Trace Element Analytical Chemistry in Medicine and Biology

Trace Element Analytical Chemistry in Medicine and Biology

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PREFACE

This year the fourth workshop on "Trace Element Analytical Chemistry in Medicine and Biology" was organized again by the GSF (Gesellschaft für Strahlen- und Umweltforschung mbH) and the working group "Trace Elements in the Life Sciences" of the AGF (Arbeitsgemeinschaft der Großforschungseinrichtungen) at Neuherberg.

Six years ago we started this series of meetings with international and multi-disciplinary character in order to intensify the mutual transfer of scientific knowledge in this field. The general theme of the first workshop was the analysis of essential trace elements. Two years later the aim of the second meeting was to stimulate discussion on the deficiency problems of essential trace elements and their analysis. At the 3rd workshop a lot of time was devoted to the increasing importance of the trace element selenium and recent developments in the field of some "newer trace elements".

It was the aim of the present workshop to consider modern nutritional aspects of trace elements as well as their role in clincal states. The invited experts dealt with the important aspects of bioavailibility (A.E. Harmuth-Hoene), dietary requirements (L.M. Klevay) and recommendations (R. Schelenz), interactions (R.J. Shamberger) and toxic action (M. Anke) of trace elements as well as their role in metabolic processes (M. Kirchgessner, C.F. Mills), infant nutrition (H.B. von Stockhausen), parenteral nutrition (K. Ladefoget, W. Prellwitz) diagnosis and therapy of cancer (G.N. Schrauzer) and psychiatric disorders (C.J.M.van Tiggelen). Each of the short contributions was presented in the auditorium and extensively discussed.

It is clear that our knowledge of trace element distribution in the body, the delineation of deficiency situations of the organism, and the identification of trace element-containing biologically active molecules depend on the contemporary state of the art in trace element analytical chemistry. Although at this workshop the analysis of samples did not feature prominently, this aspect will be a major feature at the next meeting. Now as before it is our hope that developments in analytical chemistry will help to extend the research frontiers in biochemistry, medicine, biology and nutrition related to trace elements.

This book contains the 56 papers accepted by the scientific committee. As it was again our aim to produce the book fairly rapidly, the manuscripts were not extensively edited. We are indebted to all the participants, for their presentations and for their valuable support of interdisciplinary research.

Our thanks are also due the chairmen, the referees of the poster session and all who contributed to the discussions.

October 1986

P. Brätter

P. Schramel

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C.F. Mills

Chairman:

L.M. Klevay

TRACE ELEMENT REQUIREMENT MODELS BASED ON PHYSIOLOGICAL AND METABOLIC PROCESSES

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Introduction

Because of essential functions of trace elements in metabolism, mainly as integral constituents or as effectors of enzymes and hormones, their nutritional supply must be sufficiently high to meet the metabolic requirements. The supply status of a trace element may be divided into five ranges (see Kirchgeßner and Reichlmayr-Lais 1985):

1. deficient: characterized by clinical symptoms

2. suboptimal: there are no conspicuous clinical symptoms, however biochemical responses are measurable

3. optimal: optimal functioning of metabolism, promoting normal health and performance

4. subtoxic: intake in excess of the optimal supply characterized by biochemical changes without manifest clinical symptoms

5. toxic: characterized by clinical symptoms.

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Over a certain range of supply, which differs from element to element, the body is capable by regulating absorption, turnover and excretion of maintaining stable trace element concentrations that are functionally necessary and physiologically tolerable for cells and their subcellular compartments. This drive for homeostasis may also be interpreted as the measures for matching the nutritional supply with the current metabolic needs. The analytically determined amount of a trace element in the food cannot be accounted as fully available for meeting the functional requirements, because "losses" occur during absorption and intermediary metabolism. Dietary allowance must, therefore, consider the efficiency with which a trace element is utilized for its functional roles (Kirchgeßner et al. 1974). For this very reason, a distinction is made between the gross and net requirement. The gross requirement refers to the dietary quantity of intake of the trace element that is adequately

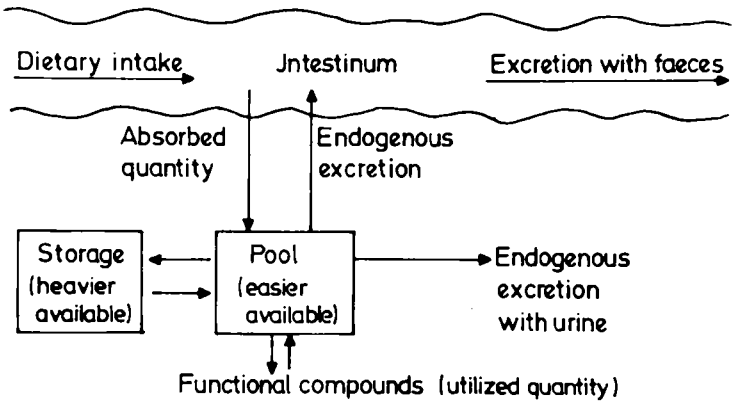

Fig. 1: Model for metabolism of trace elements

high, accounting for losses, to meet the net requirement, i. e. the functional needs at the molecular level. In Fig. 1 a simplified model of the metabolism of trace elements is shown. It elucidates that losses may be encountered not only during absorption from the digestive tract but also during intermediary metabolism. This makes evident the complexity of trace element utilization and hence of assessing requirements.

In the following, three concepts of assessing trace element requirements are presented and evaluated.

1. Balance Studies

In balance studies, intake is set against excretion. In the assessment of trace element excretion, only the amounts eliminated in feces and urine are generally considered. Losses via sweat, epithelial slouyhing or hair are largely neglected, in part, because their separate collection demands additional efforts. Neglecting these surface losses seems justified only if they are very small compared with the amounts excreted in feces and urine.

In the adult human or animal, the balance is zero under steadystate conditions, that is intake equals excretion. In the state of production, in particular body growth or gravidity, trace element balance is positive provided that the dietary supply is adequate, this is to say that retention occurs. Of the adult body the requirements are surely not met if the balance is zero or even negative in productive states of the body or if the balance is negative in the state of maintenance. The task of spezifying the required intake on the basis of balance data is aggravated by the homeostatic regulation of trace element metabolism. Within certain bounds of supply, the body attempts to compensate for a reduction in dietary intake by increasing the efficiency of absorption and/or lowering the

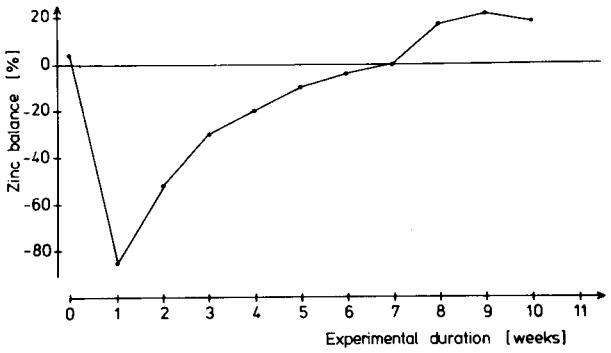

Fig. 2: Effect of depletion of Zinc on Zinc balance

excretion rate, and, conversely, attempts to counterbalance an increased supply by reducing the absorption rate and/or raising the excretion rate. In studies with dairy cows, Kirchgeßner und Schwarz (1976) observed that Zn balance (including the output in milk) approached the zero level after an initial period of negative balance in response to dietary Zn depletion even though the cows ultimately showed clinical signs of Zn deficiency (Fig. 2). Evidently, the body is capable of attaining a zero balance at different levels.

Also, time plays a role in reaching a steady balance. Interpretation of balance data, therefore, must take into account the duration of the study, in particular the length of the preliminary and the collection periods. If, for examples the length of the experiment is too short, balance may not get be fully adapted to the particular supply level and the

findings may be misleading. Therefore, aspects of the length of the preliminary and collection periods are of particular importance in situations were trace element supply was inadequate before the balance study. Consequently, the supply status of the trace element under study must be evaluated critically when results of balance studies are interpreted.

Additional difficulties arise in the determination of the requirements for growth and reproduction. The optimal extent of positive balance in these stages of production is difficult to decide upon. Closely associated with the positive retention is the question of the desirable extent of trace element storage. In this context, superretention during gravidity is also of concern. This extra retention in maternal tissues outside the reproductive organs leads to reserves that are depleted again in the course of lactation. Superretention has been found to be particularly pronounced, provided the trace element supply was adequate, in the case of copper more than in the case of other trace elements investigated (Kirchgeßner et al. 1978).

Further limitations to the specification of trace element requirements by balance studies are imposed by analytical aspects. Since balance data are computed as the difference between intake and excretion and its values are often very small compared with its determining quantities, it is frequently impossible to assess its magnitude with satisfactory precision. The large variation mainly results from the summation of random, and perhaps, even systematic error sources encountered in the determination of intake and excretion. A change in matrix is also involved. In assessing intake and excretion, utmost care is needed to accurately determine these quantities and to avoid contaminations in processing the samples. Accordingly, it is commonly nessessary to use materials low in trace elements and to pretreat them as an additional precaution. Of particular relevance are these analytical aspects in the case of the so-called ultra trace

elements which are present in foods, the body and excreta only in minute concentrations or are particularly difficult to analyze.

2. Dose-response Relationships

In the derivation of requirements from dose-response relationships, the response of a suitable criterion is investigated in relation to the dose over a relatively wide range of supply. As a rule, the relationships between criterion and dose are exponential and may be analyzed by regression analysis. Fig. 3 shows ideal dose-response relationships. An optimal level of supply of the trace element is reached if the differential response to the further increase in the supply of the element is relatively small or even absent. The response

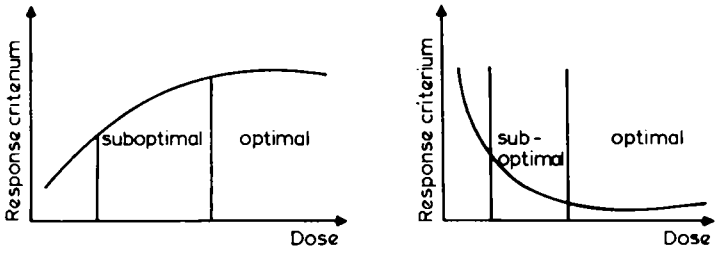

Fig. 3: Dose-response relationships

criteria to be chosen have to be specific for and sensitive to the particular trace element under study. A criterion is sensitive if it reveals latent states of deficient supply and thus responds to even slight changes in supply. Suitable criteria are for the most part biochemical parameters showing adverse changes in response to suboptimal supply as compared to optimal intake. The intensity of suboptimal supply of a trace element may be subdivided into two stages:

Degree 1: characterized by

a) an increase in fractional retention of the trace element as the result of an improved efficiency of true absorption and/or decreased rate of excretion from endogenous sources

b) a mobilization of the trace element from depot compounds, leading to a depletion of the analytical content of these depot compounds,

c) an increase in the binding capacity of transport proteins.

Degree 2: characterized by

a decrease in functional compounds, for example, a reduction in the activity of enzymes.

While the biochemical changes that indicate latent states of an adequate trace element supply are also prevalent in the case of a conspicuous deficiency, there are no manifest clinical symptoms in the situation of suboptimal supply.

There are several advantages to studies of dose-response relationships in which the dose level starts with a suboptimal supply level. First, it is not necessary to compound diets

severely deficient of the element concerned, which task may be particularly arduous in the case of ultra trace elements. Secondly, this approach, avoiding severe depletions may also be acceptable for studies with humans.

Proceeding from these boundaries of the suboptimal supply range, a variety of response criteria may be chosen such as retention, absorption, excretion of the trace element, mobilization of storage compounds, the binding capacity of transport proteins and the activity of functional compounds. In the following a few examples shall be presented for these parameters.

In the case of nickel, a specific functional compound is not yet known for the animal or human body. It has, therefore, been attempted to estimate the requirement of this trace metal on the basis of its fractional retention in relation to intake

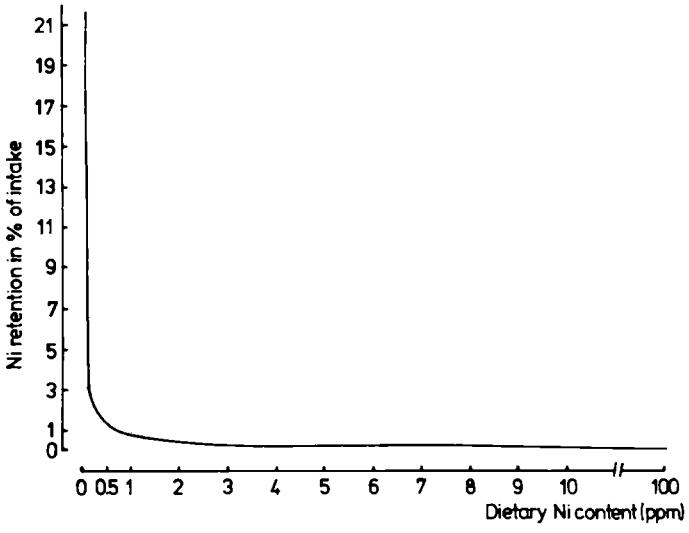

Fig. 4: Relative Ni-retention in relation to dietary Ni-content

(Kirchgeßner et al. 1984). Fig. 4 shows the relative retention of nickel by groups of growing rats fed a diet of different Ni concentrations. On the basis of this response curve, it is proposed that the growing rat requires between 1 and 10 ppm Ni in the diet.

An example of deriving the dietary requirement of a trace

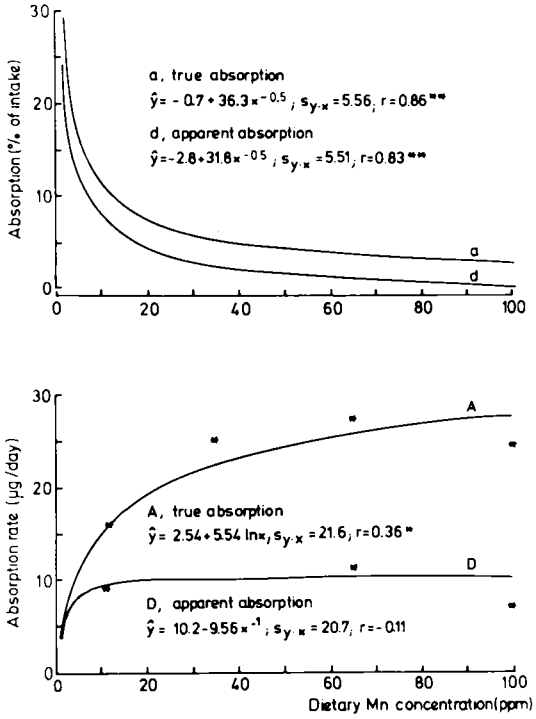

Fig. 5: Relative and absolute true and apparent absorption of Mn dependent on dietary Mn supply

element from absorption data determined in response to the dietary supply of the element is presented in the case of the Mn requirement of the growing rat. Fig. 5 shows both the apparent and true absorption of manganese as daily rate and as percentage of intake in relation to the dietary Mn concentration (Weigand et al. 1986). The response curve of the percentage absorption is similar to that of the relative Ni retention. Fig. 5 illustrates that the true and apparent Mn absorption, expressed as daily rate, have a steep incline over the low range of dietary Mn supply. Above a Mn concentration of about 15 ppm in the diet, the response of the true absorption rate rapidly falls and the apparent absorption rate varies around a plateau value of somewhat less than 10 ug Mn/day. The difference between the curves of true and apparent absorption reflects the fecal excretion of manganese from endogenous sources. The increasing rate of endogenous excretion is clear evidence that the Mn supply status is controlled not only by a pronounced change in the absorptive efficiency but also by a responsive endogenous excretion on the metal. The minimum dietary Mn requirement of the fast growing rat is approximately at the dietary Mn concentration where the apparent absorption rate approaches its plateau value. This indicates that the requirement is already met at a dietary level where percentage absorption has not yet reached the right section of the response curve where it assumes an asymptotic course. Accordingly, the asymptote approach of the relative absorption curve would estimate the requirement to be higher. This situation also has to be clarified for other elements. As an example of a functional compound serving as a response criterion, the activity of the glutathione peroxidase of the liver in relation to different dietary Se supply is presented in Fig. 6 according to a study by Sunde et al. (1981). Apart from the activity of the glutathione peroxidase in liver, the response of the activity of this enzyme may also be analyzed in whole blood, plasma, erythrocytes or platelets in relation to Se nutrition.

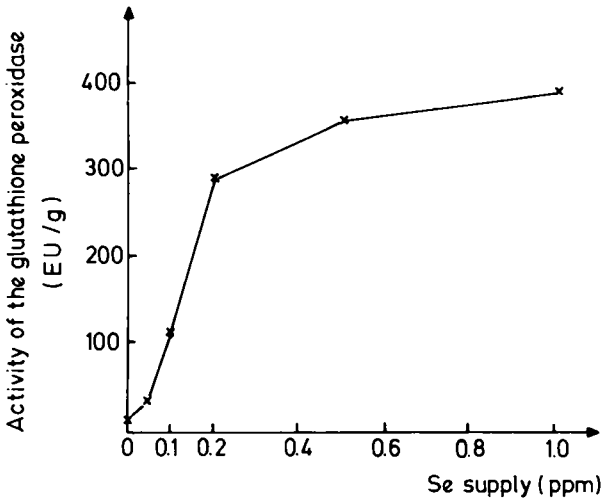

Fig. 6: Activity of the glutathione peroxidase in liver of rats given different Se supply

Generally, model studies must be conducted for each element to find response criteria that are most suitable for assessing the dietary requirement. It must be considered that certain criteria, for example, enzyme activities may respond differently depending upon organ or tissue. In all cases, however, it may be advisable to investigate several criteria for the derivation of requirements by dose-response relationships.

Two different approaches may be followed in the experimental design of studies for deriving dose-response relationships:

1. The response criterion is analyzed after the dietary supply of the trace element has been varied over a wide range, including suboptimal to optimal levels.

2. Following a period of depletion, perhaps to the point of clinical deficiency symptoms, the effect of repletion at graded dose levels on the response criterion is investigated, or the element is supplied in successively higher doses until a level is reached at which the criterion no longer responds.

In the case of either method, care must be taken that the response criterion is analyzed at the time when it has adjusted completely to the particular dose level in order to avoid misinterpretations of the data. This aspect of proper timing is especially important when the dose-response curve is assessed after a deficiency may exert on absorption, metabolism and excretion of the trace element.

Some major points have to be taken into account when requirements are derived by the dose-response concept:

1. The method leads to estimates of the gross requirement. The response criterion may reach its plateau value at different levels of dietary intake depending upon the efficiency with which the element may become available for absorption and metabolism. Exogenous factors influencing trace element utilization include the type of chemical compound and interactions with other dietary constituents. Strictly speaking, estimates of the requirement are valid only for the situation under which they have been determined.

2. The net requirement depends upon the intensity of production of the body, for example, the rate of live weight gain. Accordingly, the response criterion may plateau at a different level of intensity of the response criterion depending upon the height of the net requirement. Therefore, dose-response relationship have to be reassessed whenever productivity materially affects the net requirement.

3. Different response criteria may reach their response optimum at different dose levels. The requirement is assumed to be met when even the most sensitive criterion has reached its optimum.

3. Factorial Model for Deriving Requirements

According to the factorial model for deriving trace element requirements, the total net requirement (N) is comprised of a maintenance component (N_m) and a production component (N_p) :

$$N = N_m + N_p.$$

The net requirement for maintenance accounts for the inevitable losses of the trace element which occur during the continuous turnover of functional compounds and renewal of epithelia. Experimentally, the net requirement for maintenance is most often determined under the nutritional situation of suboptimal or even deficient supply of the trace element of concern in order to avoid the additional excretion of the element from endogenous sources which occurs in response to higher intakes for homeostatic reasons and is regarded as a quantity affecting the efficiency of metabolic utilization. The inevitable losses are usually considered as constant for a given situation. Certainly, these are not really constant, but are most likely to vary with the supply status. It may be assumed that the mean turnover rate of functional compounds is slowed with the advance of a nutritional depletion of the trace element and that a higher portion of the element is reutilized in metabolism. Otherwise, the inevitable endogenous losses may be related to metabolic body size or body mass as has been demonstrated, for example, in the case of zinc (Weigand and Kirchgeßner 1977).

The net requirement for production is determined by the type and intensity of productive functions beyond body maintenance. It is frequently estimated by analyzing the retention of the trace element in the products formed. There are restrictions to the applicability of this approach to human studies. For example, the net requirement for pregnancy could be assessed only by analyses of stillbirths or aborted fetuses. Estimation of the net requirement for production by analyzing the retention of the trace element in the products is not without problems, because the retention per unit of product formed is not necessarily constant, but may vary in response to a number of factors including, for example, the supply status or interactions with other elements.

The efficiency of utilization of the particular trace element is needed to compute the gross requirement (G), i.e. the necessary daily intake to meet the net requirement (N). The computational formula for the gross requirement is $G = N/c$, where c defines the efficiency of total utilization, which may vary within the boundaries of $0 \leq c \leq 1$. Total utilization may be influenced by numerous factors not only affecting absorption, but also intermediary metabolism and excretion of the trace metal. Such factors are, for example, level of intake, supply status, chemical nature of the element, dietary composition, interactions with other dietary constituents, pH and ionic conditions in the gastrointestinal tract etc. In this factorial concept of deriving the gross requirement, the overall efficiency of utilization or availability is not equated with absorbability as it has been done in other models (e.g., NRC). The concept presented here and previously (Kirchgeßner et al. 1974, Weigand and Kirchgeßner 1977/78) is baced on the fact that the absorbed amount of a trace element is not a priori fully available for the biosynthesis of functional compounds. Accordingly, the model concept of total utilization (c) entails its partitioning into two partial efficiencies, (1) the absorbability (a) and

(2) the metabolic efficiency (q), whereby the relationship $c = a \cdot q$ holds. This concept thus also takes factors into account which exert their effect on trace element utilization at the site of intermediary metabolism. Besides, trace elements are often absorbed in amounts exceeding the net requirement and hence the excessive quantity is then either stored or excreted because of homeostatic regulations and is consequently not used for the synthesis of functional compounds. Overall, this factorial model of total utilization represents a concept of a broader and more general applicability.

The partial efficiency termed absorbability of fractional true absorption is defined as the ration of the truly absorbed amount (A) over intake (I):

$$\text{absorbability} = \frac{A}{I}$$

The metabolic efficiency is defined as the ratio of the amount of the element utilized for metabolic functions (Q) over the amount truly absorbed (A):

$$\text{metabolic efficiency} = \frac{Q}{A}$$

The total utilization defining the overall efficiency represents the product of absorbability and metabolic efficiency or, correspondingly, the ratio between the amount utilized in metabolism and the amount of intake:

$$\text{total utilization} = \frac{A}{I} \cdot \frac{Q}{A}$$

and hence

$$\text{total utilization} = \frac{Q}{I}$$

A major obstacle of deriving gross requirements by the factorial concept lies in the practical difficulties of

determining total utilization. Theoretically, it would be desirable to obtain a direct integral measurement of the quantity of the trace element used for functional roles per unit of time. This approach however, proves impracticable for the simple reasons that, first, there is still a lack of information on the biochemical role for a number of elements and that secondly, trace elements frequently have functions in numerous compounds which cannot be integrated, at the present, to an overall quantitative value.

In certain cases, total utilization may be approximated from determinations of apparent absorption or retention (Weigand and Kirchgeßner 1979, 1980). This shall be demonstrated by the following sequence of equations, in which capital letters express absolute quantities with the dimension of mass permit of time:

$$D = I - F$$

$$d = \frac{D}{I}$$

$$R = I - F - U$$

$$r = \frac{R}{I}$$

$$Q = I - F - U - S + F_m + U_m$$

$$Q = D - U - S + F_m + U_m$$

$$c = d - \frac{U+R}{I} + \frac{F_m+U_m}{I}$$

D = apparent absorption

r = apparent retention

I = intake

F = fecal excretion

U = urinary excretion

d = fractional apparent absorption

r = fractional apparent retention

F_m = inevitable endogenous fecal losses

or, alternatively,

$$Q = R - S + F_m + U_m$$

$$c = r - \frac{S}{I} + \frac{F_m + U_m}{I}$$

U_m = inevitable endogenous urinary loss

S = retention without being used for functional roles, i. e. storage

If it holds, for example that $R \geq S$, F_m and U_m, it follows that $Q \sim R$ and hence $c \sim r$. This is most likely the case in situations of high productivity, for instance rapid growth rate. The values of R and r can be estimated by conventional balance studies or, in the case of animal studies, also by carcass analysis.

This way illustrate the practical application of the factorial concept for deriving requirements. At the present stage of trace element research, however, the application of this model concept may be limited for the most part to situations of high productivity. The approximation of total utilization in these cases seems acceptable from the standpoint that recommended dietary allowances should contain safety margins. It should be ascertained, however, that such safety margins do not approach subtoxic levels of supply. Also, safety margins should not be so ample as to visk imbalances.

Conclusion

The net requirement of trace element is not of constant magnitude, but depends upon the height of the maintenance requirement and the type and intensity of production.

Accordingly, the determination of requirements must take into account the particular situation of life. The gross requirement, which specifies the necessary dietary intake, must be sufficiently high so that the net requirement is adequately net. Quantitative information on trace element utilization is a major prerequisite for computing gross requirements. The efficiency with which trace elements are utilized depends upon numerous factors such as, for example, chemical nature of the trace element, dietary composition, interactions with other dietary constituents, especially with other mineral elements, supply status, pH conditions within the gastrointestinal tract. Because of the multiplicity of influencing factors and the complexity and dynamics of trace element metabolism, the experimental determination of trace element utilization represents a challenging, multifaceted task that can be solved only approximately even under strictly standardized nutritional situations. For reliable recommendations of dietary allowance it is, never the less, necessary to invest special research efforts into the metabolism and utilization of trace elements. Under practical situations, the specification of the efficiency of utilization is thwarted especially because of a continual change in dietary composition. It is therefore necessary to rely on the approximation of assuming average values of utilization.

In the derivation of requirements, the theoretical concept of the factorial method fits best the fundamental concepts of trace element metabolism. The limits in the determination of the efficiency of utilization set at the same time the limits to the applicability of this method. Estimating requirements by the factorial method may be an especially sensible approach whenever the requirement for production is very much higher than that for maintenance, so that total utilization or may be approximately equated with apparent absorption apparent retention.

Considering the present state of knowledge of trace element metabolism and the methodological prerequisites, the dose-response relationship may currently appear as the method of choice for determining requirements. It is, however, recommended to analyze several response criteria. Model studies must be conducted to find response criteria suitable for estimating requirement, suitable even in studies with humans. However, continual changes in dietary composition again impose a very serious obstacle. It is therefore necessary that experimental estimates of the requirement are provided with a certain margin of safety for the specification of a recommended dietary allowance. The margin of safety must however, not be so high so as to impose the situation of trace element imbalances or subtoxic effects. Safety margins are needed not only because of intrapersonal variability, but also because of interpersonal variability.

In view of the fact that, within certain boundaries of trace element intake, the body is capable of responding to changes in the dietary supply with equivalent changes in the excretion, balance data are very difficult to interpret and, therefore, should no longer be applied for estimating requirements.

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MODELS FOR TRACE ELEMENT METABOLISM IN HUMANS

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Introduction

Appreciation of the significance of the essential and toxic trace elements for health has grown rapidly during the last 20 years. During that time, studies with experimental animals have gradually changed in emphasis from demonstrations of the gross clinical effects of deficiencies and toxicities to investigations of early pathological responses. From these we have come to appreciate that the absence of diagnostically specific clinical signs is no guarantee that tissue damage is not occurring and, furthermore, that when external evidence of a pathological response eventually appears, its causes are often difficult to identify from the clinical picture alone. In such situations, the ability to resolve the problem depends upon the availability of information from experimental studies describing relationships between trace element intake and animal response, with the latter defined either from evidence of physiologically significant anomalies in tissue trace element patterns or biochemical evidence of characteristic metabolic defects.

The experimental approaches needed to define the tolerable extremes of trace element exposure and to devise more appropriate procedures for detecting pathological responses involve deliberate depletion or excessive loading with the element under investigation. Ethical considerations will frequently

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preclude such studies with human subjects, particularly during pregnancy or early postnatal development when the risks of incurring permanent damage are particularly high. Since high risk situations are of particular interest it is inevitable that much of the information upon which risk assessment is based will continue to be derived from studies with experimental animal models, the advantages and limitations of which must be kept continually under review.

At the molecular or functional level there is a remarkable consistency in the evidence derived from a wide range of species that the essential trace elements serve identical and often highly specific functional roles. Typical examples are the respective roles of copper, zinc and selenium in the enzymes cytochrome oxidase, alkaline phosphatase and glutathione peroxidase, all of which irrespective of species, decline in activity as tissue deficiency of their inorganic component develops. However, much more relevant to the problem of selecting an appropriate animal model is that both the sensitivity to and the pathological expression of such changes can differ both between species and within a single species at differing stages of development (1). Such factors, together with species differences in sensitivity to interactions involving dietary constituents influencing trace element absorption and tissue redistribution, are all relevant to the selection of an appropriate model intended to simulate the response of man to changes in element supply.

Objectives of modelling

Our objectives in attempting to develop animal models of trace element metabolism in man are frequently ambitious. They can include:-

(i) attempts to focus attention on the principal pre-clinical and clinical pathological consequences of deficiency or excess in human subjects,

(ii) to assist with the development of more appropriate direct or indirect biochemical criteria from which to achieve early diagnosis of such problems and,

(iii) to explore, tentatively, the likely limits of tolerable exposure to element deficiency or excess and to identify the principal dietary and other variables likely to modify minimal requirements or tolerance.

This paper will illustrate some of the successes achieved and some of the problems encountered in attempts to meet such objectives by animal modelling of the effects caused by zinc and copper deficiencies and lead excess.

Comparative aspects of trace element availability

Evidence that trace element absorption and tissue utilisation can be influenced sufficiently by a range of common dietary constituents to induce pathological responses is much more firmly established for non-human species than for man (2). Whether the data derived from animal studies have implications for the trace element nutrition of man depends upon adequate consideration of both physiological and dietary differences as indicated by the following examples.

For most monogastric animals, the supply of physiologically available Zn is dictated both by Zn intake and by dietary content of antagonists to Zn absorption such as the phytic acid associated with components of the fibre fraction of cereals and the protein fraction of some legume seeds. Studies with pigs, chicks and rats indicated more than 20 years ago that Zn requirement was directly proportional to phytate intake. It

was also recognised that the antagonistic effects of phytate were potentiated by increases in dietary calcium and possibly ameliorated by increased dietary protein. That the antagonistic potency of phytate can thus vary has not only been ignored in many attempts to define dietary zinc availability to animals but has contributed to the controversy currently surrounding the significance of phytate-containing diets in the aetiology of zinc deficiency in man. In this context, the rat may be a reasonable model for man only if efforts are made to equate the normally differing dietary contents of the principal potentiators of the phytate-Zn antagonism, i.e. calcium and probably magnesium. Neglect of this precaution by the use of dietary regimes which, for the rat, normally contain more than twice the level of Ca in human diets quite understandably yields data which grossly overestimate of significance of phytate in the Zn nutrition of man. For the rat model, the ratio $\frac{[\text{phytate}][\text{Ca}]}{}$ (moles/kg diet) in excess of 3.5 realistically describes diets in which available Zn is insufficient to maintain optimum growth (3). While the validity of this model for man has yet to be established retrospective analysis of data from a range of Zn-balance trials suggests that a ratio exceeding 0.5 may provoke negative zinc balance. This discrepancy may be more apparent than real in view of the differing criteria used in rat and human trials to detect adverse effects on Zn utilisation.

The above simple example of the need for rigorous control and simulation of dietary conditions when attempting to "model" responses to variations in trace element supply and availability is not unique. Compositional variables, also involving dietary phytate, Ca (4) and possibly protein, certainly modify lead uptake by the rat and, according to recent evidence, probably affect Pb retention by man (Rose, H.E. personal communication). Additional evidence that the physical characteristics of protein precipitates formed during intragastric

and intraduodenal digestion (5) influence the uptake of zinc, cadmium and possibly other metals, emphasises the necessity of rigorous control of dietary protein sources in model studies intended to characterise availability phenomena. This requirement extends even to the selection of homologous or heterologous milks when investigating the uptake of adventitious contaminants from a milk diet.

Failure to take account of such variables may well have contributed to the difficulty in devising reliable animal models of Pb toxicity and, particularly, in attempts to compare dose/response relationships with man. That differences in dietary composition are highly relevant is indicated by evidence that the remarkable differences in the efficiency of Pb absorption exhibited by the rodent and the human subject (approx. 0.1-1% efficiency, rodent; 10% efficiency, man) are eliminated if the rodent is given a low fibre semi-synthetic diet. Rodent "modelling" with adequate dietary control has highlighted the significance of low dietary Ca, P (6) and Fe (7) in promoting Pb uptake (all of which effects have been confirmed in human subjects (8,9)). Additionally such models indicate that Pb uptake is enhanced strongly by a liquid milk diet; a possibility which has yet to be investigated in human subjects.

Major differences in gut physiology which, through their influence on digestive processes, influence the forms in which elements are presented to the gut mucosa, can have a major influence on model validity. Typical of such are the microbial events within the rumen or caecum which modify the forms of dietary Cu, Mo, Se and S (2) species ultimately presented to the gut mucosa. Such chemical transformations impose severe limitations upon the human relevance of absorptive studies based upon the lamb, guinea pig or rabbit. In these species, microbiological processes within the gut not only modify the

valency state of transition elements but also by reductive generation of sulphide or selenide, radically increase the opportunities for formation of insoluble metal derivatives of these particular anions and thus tend to reduce metal bioavailability, particularly of Cu, Mo and Pb. An interesting exception to this generalisation is that for diets containing Hg or Pb, there are indications that microbial methylation within the caecum can enhance bioavailability and the tissue incorporation of methyl mercury (10) or tetramethyl lead (11).

Remarkably few guidelines exist from which to develop appropriate animal models to predict the changes in trace element absorption which may occur postnatally in the human infant. Studies with rats indicate that prior to gut "closure" at approximately 16 days of age, absorption of the essential elements Fe and Cu is probably pinocytic and, in contrast to the older animal is not homeostatically regulated (12). Highly efficient pinocytic absorption of lead and of cadmium also occurs at this stage (for review, 13). If to this we add the finding that absorption of Zn by both unweaned rat and the human infant is more effective from homologous than from heterologous milk and the evidence that absorption of many trace elements from a milk diet is more efficient than from a solid diet, it will be apparent that the modelling of events at this stage of development is a particularly complex task. Since it is suspected that adventitious contamination of liquid diets offered before weaning has a disproportionally large influence on the uptake, the tissue retention and the risks of toxicity from several trace metals (e.g. Pb, Cd and Cu) it is imperative that greater attention should be devoted to the development of models appropriate both to the premature and normal human infant.

In concluding this brief account of the modelling of trace element absorptive phenomena it is important to caution against experimental approaches based solely upon administration of test doses of elements or their antagonists in drinking water or aqueous gavages except in instances where these vehicles are the specific objects of investigation. For example, evidence is emerging that the mutually competitive interactions between Fe and Zn that influence absorption of these elements are expressed much more strongly when imbalance is induced by administration of aqueous solutions rather than by modifying the Fe and Zn content of the diet (15). Similar considerations may influence the antagonistic effects of high intakes of Zn, Fe and Cd on Cu absorption. Furthermore the absence of food from the proximal gut of the rat enhances between 2 and 10 fold the carcass uptake of Zn, Cu, Fe, Pb and Hg and enhance the proportion of the retained dose present in gut tissue (14). That similar variables influence the fate of elements in man is typified by data from one study indicating that the half-time for faecal excretion of a dose of ^{65}Zn given in solution to starved subjects was approximately 13 days whereas, when given with food was only 3.5 days. Rigid compatibility of experimental protocols is thus essential both for comparative studies and to define the intrinsic properties of absorptive processes within a single species.

Modelling of pathological responses

Effective animal modelling of human responses to anomalies in trace element supply serves two objectives. It can alert investigators to subtle pathological changes that are not immediately evident from a clinical examination of human subjects at risk and can provide guidelines for discriminating effects attributable to short or long-term exposure to anomalies in trace element supply. Furthermore, if accompanied

by biochemical studies, such modelling can frequently indicate the likely value and limitations of diagnostic or monitoring procedures based on measurements of tissue trace element content or the presence of abnormal enzyme or metabolite concentrations in plasma or urine. Typical aspects of the value of such modelling are considered below.

Zinc deficiency or excess

Under the closely controlled protocols usually applied in studies with experimental animals, measurements of changes in plasma or hair Zn or of plasma alkaline phosphatase activity can provide a reasonably reliable indication of Zn status. However, such studies have also revealed limitations to these approaches. Thus, plasma Zn, even in Zn-deficient animals increases rapidly if tissue growth is limited by other nutritional insults and is depressed markedly by concurrent infection or other stress. Under extreme situations these effects may well be paralleled by corresponding changes in the pathological manifestations of deficiency. Thus, the skin lesions (parakeratosis) of Zn deficiency in the animal model are provoked by physical abrasion or ectoparasite activity; in contrast, such lesions may never appear if the concurrent intake of protein is low. Animal modelling has also shown that the teratogenic consequences of Zn deficiency in the rat are reduced or eliminated if skeletal resorption is promoted by a low Ca diet (16) or if tissue catabolism is provoked by a low food intake (17).

Virtually all these situations influencing the interpretation of data for plasma and hair Zn have been found to have their parallels in studies of Zn-responsive conditions in man. Nevertheless, species differences in response do arise as typified by the work of Hurley and her colleagues (18,19) on zinc deficiency in rodents, ruminants and laboratory primates.

These and other comparative studies have particularly highlighted marked species differences in susceptibility to teratogenic effects of Zn deficiency during pregnancy which, superficially at least, appears to be related directly to total maternal anabolic activity during pregnancy. Thus susceptibility is high in the rat but, on current evidence, virtually non existent in the primate although late foetal growth is affected in all species.

Comparative studies suggest that the critical threshold values for plasma Zn may be higher for the laboratory primate or human subject than for other species. However, studies with all primates, including man, illustrate a complexity of relationships between plasma Zn concentrations and the pathological expression of Zn deficiency that is not always revealed in studies with other animal species maintained under closely controlled experimental conditions. Among such findings are an initial decline in plasma Zn when the growth of deficient infants resumes after Zn supply is increased and evidence of an inverse relationship between plasma Zn and growth of the human foetus, infant and laboratory primate judged to have a "marginal" Zn status as defined by other biochemical and pathological criteria (18-21).

Such ostensibly paradoxical findings indicate, firstly, the need for better understanding of factors governing the equilibria between Zn in plasma and other tissues and, secondly, the need for better criteria than plasma Zn for assessing Zn status. Work with rats suggests that plasma concentrations of the Zn-induced protein, metallothionein, may offer a more suitable and physiologically relevant alternative (22,23). Its validity as a marker of the Zn status of man has yet to be assessed. Other inadequately explored aspects of the potential relevance of data from animal models also exist. Thus there are clear indications from studies with rats that the

efficiency of Zn absorption is enhanced markedly during the last third of pregnancy and during early lactation (24). This finding may well be relevant to growing evidence that past predictions of Zn requirements for human pregnancy and lactation are gross overestimates when derived by a factorial approach assuming no adaptive increase in the efficiency of Zn absorption. Also widely ignored is evidence from work with rats and ruminants that while Zn is normally well tolerated, susceptibility to Zn intoxication is particularly high during the terminal stages of pregnancy when the element is rapidly translocated to the developing foetus (25,26). Indications of the possible relevance of this finding are unfortunately provided by case histories of abortions in women given 75 mg Zn orally/day - a dose rate which although high would normally be well tolerated by non-pregnant subjects (25).

Copper deficiency

The growth of studies of copper deficiency in man provides clear examples of the value, the limitations and, sometimes, the misinterpretation of data derived from work with experimental animals. Initial studies with animal models concentrated on the origins and characteristics of the anaemia of Cu deficiency, paid little attention to adverse effects on growth and virtually ignored other aspects. The failure of one group of investigators to produce anaemia in infants subjected to a period of Cu depletion (27), coupled with a wider appreciation of the extensive precautions to exclude contamination that are needed to induce Cu deficiency anaemia in rats and swine, established the belief that Cu deficiency was unlikely to arise in human subjects.

Although one case report later described the development of a Cu-responsive anaemia in socially deprived infants offered diluted cow's milk, the wider pathological implications of Cu

deficiency in man were only appreciated after more extensive studies with laboratory animals. Such reports of the effects of Cu deficiency on skeletal and connective tissue development, cardiovascular and CNS integrity, hair structure and pigmentation and foetal survival all pointed very clearly to the fact that anaemia is a relatively late consequence and is thus unlikely to be the principal pathological feature of human Cu deficiency.

Features common to nutritional Cu deficiency in human infants, adolescents and many experimental animal models include defects in bone connective tissue (elastin and collagen) biosynthesis and in neutrophil development or function (for review see 28). Data from animal models indicate that the metabolic defects responsible for all these lesions can develop before overt signs of deficiency appear.

The significance for man of many other features of Cu deficiency in experimental animals has not yet been explored adequately. These include cardiomegaly and its associated connective tissue changes, decreased energetic efficiency of the heart, defects in catecholamine metabolism in the heart (29), intestine and other tissues and metabolic lesions influencing mitochondrial energy metabolism in a variety of tissues. Some lesions, such as defects in aortic elastin morphology (30) and those responsible for foetal death and resorption which develop in young rats given diets only "marginally" deficient in Cu (e.g. 2 mg Cu/kg diet), merit closer investigation with respect to the effects of Cu deficiency in other species, including man.

In remarkable contrast, virtually all the Cu deficiency lesions demonstrated in experimental animals have now been detected in infants with Menkes' disease (28). Indeed, the identity of this disease as a genetic defect in Cu metabolism was first suggested from the similarity of structural defects

in the hair of affected subjects with those of the wool of Cu deficient sheep. The other major characteristic of Menkes' disease, its clinical manifestations of neuropathological defects, appears superficially similar to that caused by degenerative lesions of the CNS in neonatal Cu-deficient lambs. However, there are suspicions that the pathogenesis of these two disorders may differ with cerebral vascular lesions restricting oxygen supply playing a much more prominent role in Menkes' disease.

The cardiac lesions and cardiovascular functional abnormalities of Menkes's disease also resemble those found in Cu deficient rats. The extent to which such lesions also develop as a consequence of nutritional Cu deficiency in man is a topic currently exciting attention (31,32). The heart of the Cu deficient rat is enlarged, fragile, flaccid, becomes oedematous under workload and exhibits arrhythmia and other ECG abnormalities (29,34). Biochemical studies indicate changes in collagen type, decreased concentrations of noradrenaline and nucleotide triphosphates and a reduced energetic efficiency. For reasons unknown as yet, susceptibility to cardiac damage appears to be exacerbated by diets high in fructose (33). It is also claimed that a concurrent deficiency of selenium or a high dietary Zn/Cu ratio increases mortality from myocardial rupture in Cu deficient rats.

For obvious reasons, much less is known of any cardiopathological changes in human subjects with a low Cu status. However such consequences must be considered more closely in the light of recent evidence of the development of tachycardia and other ECG abnormalities in 5 of 25 human subjects offered diets providing approx. 0.36 mg Cu/megacal for periods between 7 and 15 weeks (31,32). Appraisal of the validity of the rat or pig model of such possibly Cu-related cardiovascular phenomena is probably contingent upon closer in vivo monitoring of

cardiac output and related electrophysiological changes in human volunteers undergoing Cu depletion.

Pathological aspects of the modelling of lead intoxication

A wide range of neurological lesions has been reported from studies of lead toxicity conducted with laboratory animals exposed to relatively high doses of Pb in food or drinking water. Biochemical findings in such models include depression of acetyl choline release, changes in catecholamine metabolism and depressed oxidative metabolism of the brain cortex. However, the doses of Pb required to provoke such changes or to produce consistent behavioural abnormalities are substantially higher than those claimed to provoke adverse effects in man. The possible relevance of differences in dietary composition influencing the dynamics of Pb absorption in studies with man and laboratory animals has been commented upon earlier in this paper. However it is also evident that the typical blood Pb concentrations associated with such pathological responses in laboratory rodents are frequently 2-3 fold higher than in human subjects believed to be at risk.

These and other limitations of rodent models of Pb toxicity have been considered in extenso elsewhere. The possibly greater relevance of laboratory primate studies is suggested by work with rhesus monkeys with blood Pb in the range 0.4-0.6 mg/l thus simulating blood Pb levels in children believed to be at risk. Young monkeys so treated developed cognitive defects which persisted upto 3 years after Pb withdrawal (35). It is still not clear whether the limitations of the rodent model reflect uncharacterised physiological or metabolic differences in the response to Pb or limitations in our ability to detect in the rodent the subtle behavioural and cognitive changes that are considered significant in Pb-exposed children

Conclusion

Although necessarily restricted in scope, this paper will have indicated aspects of both the potential and the limitations of animal modelling of human trace element metabolism. For reasons outlined in the introduction it is clear that reliance on such approaches must continue. Thus it is essential to emphasise that one of the most significant limitations to progress arises from the frequently inadequate incremental approaches to the definition of dose/response relationships and the failure to define changes in these relationships at differing stages of physiological development of possibly useful model species. Tedious though such systematic studies may appear, they are essential if the potential of models is to be explored adequately. They provide the only means of defining the change in sensitivities to trace element-dependent processes that occur as synthetic and metabolic activities change during growth and development.

Obviously, it is unrealistic to expect that any animal model can reflect all the relevant aspects of human trace element metabolism for which information is required to define tolerance or pathological risks. Nevertheless, with careful interpretation of adequate data derived from models, our ability to predict and anticipate human responses is increasing rapidly.

Animal models have contributed substantially to understanding of the genetic origins of defective Cu and Zn metabolism in Menkes' disease and acrodermatitis enteropathica in man as well as suggesting approaches to clarifying the pathologies arising from dietary deficiencies of Cu, Zn and Mo deficiencies in man (36). Less satisfactory is the status of animal modelling of the claimed effects of selenium deficiency as the sole relevant variable in the aetiology of Keshan disease in

man - a situation which should perhaps be interpreted as suggesting an inadequate understanding of the causes of this "nutritional" disease rather than of inadequate modelling.

The future development and application of animal modelling depends greatly upon a wider appreciation of those physiological and dietary variables that modify the response of individual species to changes in trace element intake. Included among these is genetic variability, operating even within species, that influences both the kinetics of element absorption and retention and the pathological response to deficiency or excess. Apart from situations such as the dramatic clinical consequences of acrodermatitis enteropathica and Menkes' disease in the infant, little is yet known of the significance of such genetic variability in trace element metabolism in man. Nevertheless, evidence of its relevance to animals is growing rapidly from studies such as those demonstrating marked genetic differences in susceptibility to both deficiency and toxicity of copper in sheep and corresponding evidence of variability in responses to copper and manganese exposure in rats and mice.

Although much remains to be done, the achievements of modelling have already been substantial. Furthermore, there is clear evidence of a failure to explore the possible significance of some findings that may have relevance to man. Thus, epidemiological studies of Pb and Cd "toxicities" have almost totally disregarded the weight of evidence from animal models that dietary composition modifies toxicity. Possibly of much wider significance is the inadequate exploration of findings that a marginal Fe status, totally irrelevant from the standpoint of its minor haematological consequences, has a marked influence on the voluntary activity of rats (37).

Such considerations and the progressively increasing demand for methods for biological monitoring of the effects of changing dietary patterns or the growing availability of "novel" foods on trace element metabolism in man indicate the opportunities that exist for the development of animal modelling techniques whose applicability and limitations are responsibly considered and clearly defined.

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Session II

TRACE ELEMENTS IN NUTRITION

Plenary Lectures given by:

L.M. Klevay

H.B. von Stockhausen

R. Schelenz

A.E. Harmuth-Hoene

Chairman:

M. Kirchgeßner

M. Anke

Poster Reports:

M. Anke

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DIETARY REQUIREMENTS FOR TRACE ELEMENTS IN HUMANS

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Introduction

Study of the essentiality of nutrients is largely a phenomenon of the twentieth century. After Aristotle recorded the toxicity of arsenicals (1) and Holmes (2) and Semmelweis (3) showed how to avoid puerperal fever, physicians were well acquainted with the concept that diseases could be caused by external agents. Acceptance of the concept that diseases could occur because an agent was absent required general reevaluation of medical thought.

The origin of the deficiency concept is widely associated with Lind (4) who cured scurvy with citrus fruit. However, Boyle concluded approximately a century earlier that animals dying in a vacuum perished from the lack of an essential substance (5). Funk (6) promoted the concept of "vitamines". Vitamins were renamed by the excision of the terminal e after it was realized that not all of them were amines.

Although there may be thousands of chemicals in foods, the number of essential nutrients probably is not much greater than fifty if one counts the amino acids, the minerals, the vitamins, water and a few trace elements currently under study. Often the experiments that prove essentiality provide the first estimates of requirements.

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Nutritional Essentiality

A substance is considered a nutritional essential for an organism if the organism can neither grow nor complete its life cycle in the absence of the substance. This concept was best outlined by Bowen (7) who extended Arnon's (8) criteria for plants to animals. It is difficult, perhaps impossible, to obtain evidence in conformity with this rigid definition, even for plants or animals. Essentiality for humans sometimes is based on inference. It is unethical to satisfy this definition by subjecting people to severe experimental conditions.

Surprisingly little has been written on the definition of nutritional essentiality (9-25). Table 1 contains a summary of criteria suggested by several authors. Criteria are classified as definitive or inferential; criteria for which there was little agreement among authors are omitted.

TABLE 1. CRITERIA OF NUTRIENT ESSENTIALITY

Definitive	
Restricted Diets:	Supplemented Diets:
interrupt the life cycle	prevent pathology
impair function	provide relief

Inferential
Atomic number is low
Biological activity
Homeostatic control
Toxicity is low
Ubiquitous

Definitive criteria

The criteria (Table 1) illustrate the concepts of the authors but do not necessarily reflect the words used by the authors. For example, Bowen and Arnon are quoted above regarding the definitive criteria. Hegsted (16) referred to a requirement "...for normal growth and development...". Mertz (19) wrote "absence...is incompatible with life" and Frieden (25) defined essential elements as those "required for the maintainance of life" and quoted Bertrand's work of 1912 regarding less severe deficiency states.

Several of the authors noted that function is impaired if too little of an essential nutrient is ingested. Cotzias (15) mentioned "structural and physiological abnormalities". Some authors noted that more than one function could be impaired by deficiency of one nutrient.

McCollum (9) referred to a "diet which causes malnutrition" and the identification of "protective foods". Mertz (18) referred to "supplementation" and "therapeutic trial" as means of improving an impaired function. Later he mentioned prevention or cure by an essential element (20). Underwood (13) mentioned "responses in growth" and Cotzias (15) wrote that an essential element "either prevents or reverses" abnormalities and included biochemical improvement as appropriate evidence of essentiality. Others (24) refer to "normal function and health".

In summary, anatomical, biochemical and physiological abnormalities produced by restricted diets and relieved by supplemented diets are acceptable as evidences of essentiality. A nutrient can have more than one function. Death and improved growth are mentioned specifically; other impairments are permissible but are not specified.

Inferential criteria

The definition of essentiality is simple in theory and severe in practice. Many experiments have been done with nutrients that satisfy the definitive criteria. Some characteristics of essential nutrients have been inferred from these experiments. The inferential criteria are not very helpful in predicting which elements will be found essential after appropriate experiments.

Schwartz (23) noted that no elements with atomic number greater than that of iodine has been proved to be essential. Schroeder (11) suggested lanthanum as an upper limit. Underwood (22) suggested that gallium and germanium may be worthy of study for essentiality because a substantial number of essential elements have atomic numbers between 23 and 34. Consideration of atomic number is peculiar to trace elements; however, other nutritional essentials usually have low molecular weights.

Mildvan (26) has estimated that more than 27% of known enzymes "have metals built into their structures, require added metals for activity, or are further activated by metal ions". Underwood (13) quoted Green (27) as saying that "enzymatic catalysis... is the only rational

explanation of how a trace substance can produce profound biological effects". Frieden (25) however, cautioned that stimulatory effects are not always evidence of essentiality and that a decreased amount of an essential nutrient may be beneficial. This latter situation can arise if the dose of the nutrient is too large or if the dose is sufficient to interfere with the utilization of another nutrient.

Several authors have emphasized that essential elements are under homeostatic control. Perry et al. (12) noted that the value for the 90th percentile for metal concentrations in organs divided by that of the 10th percentile (80% range) was greater for nonessential than for essential elements. A quotient of 7.0 separated the two groups. Liebscher and Smith (17) proposed several distinctions, the simplest of which was that essential elements have normal distributions and nonessential elements have log-normal distributions.

Schroeder (11) suggested that the dose of an essential element equal to that of the body burden is readily tolerated. Schwarz (23) believed that toxicity does not mitigate against essentiality.

Several authors have suggested that essential elements are ubiquitous. Cotzias (15) emphasized presence in healthy tissues. However, contemporary analysts can measure small amounts and low concentrations accurately and with such ease (28),that general detectibility is not very helpful in distinguishing between essential and nonessential elements. Vallee (10) has warned that the "concentration of an element in biologic matter is <u>not</u> the sole criterion...of importance... in health and disease...". Underwood (13) and Mertz (19) warned against uncritical reliance on analysis in establishing deficiency. One can infer that similar caution is desirable in establishing essentiality.

In addition to the inferential criteria listed in Table 1, numerous other criteria have been suggested (9,17,19,21). Although some of these may be helpful, these criteria have received less attention. Careful reading permits the conclusion that exceptions are numerous.

After essentiality has been established, measurement of nutritional requirements becomes of prime importance. Experiments with animals are more likely to yield rapid progress than those with humans.

Zones of nutriture

Dann and Darby (29) provided instruction in the search for impaired function. Tracing the history of nutritional appraisal to earlier study of protein, they emphasized vitamins. Their five zones of nutriture, which are equally applicable to trace elements, are summarized in Table 2. Saturation implies maximal storage of the nutrient without early toxicity. Potential deficiency exists when departure from saturation is sufficiently great that a new stress on the organism will induce illness or when a suitable test reveals reduced functional capacity. Latent deficiency disease is the mildest clinically detectable form of deficiency disease. This zone is characterized by vague, nonspecific symptoms that do not permit a definite diagnosis. Improvement occurs on nutritional replacement. Clinically manifest disease may be mild or severe. Severe illness can be diagnosed without the assistance of the laboratory or therapeutic trial. Some examples of recent research that illustrate these principles are provided.

TABLE 2. ZONES OF NUTRITURE*

1. Saturation
2. Unsaturated, but functionally unimpaired
3. Potential deficiency disease
4. Latent deficiency disease
5. Clinically manifest deficiency disease

*After Dann and Darby (29).

Guiding Principles

Animal studies

Many of characteristics are useful in assessing vitamin nutriture and establishing nutritional requirements. Methods of assessing trace element nutriture and establishing requirements are less well developed. Experiments with vitamins can be useful guides.

The principles which guide the measurement of the nutritional requirements of humans are similar to those used for animals. A few years ago I determined the biotin requirement of rats fed egg white (30). Many students of trace element metabolism use egg white as a dietary protein because it is of high nutritive quality and it is low in copper and zinc. Unfortunately one of the proteins in it interferes with the metabolic utilization of biotin. Weanling rats fed 1.0 or 1.5 mg of biotin per kg of diet weighed 45 and 72%, respectively of those fed 2.0 mg/kg after sixty days (30). This impairment corresponds to

the fifth zone of nutriture (Table 2). Growth in response to the lower amounts of biotin was clearly inferior; greater amounts of biotin gave no benefit. The requirement was set at 2 mg/kg.

We also assessed several characteristics of copper deficiency in a group of rats (31). Table 3 summarizes the data. The rats were equally malnourished as a group; variation between rats sometimes was considerable. Some animals were ill (zone 5, Table 2); others had functional impairment (zone 3, Table 2). Variation among the characteristics was even greater. Liver iron was nearly three times normal; body weight was decreased by one fourth. Many of these characteristics cannot be measured on people. They illustrate some types of useful measurements, however. In principle functional tests based on cardiac catecholamines can be devised. There is no theoretical obstacle to those tests being validated.

TABLE 3. INDICES OF COPPER DEFICIENCY IN RATS

Greatest Change	Other Findings:
Liver iron	Abnormal electrocardiograms
Heart dopamine	Achromotrichia
Liver copper*	No anemia
Plasma cholesterol	Ventricular aneurysms
Heart weight	
Heart norepinephrine*	
Body weight*	
Least change	

*Decreased, others are increased

After Klevay, et al. (31)

The chemical reactivity of transition elements makes probable the existance of several chemical forms of these elements in both diets and in organs. Vallee (10) suggested that if it had been hypothesized --- prior to the discovery of vitamin B_{12} --- that cobalt is a significant factor in pernicious anemia, pharmacologic experiments with cobalt would have been negative. Measurement of cobalt in organs would have been similarly uninformative.

Underwood (13) noted that it often is difficult to correlate the deficiency state with "subnormal levels of the elements in the blood or organs". Mertz (20) expanded on this view. Copper deficiency produced decreases in cardiac copper ranging from 26 to 42% in three replications of an experiment (32) with rats. However, the highest control value for cardiac copper was 2 1/2 times the lowest value.

Funtional impairment as a result of dietary deficiency usually is demonstrated by the methods of biochemistry. In principle, the techniques of anatomy or physiology are equally useful.

Human studies

Studies of vitamin nutriture are more complete than those on trace elements and can be a useful guide. Theone, Mock and Kien, et al. (33-36) have studied the biochemical characteristics of human biotin deficiency. Table 4 summarizes some of their measurements. Underwood (22) and others (24) have emphasized that judgement is necessary in interpreting data on requirements. Data for some nutrients often are