In contrast, an increase in fractional absorption with identical phytate reduction was not observed in relatively well Malawian children whose baseline fractional absorption was similar to that of the malnourished children. This difference in response may most readily be explained by host differences in physiologic requirements. Because the requirements

were relatively low in the well children, it was hypothesized that an inhibitory effect of phytic acid on efficiency of utilization was not detectable, in contrast to the recovering malnourished children who likely needed a higher fractional absorption to meet requirements. This higher absorption could be achieved only when the inhibitory effect of high dietary phytate was removed. Caution is, however, required in interpreting these data as the subject numbers were small and the study was not designed prospectively to address this question.

There are other data from studies of the inhibitory effects of phytic acid on zinc absorption that can be plausibly explained by an effect of zinc 'status' on fractional absorption of zinc. Specifically, when healthy subjects whose habitual diets contain relatively little phytic acid are fed a high phytic acid test meal (Sandstrom & Sandberg 1992) or high phytic acid meals for a single day (Adams *et al.* 2001), fractional zinc absorption is relatively low. Recent observations in Malawi (Manary *et al.* 2000), (Manary, unpublished data) in subjects whose habitual diet is high in phytic acid suggest, however, that humans may be able to up-regulate absorption over time.

Some evidence also suggests that fractional absorption of zinc is not affected by zinc status. For example, we have observed that three weeks on a moderately zinc restricted diet was not associated with an increase in fractional absorption when an identical test meal was given for the two periods (Krebs *et al.* 2001). The lack of correlation between the size of EZP and fractional absorption in studies in both adults and infants also argues against a specific effect of 'status' on absorption (Krebs *et al.* 2000a; Lei *et al.* 1996).

Prospective human tracer studies carefully designed to address the factors affecting absorption are needed. Specific issues to be clarified include the effects of host factors, such as zinc status and physiologic state, vs. intralumenal factors, such as the amounts of zinc, phytic acid, and other dietary components. Clearly, there may also be interactions among these factors. It is apparent that parallel progress at a sub-cellular/molecular level and a human physiology level will be mutually invaluable in attaining this goal.

Total absorbed exogenous zinc

Although fractional absorption has been given considerable attention, especially since it is typically the variable that is actually measured with extrinsic labelling tracer techniques, it is the quantity of zinc absorbed per day, rather than the fractional absorption that seems to be of most practical importance. The total absorbed zinc (fractional absorption $\times$ zinc intake) is the variable that is directly impacted by changes in intake of available zinc. Beyond a certain level of zinc intake (which may correspond to approximate dietary requirements), increases in absorption of zinc are limited. Homeostatic mechanisms, however, do not prevent a small but progressive increase in absorption with increasing intake (Hambidge & Krebs 2001; Weigand 1983). At low intakes, total absorption progressively and relatively rapidly declines directly with the severity of zinc restriction. This is despite progressive increases in fractional absorption of the available zinc, which appear inadequate to maintain homeostasis alone even with relatively mild degrees of dietary zinc restriction. The direct relationship between daily zinc absorption and ingested zinc has been depicted in both animal models (Weigand 1983) and in humans (Food and Nutrition Board 2001, pre-print; Hambidge & Krebs 2001).

Excretion of endogenous zinc

Endogenous zinc is excreted via several routes, including the intestine, kidneys, integument, and semen. The intestine is not only the major route, but also the only one that is clearly subject to regulation at typical as well as at extreme levels of intake. Endogenous zinc excreted in the feces is typically at least twice that excreted via all other routes and can be several-fold higher. The quantity of endogenous zinc excreted via the intestine, i.e. in the feces, depends on both recent (Jackson *et al.* 1984; Johnson *et al.* 1993; Taylor *et al.* 1991) and long-term (Lee *et al.* 1993; Lei *et al.* 1996) zinc intake over a wide range of ingested zinc (Hambidge & Krebs 2001). The quantity can vary by an order of magnitude depending on zinc intake.

In contrast to fractional absorption, excretion of intestinal endogenous zinc is apparently regulated in response to changes in the zinc 'status' of the host over a wide range of typical dietary intake. Regulation appears to be quite rapidly responsive to changes in zinc 'status' and may be sensitive to minor changes. Adjustments in excretion of intestinal endogenous zinc to changes in zinc intake are maintained over prolonged periods (Lee *et al.* 1993; Lei *et al.* 1996). These characteristics identify endogenous intestinal zinc as a variable of zinc homeostasis that is of cardinal importance.

The quantity of endogenous zinc excreted in the feces is the difference between that secreted and that reabsorbed. With the anticipated rapid exchange of most zinc ligands (Williams 1989), it is difficult, although not impossible, to hypothesize differential reabsorption of endogenous zinc compared to absorption of exogenous zinc from the intestinal lumen. Therefore, if fractional absorption of dietary zinc is not regulated by zinc 'status', this is likely to be equally true for reabsorption of endogenous zinc. If this is so, the effects of zinc 'status' on the regulation of intestinal excretion of endogenous zinc should then be directed to the quantity of endogenous zinc secreted.

Relatively small amounts of endogenous zinc are secreted into the gastrointestinal tract in saliva, gastric juices and bile (Finley *et al.* 1994; Sullivan *et al.* 1965), with more possibly secreted through the small intestinal mucosal cells (Sturniolo *et al.* 1999), although the documentation in the human of the latter is quite limited. There is evidence, particularly from animal studies, to support the pancreatic secretions as a major source of endogenous zinc in the intestinal lumen (Adler *et al.* 1980; Birnstingle *et al.* 1956; Dijkstra *et al.* 1991; Lee *et al.* 1990; Van Wouwe & Uijlenbroek 1994). Results of intestinal aspiration/perfusion studies in humans are compatible with this conclusion, although none have distinguished pancreatic from biliary secretion (Krebs *et al.* 1999, 1998b; Lee *et al.* 1990; Sullivan *et al.* 1965). As noted earlier, both intestinal aspiration of labeled endogenous zinc and compartmental modeling suggest that the majority of endogenous secretion is quite proximal (Figure 3), i.e., consistent with pancreaticobiliary secretions into the duodenum (Krebs *et al.* 1999, 1998b). It is tempting to speculate that the rapid induction of metallothionein in response to zinc administration, including metallothionein in the pancreas, may have a role in the regulation of zinc secretion (Andrews *et al.* 1990; Dalton *et al.* 1996; De Lisle *et al.* 1996; Kelly *et al.* 1996). Recent detection of metallothionein in pancreaticobiliary secretions in the human duodenal lumen is consistent with such a hypothesis (Krebs, unpublished data).

The quantity of endogenous zinc secreted with a test meal appears to be substantial relative to the daily fecal excretion of endogenous zinc (Krebs *et al.* 1999, 1998b; Matseshe *et al.* 1980). To maintain normal zinc homeostasis, it thus seems probable that reabsorption of some endogenous zinc is essential. Based on the calculated net endogenous zinc flow at the most distal aspiration site (proximal jejunum) in our intestinal perfusion/aspiration studies and the resultant compartmental model (Figure 3), a large portion of the reabsorption is likely to occur in the more distal small bowel (Krebs *et al.* 1999, 1998b). The role of jejunum and ileum in reabsorption of endogenous zinc is further supported by the strong positive correlation observed between endogenous fecal zinc and fecal fat in infants with pancreatic insufficiency due to cystic fibrosis and with consequent fat malabsorption (Krebs *et al.* 2000b). This observation is discussed in more detail in a later section.

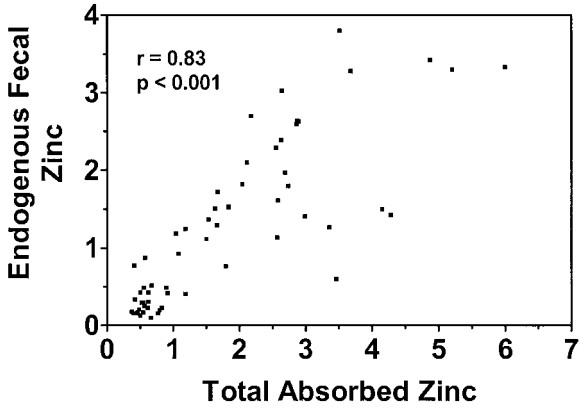

Figure 5. Correlation between total absorbed zinc and endogenous fecal zinc. Data points represent individual subjects from zinc stable isotope studies in infants and adults. Specific references are provided in the text.

Despite recent advances in characterization of zinc transporters and possible interaction with metallothionein, the molecular mechanisms and cellular processes in the gastrointestinal tract that are responsible for regulation of absorption of exogenous zinc and secretion and reabsorption of endogenous zinc await further clarification. Meanwhile, studies of the central role of the gastrointestinal tract in maintaining human zinc homeostasis at a whole body level are giving perspective to the quantitative importance of the key processes, their specific roles, and their gross anatomic localization.

Interrelationships between key variables of zinc homeostasis

As an increasing body of knowledge accumulates from the application of zinc stable isotope techniques, it is becoming apparent that there are some consistent and predictable interrelationships between key variables of zinc homeostasis under normal circumstances. No-

table among these is the positive correlation between endogenous fecal zinc and total absorbed zinc, illustrated across a range of absorbed zinc in infants and adult subjects in Figure 5 (Krebs & Westcott 2001, in press; Lei *et al.* 1996). Of considerable practical importance is the growing yet incomplete evidence that this relationship holds not only when absorption exceeds physiologic requirement, but also at very low levels of absorption (Hambidge & Krebs 2001). The direct relationship between these two variables is clearly of central importance to zinc homeostasis and the achievement of zinc balance. It demands close attention in any factorial approach to calculating dietary zinc requirements (Food and Nutrition Board 2001, pre-print).

This association suggests that the quantity of endogenous zinc excreted in the feces is responsive to recent and habitual absorbed zinc (the quantity of, not the fraction of). If this is mediated through the effect of recent zinc absorption on zinc 'status', the effect on endogenous secretion is rapid enough (Jackson *et al.* 1984) that it is likely to be triggered by an increase in a component of the rapidly exchanging zinc pools (Miller *et al.* 1994). It was concluded by Chesters many years ago that the effects of zinc deprivation on feeding patterns and growth in mammalian models were so rapid that they must result from subtle, but physiologically important changes in the quantity of zinc in one or more rapidly exchangeable pools. Moreover, the quantity of zinc in this pool(s) must be very sensitive to dietary zinc (Chesters 1982). An observation that fits with this hypothesis is the positive correlation that has been observed between dietary zinc (Miller *et al.* 1994), and especially, total absorbed zinc and the size of the EZP (Krebs *et al.* 2000a; Lei *et al.* 1996).

The size of the EZP is also normally positively correlated with the quantity of endogenous zinc in the feces, consistent with the conclusion that it is some component of this rapidly exchanging system that is responsible for the regulation of the quantity of endogenous zinc secreted into and eventually excreted via the intestine. Incidentally, parallel correlations with plasma zinc have not been a consistent observation and there is some evidence that homeostatic mechanisms may maintain plasma zinc in circumstances that are associated with reduction in the size of the EZP (Lei *et al.* 1996).

Alterations in zinc metabolism during disease states

In general, understanding is limited for the specific metabolic processes underlying the observed changes in zinc transport and distribution in the setting of various pathologic conditions. Furthermore, the interplay between disease and dietary zinc deficiency has not been well characterized, particularly not in the human.

As described by Beisel, infection-induced malnutrition, the most common form of cytokine-induced malnutrition, occurs from the actions of proinflammatory cytokines, which initiate the acute phase response (APR) (Beisel 1995). In addition to the systemic symptoms (fever, malaise, myalgia etc.), a number of metabolic-nutritional responses also result from the APR, including protein catabolism, stimulation of metallothionein synthesis and sequestration of zinc, and many endocrinologic changes (Beisel 1995; Gabay & Kushner 1999). Extensive experimental work has demonstrated that hepatic metallothionein is involved in the response to stress. This can be induced by infusion of dexamethasone or other glucocorticoids, endotoxin and cytokines, as well as to a number of hormonal stimuli, including glucagon and epinephrine (Bremner & Beattie 1990; Cousins *et al.* 1986; Hernandez *et al.* 1996; Lehman-McKeeman *et al.* 1988; McCormick *et al.* 1981; Prasad 1993; Quinones & Cousins 1984; Schroeder & Cousins 1990). In hepatic tissue, the increase in metallothionein mRNA and metallothionein itself are also strongly correlated with increased hepatic zinc, and corresponding reduction of circulating zinc. In experiments with rats, the effects of stress and/or endotoxin on hepatic metallothionein synthesis were found to be significantly and synergistically enhanced by pretreatment with zinc, whether administered parenterally or enterally (Hernandez *et al.* 1996), emphasizing the ability of zinc itself to induce metallothionein synthesis. What is not by any means clear either from subcellular or whole animal, and certainly not from human research, is what role these profound changes in zinc metabolism have in combating stress and infection. Nor is it apparent what the end result of these changes in zinc metabolism is on zinc homeostasis and 'status'. It is unknown for example, if endogenous zinc losses are decreased, increased or unchanged.

The impact of disease state on zinc homeostasis can be considered under the broad headings of excessive losses (e.g. gastrointestinal tract and kidney), increased requirements (e.g. rapid growth, 'catch-up',

tissue repair, immune stimulation), and redistribution (e.g. inflammation, closed head injury, Down syndrome, possibly Alzheimer disease). In general, tracer techniques have not been applied to systematically and comprehensively study zinc homeostasis under these clinical conditions.

Excessive losses

The dominant role of the gastrointestinal tract in normal zinc homeostasis has been described. It is thus not surprising that involvement of this organ system can result in significant perturbation of zinc homeostasis. A circular relationship of zinc deficiency and diarrhea is well recognized: severe zinc deficiency causes diarrhea and diarrhea may cause zinc deficiency. Proposed mechanisms for the diarrhea associated with zinc deficiency have included induction of certain proteins that result in increased fluid and possibly zinc secretion into the gastrointestinal tract. Examples include uroguanylin, cholecystokinin, and inducible nitric oxide synthase, all of which have increased expression during zinc deficiency (Abou-Mohamed *et al.* 1998; Blanchard & Cousins 1997; Wapnir 2000). Zinc deficiency is also associated with immune dysfunction. Impairment of the extensive immune system in the gastrointestinal tract may predispose to invasion by microorganisms as well as alter systemic immune responses (Scott & Koski 2000). Diarrhea from nonnutritional causes may cause excessive zinc losses and predispose to zinc deficiency by altering transit and/or the absorptive surface and thus impacting both absorption and reabsorption of exogenous and endogenous zinc. Despite the limitations in understanding of the complexities of zinc physiology in the setting of diarrheal disease, the results of a recent meta-analysis emphasize the remarkable benefit of zinc supplementation in the treatment and prevention of diarrhea in developing countries (Bhutta *et al.* 1999).

Cystic fibrosis represents a specific example of a disease with perturbed zinc homeostasis. Although pathological changes are discernible throughout the gastrointestinal tract, the outstanding pathophysiologic feature in the gastrointestinal system of this autosomal recessively inherited disease is pancreatic insufficiency. Effects on zinc metabolism, even in young infants at early stages of disruption of exocrine pancreatic function, include impairment of absorption of exogenous dietary zinc and excessive intestinal excretion of endogenous zinc (Easley *et al.* 1998; Krebs *et al.* 2000b). The quantity of the endogenous zinc excreted in the feces is positively correlated with fecal fat, which is typically excessive in this disease due to lipase deficiency. Since fat is absorbed primarily in the ileum, these findings suggest that this region of the intestine normally has a substantial role in the reabsorption of endogenous zinc that is secreted post-prandially. This observation serves as a further reminder of the need to evaluate all regions of the intestine, especially the small intestine, in investigating the mechanism(s) responsible for zinc absorption and reabsorption. The fat malabsorption associated with pancreatic insufficiency, as in cystic fibrosis, and the accompanying excessive losses of endogenous zinc, would certainly predispose to zinc deficiency if persistent. Indeed, we have reported that in infants identified by newborn screening to have cystic fibrosis, approximately one third have hypozincemia, most likely representing zinc deficiency (Krebs *et al.* 1998a).

A number of conditions are associated with hyperzincuria, but the underlying mechanism has not been characterized, nor is it clear whether there may be more than one mechanism. Hyperzincuria is associated with many chronic inflammatory states, including especially liver disease (Hambidge *et al.* 1987; Narkewicz *et al.* 1999; Sullivan & Lankford 1965), but also inflammatory bowel disease (Fleming *et al.* 1981), closed head injury (McClain 1990), skeletal trauma (Askari *et al.* 1982), cancer (Melichar *et al.* 1994), and diabetes (Chausmer, 1998). Whether there is any relationship between metallothionein in the kidney and hyperzincuria in these clinical conditions has not been reported. It is also tempting to speculate that one of the recently characterized zinc transporters, such as ZnT-1, which has been suggested to have a zinc exporting function, may be induced by the inflammatory response (Palmiter & Findley 1995). The relatively rapid normalization of the hyperzincuria observed after liver transplant in patients with chronic liver disease also suggests that there may be systemic signals, such as cytokines, that drive the hyperzincuria (Narkewicz *et al.* 1999).

Increased requirements

Several clinical conditions are characterized by tissue proliferation and by relatively high zinc requirements. Early infancy and childhood, adolescence, and the reproductive cycle are obvious times during the normal life cycle when zinc requirements are increased (Food and Nutrition Board 2001, pre-print; King 2000; King & Turnlund 1989; Krebs & Hambidge 1986). Zinc

deficiency has been documented in all of these conditions (Brown *et al.* 1998; Caulfield *et al.* 1998, 1999; Goldenberg *et al.* 1995; Hambidge *et al.* 1972; Prasad *et al.* 1961; Walravens & Hambidge 1976).

Infants born prematurely have a particularly high requirement for zinc absorption and retention to achieve intrauterine accretion rates. Stable isotope methodology, including compartmental analysis, has been applied to this population to characterize variables of zinc homeostasis (Ehrenkranz *et al.* 1989; Friel *et al.* 1996; Jalla *et al.* 1997; Wastney *et al.* 1996, 1999). Results of these studies have generally concluded that healthy growing premature infants can achieve *in utero* zinc accretion rates (Ehrenkranz *et al.* 1989; Jalla *et al.* 1997; Wastney *et al.* 1999). Further, we found a significant positive correlation between average daily rate of weight gain and net absorbed zinc, emphasizing the importance of optimizing zinc retention (Jalla, unpublished data). To date, the rigorous demands of the application of tracer methods has limited their use to relatively stable preterm infants. Given the critical role of zinc in normal growth and development, such techniques are likely to offer important insights into potential differences in zinc homeostasis between normal and growth retarded neonates.

Other less well characterized clinical circumstances in which zinc requirements are exceptionally high are traumatic and surgical wound healing, conditions which are often complicated by considerable inflammatory response and concurrent increased zinc losses (Agren 1990; Iwata *et al.* 1999). Activation of the immune response is associated with an increase in the need for zinc, due to its involvement with cell replication and lymphocyte clonal expansion, as well as with lymphocyte activation (Fraker *et al.* 2000; Shankar & Prasad 1998). There is ample documentation of the detrimental effect of zinc deficiency on the immune response, but little is known about the impact of immune stimulation on whole body zinc homeostasis, that is, in changes in distribution and exchange rates between tissues, in uptake and excretion. Zinc concentration in the circulation has been proposed to be especially important, with both low and high levels impacting leukocyte responsiveness (Rink & Kirchner 2000). Clearly the complexity of the immune system presents significant challenges to the application of tracer techniques, but likewise, whole body studies may provide important complementary insight to in vitro studies of small components of this system.

Redistribution

The shifts in zinc distribution that occur in inflammation and the development of the acute phase response have been described above. Patients with Down Syndrome (DS, Trisomy 21) have been repeatedly found to have, on average, low plasma zinc levels despite dietary zinc intakes that are unremarkable (Chiricolo *et al.* 1993, 1994a; Licastro *et al.* 1994b; Napolitano *et al.* 1990; Stabile *et al.* 1991; Sustrova & Strbak 1994). Zinc supplementation has been undertaken in several trials, with positive effects on thyroid function (Napolitano *et al.* 1990; Sustrova & Strbak 1994), growth (Napolitano *et al.* 1990), humoral and cellular immune function, and apoptosis in peripheral lymphocytes (Antonucci *et al.* 1997). Altered zinc metabolism has also been proposed to be at least part of the basis of the accelerated aging in the DS population (Licastro *et al.* 1994b). No studies have been undertaken to utilize tracer techniques to study variables of zinc homeostasis or pool sizes. Thus it is not known whether there are differences in uptake and retention of exogenous zinc or whether the apparent zinc deficit is the result of differences in the exchangeable pool sizes or in total body zinc. Although there are significant challenges to applying stable isotope techniques to this population, the information that could be gleaned from such studies is potentially invaluable to advance understanding of zinc metabolism in this specific population and in general.

Future directions

The potential rewards of synergy in zinc research between cellular biology and human physiology and nutrition are becoming increasingly apparent as progress in each of these areas accelerates. Those of us involved in human zinc research are dependent on parallel advances in research directed to the cellular biology of zinc.

Such advances at the subcellular level are essential to achieve an adequate understanding of zinc homeostatic mechanisms, their interrelationships and limitations. This, in turn, is necessary if, for example, we are to really understand dietary zinc requirements and the limitations of homeostasis beyond which zinc deficiency or toxicity will occur. The considerations in this paper highlight the gastrointestinal tract and its associated organs which have a central role in the maintenance of human zinc homeostasis. Acceleration in zinc tracer research, supplemented by special

techniques such as intestinal intubation/perfusion or by regional scanning of the distribution of radio-zinc, is now at least starting to provide clearer insights into the regulation of major variables of zinc homeostasis and into the interrelationships between these variables. Temporal and anatomic aspects of homeostasis are recognized, although not yet totally clarified, especially the regulation of endogenous zinc excretion. Future progress in these areas can assist in guiding the direction of cellular and molecular research on the mechanisms and regulation of zinc absorption and excretion.

Advances in the cellular biology of zinc alert the human nutrition researcher to the remarkable scope and diversity of zinc-dependent biology and metabolism. These range from more generalized functions, including those related to transcription, cellular growth, and the diverse roles of metallothionein, to highly specific functions such as the role of zinc in synaptic signaling in the central nervous system. These advances also hold out hope of new biomarkers of zinc status, for which there is a real need. Simultaneously, progress in our understanding not only of the clinical but also of the global public health importance of human zinc deficiency, highlights those directions in which advances in cellular biology are likely to have special immediate relevance to human health. These include, for example: cellular growth and differentiation, the biological roles of zinc in the immune system, and other aspects of host defense mechanisms and the role of zinc in cognitive function. Finally, despite recent advances, wide gaps remain between recent advances in our understanding of the cellular biology of zinc and specific links with the clinical features of zinc deficiency.

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