Animal Models for Assessing Bioavailability of Essential and Toxic Elements[1]

M. R. SPIVEY FOX, R. M. JACOBS, A. O. L. JONES, B. E. FRY, Jr., M. RAKOWSKA,[2] R. P. HAMILTON, B. F. HARLAND, C. L. STONE, and S.-H. TAO, Division of Nutrition, Food and Drug Administration, Department of Health and Human Services, Washington, DC 20204

ABSTRACT

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Mineral bioavailability problems are increasingly recognized as important in assessing the nutritional quality or safety of a dietary supplement, food, or diet. The chemical species of the element and other components of the test substance, food, or diet may influence bioavailability of the element. These factors affect toxic elements, such as lead and cadmium; the established essential elements; and probably the "new" trace elements, such as nickel, vanadium, and silicon. Improvements are needed in designing animal experiments to mimic more closely human dietary practices and problems and to improve the sensitivity and specificity of assessing nutritional effects so that animal studies can provide data more relevant to humans. From the large number of experimental variables that need to be studied in animals, minimal conditions to produce adverse effects need to be identified and investigated in detail for biochemical mechanisms and for making direct comparisons between animals and humans.

Recognition of the need to evaluate bioavailability of inorganic elements under various dietary conditions has evolved from study of existing or potential problems in humans. These include deficiences of iron (DHEW 1972, Owen and Lippman 1977, Abraham et al 1979) and zinc (Hambidge and Nichols 1978, Prasad 1979), the presence of toxic elements in foods (Mahaffey et al 1975), the use of dietary supplements high in minerals (National Nutrition Consortium 1978), and new technologies that have led to vegetable-base products, such as those from soy, as substitutes for conventional protein foods (Wilcke et al 1979). Studies in animals have shown that bioavailability differs markedly with different concentrated forms of an element (Ammerman and Miller 1972, Peeler 1972), with elements supplied by different foods (O'Dell et al 1972, Momčilović and Shah 1976), and with the same elemental form fed in different diets (Forbes and Yohe 1960).[3] Interactions among minerals (essential and toxic) and between minerals and other nutrients and dietary components have been observed (Underwood 1977).

The gradual emergence of these varied types of bioavailability problems, changes in the food supply, and changes in human dietary habits have contributed to the need for obtaining a wide range of information on the nutritional adequacy of foods and diets. The purpose of this article is to examine various facets of experimental design and response evaluation and to suggest improvements in animal models for predicting bioavailability of inorganic elements for humans. Consideration is given primarily to studies within nutrient deficiency and optimal health ranges; however, some examples of responses in the range of toxicity are also presented.

DEFINITIONS

Dietary requirement is the minimal quantity of a nutrient (incorporated into a given diet) needed to support normal structure and physiological functions for optimal health at designated segments of the life cycle.

Bioavailability is a quantitative measure of the utilization of a nutrient under specified conditions to support the organism's normal structure and physiological processes.

Relative biological value (RBV) is the bioavailability of a test source of a nutrient, expressed as a percentage of the value obtained when the nutrient is fed in the form of a reference material.

A reference material for bioassays is a substance that contains the nutrient of interest. It should be chemically stable, commercially available in a standardized form, and have been shown to be utilized satisfactorily and reproducibly by several species under various conditions in several laboratories. If reference materials are used for physiocochemical analyses of an element, they are often nonpurified; they must be homogeneous and stable under storage and be certified for elemental content by two or more reliable methods.

A test substance may be any material that could be an appropriate dietary source of a given nutrient. It could be a specific chemical, a partially purified material, or a nonpurified material.

Primary indices of response include quantifiable levels of morphology or physiological function that indicate health status. Examples are measures of growth (height, weight, head circumference, etc), skeletal development (bone size, conformation, and mineralization), hematopoiesis, circulatory function, respiratory function, ability to perform work, immunocompetence, etc.

Secondary indices of responses are quantifiable responses that are not direct measures of health status but must be correlated with primary indices under defined conditions. Examples include the whole body retention of elements or the levels of inorganic elements, metabolities, enzymes, or hormones in tissues, body fluids, or excretory products.

INDICES FOR ASSESSING BIOAVAILABILITY

The selection of indices for evaluating bioavailability is based on knowledge of nutrient requirements, metabolism, and function. The sensitivity of measurable responses will control the flexibility possible in the nutritional design. Types of theoretical response curves that may be obtained with graded levels of dietary elements in the deficiency and optimal health ranges are shown in Fig. 1.

The upper panel of this figure relates to health indices, or primary responses. In the deficiency range, various indices may not be equally sensitive to an inadequate supply of the nutrient, as illustrated by curves A–C. A response that changes over a wide range of deficient dietary intakes of reference material and reaches an asymptote at requirement provides the greatest opportunity for obtaining values at several points with a test substance, thus permitting calculation of an RBV. Curve B most closely approximates this. Once requirement is met, all health indices (Fig. 1D) remain the same until the dietary level reaches the toxicity range, at which point one or more measurements may decline. Therefore, primary indices can only be used in the deficiency and toxicity ranges.

The concentrations of inorganic elements in tissue and body fluids often provide the most versatile means of assessing status and bioavailability over a wide range of intakes, as shown in the lower part of Fig. 1. The concentration of elements in tissue may remain

constant irrespective of intake (Fig. 1F and M). In rare instances, tissue concentrations may be elevated in severe deficiency (Fig. 1E); however, the typical effect is a marked decrease (Fig. 1G–I). Curve I represents a threshold effect that would pose problems in identifying exact ranges of intakes for the test source of the element. From correlation of tissue element levels with health indices, element levels alone can be used for preliminary assessment of health status.

Within the optimal health range of nutrient intakes, tissue mineral levels and whole body retention (Fig. 1J–L) extend the capability for assessing bioavailability. Investigation within this health range has the advantage that health status, food intake, and growth rate are the same for all groups of animals. Identification of tissue element concentrations at the requirement level and at the beginning of the toxicity range are particularly important for assessing the levels of protection against deficiency and safety from toxicity, respectively.

Concentrations of metabolites, enzymes, hormones, etc. may also be related to dietary intake of a nutrient. These response curves may resemble either those for primary health indices or for tissue element concentrations (Fig. 1) and thus be useful for assessing bioavailability.

Figure 2 illustrates the different types of response curves obtained by Leucke et al (1970) in an experiment with rats fed a zinc-deficient egg white diet. The advantage of the bioassay based on growth is that it requires very low levels of zinc intake. Serum zinc could be useful both above and below requirement. The femur zinc concentration has a more extended useful range, including dietary levels above requirement.
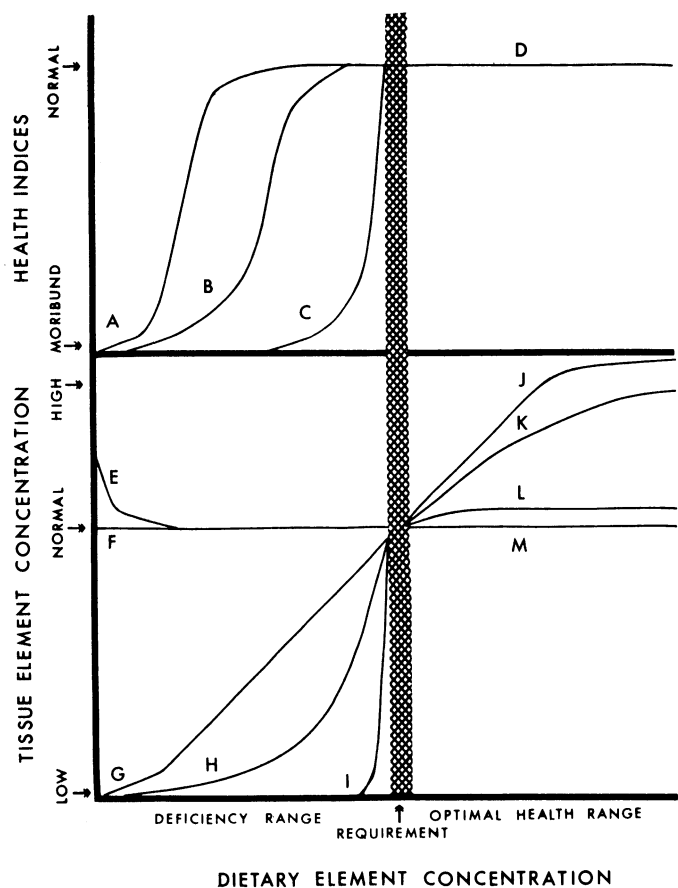

Fig. 1. Responses to dietary concentrations of essential elements in the deficiency and optimal health ranges. **Upper,** curves of health indices (primary responses). **A–C,** Curves of varying sensitivity; **D,** unchanged indices between requirement range and toxicity range. **Lower,** curves of tissue element concentration (secondary responses). **E,** Rare curve of severe deficiency causing elevation of concentration; **F** and **M,** curves of constant concentration; **G–I,** typical deficiency curves; **J–L,** curves showing increased element concentration beyond requirement range.
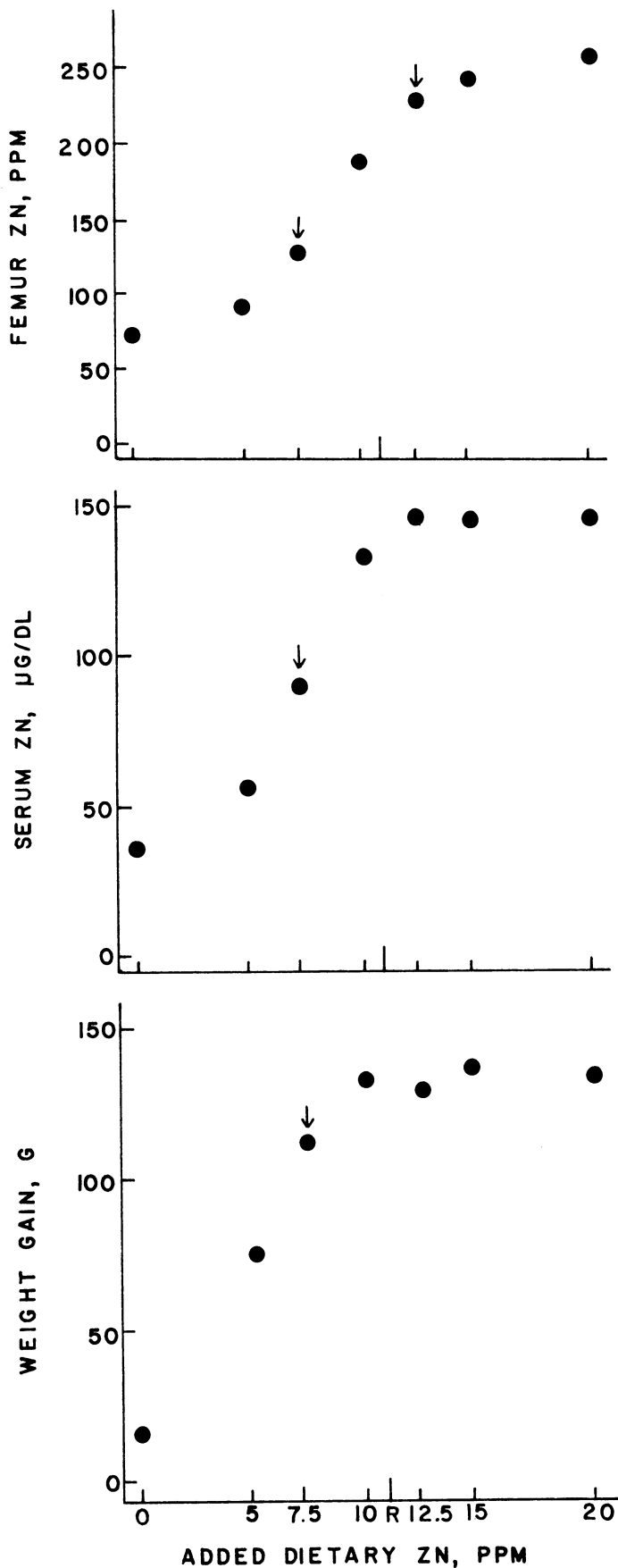

Fig. 2. Response curves showing effects of graded levels of dietary zinc added to a zinc-deficient egg white diet on weight gains and serum and femur zinc (fat-free dry weight basis) concentrations of rats (Leucke et al 1970). Zinc intake R = requirement. Arrows indicate the first level of added zinc that produced a significant difference ($P < 0.001$) either below or above the value for the control group (10 ppm added zinc).
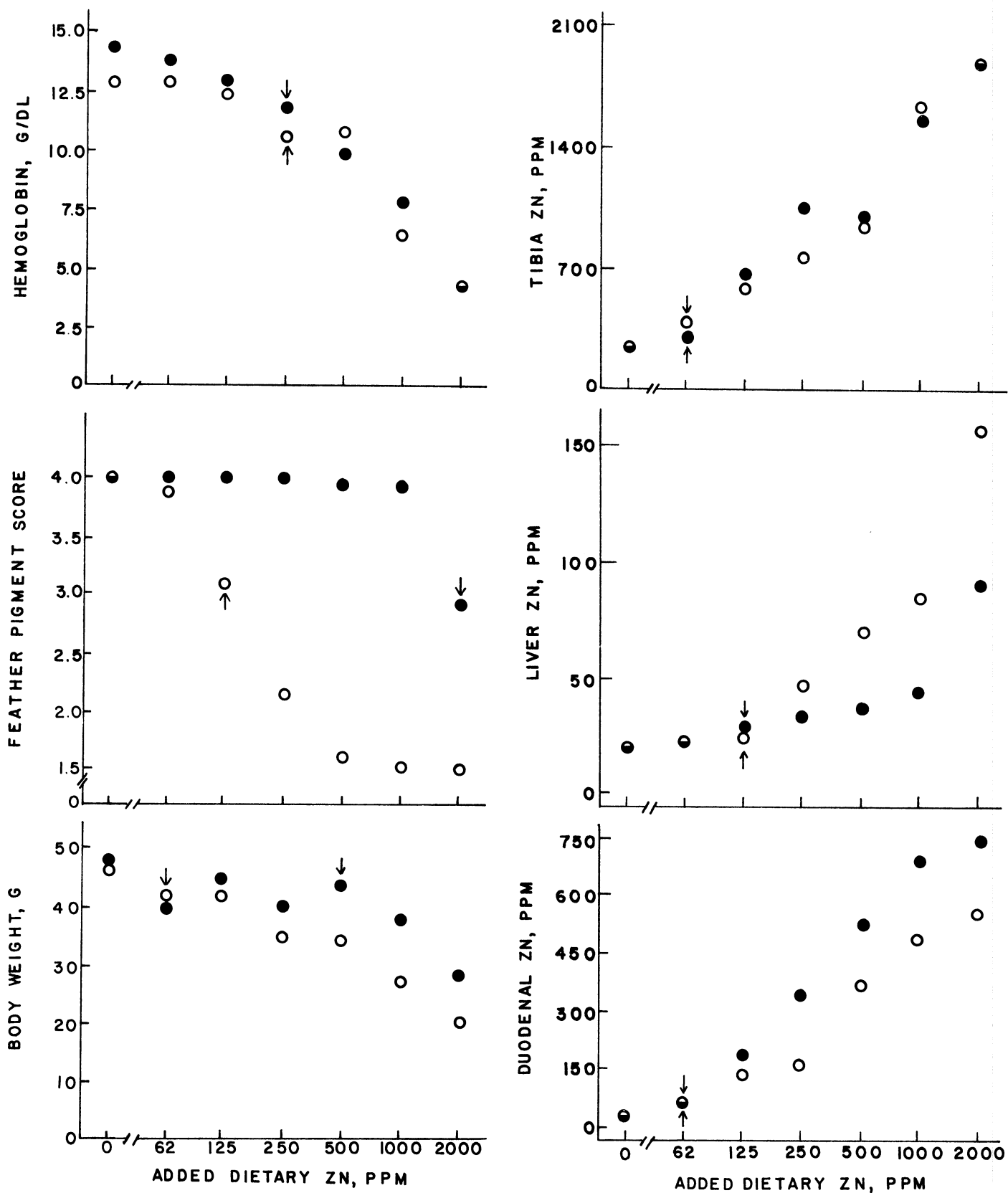
Fig. 3. Response curves showing effects of graded levels of dietary zinc added in excess of requirement (20 ppm zinc) to a casein-gelatin diet on body weight, feather pigment score (4 = normal, 0 = maximal lack of pigment), hemoglobin, and duodenal, liver, and tibia zinc (fat-free dry weight basis) concentrations in Japanese quail (Hamilton et al 1979 and R. P. Hamilton, unpublished data). o = Diet marginally deficient in copper (two-thirds of requirement), • = diet adequate in copper (about two and one half times requirement). Arrows indicate the first level of added zinc that produced a significant difference ($P < 0.05$) from the respective control group (no added zinc, same level of dietary copper) and that was followed by similar or greater differences from the control group with higher levels of dietary zinc.

Figure 3 shows response curves from a study in which three health indices and three tissue concentrations of zinc were investigated (Hamilton et al 1979 and Hamilton[4]). This study compared the effects of dietary zinc at graded levels at and above requirement in conjunction with low copper and copper in moderate excess. Body weight and hemoglobin were the indices least sensitive to excess zinc; however, graded respsonses in relation to dose were obtained. Feather pigmentation was particularly sensitive to excess zinc with marginal dietary copper. This would be a sensitive bioassay for zinc sources free of or low in copper. The duodenal, liver, and tibia zinc concentrations responded to changes in dietary zinc. The effects of copper were reversed between the liver and duodenum.

The exact dose-slope relationships can be expected to vary under slightly different circumstances. Each investigator must select the best indices for the purpose.

Bioassays should include at least three dietary levels of reference material and of each test substance, but this may not be feasible. Statistical analyses for estimating RBV can be based on parallel lines or slope ratio evaluations (Finney 1964, Harpley et al 1973). The appropriate method depends upon the quantitative dose-response relationships and the relative responses to reference and test materials. Even with simple test substances, each response line may have to be "fitted" through a common y-intercept before relative bioavailability is calculated, as has been reported for iron salts (Amine et al 1972). In some circumstances estimating bioavailability from only one point or from an extremely narrow dose range may be necessary.

The bioavailability of iron in experimental animals and in humans has been studied more extensively than that of any other inorganic element. Waddell (1973) summarized early information on methodology and bioavailability. The useful methods have been based on primary and secondary indices, including chemical balance (input vs output), radioiron balance (input vs output and whole body measurements of radioactivity), plasma iron absorption curves (chemical or radioisotope measurements), red cell radioiron content (single oral dose or oral and injected doses of different isotopes), and hemoglobin repletion or maintenance. More recently serum ferritin and free erythrocyte protoporphyrin have been useful indices of iron status.

DESIGN OF ANIMAL EXPERIMENTS TO ASSESS BIOAVAILABILITY OF ELEMENTS IN FOODS

Determining bioavailability of individual elements added to the diet in concentrated form is relatively simple. Assessing bioavailability of 1) an element in the same form but incorporated into a food for nutrient fortification purposes and 2) an element naturally present in a food are more difficult. Only the latter two will be considered in this section. Table I is a summary of considerations important in designing a bioassay.

Factors Influencing Bioavailability

Bioavailability can be influenced by the species of the element (valence state and molecular form) that occurs in the test food and by the levels of other elements and ligands in the food and/or the diet. The underlying mechanisms affecting bioavailability include formation of insoluble and nonabsorbable substances in the gut and facilitation or hindrance of muscosal uptake, transport, and metabolism in the body. Other inorganic elements may compete with the test element for important binding sites during these processes. O'Dell (1969, 1979) has reviewed factors that influence bioavailability of zinc.

Choice of Experimental Diets

Adequate diets composed of human foods provide the best duplication of conditions directly applicable to humans. Only purified diets provide the flexibility of formulation that permits varying the levels of individual nutrients to produce one or more deficiencies, imbalances, or excesses of specific nutrients. These changes are necessary to provide appropriate conditions for

[4]R. P. Hamilton. Unpublished data.

response measurement and to permit feeding graded levels of test food in a controlled manner. However, none of the purified proteins available for use in animal diets is sufficiently low in all minerals needed for these types of studies.

Choice of Experimental Conditions

The ideal conditions for bioassays (Table I) can seldom be attained. Nevertheless, recognition of the problems and consideration of the best possible conditions may lead to improved approaches to this very complex problem.

Processing Test Food as for Human Use. This is seldom simple except for foods of very low moisture content. Freeze-drying and fine comminution to obtain a material readily incorporated into a purified diet is a reasonably acceptable common practice. Preparation of a radioisotope-labeled food that is given as a single meal is probably the best alternative. Daily preparation of nonlabeled meals of the food for long periods of time is laborious, and reproducing the composition uniformly may also be difficult.

Mineral Content of Test Food Similar to That of Human Foods. This is a consideration of particular concern in studies of toxic elements such as cadmium, lead, or mercury, which are present at very low levels in foods for humans. The levels of interest are generally well below amounts that would produce adverse health effects in animals within a reasonable experimental period. Many plant or animal tissues with very high levels of these elements can be produced and used as foods for bioassays; however, this may alter the speciation of the toxic element in the food and change the levels of interacting essential elements.

Oysters exposed to high levels of lead so that the lead concentration in the freeze-dried tissue increased to 5,220 $\mu g/g$ contained 43% more iron, 29% less copper, 21% less zinc, and 110% more calcium than did control oysters that contained 5.5 μg lead per gram (Stone,[5] Stone et al 1979[6]). The concentrations of

[5]C. L. Stone. Unpublished data.
[6]Stone, C. L., Fox, M. R. S., and Hogye, K. S. September 1979. Biological availability of lead in oysters dosed with lead. Presented to the Div. of Agric. Food Chem., Am. Chem. Soc. Annual Meeting, Washington, DC.

TABLE I
Desirable Conditions for Animal Bioassays of Essential and Toxic Elements to Obtain Data Applicable to Humans[a]

1. Use indices for assessing bioavailability that reflect pertinent processes in the human population of concern.
2. Process test foods as for human use.
3. Use test foods with test mineral levels similar to those in human foods.
4. Use test food levels to bracket human intake ranges.
5. Adjust diet composition and nutrient levels based on animal requirements and human requirements, nutritional problems, and food use.
6. Differentiate between nutritional status and meal balance effects.

[a]See text for detailed discussion.

TABLE II
Effects of Feeding Soy Protein Concentrate in a Casein-Gelatin Diet to Japanese Quail Between 7 and 14 Days of Age[a]

Measurement	Soy Protein Concentrate, g/kg of diet	
	0	70
Body weight, g	43.8 ± 0.85	45.5 ± 1.46
Tibia		
Zn, $\mu g/g$	258 ± 6.6	239 ± 6.4[b]
Fe, $\mu g/g$	142 ± 5.0	120 ± 3.8[b]
Mg, mg/g	2.53 ± 0.11	2.32 ± 0.10

[a]Mean values ± SE; 10 birds per group. All tibia values are on the basis of fat-free dry weight. The total dietary concentrations for each diet, the amount supplied by soy protein concentrate, and the requirement with the casein-gelatin diet in quail 7-14 days of age, respectively, were as follows: ZN 30, 2.5, 12.5 mg/kg; Fe 100, 7.5, (50) mg/kg; Mg 300, 107, 300 mg/kg; protein 320, 48, (320) g/kg. Parentheses indicate estimates.
[b]Significantly different from control with no soy protein concentrate, $P < 0.05$.

cadmium, manganese, and magnesium were not significantly different. At doses above human intake levels, the lead in lead-dosed oysters was taken up by the tibia of young Japanese quail in smaller amounts than was lead as the acetate. The intercept of the curves for the reference material and the lead-dosed oysters can be interpreted to indicate that at lower dietary levels, the lead from the oysters would be more available than the lead as the acetate would be. The use of foods labeled physiologically with a radioisotopic form of the element circumvents the above problems by permitting more sensitive measurements.

Test Food Levels That Bracket Human Use Range. These are desirable to make the model valid and to avoid imbalancing the diet with nutrients or other substances in the test food. If the test food is typically eaten as one serving per day, usually ranging from 60–100 g, the corresponding level to be incorporated into an animal diet could be approximately 18 g dry weight per kilogram of diet (60 g serving, 70% moisture; Table III, footnote c). The amount of dry material would be less for a vegetable with a higher moisture content. The midlevel of a three-point bioassay might represent the best approximation of human use. If the bioassay is for a cereal product that is a dietary staple of the population of interest, much higher test levels are appropriate. The feasibility of using these test levels depends on the sensitivity of the bioassay response. Even the highest reasonable intake range by humans may be below the detection range of the bioassay.

Adjustment of Diet Composition. This is the most difficult and probably the most critical factor for obtaining realistic data. The nutrients in the basal diet should meet the animal's requirements without appreciable excess. When the test food is incorporated into the diet, several options are available for adjusting the nutrient content of the diets containing the test food or the reference material. These include 1) no correction, 2) adjustments to maintain total concentrations of nutrients with the test food at constant levels, 3) addition of nutrients equivalent to those supplied by the test food to the diets containing the reference material, or 4) a combination of the two types of adjustments (Fox et al 1978).

Several considerations are important for determining the need for and extent of such dietary adjustments. These include the required level of the test food in the animal diet, the presence of unusually high concentrations of a nutrient or other substance in the test food that could cause an imbalance, and the level of the test food used by human beings. Some of these factors were studied in

relation to the bioavailability of cadmium in foods (Fox et al 1978).

With current instrumentation for multiple element analysis, screening for bioavailability of several elements simultaneously is feasible. Table II illustrates the effect of incorporating 70 g of a soy protein concentrate per kilogram of diet on tibia concentrations of three minerals. The levels of each of these in bone are indicative of status for the respective element. The soy protein concentrate caused significant decreases in zinc and iron. Tibia magnesium concentration was not affected; however, subsequent experiments showed that with dietary magnesium concentrations of one-half to two-thirds of requirement, magnesium supplied in the form of soy concentrate was not utilized as efficiently as that fed as the sulfate (Fox et al 1976).

Differentiation Between Effects of Nutritional Status and Nutrient Balance Within a Meal. This is of great practical importance but has been studied very little. Significant documentation shows that dietary levels of several nutrients can profoundly influence the adverse effect of the toxic elements (Fox 1979, Mahaffey 1980); numerous antagonisms between essential elements have been observed with imbalanced diets (Underwood 1977). Both types of problems have been established in humans and animals with dietary intakes relatively constant over a period of time. The extent to which the same relationships may be important in a single meal needs investigation. Variation in mineral balance of meals is to be expected; some foods can contribute markedly to such an imbalance, and the net effect could be either harmful or beneficial.

Bioavailability of an element from a meal could depend on factors that affect absorption. For example, ascorbic acid greatly increases iron absorption, but this occurs only if the two nutrients occur in the same meal. Absorption of the high level of cadmium in oysters may be decreased by the high levels of zinc and copper that also occur (Fox et al 1978). On the other hand, a single high dose of cadmium is much less toxic in an animal already exposed to high levels of cadmium (Terhaar et al 1965), thus having greater levels of tissue metallothionein than does an animal not exposed. A large intake of a small chelating agent, such as the food additive (ethylenedinitrilo) tetraacetic acid or some amino acids, increase absorption of zinc (O'Dell 1969, 1979). These examples illustrate several possibilities by which the bioavailability of an element in a meal could be affected. The long-term significance would depend on the degree and frequency with which such effects occurred.

Bioavailability assessment of naturally occurring or added elements in a food presently requires the use of appropriate intrinsic, physiologically incorporated isotopic labels or extrinsic labels, respectively. A meal-dose experimental model also provides great flexibility in assessing both the bioavailability of an element from a single food and the factors that may influence the bioavailability (Jacobs et al 1976).

EXTRAPOLATION FROM ANIMALS TO HUMANS

Much less is known about the nutrient requirements of humans than about those of some experimental and domestic animals. Studies with humans are more laborious and necessarily more limited in scope than those with animals. Many complexities are inherent in assessing bioavailability of essential and toxic elements and therefore, much research relevant to humans must be carried out in experimental animals.

Table III is a summary of requirements for five essential elements. Except for phosphorus, they include all of the essential elements for which a recommended dietary allowance has been established for humans (NAS/NRC 1980). The values are expressed as amounts per kilogram of air dry diet. The basis for the conversions is given in footnote b, Table III.

Most of the requirement values in Table III are in remarkably close agreement when one considers that the values were established for each species by independent expert committees and were based on evaluation of experiments carried out for varying periods of time, with differing criteria, and without designation of a chemical form of the element for the final requirements.

The chief difference in requirements between humans and

TABLE III
Comparison of Recommended Dietary Allowances (RDA)[a] for Humans (Calculated to a Dietary Basis Similar to that of Animals) and Requirements[a] of Animals for Five Elements[b]

Nutrient	Human[c]		Rat	Chick	Pig	Calf
	20-kg Child	70-kg Adult Male				
Ca, mg	1,992	1,248	5,000	9,000	8,000	7,000
Mg, mg	498	546	400	600	400	700
Fe, mg	25	16	35	80	140	100
Zn, mg	25	23	12	40	100	40
I, μg	224	234	150	350	140	250

[a]Quantity per kilogram of diet.
[b]Requirements are estimated for a child four to six years old and a male 23–50 years old (NAS/NRC 1980). Requirements are for a young, growing rat (NAS/NRC 1978b), a chicken zero to eight weeks old (NAS/NRC 1977), a pig with 5–10 kg of body weight (NAS/NRC 1979), and a dairy calf fed a milk replacer diet (NAS/NRC 1978a). Only the pig diet calculations include fiber; requirements were for a diet containing a substantial level of fortified grain and soybean meal.
[c]Values for humans were calculated from the protein RDA, with one-third of the calories as fat, 4% of the diet as mineral salts, 10% of the diet as moisture (air-dry basis), and no fiber. The daily dietary intakes for the child and the man, respectively, were: 30 and 56 g of protein, 63 and 100 g of fat, 253 and 394 g of carbohydrate, 16 and 26 g of salts, and 40 and 64 g of water; 402 and 640 g of total food. To convert amounts per day to amounts per kilogram of diet, the amounts per day were multiplied by 2.49 and 1.56 for the child and the adult male, respectively.

animals is the higher requirement for calcium by animals (Table III); the pig also had a higher requirement for iron and zinc. Making gross extrapolations from animals to humans, based on responses to nutrient quantities per unit of diet weight, seems reasonable. The requirements for the child and the adult male were similar.

RESEARCH NEEDS

Because of the emerging knowledge of the complexity of nutrient interactions and the numbers of dietary components that can influence bioavailability of an element, large multifactorial studies of dietary component interactions are needed; studies of this magnitude can only be approached with animals. At present, bioavailability problems of practical significance are recognized for some of the elements of long-established nutritional importance; however, similar problems probably also exist for the "new" trace elements, nickel, vanadium, and silicon.

Even though requirements of the "new" trace elements are very small and deficiencies have been produced in animals only by excluding the element under extreme conditions, factors such as those shown for other elements could possibly decrease bioavailability and make the dietary level of these elements of practical importance to humans.

Limited bioavailabilty studies of many nutrients within the optimal or mildly deficient ranges can be carried out in humans. Short-term studies of more severe nutrient deficiencies and toxicities can be carried out in humans, and useful information on metabolism and bioavailability could be expected, particularly with the use of stable isotopes. Many studies of the toxicity of essential elements and of the nonessential toxic elements cannot be carried out in humans because of health risks.

Impressive evidence suggests that many effects observed in animals have relevance for human beings. Greatly needed is the development of more refined animal models patterned after human dietary practices and nutritional deficits, excesses, and exposure to environmental toxicants. For this work, more precisely defined purified and nonpurified reference diets for animals are needed.

To assess the nutritional significance of diet modifications, more rapidly responding animal models and more sensitive and simply quantitated primary and secondary responses are needed. Speciation of the element in selected tissues could be useful. This would permit investigation of a wide range of experimental variables before selection of the minimal adverse conditions needed for detailed investigation of biochemical mechanisms and for direct comparisons between animals and humans.

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