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Some Outstanding Problems in the Detection of Trace Element Deficiency Diseases

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Some outstanding problems in the detection of trace element deficiency diseases

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Knowledge of the functional roles of many essential trace elements has grown rapidly. Despite this, it is rarely possible to relate this information to observed pathological consequences of deficiency. Few studies of the effect of deficiency upon enzyme activity have attempted to determine whether such changes influence substrate–product relations and thus may have pathological significance.

Evidence that the differing susceptibility of tissues to deficiency may reflect metabolic activity or the lifespan of cells and their organelles is considered.

The need is growing for more effective biochemical diagnostic techniques for the early detection of covert pathological changes in trace element deficient subjects. Progress towards satisfying this need will reflect the future availability of information from which to predict the nature of rate-limiting metabolic defects in sensitive populations of cells.

Introduction

In the paper that he presented to the First International Symposium on Trace Element Metabolism in Man and Animals at Aberdeen in 1969, the late Professor Eric Underwood wrote:

> . . . embarrassingly little is yet known of the precise metabolic roles of the trace elements or of their specific functional relationships to the clinical and pathological disturbances which accompany deficiencies and toxicoses. It is rare indeed to be able to relate any significant change in enzyme activity in tissues to the clinical and pathological picture presented by the trace element deficient animal. Bridging of the gap between the findings of the nutritionist and those of the enzymologist is the most urgent of our tasks if the physiological functions of the trace elements are to be understood.

Since then, progress with the isolation and identification of metalloproteins has been particularly rapid and has yielded a rich harvest. Despite this, the problems that Underwood defined so clearly remain with us and progress towards their resolution has been much less spectacular.

A wide range of biochemical techniques is now available to support diagnosis of overt deficiency disease. However, much greater emphasis is now placed upon the need for techniques that will indicate whether covert pathological changes attributable to suboptimal trace element supply are present or likely to arise. The present trend in veterinary and human medicine and in the development of nutrition policies for animals and man is towards prevention rather than cure. With respect to trace element deficiency diseases, this change in emphasis is revealing the inadequacies of the many investigative techniques based solely upon the analysis of accessible body tissues or fluids. We have little difficulty in determining when trace element 'status' is low, but we face much greater problems in assessing the pathological relevance of such a finding. Our success in developing less equivocal and more sensitive biochemical diagnostic

techniques will reflect the facility with which we can identify, with greater certainty, the tissues most sensitive to trace element deficiency and understand the metabolic origins of their pathological changes. This paper will consider some of the problems encountered in such studies.

Tissue trace element content and the detection of deficiency

Accessibility for sampling is frequently the sole consideration dictating the selection of whole blood, plasma, liver or hair for trace element analysis. The enormity of the assumption that the trace element content of such tissues reflects that at cellular sites most sensitive to depletion will

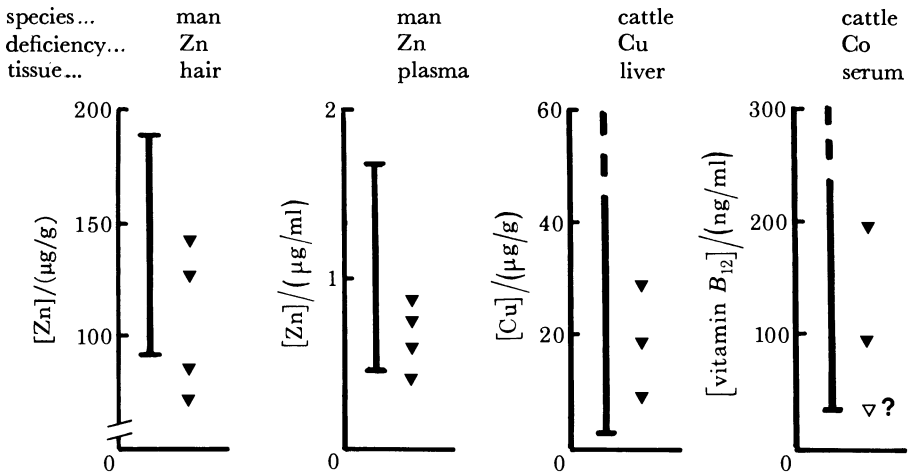

Figure 1. Suggested diagnostic 'threshold' values (▼) for detection of zinc, copper and cobalt deficiencies Vertical bars indicate ranges of values typically found in clinically normal subjects and thus illustrate diagnostic limitations of these indices.

not require emphasis! However, the validity of the generalization differs between elements and between tissues. Thus, except when molybdenum enhances the affinity of plasma proteins for copper (Mills *et al.* 1980*a*, *b*), plasma concentrations of copper reflect the adequacy of hepatic reserves of this element. In contrast, changes in plasma zinc reflect not the adequacy of reserves but the rate at which these are being mobilized if intake is deficient. No mobile cellular reserve of zinc has been identified; only very small changes in zinc content occur in tissues suffering degenerative change during zinc deficiency.

Neither for copper nor for zinc, nor for any of the other essential trace elements, is there evidence that subnormal content of the element in a gross tissue sample invariably reflects the presence of a pathologically relevant metabolic lesion. Reduced concentration simply suggests the possibility of pathological change. For example, surveys of plasma copper in beef cattle in Wales indicate that more than 53% have hypocupraemia (Davies & Baker 1974). This proportion of the cattle population certainly do not exhibit clinical signs of deficiency, but the number of these in which covert pathological changes may exist cannot be determined. Similar uncertainty attributable to the lack of suitable indices of pathological change hinders the interpretation of the finding that whole blood selenium is abnormally low in residents of New Zealand (Rea *et al.* 1979) and in infants with cystic fibrosis (P. J. Aggett & J. R. Arthur, personal communication). Whether or not the relatively low plasma zinc concentrations of

women taking oral contraceptives, of animals in acute phases of infection or during recovery from surgery has pathological relevance is also unknown. The dangers of misinterpreting such evidence are apparent from two recent reports. Attempts to maintain the zinc status of women during the terminal stages of pregnancy by oral administration of zinc have resulted in abortion or stillbirths (Kumar 1976). Furthermore, Chesters & Will (1981) found that regulated infusion of zinc to correct the hypozincaemia of pigs suffering from *Escherichia coli* endotoxin shock, far from alleviating the syndrome, enhanced both the shock response and mortality. Such problems in no sense reduce the primary value of gross tissue analysis, namely the evidence that it may provide that trace element status is subnormal – at least in the tissues sampled. However, such data are of limited value for assessing the possibility that disease exists or will develop. Continuing controversy over the diagnostic validity of suggested threshold values for the inorganic composition of blood plasma or other accessible tissues (see figure 1) is unlikely to eliminate this difficulty. What limitations are therefore restricting progress towards more useful indices of deficiency?

Pathological relevance of local changes in trace element content

Since clinical responses to trace element deficiency sometimes suggest the involvement of a specific target tissue it can be argued that data on the inorganic composition of such tissues or cells might be less difficult to interpret. Such an approach did much to promote early work on the relevance of iodine deficiency to goitre. A further example arises from examination of relations between plasma, liver or brain stem copper concentrations and the clinical manifestations of ataxia in copper deficient lambs (Mills & Williams 1962 and unpublished data). Although both plasma copper and liver copper in the population of lambs investigated were below generally accepted normal values (figure 2*a*, *b*) no relation existed between the copper content of these tissues and the incidence of ataxia. In marked contrast, the incidence of clinical disease was very clearly related to brain stem concentrations of less than 4 mg Cu/kg d.m. (figure 2*c*). This analytical approach was suggested after considering the probability that motor defects were attributable to degenerative changes in motor neurons of the brain stem (Fell *et al.* 1965). Despite the heterogeneous population of brain stem cells in the samples analysed, such results indicate the greater discriminative value of analysis of a pathologically sensitive tissue. They also suggest that variables inhibiting the accumulation of copper by this population of motor neurons are more closely involved in the aetiology of this disease than is the generally low copper status of many other tissues of the lambs at risk.

Much more effective discriminatory analysis should be feasible as the sensitivity of analytical microprobe techniques increases. The few relevant data so far available relate to changes in the zinc content of subcellular components of cells of the prostate during zinc depletion of the rat. They indicate, perhaps unexpectedly, that the nucleus and nucleolus, although apparently remote from interstitial fluids, suffer marked decreases in zinc content (J. R. Chandler, personal communication); this observation is of particular interest in view of suggestions that zinc deficiency modifies the efficiency of genetic expression (Chesters 1978; Vallee & Falchuk, this symposium).

The relevance of anomalies in intracellular metal distribution to the development of metabolic and pathological defects is now evident from several studies. Thus, thio- and oxythiomolybdates act in rats as systemic antagonists of copper metabolism by depressing the proportion of cyto-

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solic copper in liver and kidney cells. These compounds also depress the proportion of copper accounted for as metalloenzyme-containing fractions (Mills *et al.* 1981 *a*, *b*). Furthermore, it is known that cytosolic and mitochondrial fractions of liver suffer a much greater loss of copper than other cell components during the development of copper deficiency in rats. Although, typically, liver copper may fall by 50–60%, cytosolic and mitochondrial copper in liver cells have been found to decline by 85 and 92% as clinical signs of deficiency develop (Williams *et al.* 1976; Mills & Mitchell 1971).

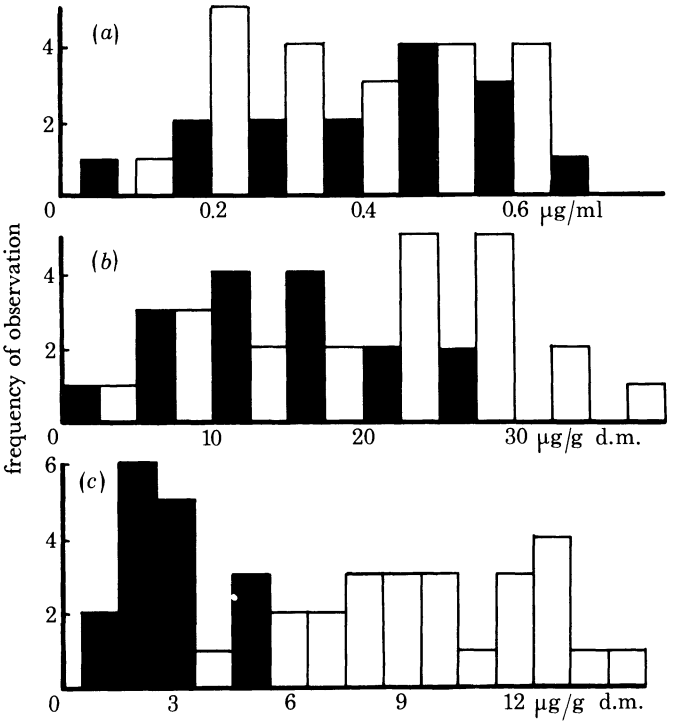

FIGURE 2. Plasma (*a*), liver (*b*) and brain stem (*c*) copper in copper-deficient lambs 1–3 months of age. Vertical bars indicate frequency of observations in clinically normal lambs (□) and lambs exhibiting ataxia ('sway-back') (■).

The study of Williams *et al.* (1976) is one of many illustrating both the involvement of copper-dependent processes in the metabolism of iron and the influence of this interdependence of subcellular distribution of metals. Conflicting views on the metabolic origin of defects in iron metabolism during copper deficiency have been discussed elsewhere (Mills 1980; Frieden 1980). The most significant feature of the Cu–Fe relation is that abrupt increases in mitochondrial non-haem iron content and, later, histochemically evident deposition of iron in hepatocytes and enterocytes occur before the appearance of overt signs of copper deficiency. Thus, simultaneous monitoring of liver iron and copper concentration offers the first practicable method of predicting the probable outcome of chronic copper deficiency. However, we have only a superficial understanding of either the metabolic origins of this 'secondary' effect of copper deficiency or of its relevance to concurrent mitochondrial damage and gross pathological changes.

Although not likely to become routine diagnostic methods, subcellular trace element analysis may facilitate identification of cell types most sensitive to suboptimal trace element supply. Where, however, a significant pathological lesion is initiated by loss of the element from a

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readily dissociable metal–enzyme or other functional complex while its concentration in other intracellular locations remains virtually unchanged (as may occur in zinc deficiency (Chesters 1978)), microanalytical techniques will have little value and alternatives must be sought.

Enzyme activity as an indicator of deficiency

Unequivocal evidence for functional or structural roles of seven essential trace elements in at least 48 mammalian metalloproteins now exists. The list of mammalian enzymes for which there is less complete evidence of roles for trace elements is growing rapidly. Much less spectacular progress is being made towards the identification of metalloenzymes, the activity of which *in vivo* is sufficiently changed by decreases in tissue metal concentration for them to become rate-limiting and relevant to pathological defects. The few deficiencies and enzymes for which such relations are firmly established, rather than presumed, are listed in table 1*a*. Some typical presumed relations are indicated in table 1*b*.

Table 1. Trace element deficiency, metalloenzyme activity and metabolic defects in mammalian tissues

(*a*) *Evidence available that deficiency influences both enzyme activity and substrate–product relations*

deficiency	enzyme activity depressed	metabolic defect
copper	lysyl oxidase	inhibited cross-linking of elastin and collagen; accumulation of soluble precursors
cobalt†	methylmalonyl-CoA mutase	inhibited metabolism of propionate and accumulation of methylmalonate
cobalt†	tetrahydropteroylglutamate-Me transferase	catabolism of histidine interrupted with accumulation of formimino-glutamate

(*b*) *Some presumed relations between metalloenzyme activity and metabolic consequences of trace element deficiency*

deficiency	pathological defect	enzyme tentatively implicated
copper	defective melanogenesis	monophenol monooxygenase
copper	enhanced retention of urate	urate oxidase
manganese	glycosaminoglycan and glycoprotein synthesis	'UDP-glycosyltransferases' [Mn-activated]
nickel	impaired utilization of non-protein sources of nitrogen by ruminants‡	urease [rumen bacterial]
selenium	enhanced susceptibility of lipids to peroxidation	glutathione peroxidase

† Effects of Co deficiency specific to ruminants and reflect incorporation of element into cobalamin cofactor by rumen microorganisms and role of cobalamin in the enzymes indicated.

‡ Effect of Ni deficiency attributed to inhibited microbial degradation of endogenous or exogenous sources of urea.

The value of defining such relations is best illustrated from studies of the metabolic effects of cobalt deficiency in ruminants, a disease particularly difficult to diagnose by clinical investigation and which has particularly complex biochemical origins (see Underwood 1977). This success story, born of close collaboration between biochemists, pathologists and clinicians, is almost unique. A similar example relates to the growing evidence that connective tissue defects arising at early stages of the copper deficiency syndrome are attributable to rate-limiting decreases in the activity of lysyl oxidase, the copper enzyme involved in cross-linking of collagen and elastin (Harris *et al.* 1980; O'Dell, this symposium).

Despite the plethora of zinc-containing enzymes now known to exist in microorganisms and mammalian tissues, we have still not identified, or perhaps discovered, those in which a decline

in activity has pathological relevance. As indicated by Hambidge (this symposium), we still lack effective biochemical procedures for the detection of zinc deficiency in man. If the findings of Vallee & Falchuk (this symposium), that zinc deficiency influences gene activation or repression in *Euglena gracilis*, are found to be applicable to mammalian zinc deficiency, new lines of enquiry will develop. These may explain why pathological effects of zinc deficiency are so severe in most proliferating tissues. Meanwhile, we have no biochemical diagnostic criteria by which we can discriminate subjects with merely a low tissue zinc status from those in which clinical deficiency is incipient. This conclusion is equally applicable to deficiencies of selenium, manganese and, with the possible exception of chromium, to most of the 'newer' trace elements (Davies, this symposium).

Despite this situation, the information that an essential trace element has more than one functional role can offer a means of monitoring the progress of deficiency. For example, during the development of bovine copper deficiency, decreases in the activity of copper-dependent enzymes in blood follow the sequence: (i) plasma caeruloplasmin (EC 1.16.3.1), (ii) plasma amine oxidase (EC 1.4.3.6) and (iii) erythrocyte (Cu)-superoxide dismutase (EC 1.15.1.1) (Mills *et al.* 1976; McMurray 1980). Other examples are the earlier loss of serum alkaline phosphatase (EC 3.1.3.1) than of erythrocyte carbonic anhydrase (EC 4.2.1.1) activity during the development of zinc deficiency (for review see Kirchgessner *et al.* 1976). Although the pathological relevance of any individual change may be questionable, data indicating the extent of inhibition of systems differing in susceptibility are capable of differentiating between subjects with brief or prolonged exposure to deficiency. The potential value of this approach is not widely appreciated. At present, it is being applied only in the diagnosis of copper and cobalt deficiencies in farm animals.

Variables influencing cellular responses to trace element deficiency

From impressive lists of the many trace metal-dependent enzymes and very limited knowledge of the variables influencing metal transport to their sites of synthesis, the biologist is often enjoined to construct metabolic models of deficiency syndromes that will both rationalize pathological findings and suggest less equivocal diagnostic criteria than information on the trace element content of accessible tissues.

Kinetic models describing competitive interactions have proved useful in providing a basis for understanding the adverse effects of trace element imbalances and antagonisms (see, for example, Hill & Matrone 1970). Their potential for predicting metabolic events that could promote a physiologically significant redistribution of trace metals and provoke changes in metalloenzyme activity without a corresponding change in total metal content of the cell is now being emphasized (Williams, this symposium).

However, the most important limitation to the validity of such models is that they cannot yet take adequate account of relations between supply and demand in cells and in subcellular components differing in function and activity. Some relevant processes are indicated in figure 3. Evidence of their relevance to the appearance and tissue distribution of pathological defects of deficiency is now considered.

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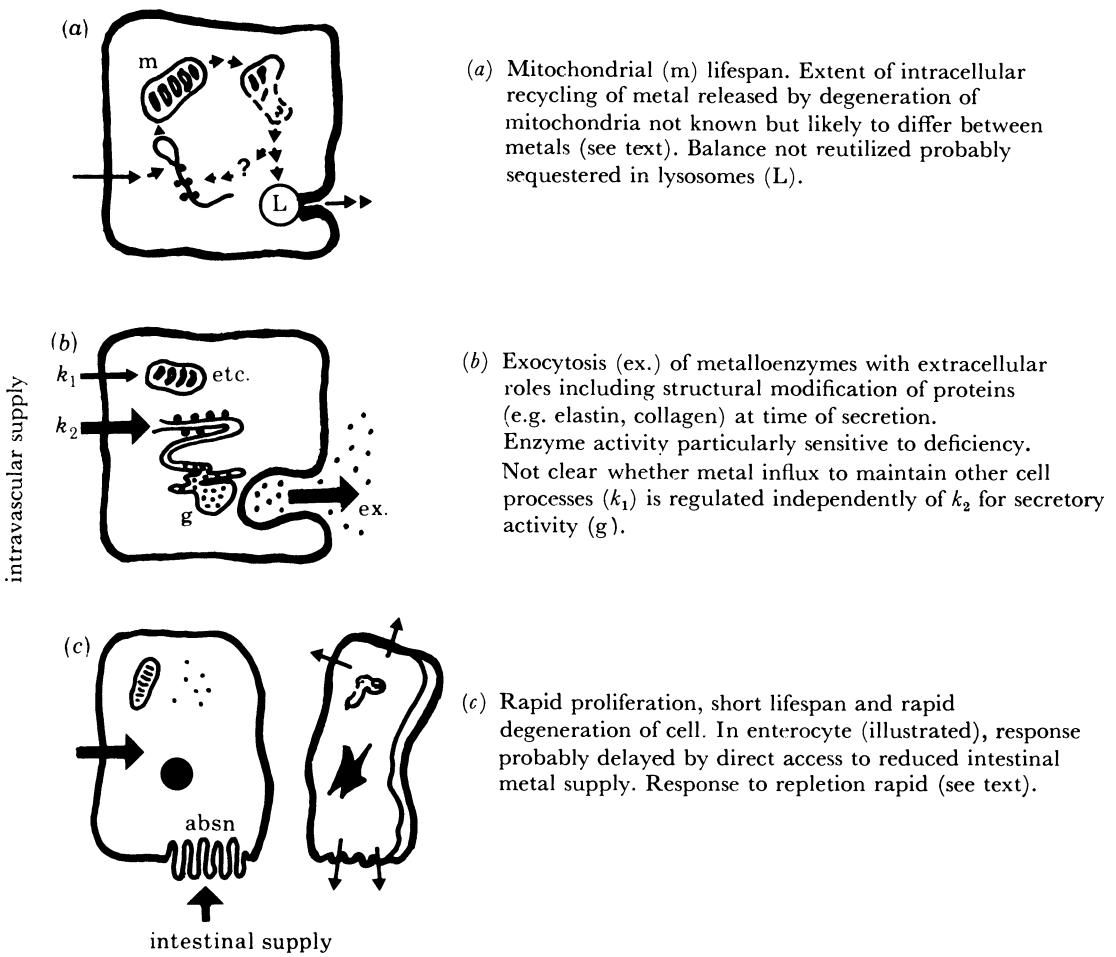
(*a*) *Anabolic–catabolic balance and trace element 'mobility'*

Studies of responses to infection, to stress and to variations in the dietary supply of limiting essential amino acids all indicate that such challenges can influence the metabolism of zinc, copper and iron. Thus plasma zinc in zinc-deficient rats increases when dietary protein content is reduced (table 2) and declines abruptly when the supply is restored (Mills 1973). This and evidence of significant inverse relations between daily food intake and plasma zinc (Chesters & Will 1973) suggest that zinc flux into tissues is enhanced by anabolic processes, and that even



(*a*) Mitochondrial (m) lifespan. Extent of intracellular recycling of metal released by degeneration of mitochondria not known but likely to differ between metals (see text). Balance not reutilized probably sequestered in lysosomes (L).

(*b*) Exocytosis (ex.) of metalloenzymes with extracellular roles including structural modification of proteins (e.g. elastin, collagen) at time of secretion. Enzyme activity particularly sensitive to deficiency. Not clear whether metal influx to maintain other cell processes (k_1) is regulated independently of k_2 for secretory activity (g).

(*c*) Rapid proliferation, short lifespan and rapid degeneration of cell. In enterocyte (illustrated), response probably delayed by direct access to reduced intestinal metal supply. Response to repletion rapid (see text).

FIGURE 3. Some physiological processes influencing the response of metalloenzymes to essential trace metal deficiency.

TABLE 2. INFLUENCE OF CHANGES IN DIETARY PROTEIN CONTENT ON PLASMA Zn IN Zn-DEFICIENT RATS

	dietary protein (%)	plasma Zn mg/l
protein reduced		
initial	20	0.52 ± 0.08
final	5	0.79 ± 0.07
protein increased		
initial	5	0.71 ± 0.05
final	20	0.29 ± 0.01

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brief periods in which catabolism predominates promote its efflux into plasma. The pathological relevance of such processes is illustrated by the finding that reducing calcium supply to zinc-deficient pregnant rats markedly decreases the incidence of foetal malformations, presumably by facilitating utilization of skeletal stores of zinc (Tao & Hurley 1975).

(*b*) *Tissue topography and the response to deficiency*

The possibility that cells relatively remote from the vascular or capillary bed may suffer proportionally greater depletion as the concentration of 'exchangeable' element in plasma declines is rarely considered. If remoteness from vascular supply sufficiently depresses concentrations of the limiting element in interstitial fluids for cellular uptake to become inadequate pathological changes should be more strongly evident in such cells. Such topographic relations have been clearly demonstrated in the work of Westmoreland *et al.* (1969, 1971) on the origins of long-bone defects in zinc-deficient chicks. Within the epiphyseal growth plate of such bones, the loss of chondrocyte alkaline phosphatase activity induced by zinc deficiency, the frequency of degenerative nuclear lesions in chondrocytes and the extent of morphological changes in the extracellular matrix were all directly related to the distance of the cell from blood vessels penetrating from the diaphysis.

The extent to which the distribution of neuronal lesions developing in the brain of copper-deficient foetal lambs and of children with Menkes's disease similarly reflects a localized deficiency of copper is not known. Cerebral vascular lesions are however a prominent feature of Menkes's disease and, as in lambs suffering from 'swayback', brain copper content is decreased, proportionally, to a much greater extent than that of most other soft tissues. Furthermore, it is noteworthy that cerebral vascular lesions are absent, degenerative changes do not occur and brain copper content is much less influenced when copper deficiency develops postnatally in children or lambs. The similarity between such developmental changes in response certainly suggest that unidentified variables influencing the access of copper to specific populations of cells within brain tissue affect the pathological outcome of copper deficiency syndromes. Studies with rats in which the relations between low dietary copper and reduced activity of the copper-dependent enzymes, cytochrome oxidase (EC 1.9.3.1) and (Cu)-superoxide dismutase (EC 1.15.1.1) in brain were examined, also support the view that equilibria influencing the distribution of copper between cerebral tissues and the rest of the body change rapidly after weaning (Paynter *et al.* 1979).

Cell activity and the response to deficiency

The susceptibility of some cell types towards the development of pathological changes during trace element deficiency often reflects either a specialized functional role or an unusually high metabolic activity. Thus, the earliest pathological defects so far detected in selenium- or copper-deficient animals arise in neutrophils challenged with foreign organisms. Phagocytic activity remains unimpaired but the capacity of the neutrophil to kill ingested organisms declines (Serfass & Ganther 1975; Boyne & Arthur 1979). Both (Se)-glutathione peroxidase (EC 1.11.1.9) and (Cu)-superoxide dismutase are closely involved in the reactions described by Hill (this symposium) that influence the balance between hydrogen peroxide, hypochlorite and the free-radical derivatives of reduced oxygen involved in phagocytic defence mechanisms, but their degree of involvement in events leading to this failure of microbicidal activity is not yet understood. Nor can we yet explain the particular susceptibility of ruminant skeletal and

cardiac muscle to degenerative changes during selenium deficiency. Even so, there are clear indications that either the physical or metabolic consequences of unaccustomed exercise enhance susceptibility.

Decreases in glutathione peroxidase activity in erythrocytes, neutrophils and cardiac and skeletal muscle accompanying these pathological defects clearly indicate that the selenium status of the tissues is declining but that relationships between enzyme activity and tissue damage are not close. Again we are in the position of having a reasonable indicator of risk but not of the existence of pathological defects – a situation that, not surprisingly, is provoking controversy on the levels of glutathione peroxidase activity that should be regarded as pathologically significant.

The generalization that zinc deficiency disease is a disease of tissues with a high frequency of cell division and of cells with highly developed secretory functions is widely applicable. Aspects of deficiency reflecting secretory activities will be considered later. Gross clinical signs of deficiency, not apparent when the potential for growth is low or is not expressed (e.g. when the supply of other nutrients is suboptimal), often appear when growth is resumed. They may develop locally, for example in epidermal tissues undergoing repair after injury or infection, even in subjects in which plasma zinc, although low, would not be considered indicative of frank deficiency (Demertzis & Mills 1973). The latter finding suggests that a reduced influx of zinc through a a damaged capillary bed may be relevant to the appearance of such lesions in animals only 'marginally' deficient in zinc. This suggestion, supported by other evidence of localized deficiency in tissues, may well explain why it is frequently difficult to predict the potential therapeutic value of zinc supplements for accelerating wound repair when plasma zinc is the only available criterion of body zinc status.

The incidence of pathological lesions in zinc-deficient animals, frequently greater in males than females, reflects growth rate before a decrease in zinc supply. The distribution of lesions again emphasizes the greater susceptibility of rapidly proliferating tissues.

Growing evidence of the role of zinc in DNA and RNA polymerases and in modifying genetic expression in bacteria and protozoa has suggested that similar defects may arise in mammalian zinc deficiency and account for such differences in tissue susceptibility. However, even the mitotic response to zinc deficiency differs between tissues (Fell *et al.* 1973). Thus it is particularly difficult to reconcile the marked increase in mitotic index and the severity of the pathological lesions of the zinc deficient oesophagus with the trivial lesions of the equally proliferative mucosa of the small intestine. Topographical relations influencing access of the limited dietary supply of zinc to these tissues may influence their response.

Cardiomegaly, a very early consequence of copper deficiency, developing before any external manifestations of deficiency arise, is a further example of the sensitivity to deficiency of an actively metabolizing tissue. Initially dismissed as an example of 'work hypertrophy' resulting from the anaemia of copper deficiency, it is now apparent that the mitochondrial lesions, increased myocardial fragility and changes in collagen structure and type (including the abnormal persistence of type III collagen) in the copper-deficient neonatal rat result from lesions unrelated to the subsequent anaemia (R. Dawson & R. B. Williams, personal communication). No diagnostic criteria yet available indicate the existence or absence of such defects and, apart from the increased incidence of aortic ruptures in chicks, rats and cattle (O'Dell *et al.* 1961; Mills *et al.* 1976) and the frequency of degenerative lesions in the ventricular cardiac apex of the rat (Paynter & Martin 1980), the time of onset and pathological significance of these lesions in other species is not known.

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There is little evidence that fluctuations in hypertrophic growth rate have a marked influence on the biochemical consequences of other trace element deficiencies. In contrast, evidence that other events within the cell influence the outcome of deficiency is more extensive.

(*a*) *Intracellular response to deficiency*

It is often difficult to determine whether the particular sensitivity of a cell population to deficiency of an essential trace metal is attributable to processes that have discriminated against metal uptake or whether the 'susceptible' cells have an unusually high requirement. For example, in the brain stem motor neurons of copper-deficient lambs, a marked loss of copper-dependent cytochrome oxidase activity is evident, whereas that of perineuronal tissues remains unaffected (Fell *et al.* 1965). To explain such relatively specific effects, neither unique to copper deficiency nor to neurons, the following information is needed: (i) evidence of discrimination against susceptible cells and preferential retention of the depleted metal supply by neighbouring tissues, and/or (ii) evidence of a high requirement for metal resulting from either a short lifespan of either the entire susceptible cell or its components. The difficulty of ascertaining (i) has already been discussed; the influence of other variables upon suceptibility to deficiency will now be considered.

Few data exist on the relation between the half-life of entire cells or of their organelles and the rates at which their activities decline when trace element supply becomes limiting. The neatly 'packaged' activities of (Cu)-superoxide dismutase and (Se)-glutathione peroxidase in erythrocytes reflect tissue copper and selenium status, retrospectively, at the time of erythrogenesis. However, such discrete packaging of metalloenzymes in which activity reflects both the availability of trace metal supply at the time of synthesis and of the lifespan and rate of replacement of the organelle is certainly not confined to erythrocytes, as the following examples indicate.

(*b*) *Cell longevity*

The sensitivity of bovine gastrointestinal function to copper deficiency and the particular rapidity of its response to therapy have been noted in several studies (e.g. Ferguson *et al.* 1943; Mills *et al.* 1976). Diarrhoea, the severity of which is influenced by factors not yet identified, but which is always accompanied by a loss of cytochrome oxidase from, and structural defects in, enterocyte mitochondria (Fell *et al.* 1975), terminates abruptly when copper is given orally in quantities insufficient to influence either copper-dependent enzymes in, or the copper content of, other body tissues. Although the rate of decay of cytochrome oxidase activity from bovine intestinal mucosa during copper deficiency is not known, experiments with rats indicate that activity declines rapidly in this tissue (Dallman 1967; N. T. Davies & C. F. Mills, unpublished data). Figure 4, derived from these experiments (and from Paynter 1979) typifies such responses during depletion and repletion. Particularly noteworthy are, first, the rapid of rate recovery of intestinal cytochrome oxidase activity on copper repletion, and secondly, the asymetry of the depletion–repletion curve. In contrast to tissues with a longer cell lifespan, the rate of cell differentiation (and thus of mitochondrial differentation) and cell replacement in the mucosa is clearly the primary determinant of the rate of recovery of cytochrome oxidase after copper repletion. (The relatively slower decline of activity in the mucosa during depletion probably reflects the accessibility of the traces of copper remaining in the deficient diets used in these studies.)

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The predominant influence of the rate of mucosal cell replacement (figure 3*c*) over other processes normally influencing intracellular metal distribution is also evident from the work of Johnson *et al.* (1977) on the apparent decay rates of the molybdenoenzymes xanthine oxidase (cytosolic) and sulphite oxidase (mitochondrial) in molybdenum-deficient rats. In intestinal mucosa the decay rates of xanthine oxidase (EC 1.2.3.2) and sulphite oxidase (EC 1.8.3.1) were virtually identical and similar to the estimated half-life of the mucosal cell population (1 day). In other tissues such as liver, the apparent decay rate of mitochondrial sulphite oxidase closely reflected mitochondrial half-life (5 days) (Aschenbrenner *et al.* 1970), where xanthine oxidase activity in liver cytosol declined more than twice as rapidly. Information on such patterns of response in a wider range of tissues could help to resolve present uncertainty about the nature of metabolic defects arising in molybdenum-deficient animals (Mills & Bremner 1980).

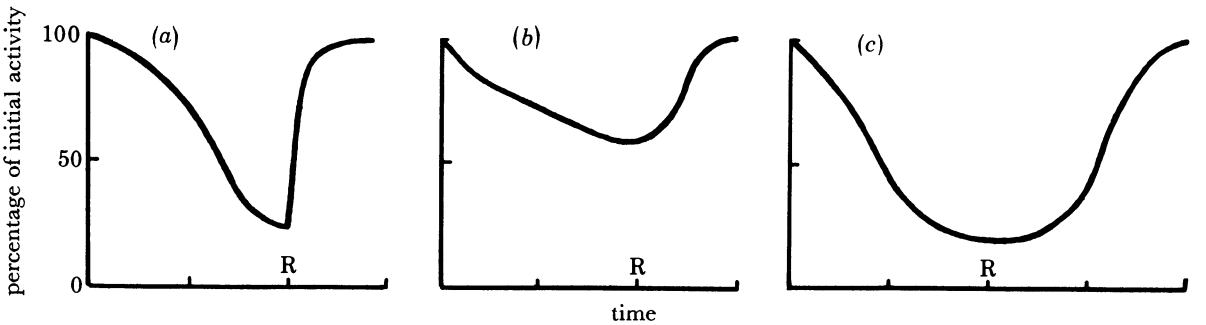

FIGURE 4. Tissue differences in the response of cytochrome oxidase activity to the induction and recovery from copper deficiency in rats. (*a*) Intestinal mucosa; (*b*) liver; (*c*) heart. Repletion commenced at point R. Data sources: depletion period, Paynter (1979), Davies & Mills (unpublished); recovery period, Dallman (1967), Paynter (1979). Depletion periods differed between 6 and 7 weeks; all deficient diets provided less than 0.8 μg Cu/g. The data of Paynter (1979) indicate that the response of kidney resembles that of liver (*b*) and skeletal muscle, that of heart (*c*).

(*c*) *Intracellular responses*

Even within the intracellular matrix, the response of individual metalloenzymes to a depleted pool of metal cofactor is not constant from tissue to tissue as indicated by a recent study by Paynter (1979). During the development of copper deficiency in rats, the activity of (cytosolic) (Cu)-superoxide dismutase declined more rapidly than that of cytochrome oxidase (mitochondrial) in many tissues. Recovery rates on repletion with copper differed correspondingly. However, in heart and skeletal muscle, both the rate and the extent to which cytochrome oxidase activity declined far exceeded that in other tissues, but superoxide dismutase activity declined only slightly. In the absence of any evidence that differences in tissue copper content caused such differing responses, it must be presumed either that tissue differences exist in the rate of entry of copper to the sites of cytochrome oxidase synthesis or, more probably, that the mitochondrial half-life of skeletal and cardiac muscle is appreciably shorter than that of most other tissues. Evidence relevant to the latter possibility is awaited with particular interest. Whatever the origin of these differing responses may be, it is reasonable to anticipate that tissue susceptibility to mitochondrial and other damage (see Hill, this symposium) will differ correspondingly.

It is not yet feasible to determine whether metal released by intracellular degenerative changes or the normal turnover of an organelle can be reutilized for intracellular synthesis (figure 3*a*). In molybdenum deficiency, this appears unlikely from the work of Johnson *et al.*

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(1977) on the rapid rate of decay of mitochondrial sulphite oxidase. Intracellular 'scavenging' or recycling of manganese appears much more probable. Paynter (1980) has shown that the manganese contents of liver, kidney and heart tissue decline rapidly in the manganese-deficient rat. In heart tissue the rate of decline in manganese content and (mitochondrial) (Mn)-superoxide dismutase activity were similar. However, in manganese-depleted liver and kidney, there are strong indications of conservation of (Mn)-superoxide dismutase with a pronounced lag in the decay of activity which, in liver, greatly exceeds the estimated lifespan of hepatocyte mitochondria. Differences in the apparent decay rate of copper-dependent mitochondrial and cytosolic enzymes in the liver also suggest that some conservation of copper for mitochondrial function may occur.

With the exception of the liver, little is known of the biological half-life of the organelles within which many such metal-dependent processes operate. The scarcity of information on the rates of degradation and replacement of whole cells and their organelles in different tissues probably accounts for our present inability to predict the cell types and cell functions most likely to suffer significant pathological changes as deficiency develops.

(*d*) *Extracellular responses to deficiency*

Plasma alkaline phosphatase activity often declines rapidly when dietary zinc supply is reduced. The principal sources of the enzyme in plasma are bone, liver, intestinal mucosa and placenta, the proportions of total plasma activity derived from these tissues differing between species. The influence of zinc deficiency on the response of individual isoenzymes in plasma has not yet been determined, but other studies indicating that activity in bone (Kirchgessner *et al.* 1976) and intestinal mucosa (Davies *et al.* 1978) declines rapidly during deficiency suggests that, irrespective of its origin, the level of activity in plasma should provide a useful index of tissue zinc status. This view is contestable, however, first because there is no evidence that the loss of alkaline phosphatase can be implicated in the metabolic defects leading to growth failure and, secondly, because changes in activity often parallel those of plasma zinc so that its diagnostic or predictive value is felt to be no greater.

Similar arguments surround the interpretation of the decrease in plasma caeruloplasmin activity at early stages of copper deficiency. Again, it closely follows plasma copper and does not appear to be related to the most significant metabolic defects that arise. A virtually complete loss of activity from plasma provides no indication of the imminence, or otherwise, of gross clinical signs of deficiency. Before discarding such indices of deficiency as useless, it is appropriate to consider what relevance they may have to changes in the activity of other metal-dependent systems in the cells from which these enzymes originate.

It is becoming apparent that the secretion of metalloenzymes and of other proteins from tissues (figure 3*b*) is a process particularly sensitive to deficiency of several essential trace elements. Processes affected include not only synthesis of alkaline phosphatase and caeruloplasmin, but also of carboxypeptidase A (zinc deficiency), lysyl oxidase, melanin and keratin (copper deficiency) and of glycosaminoglycans (manganese deficiency). Defects arising during the synthesis of collagen in copper-deficient animals clearly illustrate the differing sensitivities of intracellular and extracellular processes to deficiency. Thus intracellular formation of disulphide cross-links in its precursor, procollagen, proceeds normally despite evidence that S–S formation in the extracellular maturation of keratin in other tissues is impaired. Instead, the primary defect in collagen (and elastin) biosynthesis occurs at, or shortly after, exocytosis of

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collagen fibrils. It reflects the loss of Cu-dependent lysyl oxidase normally discharged concurrently (for review, see Harris *et al.* 1980) and in close proximity to its substrate(s) collagen and elastin.

Many of the other metalloenzymes particularly sensitive to trace element deficiency are similarly translocated in that they are discharged from ribosomes of the endoplasmic reticulum into cisternae, followed by 'export' via the Golgi complex.

It seems improbable that the rapid decline in the activity of enzymes with such diverse origins and distribution as alkaline phosphatase, carboxypeptidase and lysyl oxidase could be attributable solely to high rates of extracellular degradation and inefficient 'recycling' of their metal components. Such a concept would not account for the longer immunity from the effects of deficiency of many intracellular processes. The alternative, that metal transport into organelles committed to 'exporting' activity is governed by equilibria differing fundamentally from those influencing distribution to other functional sites, is a possibility that we may have to consider. Unless evidence of such a discontinuous equilibrium in metal distribution becomes available, we can hardly disregard the early warning of deficiency provided by the susceptibility of these processes, nor should we fail to recognize the need for a more adequate study of their pathological relevance.

Conclusion

Both for veterinary and medical diagnostic purposes and to support experimental investigations of the aetiology of disease, the need exists for sensitive and unequivocal indices of trade element deficiency. Instances of overt clinical deficiency cause few diagnostic problems. In contrast, we have very few specific indices of covert pathological changes in animals suspected to be at risk. For example, many tentative estimates of the trace element requirements of man and of farm animals are now available (World Health Organization 1973; Agricultural Research Council 1980), but few have yet been validated adequately by studies of relations between dietary supply and response because of the lack of specific biochemical 'markers' of pre-clinical pathological changes.

As pointed out by Riordan & Vallee (1976) when discussing the biological role of zinc, more effective progress towards the earlier recognition of deficiency disease demands closer collaboration between the biochemist and the pathologist. The most important objectives are to identify the functional roles of the elements and to predict in which tissues metabolic defects arising from deficiency are likely to have the greatest pathological significance. Such objectives are not overambitious and are very likely to be attained in the study of the pathogenesis of copper and cobalt deficiencies in the near future.

The arguments considered in this paper may well have suggested that the metalloenzyme chemist has already made adequate contributions. With the exceptions that we still seek the identity of the essential biochemical roles of chromium, nickel, vanadium, fluorine, silicon and arsenic, this may well be true! However, it is clear that the rate of progress of studies of metalloenzyme structure and function has far outstripped that of processes determining metal distribution between and within tissues or that describing rates of cell and organelle lifespan and turnover, all of which must determine sensitivity to trace element deficiency. Fortunately, and sometimes for reasons quite unrelated to the trace elements, relevant biological data are now beginning to appear and will certainly be of value in narrowing the search for biochemical defects that have pathological relevance at early stages of deficiency.

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