NUTRITION: BASIC AND APPLIED SCIENCE

A SERIES OF MONOGRAPHS

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Nutritional Toxicology

VOLUME III

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ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers
San Diego New York Berkeley Boston
London Sydney Tokyo Toronto

Academic Press, Inc.
San Diego, California 92101

United Kingdom Edition published by
Academic Press Limited
24–28 Oval Road, London NW1 7DX

Library of Congress Cataloging in Publication Data

(Revised for vol. 3)

Nutritional toxicology.

(Nutrition, basic and applied science)
Includes bibliographies and indexes.
1. Nutritionally induced diseases.
2. Nutrition. 3. Food additives--Toxicology.
I. Hathcock, John N. [DNLM: 1. Food contamination. 2. Food additives--Adverse effects. 3. Food poisoning. WA 701 N976]
RC622.N894 1982 616.3'99 82-4036
ISBN 0-12-332601-X (v. 1)
ISBN 0-12-332603-6 (v. 3)

Printed in the United States of America
89 90 91 92 9 8 7 6 5 4 3 2 1

Preface

Many problems in food safety and nutritional adequacy involve interactions of diet and toxicants, substances that have basically opposing influences on living organisms. Nutritional substances provide the essential structural, catalytic, and energetic support for life processes, whereas toxic substances disrupt these functions. Nutrients and toxicants thereby show extensive mutual modulation of effects. Under some circumstances, diet can be a significant source of toxicants. At sufficiently high intakes, nutrients themselves become toxic.

This simple but fundamental relationship between nutrition and toxicology is exemplified by numerous problems that have been researched extensively, but many remain unsolved. This book provides a review and interpretation of several areas of research involving issues with potential public health or regulatory importance. Understanding the basics behind such issues is fundamental in protecting and promoting public health and in providing a sound scientific basis for policy and regulatory decisions. This third volume of "Nutritional Toxicology" was designed to further our understanding of these problems. The topics included complement and extend those in Volumes I and II of this treatise.

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Contents of Previous Volumes

Volume I

Volume II

1

Food Packaging Materials: Health Implications

D. C. Kirkpatrick, R. A. Ripley, and M. A. Pelletier

I. INTRODUCTION

Protecting foods against infestation and general contamination as well as the ingress of light, moisture, and oxygen, which can induce chemical degradation of food constituents, is a major challenge to the food industry. Food packaging provides a means of responding to this challenge; and its role in the preservation, distribution, and sale of foods is of paramount importance. Without today's packaging materials the availability of the variety of foods found in the mar-

NUTRITIONAL TOXICOLOGY, VOL. III

ketplace would be severely curtailed. Food packaging encompasses a broad spectrum of food contact articles—from the bottles, jars, tubs, cans, trays, cartons, bags, boxes, tubes, closures, and film wraps used at the retail level to the drums, barrels, pails, crates, tote boxes, baskets, bags, holding tanks, and transporter vehicles used for the commercial handling of bulk foods. The materials used to fabricate these articles range from wood, plant fibers, and glass, which have been used for centuries, to those of more modern origin such as paperboard, steel, tinplate, aluminum, and the myriad of plastics that have been developed (Sacharow and Griffin, 1980).

Packaging materials are generally in intimate contact with the foods they protect, often for extended periods of time and in some instances at elevated temperatures. Since such conditions are conducive to the migration of constituents of the packaging materials to foods, many countries have enacted regulations or established codes of practice to control food-packaging materials to ensure that migrating constituents that enter the food supply do not pose a health risk to consumers (Briston and Katan, 1974).

II. CURRENT TRENDS IN PACKAGING

Although traditional materials such as glass, steel, tinplate, paper, and paperboard are still the workhorses of the food-packaging industry, changing consumer eating-patterns and demands for improved food-handling convenience, coupled with changing food-processing technology, have created a need for more technically sophisticated food-packaging materials. This need is being met increasingly by plastic-based materials. Thus, for example, consumer acceptance of microwave oven cooking created a need for trays, for packaging frozen entrees, that have low gas permeability, thermal stability at cooking temperatures, and high microwave transmission. Trays manufactured from a variety of plastics including crystallized poly(ethylene terephthalate) (CPET), filled thermoset polyester, poly(ether sulfone) and coextruded poly(etherimide)/polycarbonate are now used for this application. Other examples include poly(ethylene terephthalate) (PET) bottles for soft drinks, beer, and liquor, PET/aluminum/polyolefin laminate pouch structures for retorted food products, coextruded multilayer flexible bottles based on polypropylene and ethylene–vinyl alcohol copolymer for oxygen-sensitive foods such as ketchup, and similarly structured microwaveable multilayer trays and bowls for thermally processed shelf-stable soups (Dilberakis, 1987; Knill, 1987).

This trend in food packaging is expected to continue in the future; in the United States, for example, the number of plastic food containers used is projected to increase at a rate of 11% annually over the next decade, compared with annual rate changes of +6.2%, −0.2%, and −2.5% for paperboard, metal, and

glass, respectively. Major plastic food container growth areas are expected to be in dual ovenable entree trays and coextruded, high barrier multilayer bottles, jars, and cans (Nazarenko, 1987). Thus, the share for plastics of the food-packaging market in the United States is projected to increase from 17% of an estimated $19 billion market in 1985 to 42% of the estimated $44 billion market in the year 2000 (DuPont Co., 1987).

Because of this expanding use of plastics in food packaging and the increased potential exposure of consumers to migrating substances in the food supply, regulatory agencies responsible for health protection have focused special attention on the safety of plastic-based food-packaging materials. This chapter will address some of these health implications.

III. POTENTIAL MIGRANTS FROM PLASTICS

There are three principal sources of migrants from plastics: base polymers, processing aids, and end-service additives.

TABLE I

Polymers Used in Food-Packaging Materials[a]

Polyethylene (C_{2-8} 1-alkene comonomers)
Polypropylene (ethylene comonomer)
Ethylene-vinyl acetate copolymers
Ethylene-vinyl alcohol copolymers
Ethylene-methylacrylate copolymers
Ionomers (ethylene, vinyl acetate, isobutylacrylate, methacrylic acid salt copolymers)
Acrylonitrile copolymers (styrene, butadiene, methylmethacrylate comonomers)
Polystyrene (butadiene, maleic anhydride comonomers)
Poly(vinyl acetate)
Poly(vinyl chloride) (ethylene, propylene, vinyl acetate comonomers)
Poly(vinylidene chloride) (vinyl chloride, acrylonitrile, methylacrylate comonomers)
Polyisobutylene
Poly(*p*-methyl styrene)
Polyamides (Nylon 6:6, 6:10, Nylons 6, 11, and 12)
Poly(ethylene terephthalate)
Poly(ether sulfone)
Polysulfone
Polycarbonate
Polyurethanes
Polyesters (unsaturated)
Polyacetal

[a]This is not intended to be an exhaustive list. For more examples see Food and Drug Administration (1987) and British Plastics Federation (1980).

A. Polymer Sources

The principal base polymers used in food-packaging applications are listed in Table I.

Potential migrants from base-polymer sources include low molecular weight polymer fractions, oligomers, monomers, and monomer impurities (e.g., ethylbenzene in polystyrene). Other potential migrants include residues of polymerization initiators (e.g., aluminum and titanium oxides from Ziegler catalysts in polyolefins), process solvents (e.g., monochlorobenzene in polycarbonates),

TABLE II

Food-Packaging Material Processing and Service Aids[a]

Technical function	Example	Use levels: wt% (polymer)
Antioxidant	Tetrakis[methylene (3,5-di-*tert*-butyl-4-hydroxyhydrocinnamate)]methane	0.25 (polystyrene)
	Tris(2,4-di-*tert*-butylphenyl) phosphite	0.2 (polyolefins)
Stabilizer	Di(*n*-octyl)tin *S,S'*-bis(isooctylmercaptoacetate)	1.5 (PVC)
	Epoxidized soybean oil	6 (PVC)
	Stearoylbenzoylmethane	0.5 (PVC)
	Cuprous iodide	0.01 (Nylon 6,6)
Plasticizer	Di(2-ethylhexyl) phthalate	40 (PVC)
	Di(2-ethylhexyl) adipate	20 (PVC)
	Acetyltributyl citrate	5 (PVDC)
Lubricant	*N, N'*-Ethylenebisstearamide	1.0 (PVC)
	Pentaerythritol adipate-stearate	1.0 (PVC)
Processing agent	Styrene/butadiene/methacrylate copolymer	2.0 (PVC)
Melt fracture eliminator	Vinylidene fluoride-hexafluoropropylene copolymer	0.1 (polyethylene)
Slip agent	Fatty acid amides (erucamide, oleamide)	0.2 (polyolefins)
Antistatic agent	*N, N'*-Bis (2-hydroxyethyl)alkyl-C_{14-18}-amine	0.15 (polyolefins)
Blowing agent	Azodicarbonamide	0.15 (polyethylene)
Antiblock agent	Silica, talc	0.2 (polyethylene)
Impact modifier	Butadiene/styrene/methacrylate copolymers	10 (PVC)
Clarifying agent	Dibenzylidene sorbitol	0.25 (polyolefins)
Light stabilizer	2-Hydroxy-4-*n*-octoxybenzophenone	0.5 (polyolefins)
	Dimethylsuccinate-(4-hydroxy-2,2,6,6-tetra-methyl-1-piperidyl)ethanol polycondensate	0.25 (polyolefins)
Coupling agent	3-(Triethoxylsilyl)propylamine	0.5 (Nylon 6,6)
Filler, extender	Calcium carbonate, clay, talc	>5 (Various polymers)
Reinforcing agents	Glass fiber, mica, calcium silicate	>5 (Various polymers)
Colorant	Titanium dioxide, ferric oxide, carbon black, ultramarine blue, phthalocyanine blue	0.1–5 (Various polymers)

[a]For more examples see Food and Drug Administration (1987) and British Plastics Federation (1980).

chain-transfer agents (e.g., *n*-dodecyl mercaptan in acrylonitrile copolymers), emulsifiers [e.g., alkyl aryl sulfonates in poly(vinyl chloride), PVC], and suspension agents (e.g., hydroxyethylcellulose in PVC).

B. Processing Aid Sources

Various additives are blended with the base polymers to facilitate processing into the desired food-packaging material. Some polymers such as poly(ethylene terephthalate) polyesters (PET) require little or no additives whereas others such as poly(vinyl chloride) (PVC) require several in substantial quantities. Included in this category are such additives as antioxidants, heat stabilizers, plasticizers, antiblock agents, antistatic agents, slip agents, and lubricants. Some examples of processing aids and levels of use in food-packaging plastics are given in Table II.

C. Packaging End-Service Aid Sources

Additional substances can be incorporated with the polymer/processing aids to perform specific technical end-service functions in the final food-packaging materials. In some instances the same additive may perform dual processing and end-service aid functions. End-service aids include heat stabilizers, light stabilizers, antiblock agents, antistatic agents, lubricants, blowing agents, impact modifiers, clarifying agents, fillers, extenders, reinforcing agents, coupling agents, and colorants. A few examples and typical use levels are included in Table II.

IV. ASSESSMENT OF DIETARY EXPOSURE

To assess the human health implications of any chemical substance that may be present in foods, it is necessary to have information on potential dietary exposure to the substance and then to relate this to the toxicological profile of the substance.

The estimation of dietary exposure to a substance requires information on where the substance may be present and at what levels, as well as data on the intake of those foods containing the substance. Approaches for estimating dietary exposure of consumers to chemical substances have been described in a number of publications (World Health Organization, 1985; Bunyan, 1985; Gunner and Kirkpatrick, 1979).

In the case of food-packaging materials, data on migrant levels in foods can be obtained either directly by analysis of foods or indirectly using food simulants. In this regard, it is necessary to ascertain (1) the soundness of the analytical methodology so that chemical identity of the migrant is confirmed, interferences and

artifacts are excluded, and so on, and (2) the adequacy of detection limits and sensitivity of the method to enable a meaningful estimate of migrant intake to be determined. The latter consideration is crucial to the determination of actual residues in food of toxicologically undesirable substances such as acrylonitrile, and vinyl chloride.

The use of food simulants to determine levels of potential migrants from food-packaging materials has found widespread application, particularly in the context of regulatory preclearance assessment of such materials. This situation has arisen largely because of practical problems in analyzing actual foods for trace migrant levels, including analytical sensitivity limitations, interfering food constituents, and food instability problems.

This indirect approach involves exposing test packaging-material substrates containing the potential migrant to certain prescribed food simulants under stipulated surface-to-volume exposure ratios and under time/temperature test conditions chosen to reflect the intended end-use, and then analyzing the simulants for migrant levels. The food simulants that are usually accepted by regulatory agencies as surrogates for aqueous, acidic, and alcoholic foods are distilled water, acetic acid solutions, and ethanol solutions, respectively (Food and Drug Administration, 1976; European Economic Community, 1982). At the present time, there is no clear consensus on a suitable fatty food simulant. Although several media have been proposed, and numerous studies have been conducted to find the most suitable fatty food simulant (Schwartz, 1983; de Kruijf *et al.*, 1983), there is still disagreement among regulatory agencies, evidenced by the various extraction study requirements prescribed in food-packaging regulations throughout the world. The United States Food and Drug Administration (FDA), for example, has for many years accepted *n*-heptane (Food and Drug Administration, 1976) and more recently has considered others, including 100% ethanol or 50% aqueous ethanol (Schwartz, 1983), whereas the European Economic Community (EEC) has adopted the use of olive oil, sunflower oil, or a mixture of synthetic triglycerides (European Economic Community, 1982). Differences also exist among regulatory agencies with respect to the conditions under which to run the extractions. Although the FDA requires that extractions for a specific migrant be run to equilibrium (Food and Drug Administration, 1976), the guidelines prescribed by most EEC countries specify actual time limits, usually up to 10 days (European Economic Community, 1982).

Considerable research effort has also been directed at developing theoretical models and equations that might help predict the extent of migration of substances to foods based on calculations (Smith *et al.*, 1981; Reid *et al.*, 1983).

As is the case with determining levels of packaging-material migrants in foods, there are two general approaches to obtaining food-intake data. Details of these have been described in a number of publications (Pekkarinen, 1970; Marr, 1971; World Health Organization, 1985). The first of these, the indirect ap-

proach, is commonly applied and makes use of regional food-disappearance figures. Such figures take into account imports, exports, and other factors such as waste prior to retail sale. Per-capita consumption of a food is obtained by dividing the annual disappearance figure for that particular food by the total population of the region. This approach provides only an approximate average intake for a given food because it suffers from a number of inherent weaknesses. In particular, food-intake figures so derived do not take into account atypical consumers or particular segments of the population, such as infants and pregnant women, that may warrant special consideration.

The direct approach makes use of data on actual amounts of food consumed by an individual. The data are obtained from detailed dietary surveys of a population, taking into account variables such as age, sex, cultural background, and socioeconomic status. Such food-monitoring surveys can be accomplished in a number of ways, for example, using a diary to record types and amounts of food consumed or employing dietary recall. They can involve consumption tracking or recall for different durations, for example, 24 hours or 7 days.

In contrast to the indirect approach, data from actual dietary surveys (i.e., the direct approach) can be used to target those segments of the population who, because of their dietary habits, may be at higher risk from exposure to a particular substance. For example, from the food-consumption portion of the Nutrition Canada Survey (Nutrition Canada, undated), the mean intake of alcoholic wine beverages by *eaters only* is 650 g/person/day. Based on food disappearance figures (i.e., the indirect approach) the per capita consumption of wine in Canada is 26 g/day (Statistics Canada, 1984). Thus, the estimation of intake of a food-packaging migrant present in wine based on the disappearance figure for wine would lead to a gross underestimate.

In estimating dietary exposure of vinyl chloride, the United Kingdom Ministry of Agriculture, Fisheries and Food (MAFF) conducted surveys to determine PVC food-packaging use patterns, vinyl chloride levels in PVC food-packaging materials, and vinyl chloride levels in targeted marketplace foods (Ministry of Agriculture, Fisheries and Food, 1978b). To simplify the task, MAFF considered only foods that were generally packaged in PVC, foods that made a significant contribution to the diet, and foods that contained measurable amounts of vinyl chloride. Dietary exposure of vinyl chloride was then estimated using the analytical data on vinyl chloride levels in those foods and United Kingdom food-intake statistics. Using this approach, dietary exposure to vinyl chloride was estimated to be 1.3 μg/day/person in 1974. MAFF used similar survey approaches to estimate dietary exposure of acrylonitrile (1982), vinylidene chloride (1980), and styrene (1983) from plastic food-packaging sources.

Another more systematic procedure for estimating potential exposure to food-packaging migrants is that employed by the United States FDA for the regulatory preclearance assessment of food-packaging materials (Food and Drug Admin-

istration, 1981), which uses information on the use patterns of food-packaging materials obtained from direct industry survey data (Food and Drug Administration, 1979).

In the FDA approach, the likely concentration M of the migrant in foods that contact packaging materials containing it is derived from the formula:

$$M = f_{\text{aqueous}}\,(M_{H_2O}) + f_{\text{acid}}\,(M_{\text{HOAc}}) + f_{\text{alcohol}}\,(M_{\text{EtOH}}) + f_{\text{fatty}}\,(M_{\text{heptane}})$$

where f is the *distribution value* for each food type and M simulant is the migration value into each food simulant. The f values are used to account for the varying nature of food contacting each packaging material. Each f value thus represents the fraction of the food in that category (i.e., aqueous, acidic, alcoholic, or fatty) that will contact the polymer.

From the concentration M, the estimated daily intake (EDI) of the migrant is then determined using the following equation:

$$\text{EDI} = 3{,}000 \text{ g/day} \times M \times CF$$

where CF is the appropriate consumption factor for the polymer under consideration. This factor describes the portion of the total diet (3000 g/day) that is likely to contact a specific packaging material.

V. MIGRANTS OF POTENTIAL CONCERN

The human health implications of dietary exposure to food-packaging material migrants can be determined only on the basis of available biological data. Such data may consist of results from studies conducted to determine the migrant's biochemical properties (e.g., its absorption, distribution, and excretion characteristics) and its toxicological effects (e.g., its short- and long-term toxicity and its potential reproductive, teratogenic, and carcinogenic effects).

The following discussion of several specific migrants of particular concern to health authorities highlights some of the considerations involved in the safety evaluation and regulation of food-packaging materials.

A. Vinyl Chloride

Vinyl chloride polymers (PVC) in various forms have been used worldwide for many years in the manufacture of food-contact articles. For example, plasticized flexible PVC is used for fabricating food-wrap film and beverage bottle-cap liners; semirigid PVC sheet is used to fabricate processed meat blister packs and portion packs for jams and jellies; and rigid PVC bottles are used for

packaging liquor, wine, vinegar, vegetable oils, and mineral water (Codex Committee on Food Additives, 1984).

In the early 1970s it was discovered that liquor stored in PVC bottles contained up to 20 ppm vinyl chloride (Food and Drug Administration, 1973). Subsequent investigations conducted in Canada found vinyl chloride levels up to 3.29, 8.4, 1.6, and 0.84 ppm in peanut oil, vinegars, distilled spirits, and wine, respectively, that were packaged in PVC containers (Williams and Miles, 1975). When it was later shown that vinyl chloride monomer was a carcinogen in animals (via both inhalation and ingestion) and in humans (via inhalation) (International Agency for Research on Cancer, 1974), governments acted to protect consumers against residues of this monomer in the food supply. For example, the United States FDA (1975a) proposed a ban on rigid and semirigid vinyl chloride polymers for food-contact use, and Canada prohibited the use of food-packaging materials contributing detectable amounts of vinyl chloride to foods (Health Protection Branch, 1976).

Using improved manufacturing technology, PVC producers and compounders were successful in effecting dramatic reductions in residual monomer levels in their products. Monitoring conducted in the United Kingdom over the period 1973–1976 demonstrated that maximum vinyl chloride levels in PVC resins dropped from 1000 to 50 ppm, with corresponding reductions from 100 ppm to 1 ppm in monomer levels in PVC bottles used for packaging foods (Ministry of Agriculture, Fisheries and Food, 1978b). These improvements reduced vinyl chloride levels from >0.2 ppm to <0.01 ppm in cooking oils, concentrated fruit drinks, and soft margarine, with a resultant decrease from 1.3 to 0.1 μg/day in the estimated dietary intake of vinyl chloride from food packaging.

Additional improvements in PVC manufacturing/compounding technology further reduced residual monomer levels in PVC compounds to the point that in 1986 the FDA withdrew its 1975 proposed restrictions and proposed regulatory limits for vinyl chloride in PVC food-contact articles in the range of 5 to 50 ppb. At the time, it was estimated that usage of such materials would result in a dietary intake of less than 25 ng/day/person, and it was concluded on the basis of a quantitative risk assessment that there was "a reasonable certainty of no harm" to consumers from such exposure. Details of the risk assessment procedure used by the FDA have been published (Food and Drug Administration, 1986).

Dietary exposure to vinyl chloride is now controlled in several other countries either by limiting monomer content of food-contact materials (typically <1 ppm) or limiting monomer migration to foods to nondetectable levels (typically <0.01 ppm) or both (European Economic Community, 1980a). It would appear that the overall thrust of such controls is to keep migration of vinyl chloride to foods from packaging materials to the lowest level technologically achievable. This is in concert with the FAO/WHO Joint Expert Committee on Food Additives (JECFA) recommendation (1984) for this migrant.

B. Acrylonitrile

Acrylonitrile copolymers of low acrylonitrile content (<30%) such as acrylonitrile/butadiene/styrene (ABS) have been used for many years to fabricate such diverse food-contact articles as refrigerator liners, cake packaging inserts, and tubs for margarine, dairy products, and salads (Codex Committee on Food Additives, 1984). Development in the 1970s of copolymers with up to 70% acrylonitrile content based on styrene, butadiene, and methacrylate comonomers, and with exceptional gas-barrier characteristics, led to their use in the manufacture of bottles for carbonated beverages and other oxygen-sensitive foods such as vegetable oils. Barrier coatings based on acrylonitrile/vinylidene chloride have also been used for a number of years on poly(ethylene terephthalate), polypropylene, and regenerated cellulose films used for packaging snack foods (Codex Committee on Food Additives, 1984).

The finding in 1974 that certain high-barrier acrylonitrile copolymer bottles could contribute up to 0.24 ppm acrylonitrile to soft drinks stored in them for long periods (Food and Drug Administration, 1974), taken together with results of studies purporting that acrylonitrile monomer was an animal carcinogen, raised concern about the safety of acrylonitrile copolymer packaging materials. In 1977, FDA banned the use of acrylonitrile copolymer bottles for packaging beverages (Food and Drug Administration, 1977). Subsequent investigations in the United States and other countries provided further evidence of acrylonitrile residues in foods packaged in acrylonitrile copolymer materials. For example, a United Kindgom survey showed that residual acrylonitrile levels in ABS margarine tubs were typically 100 ppm, resulting in up to 0.35 ppm acrylonitrile in margarine (Ministry of Agriculture, Fisheries and Food, 1982). In addition, other rat bioassays confirmed that acrylonitrile monomer was a multifocal animal carcinogen (Joint Expert Committee on Food Additives, 1984).

Initiatives by governments and industry were successful in significantly reducing the residual acrylonitrile content of acrylonitrile copolymer food-packaging materials. Data from the United Kingdom showed that over the period from 1975 to 1979, average acrylonitrile levels in ABS margarine tubs decreased from 100 to 8 ppm with a resultant decrease from 0.35 to 0.04 ppm in average acrylonitrile levels in margarine. Similar reduced levels of this migrant in margarine and in other foods packaged in acrylonitrile copolymer containers were found in surveys conducted in 1980 in Canada (Page and Charbonneau, 1983) and in the United States (Breder, 1980). In 1982, Canada promulgated a regulation prohibiting the sale of foods in packaging materials that contribute detectable amounts of acrylonitrile to foods (Health Protection Branch, 1982).

Improvements in the manufacture of high-barrier acrylonitrile copolymer bottle resins resulted in FDA's reinstatement of resins containing less than 0.1 ppm

acrylonitrile for fabricating nonalcoholic beverage bottles. This revised regulation was promulgated in 1984, at which time a worst-case dietary exposure of 0.058 μg/day/person acrylonitrile from acrylonitrile copolymer food packaging was estimated. On the basis of a quantitative risk assessment it was concluded that there was "a reasonable certainty of no harm" to consumers from such dietary acrylonitrile exposure. Details of the risk assessment procedure used by the FDA have been published (Food and Drug Administration, 1984).

C. Di(2-ethylhexyl) Phthalate

Di(2-ethylhexyl) phthalate (DEHP) has been used extensively worldwide for many years as a plasticizer in flexible PVC compounds used in a variety of industrial and consumer products, including food-contact materials. The principal food-contact use of DEHP-plasticized PVC in North America is as produce film wrap, which may have a DEHP content of up to 40% by weight. It is also used for beverage bottle-cap liners and jar-lid sealants and as tubing, conveyor belting, and liners for bulk liquid holding tanks in food plants.

As a consequence of its wide usage and production volume, which was estimated to be 257.2×10^6 lb in 1980 in the United States (Consumer Products Safety Commission, 1982), DEHP is considered ubiquitous in the environment (International Agency for Research on Cancer, 1982). DEHP residues have been reported in dairy products, meats, poultry, fish, cereal products, fruits, and vegetables—at average levels, for example, of less than 0.1 ppm in eggs, 0.1 to 0.2 ppm in milk, up to 1 ppm in cheese, and more than 2 ppm in salted meat (Cerulis and Ard, 1967; Burns *et al.*, 1981; Rost, 1970; Filipic *et al.*, 1969; Kato *et al.*, 1979; Food and Drug Administration, 1975b; Chemical Manufacturers Association, 1986; Cox, 1986; Cocchieri, 1986). Unfortunately, these data do not provide definitive insights into DEHP residue levels in food emanating specifically from food packaging.

DEHP has been shown to be a hepatocarcinogen in rats and mice (National Toxicology Program, 1982). Toxicological data on DEHP, including results of studies of its carcinogenic potential, were reviewed by the JECFA (1984). This committee recommended that the level of DEHP in food-contact material and the extent of its migration into food should be kept at the lowest levels that are technologically possible.

In the early 1980s, the United States Chemical Manufacturers Association (CMA) submitted protocols for an extensive voluntary phthalate ester test program to the Environment Protection Agency, under Section 4 of the Toxic Substances Control Act (Chemical Manufacturers Association, 1981b). Part of the yet to be completed test program is intended to address health effects of phthalate esters and, in particular, to further investigate the relevance to humans

of the NTP carcinogenicity study findings in test animals. The results of studies conducted under this program should provide insights into the human health implications of dietary exposure to DEHP.

D. Di(2-ethylhexyl) Adipate

Di(2-ethylhexyl) adipate (DEHA) has a long history as a plasticizer in PVC and poly(vinylidene chloride) (PVCD) food-packaging materials. Its principal use is in PVC food-wrap film sold to consumers as household wrap or to supermarkets for wrapping such foods as fresh meats, poultry, fish, and cheese. PVC film wraps typically contain 15–25 wt% DEHA.

There is a paucity of data on DEHA residues in marketplace foods,* but migration of DEHA from PVC film to foods under controlled laboratory conditions has been demonstrated (Daun and Gilbert, 1977). More elaborate FDA-sponsored studies using radiolabeled DEHA also showed high levels of migration of DEHA to fatty foods from PVC film containing 22% DEHA when held at 4°C for 7 days (Food and Drug Administration, 1978). Migration was shown to decrease with lessening fat contact of the food, ranging from a high of 155 ppm in beef fat through 8.7 ppm in lean beef to a low of 0.73 ppm in carrots. Migration studies by the Swedish National Food Administration demonstrated that DEHA content in cheese that is held in immediate contact with PVC film for 7 days at 16°C reached levels up to 1500 ppm. It was calculated that consumers who ate cheese slices that had been in immediate contact with PVC film could be exposed to a daily intake of 7–20 mg DEHA (Sandberg *et al.*, 1982). Studies by United Kingdom health authorities showed that when cheese was stored for 24 hr in contact with PVC film containing 14.4–18.8% DEHA, DEHA migration film loss ranged from 32–49% at 2°C and 47–83% at 20°C (Anonymous, 1986). Concern over increased DEHA migration from PVC food wrap to microwave oven cooked foods prompted the United Kingdom Ministry of Agriculture and Fisheries to request PVC food-wrap manufacturers to label their products advising consumers not to use them in microwave ovens (Ministry of Agriculture, Fisheries and Food, 1986).

DEHA has been shown to be hepatocarcinogenic in the mouse but not in the rat (National Toxicology Program, 1981). DEHA was also included in the CMA's phthalate ester test program cited in the discussion in DEHP (Chemical Manufacturers Association, 1981a). Results from this program will hopefully

*Following completion of this manuscript, the United Kingdom Ministry of Agriculture, Fisheries and Food published the results of a survey of plasticizer levels in food contact materials (MAFF, 1987). While this publication covers several plasticizers, dietary exposure from DEHA in various foods receives special consideration including exposure from the use of DEHA-plasticized PVC film in microwave ovens. The possible risk to public health from the estimated intake of 16 mg/day/person of DEHA from the average United Kingdom diet was considered to be remote.

permit an assessment of any hazard posed to humans by dietary exposure to DEHA.

E. Nitrosamines

Trace levels of certain nitrosamines are present in nitrite-cured pork products as a consequence of nitrosation of the corresponding pork-derived amine precursors (Sen *et al.*, 1979). Analysis of Canadian cured pork products in 1986 found up to 29 ppb *N*-nitrosodi-*n*-butylamine and 2.4 ppb *N*-nitrosodiethylamine in those products that had been processed in elastic nettings manufactured from rubber thread (Sen *et al.*, 1987). The source of these particular nitrosamines was determined to be the dialkyldithiocarbamates used as rubber-thread vulcanization accelerators, which gave rise to the corresponding dialkylamines and thence the nitrosodialkylamines, under the pork-curing conditions. Because both nitrosamines are animal carcinogens (International Agency for Research on Cancer, 1978), thread manufacturers have since reformulated their products toward eliminating these nitrosamines in cured pork from netting sources (manufacturers' personal communication with the Health Protection Branch of Health and Welfare Canada). This and other examples of nitrosamine migration from food-contact materials to foods are described in a recent review (Sen, 1987).

VI. REGULATORY CONTROL MEASURES IN VARIOUS COUNTRIES

Most industrialized countries exercise some type of direct or indirect control of food-packaging materials aimed at protecting consumers from harmful adventitious packaging migrants in the food supply. The controls run the gamut from nonstatutory recommendations and codes of practice, through statutory controls of a general nature, to specific statutory regulations (Katan, 1983). A number of countries that have specific statutory regulations employ the concept of permitted or positive lists of substances from which food-contact materials may be manufactured to the exclusion of other substances. These positive lists are often supplemented by additional requirements such as end-use limitations, composition limits, overall migration limits, specific migration limits, and extraction procedures using specified food simulants, test conditions, and analytical procedures to verify compliance with the requirements. Examples of countries that have positive list-based legislation include the United States, Belgium, France, the Netherlands, and Italy (Katan, 1983). Of these, the United States has perhaps the most detailed regulations covering several hundred permitted food-contact article ingredients (Food and Drug Administration, 1987). Under the 1958 Food Additives Amendment of the United States Federal Food Drug and Cosmetics

Act, food-contact materials are regarded as indirect food additives. A company that wants to manufacture a food-packaging material using an ingredient that is not permitted and that can reasonably be expected to migrate to food must formally petition FDA with supporting safety documentation and request amendment of the regulations for its use.

Under Canadian legislation, food-packaging materials are not considered to be food additives and are therefore not subject to the mandatory preclearance requirements that food additives must meet under the Food and Drugs Act and Regulations. Instead, food-packaging materials are regulated as such under a general provision that makes food sellers responsible for the safety of any packaging materials used in the sale of their food products (Health Protection Branch, 1987). Notwithstanding this general provision, however, there are some specific regulations governing the use of certain packaging materials, for example, those that may contribute vinyl chloride or acrylonitrile residues to foods. Because these regulations do not employ a positive list system, food-packaging manufacturers make submissions on a voluntary basis to the Health Protection Branch (HPB) of Health and Welfare Canada for advice on the safety of their products. If the packaging material is judged acceptable, a "no objection" letter is issued for the material for a specified end-use. Australia has similar legislation, aided in its implementation by a voluntary industry code of practice (Anonymous, 1985). Swedish regulations likewise do not employ a positive list system. Instead, control is effected under a general provision in the food law and voluntary standards by the food packaging industry (Katan, 1983).

Until 1978 there was no United Kingdom legislation dealing specifically with food packaging. However, consumers were protected against harmful food-packaging migrants under the general provisions of the Food and Drugs Act 1955 and by informal controls involving the cooperation of industry and government (Ministry of Agriculture, Fisheries and Food) in such projects as surveillance programs for packaging-derived styrene (1983), vinyl chloride (1978b), vinylidene chloride (1980), and acrylonitrile and methacrylonitrile (1982) residues in foods. Also, since 1969, the British Plastics Federation (BPF), in conjunction with the British Industrial Biological Research Association (BIBRA), has published a plastics industry voluntary code of practice for food packaging, detailing lists of recommended ingredients, use-level limits, and end-use levels (British Plastics Federation, 1980). In 1978, the United Kingdom promulgated the Materials and Articles in contact with Food Regulations 1978, which implement its obligations respecting European Economic Community Directive 76/893/EEC on food-contact materials, and which now allow direct control of food-contact materials and migration of constituents therefrom into foods (Ministry of Agriculture, Fisheries and Food, 1978a).

West German legislation allows packaging constituent migration to foods only if it can be established that the migrants are safe, do not taint the food, and are

technologically unavoidable (Katan, 1983). The legislation does not in itself contain any mandatory specifications. However, the West German Federal Health Office issues detailed nonstatutory recommendations to assist manufacturers to comply with the general requirements. The recommendations include, among other things, a positive list system of ingredients, acceptable daily intakes or specific migration limits for ingredients, compositional limits, designated food simulants, and extraction procedures.

VII. INTERNATIONAL INITIATIVES

In recent years several initiatives have been taken to control food-packaging materials at the international level. These have resulted from a desire by trading countries to harmonize their legislation toward eliminating technical barriers against trade among them.

In 1978 and 1982 the Council of Europe issued a document entitled "Substances used in plastics materials coming into contact with food" (Council of Europe, 1982). This guideline document is intended for use by member countries as a basis for their respective food-packaging regulations. It lists several hundred food-packaging constituents, including monomers, polymerization aids, and polymer additives, but does not include base polymers. Based on available toxicological data, each constituent listed is assigned a food migration limit, a prohibition against its use, or an indication that further evaluation data are needed. Guidelines on procedures for approving new constituents are also described.

In 1976 the European Economic Community (EEC) member states adopted Directive 76/893/EEC respecting materials and articles intended for contact with foods (European Economic Community, 1976). This general directive provides, among other things, that such materials and articles should not transfer their constituents to foods so as to endanger human health. Since then, specific directives have been adopted respecting a labeling symbol for food-contact materials and articles (European Economic Community, 1980a), a permitted list of substances for regenerated cellulose film (European Economic Community, 1983), the establishment of vinyl chloride limits in food-contact materials and articles and foods, analytical control thereof (European Economic Community, 1978, 1980b, 1981), and basic rules for migration testing (European Economic Community, 1982).

At its 15th session held in 1983, the Codex Alimentarius Commission endorsed the initiation of a program addressing the evaluation of food-packaging materials in relation to the health risks posed by packaging migrants in foods (Codex Alimentarius Commission, 1983). In considering approaches to such a program, the commission's Codex Committee on Food Additives (CCFA) decid-

ed not to pursue a control system based on positive lists, opting instead to address specific issues on a case-by-case priority basis. At its 16th session, CCFA (1983) identified four migrants of food-packaging plastics—vinyl chloride, acrylonitrile, styrene, and di(2-ethylhexyl) phthalate—for initial investigation. Data on these migrants were subsequently compiled from participating countries (1984, 1985) respecting their use in food packaging, migration levels in foods, and resultant dietary exposure levels. These particular migrants were also evaluated in 1984 by the JECFA, which estimated a provisional maximum tolerable daily intake of 0.04 mg/kg body weight for styrene and recommended that human dietary exposure to vinyl chloride, acrylonitrile, and DEHP in foods from food-contact materials should be reduced to the lowest levels technologically achievable (Joint Expert Committee on Food Additives, 1984). In accordance with JECFA's recommendations, CCFA has recently (1987) proceeded to address approaches to limiting entry of these migrants into foods.

Given the importance of food packaging materials to the preservation, distribution, and sale of foods throughout the world and the human health implications associated with expanding applications, international interest and initiatives respecting their safety in use will likely intensify.

REFERENCES

Anonymous (1985). Food contact regulations in Australia. *Plast. News* **May,** 11–13.

Anonymous (1986). *At Your Serv.* **Spring** (11).

Breder, C. V. (1980). (Quoted). *Food Chem. News* **Oct. 20,** 21–22.

Briston, J. H., and Katan, L. L. (1974). "Plastics in Contact with Food," pp. 251–328. Food Trade Press, London.

British Plastics Federation (1980). "Plastics for Food Contact Applications. A Code of Practice for Safety in Use," rev. ed. British Plastics Federation, London.

Bunyan, P. J. (1985). The form and role of food surveillance. *In* "Food Toxicology: Real or Imaginary Problems?," (G. G. Gibson and N. Walker, eds.) pp. 66–75. Taylor and Francis, London.

Burns, B. G., Musial, C. J., and Uthe, J. F. (1981). Novel cleanup method for quantitative gas chromatographic determination of trace amounts of di-2-ethylhexyl phthalate in fish lipid. *J. Assoc. Off. Anal. Chem.* **64,** 282–286.

Cerulis, J., and Ard, J. S. (1967). Method for isolation and detection of dioctyl phthalate from milk lipids. *J. Assoc. Off. Anal. Chem.* **50,** 646–650.

Chemical Manufacturers Association (1981a). "Voluntary Research Program on Di(2-ethylhexyl) Adipate," submitted to the Food and Drug Administration by the Chemical manufacturers Association Phthalate Esters Program Panel, October 22.

Chemical Manufacturers Association (1981b). "Phthalate Esters Voluntary Test Program under Section 4 of the Toxic Substances Control Act," submitted to the Environmental Protection Agency by the Chemical Manufacturers Association, September 30.

Chemical Manufacturers Association (1986). "Analysis of Bis(2-ethylhexyl) Phthalate (DEHP) by Gas Chromatography–Mass Spectrometry in Dairy and Meat Products," report for the Chemical Manufacturers Association, October 7. Hazelton Laboratories, Madison, Wisconsin.

Cocchieri, R. A. (1986). Occurrence of phthalate esters in Italian packaged foods. *J. Food Protection* **49,** 265–266.

Codex Alimentarius Commission (1983). "Report of the 15th Session of the Joint FAO/WHO Codex Alimentarius Commission," Alinorm 83/43, p. 72. Food and Agriculture Organization of the United Nations/World Health Organization Codex Alimentarius Commission, Rome.

Codex Committee on Food Additives (1983). "Report of the 16th Session of the Codex Committee on Food Additives," Alinorm 83/12A, p. 20. Food and Agriculture Organization of the United Nations/World Health Organization Codex Alimentarius Commission, Rome.

Codex Committee on Food Additives (1984). "Report of the 17th Session of the Codex Committee on Food Additives," Alinorm 85/12, pp. 26–27. Food and Agriculture Organization of the United Nations/World Health Organization Codex Alimentarius Commission, Rome.

Codex Committee on Food Additives (1985). "Report of the 18th Session of the Codex Committee on Food Additives," Alinorm 87/12, p. 33. Food and Agriculture Organization of the United Nations/World Health Organization Codex Alimentarius Commission, Rome.

Codex Committee on Food Additives (1987). "Report of the 19th Session of the Codex Committee on Food Additives," Alinorm 87/12A, pp. 33–34. Food and Agriculture Organization of the United Nations/World Health Organization Codex Alimentarius Commission, Rome.

Consumer Products Safety Commission (1982). "Phthalates in Consumer Products," final report to Arthur D. Little, Inc., Contract No. CPSC-80-1001, July 7.

Council of Europe (1982). "Substances Used in Plastic Materials Coming into Contact with Food," 2nd Ed. Council of Europe, Strauburg.

Cox, G. V. (1986). (Quoted). *Food Chem. News* Nov. 3, 46–47.

Crosby, N. T. (1981). "Food Packaging Materials: Aspects of Analysis and Migration of Contaminants." Applied Science Publ., London.

Daun, H. K., and Gilbert, S. G. (1977). Migration of plasticizers from polyvinyl packaging films to meat. *J. Food Sci.* **42,** 561–563.

de Kruijf, N., Rijk, M. A. H., and Soethardi, L. A. (1983). Investigation of the suitability of isooctane as a fatty food simulant. *In* "The Fourth International Symposium on Migration, November 24–25," pp. 182–212. Deutsche Unilever, Hamburg, Federal Republic of Germany.

Dilberakis, S. (1987). Food Drug Packag. **51**(5), 22–32.

DuPont Co. (1987). (Reported). *Food Eng.* **59**(4), 54.

European Economic Community (1976). "On the Approximation of the Laws of the Member States Relating to Materials and Articles Intended to Come into Contact with Foodstuffs," EEC Directive 76/893/EEC, November 23.

European Economic Community (1978). EEC Directive 78/142/EEC.

European Economic Community (1980a). EEC Directive 80/590/EEC.

European Economic Community (1980b). EEC Directive 80/776/EEC.

European Economic Community (1981). EEC Directive 81/432/EEC.

European Economic Community (1982). EEC Directive 82/711/EEC.

European Economic Community (1983). EEC Directive 83/229/EEC.

Filipic, V. J., Underwood, J. C., and Dooley, C. J. (1969). Trace components of the flavor fraction of maple syrup. *J. Food Sci.* **34,** 105–110.

Food and Drug Administration (1973). Prior-sanctioned polyvinylchloride resin. Notice of proposed rulemaking. *Fed. Regist.* **38,** 12–31.

Food and Drug Administration (1974). Acrylonitrile copolymers intended for use in contact with food. Notice of Proposed rulemaking. *Fed. Regist.* **39,** 38907–38909.

Food and Drug Administration (1975a). Vinyl chloride polymers in contact with food. Notice of proposed rulemaking. *Fed. Regist.* **40,** 40529–40537.

Food and Drug Administration (1975b). "Phthalate Esters in Food Survey FY 1974. Compliance Program Evaluation," January 8. Food and Drug Administration, Washington, D.C.

Food and Drug Administration (1976). "Guidelines for Chemistry and Technology Requirements of

Indirect Food Additive Petitions," March. Bureau of Foods, Food and Drug Administration, Department of Health Education and Welfare, Washington, D.C.

Food and Drug Administration (1977). Acrylonitrile copolymers used to fabricate beverage containers; final decision. *Fed. Regist.* **42,** 48528–48544.

Food and Drug Administration (1978). "First Annual Technical Progress Report—A Study of Indirect Food Additive Migration," FDA Contract No. 223-77-2360. Arthur D. Little, Inc., Washington, D.C.

Food and Drug Administration (1979). "Food Contact Polymers and Polymer Additives Used in Packaging," FDA Contract No. 223-77-2360, Modif. No. 2, September. Arthur D. Little, Inc., Washington, D.C.

Food and Drug Administration (1981). "Guidelines for Estimating Exposure to Indirect Food Additives," June. Food and Drug Administration, Washington, D.C.

Food and Drug Administration (1984). Indirect food additives: Polymers; acrylonitrile/styrene copolymers. Final rule. *Fed. Regist.* **49,** 36635–36644.

Food and Drug Administration (1986). Vinyl chloride polymers; withdrawal of proposal. *Fed. Regist.* **51,** 4173–4188.

Food and Drug Administration (1987). "Code of Federal Regulations. Title 21," Parts 177–181 (revised annually). U.S. Govt. Printing Office, Washington, D.C.

Gunner, S. W., and Kirkpatrick, D. C. (1979). Approaches for estimating human intakes of chemical substances. *Can. Inst. Food Sci. Technol. J.* **12,** 27.

Health Protection Branch (1976). Food and Drug Regulations, amendment. *Can. Gaz. II* **110**(4), 558–559.

Health Protection Branch (1982). Food and Drug Regulations, amendment. *Can. Gaz. II* **116**(11), 2024.

Health Protection Branch (1987). "Food and Drugs Act and Regulations," Division 23. Queen's Printer, Ottawa, Ontario, Canada.

International Agency for Research on Cancer (1974). "Report of a Working Group on Vinyl Chloride," IARC Internal Technical Report No. 74/005, June 24–25. International Agency for Research on Cancer, Lyons, France.

International Agency for Research on Cancer (1978). Some nitroso compounds. *IARC Monogr. Eval. Carcinog. Risk Chem. Man* **17,** 51–124.

International Agency for Research on Cancer (1982). Some industrial chemical and dyestuffs. Di(2-ethylhexyl)phthalate. *IARC Monogr. Eval. Carcinog. Risk Chem. Hum.* **29,** 269–294.

Joint Expert Committee on Food Additives (1984). 28th Report: Evaluation of certain food additives and contaminants. *WHO Tech. Rep. Ser.* **710,** 22–26.

Katan, L. L. (1983). "Food Packaging Legislation. US/EEC Perspectives." Elsevier Int. Bull., Amsterdam.

Kato, K., Nakoako, T., and Ikeda, H. (1979). Survey of phthalic esters contamination in rice, meat and vegetables by way of distribution. *Kanagawa-ken Eisei Kenkyusho Kenkyu Hokoku* **9,** 59; *Chem. Abstr.* **93,** 184454a.

Knill, B. J. (1987). *Food Drug Packag.* **51**(4), 3.

Marr, J. W. (1971). Individual dietary surveys: Purposes and methods. *World Rev. Nutr. Diet.* **13,** 105.

Ministry of Agriculture, Fisheries and Food (1978a). "The Materials in Contact with Food: Regulations 1978," S.I. 1978 No. 1927. Her Majesty's Stationery Office, London.

Ministry of Agriculture, Fisheries and Food (1978b). "Survey of Vinyl Chloride Content of Polyvinyl Chloride for Food Contact and of Foods," MAFF Surveillance Paper No. 2. Her Majesty's Stationery Office, London.

Ministry of Agriculture, Fisheries and Food (1980). "Survey of Vinylidene Chloride Levels in Food

Contact Materials and in Foods," MAFF Surveillance Paper No. 3. Her Majesty's Stationery Office, London.

Ministry of Agriculture, Fisheries and Food (1982). "Survey of Acrylonitrile and Methacrylonitrile Levels in Food Contact Materials and in Foods," MAFF Surveillance Paper No. 6. Her Majesty's Stationery Office, London.

Ministry of Agriculture, Fisheries and Food (1983). "Survey of Styrene Levels in Food Contact Materials and in Foods," MAFF Surveillance Paper No. 11. Her Majesty's Stationery Office, London.

Ministry of Agriculture, Fisheries and Food (1986). Press release, July 22.

Ministry of Agriculture, Fisheries and Food (1987). "Survey of Plasticizer Levels in Food Contact Materials and in Foods." Surveillance Paper No. 21, Her Majesty's Stationery Office, London.

National Toxicology Program (1981). "Carcinogenesis Bioassay of Di(2-ethylhexyl) Adipate," Tech. Rep. Ser. 212, NTP-80-29; NIH Publ. 81-1968. National Toxicology Program, Research Triangle Park, North Carolina.

National Toxicology Program (1982). "Carcinogenesis Bioassay of Di(2-ethylhexyl)phthalate in F334 Rats and B6C3F$_1$ Mice (Feed Study)," Tech. Rep. Ser. 217; NIH Publ. 82-1773. National Toxicology Program, Research Triangle Park, North Carolina.

Nazarenko, L. (1987). *Mod. Plast.* **March,** 102.

Nutrition Canada (undated). "Food Consumption Patterns Report." Bureau of Nutritional Sciences, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario, Canada.

Page, D. P., and Charbonneau, C. F. (1983). Determination of acrylonitrile in foods by headspace gas–liquid chromatography with nitrogen–phosphorous detection. *J. Assoc. Off. Anal. Chem.* **66,** 1096–1105.

Pekkarinen, M. (1970). Methodology in the collection of food consumption data. *World Rev. Nutr. Diet.* **12,** 145.

Reid, R. C., Schwope, A. D., and Sidman, K. R., (1983). Modelling the migration of additives from polymer films to foods and food simulating liquids. *In* "The Fourth International Symposium on Migration, November 24–25," pp. 105–156. Deutsche Unilever, Hamburg, Federal Republic of Germany.

Rost, H. E. (1970). Contamination of fats with phthalates from plastics. *Fette Seifen Anstrichm.* **72,** 552–553.

Sacharow, S., and Griffin, R. C. (1980). "Principles of Food Packaging," 2nd Ed. AVI Publ., Westport, Connecticut.

Sandberg, A. E., Vaz, R., Albano, L. Mattsson, P., and Nilsson, K. (1982). Migration of plasticizers from PVC films to food. *Var Foeda* **34,** 470–482.

Schwartz, P. S. (1983). Migration studies for regulation of indirect food additives: Proposed changes. *In* "The Fourth International Symposium on Migration, November 24–25," pp. 157–171. Deutsche Unilever, Hamburg, Federal Republic of Germany.

Sen, N. P. (1988). Migration and formation of *N*-nitrosamines from food contact materials. *In* "Chemical Interactions between Foods and Food Packaging" (J. H. Hotchkiss, Ed.), ACS Symp. Ser.

Sen, N. P., Seaman, S. W., and Miles, W. F. (1979). Volatile nitrosamines in various cured meat products: Effect of cooking and recent trends. *J. Agric. Food Chem.* **27,** 1354.

Sen, N. P., Baddoo, P. A., and Seaman, S. W. (1987). Volatile nitrosamines in cured meats packaged in elastic rubber nettings. *J. Agric. Food Chem.* **35,** 346.

Smith, L. E., Chang, S. S., McCackin, F. L., Senish, G. A., and Wang, F. W. (1981). "Models for the Migration of Low Molecular Weight Additives in Polyolefins," Report No. 81-2264, April. National Bureau of Standards, Washington, D.C.

Statistics Canada (1984). "Apparent Per Capita Food Consumption in Canada. Part II. Catalogue No. 32-230." Supply and Services Canada, Ottawa, Ontario, Canada.

Williams, D. T., and Miles, W. F. (1975). Gas–liquid chromatographic determination of vinyl chloride in alcoholic beverages, vegetable oils and vinegars. *J. Assoc. Off. Anal. Chem.* **58,** 272–275.

World Health Organization (1985). "Guidelines for the Study of Dietary Intakes of Chemical Contaminants," WHO Offset Publ. 87. World Health Organization, Geneva.

2

Neurotoxicology and Food Safety Assessment

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I. INTRODUCTION

Determining or predicting neurotoxicity (e.g., as expressed as behavioral, neurological, or cognitive dysfunction) from preclinical toxicological data is an important and controversial issue. Because of the ubiquitous effect of diet on

nervous system functions, it is also a subject that has generated political and scientific debates in which the degree of emotion has far exceeded the degree of empirical sophistication. Our goal in this chapter is not to review in detail every agent about which there has been substantial controversy. Instead, we have two goals: to outline a scientific framework that may be useful in studying and evaluating these issues and to provide examples of how this framework has been, or could be, used with respect to specific dietary agents.

There are numerous neurotoxicological issues relevant to diet, but we have chosen to categorize these problems broadly into two classes. The first is acute or subchronic intoxication caused by ingestion of large (relative to causation of toxic effects) amounts of particular compounds. Such situations include methylmercury intoxication [e.g., from fish (Tsubaki and Irukayama, 1977) or contaminated seed (Bakir *et al.*, 1973)], lead poisoning from ceramics (Kolbye *et al.*, 1974), botulism, ingestion of toxins [such as from cycad nuts (see section IV,E)], and the like. Although such incidents have profound effects on the target population, the neurotoxicity is easily noted, and the involved mechanisms are often elucidated relatively easily. Although the conclusions reached from such studies may have wider import (Spencer *et al.*, 1987), these situations will not be emphasized in this chapter.

The second class relates to the evaluation of two types of potential toxicants: (1) compounds that are either known or suspected neurotoxicants at very high doses, but whose long-term effects of trace amounts are unclear and (2) chemicals that are deliberately ingested but the effects of which are uncertain. It is this class with which we shall be primarily concerned in this chapter, and it is the one in which the scientific conclusions are less easily derived.

It would be remiss not to note the tremendous rate of advancement in the molecular and biochemical neurosciences that has continued for more than a decade. Our increasing understanding of the molecular basis of nervous system function has already impacted neurotoxicology in general, and nutritional neurotoxicology more specifically. Despite this, one opinion should be stated explicitly: At present, it is the subdiscipline of neurobehavioral toxicology that provides the most utilitarian method of screening for potential toxicants. It is after a compound has been suggested as a candidate neurotoxicant that questions about the molecular and biochemical mechanisms of, and sequelae to, toxicant exposure become necessary. In addition to their intrinsic scientific value, it is this latter type of data that often permits full estimation of actual health hazards. We believe that the correlation of behavioral events with alterations in neuronal function is powerful for this reason. Conversely, despite the richness of these available *in vitro* methods, we do not believe that any of them alone can ultimately, *in vacuo*, provide a rapid and efficient way to screen for potential neurotoxicants (Mailman, 1987).

II. SPECIAL NATURE OF THE NERVOUS SYSTEM: OVERVIEW OF THE SITES AND MECHANISMS OF NEUROTOXICITY

As a background to a discussion of neurotoxicological risk assessment in nutrition it is imperative to understand not only the nature of our food supply but also some fundamentals of neuroscience. The complexity of the issues that impact on understanding or predicting neurotoxicity may, at first, appear overwhelming. Yet an appreciation of important cellular and biochemical loci in the nervous system is essential to provide a framework for evaluating the potential neurotoxicological impact of dietary components.

A. Neurotransmission

It has been known conclusively for most of this century that the key mechanism by which nerve cells (neurons) communicate is the release of chemicals by one cell and the recognition of these chemicals by adjacent ones. This process of neurotransmission occurs in a specialized structure called the synapse, and involves chemicals specific for each neuron. Less than 20 years ago, fewer than a dozen neurotransmitters were believed to account for most of chemical message transmission in the nervous system. Today, the list exceeds 50 and is increasing rapidly as the basic neurosciences provide more detailed understanding of the nervous system.

A brief review of the biochemistry of synaptic function is relevant for two reasons. First, changes in our diet have been hypothesized, and in some cases clearly shown, to affect nervous system function. Second, although specific scientific problems often are best addressed by specialists, it is essential that those interested in nutritional toxicology appreciate general mechanisms of neurotransmission. The short discussion that follows is designed to provide such a background.

For scientists to accept a compound as having a role as a chemical messenger (neurotransmitter), several criteria are usually required. The compound must be found in neurons, and mechanisms for its biosynthesis must be demonstrated. On stimulation of the appropriate neurons, the compound must be released from one neuron to transmit information to another. Finally, mechanisms for the inactivation of the compound are required. These can be rapid processes such as reuptake into neurons or hydrolysis (e.g., as seen with acetylcholine or catecholamines) or diffusion and slow hydrolysis (e.g., for neuropeptides). Another important series of mechanisms involves the events initiated by the transmitter. Thus, it is essential that recognition sites (receptors) for the transmitter be identified and that biochemical, cellular, and/or physiological changes caused by receptor occupation be elucidated. Recently, it has been shown that a single neuron can simul-

taneously use two or more chemicals as neurotransmitters. Although this appears to provide an exquisite degree of physiological regulation, it also complicates neurotoxicological assessment.

B. Chemical Architecture of the Nervous System

In studying the biochemical and neurochemical mechanisms related to neurotransmission, one must remember that the central nervous system (CNS) is not simply a collection of homogeneous synapses. Not only can single neurons release several transmitters; recent advances in neuroanatomy have shown differences in neurotransmission engendered by the location of both the nerve cell bodies and their terminal fields. For example, with the catecholamines, dopamine and norepinephrine, it has been known for decades that the terminal fields in various areas of brain originate from discrete groups of nuclei. Recent studies have indicated that even within a given nucleus, individual cells may innervate only portions of the target terminal field. These findings are of specific importance to nutritional toxicology since the heterogeneous nature of synaptic connections makes simplistic hypotheses about neurotoxicologic mechanisms intrinsically suspect. Some examples of this will be given later in this chapter.

C. Brain–Behavior Relationships

The utility of behavioral screening in assessing potential neurotoxicity induced by dietary constituents depends on an understanding of the relationship of specific behavioral end points to changes in neurotransmission in discrete anatomical loci. Recent technical advances in the neurosciences have provided tools that have been of great utility in studying such processes. For example, direct quantification of the extracellular concentrations of some neurotransmitters and their metabolites can be made [e.g., using *in vivo* cerebral microdialysis and *in vivo* microvoltammetry (Westerink *et al.*, 1987; Justice *et al.*, 1985)] while simultaneously measuring the behavior of freely moving animals. Microdissection techniques (Palkovits and Brownstein, 1988), coupled with increasingly sensitive analytical methods, permit investigation of the relationship between behavior and neurotransmitter function within highly circumscribed brain regions. Such anatomical resolution is also possible when relating behavioral changes to alterations in neurotransmitter receptor sites. *In vitro* quantitative receptor autoradiography permits visualization of radiolabeled ligand recognition sites using 10–20 μm brain slices. More recently, the widespread use of molecular biological techniques has widened the range of available loci, so toxicant effects on gene expression can now be studied routinely.

Advances in the study of the mechanisms by which dietary constituents might exert neurotoxic effects will depend on the sensitive measurement of specific

behaviors, the neurobiology of which have been, at least in part, elucidated. As noted, behavioral testing may be the most unambiguous and most sensitive way of detecting neurotoxicity. However, the increasingly sophisticated biochemical techniques that can be applied to the assessment of neurotoxicity require a comparable level of sophistication in behavioral methods. Without the ability to tie perturbations in specific behavioral functions to alterations in specific brain nuclei, little information is gained. It no longer suffices to find that a compound alters motor activity or the rate of schedule-controlled lever pressing in an operant chamber.

It is also important to bear in mind that toxicant-induced perturbations of the CNS often go undetected in the absence of a pharmacological or environmental challenge. For example, minor perturbations in central dopamine systems may not be apparent unless the animal is challenged with a direct-acting dopamine agonist or tested in a learning or performance task (e.g., reaction time). In designing such challenge experiments, it is imperative to be able to generate testable hypotheses about the locus of toxicant-induced changes. This will allow the investigator to select the appropriate drug, or behavioral task or environmental stimulus, to challenge that neuronal system. Much has been learned about the neurobiology of various behavioral tasks. Although many complex behaviors require the integration of multiple neuronal systems, the use of such behavioral tasks permits hypotheses to be generated about the underlying neuropathology.

D. Special Issues Relevant to Toxicology of the Nervous System

Several special characteristics of the brain must be considered in dealing with the area of nutritional toxicity. In many cases, the brain is less vulnerable to some toxicants because of the specialized structures, commonly known as the blood–brain barrier, that prevents free diffusion of toxicants. CNS toxicity depends on contact of sufficient concentrations of the chemical with sensitive sites in the brain or spinal cord. Brain capillaries, unlike those in other tissues, are not fundamentally porous and have so-called tight junctions between adjacent cells. Carrier systems permit transport of physiologically important molecules (e.g., hexoses; carboxylic acids; neutral, basic, and acidic amino acids; amines; and inorganic ions) but hinder free diffusion of other chemicals. Many toxicants (especially polar compounds) are thus excluded from brain tissue or at least are hindered in contacting sensitive sites. To understand or to predict toxicological events in the CNS, it is necessary to know whether a toxicant can pass through, or in some cases alter or damage, the blood–brain barrier.

Another major difference between the nervous system and other larger organ systems relates to the metabolism of toxicants. Lacking, in large measure, are the numerous phase I and II systems found in liver, lung, and other tissues. Unlike many peripheral tissues, specific metabolic systems with a large capacity for

structurally diverse xenobiotics are not an important consideration in the brain. However, mechanisms for transport, synthesis, and metabolism of numerous endogenous molecules (including the neurotransmitters) are present. It is possible that some toxicants act as "pseudosubstrates" for these systems, and this may influence toxicity. Probably the most well-known case is the toxicant 1-methyl-4-phenyl-2,3,4,6-tetrahydropyridine (MPTP). The toxicity of this agent requires conversion to 1-methyl-4-phenylpyridinium (MPP^+) by brain monoamine oxidase and then cell entrance via neuronal uptake systems. Thus, compounds that may be structurally analogous to known neurotransmitters must be examined in light of existing mechanisms.

Finally, it is useful to remember that the brain, despite its sensitivity to insult, often exhibits amazing plasticity. Twenty years ago, it was a commonly held belief that the nervous system was "hard-wired" during development and that the only changes possible were those due to damage and loss of function. It is now clear that a remarkable degree of modification can take place, ranging from compensatory biochemical changes to actual "rewiring." The issue of plasticity is a subject of great current scientific interest in its own right, on which many volumes have already been written. However, in some cases of examining effects of dietary components, it may be necessary to be aware that such mechanisms exist and can provide important clues toward detecting toxicity experimentally (e.g., when there is "receptor supersensitivity").

With the widespread use of neurobehavioral methods, it would be remiss not to discuss the difficulties that sometimes arise in attempting to ascertain whether a toxicant-induced behavioral change is through direct actions on the nervous system or is a secondary response to nonneural physiological changes. The latter can include immunological effects (including allergic responses or anaphylaxis), neuroendocrine changes, or response to peripheral organ toxicity. For a variety of reasons (especially with interspecies comparisons), it is essential to distinguish primary neurotoxic events from secondary or tertiary effects.

III. STRATEGIES FOR EVALUATING NEUROTOXICITY OF FOODS

A. Some Guidelines for Rational Assessment of Food Safety

As will be shown, we believe that the evaluation of the potential neurotoxicity of food constituents can be best done by adhering to essential principles of pharmacology and toxicology. Some of the generic issues that this incorporates include the following. Intrinsic to all toxicological evaluation must be an understanding of the disposition and metabolism of the material of interest. Key to this is knowing the chemical identity of the purported toxicant(s). Frequently, this is

a major scientific problem, such as with many natural products. However, when the identity of the agent is known, it is important to develop data which show the amount of toxicant deposited in target organs as a function of dose, and the time course over which deposition and subsequent excretion occurs. Thus, analytical toxicology and the development of appropriate analytical methods are often an early task.

These data provide an important basis for so-called toxicodynamic studies, in which the actual effects and mechanisms can be investigated. Again, there is a body of scientific methods for the disciplines of pharmacology and toxicology that is unfortunately sometimes ignored. As an example, dose–response relationships provide important data for the development of hypotheses related to mechanisms of action. Moreover, dose–response data are critical for interspecies extrapolation and prediction. As an example, the Food Chemicals Codex contains many compounds that are clearly neurotoxic (e.g., acetaldehyde). Yet the actual danger of these agents is generally insignificant: The doses at which they are added to foods (and at which they occur as natural flavors) are orders of magnitude below those at which toxicity can be demonstrated. We shall give examples of the use and misuse of such methods later in the chapter.

Two other points should be mentioned. The first deals with the issue of risk assessment and low-dose extrapolation. This has been an extremely controversial area, especially in the fields of carcinogenesis and teratogenesis, and there are several excellent recent reviews. A point germane to this chapter, however, is that the best models for low-dose extrapolation can be derived when as many as possible of the relevant biological mechanisms are known; it is not surprising that as doses are increased (or decreased), one can see qualitative as well as quantitative changes. Finally, the ultimate test of human health effects is often a clinical trial. In several cases, this actually has been done with agents of dietary importance. Although it should be obvious, such clinical studies must, like the basic biological studies, adhere to fundamental issues of experimental design and analysis, including such factors as selection of tested hypotheses and alpha level and designs that are double-blind and placebo-controlled.

B. Neurobehavioral Screening

A variety of behavioral methods have been used to screen for the neurotoxicity of various compounds. Detailed descriptions of these methods can be found in many books, chapters, and review articles on behavioral methodology (e.g., Norton, 1982; Iversen *et al.*, 1977; Annau, 1986). The purpose of this section is to summarize some methods and to discuss ways in which they can be used to answer questions about nutritional neurotoxicology.

Several factors must be taken into consideration in choosing any behavioral test as a screen for neurotoxicity. First, the test must be sensitive to the toxicant,

and the results of the test must be reliable and valid. Second, if possible, the test should provide some information about the mechanism through which the toxicant may act. During the past decade, a great deal of progress has been made in identifying the neurochemical mechanisms associated with specific behavioral measures, and consequently, the behaviors can be used as markers for particular kinds of neurotoxicity. For example, reaction-time tasks and behavioral stereotypies are sensitive measures of perturbations in dopaminergic pathways (Amalric and Koob, 1987; Lewis *et al.*, 1985), and radial arm maze behavior has been used to study cholinergic and opiate mechanisms of memory (Gallagher *et al.*, 1983). Finally, it should be kept in mind that the effects of some toxicants may not become apparent until the animal is given a task or challenged with a drug, either of which requires a greater degree of neural response.

Direct observation is one of the most flexible and sensitive ways to measure spontaneous, ongoing behaviors such as grooming, locomotor activity, exploration, and behavioral stereotypies. Because it is one of the most useful methods for correlating behavioral changes with biochemical changes induced by toxicants, a variety of procedures have been developed for recording spontaneous behaviors [see Norton (1977) for a review]. In general, animals are placed in observation chambers or open fields, and their behavior is observed and recorded at regular intervals. The behaviors that are recorded and the time intervals used may vary depending on the toxicant being studied. Lewis *et al.* (1985) have developed an observational method in which microcomputers are used to record behaviors (rearing, grooming, locomotion, sniffing, licking, gnawing, etc.) that occur during each 15-sec interval of a 1-min observation period, with observation periods occurring once every 5 min over the course of 1 hr. The percentage of intervals in which a particular behavior occurred is then calculated for each observation period for each animal, yielding interval scale data that can be analyzed via analyses of variance. In using observational methods such as this, it is important to define operationally the behaviors measured, to use designs that avoid observer bias, and to train observers to a high degree of interobserver agreement.

Although behavioral observation requires more time and manpower than automated procedures, it is much more flexible and sensitive in detecting distinct patterns of behavior associated with particular toxicants. This method also can be used to characterize social interactions between animals (Mackintosh *et al.*, 1977), and behavioral observation can be combined with *in vivo* cerebral dialysis to yield a powerful way of correlating biochemical and behavioral changes.

Locomotor activity is probably the most frequently used behavioral screen for detecting neurotoxicity, and there are a variety of automated methods for recording it. The most widely used of these is the photocell cage, in which photocells are placed in parallel or at right angles along the wall of the cage so that the movement of the animal breaks the photocell beam. Interruptions of the beams

can be recorded by counters to give overall activity levels, and, with computer support, the order of the interruptions can be recorded to give information about the spatial and temporal pattern of locomotor behavior. Other methods of measuring locomotor activity include jiggle cages and running wheels; these tend to produce less stable baselines, though, because the movement of the apparatus is reinforcing to the animal.

Although locomotor activity is a widely used measure of neurotoxicity, its use should be carefully evaluated. In one of the few studies comparing the sensitivity of various behavioral measures to eight different toxicants, Pryor *et al.* (1983) found that locomotor activity as measured in photocell cages was not responsive to any of the neurotoxicants used, though it was sensitive to arsenic, which is not believed to be a neurotoxicant in rats. Furthermore, there are some conditions in which the effects of toxicants on activity levels are not apparent until the animal is challenged with a specific pharmacological agent. For example, neither lead nor acrylamide alter spontaneous motor behavior compared with controls, though both toxicants produce changes in activity compared with controls when animals are challenged with amphetamine or apomorphine (Rafales *et al.*, 1979, 1983.) These studies illustrate the importance of using appropriate behavioral measures to test specific hypotheses about the toxicants that are being studied.

In addition to the measures of activity and ongoing behavior just described, a variety of methods exist for measuring the effects of toxicants on very specific sensorimotor behavior. These include the rotarod test, screen tilt test, righting reflex, grip strength test, startle response, negative geotaxis, and the thermal sensitivity test [see Norton (1986) for descriptions].

Schedule-controlled behavior has developed into a specialized area of behavioral toxicology, and descriptions of the methodological issues and techniques have been published elsewhere (Laties and Wood, 1986; Evans and Daniel, 1987). The power of using schedule-controlled behavior lies in its flexibility in developing specific and highly controlled tests of complex behaviors such as reaction times, perceptual thresholds, and ability to discriminate between stimuli. For example, Amalric and Koob (1987) have developed a reaction-time task that is sensitive to dopamine perturbations in one specific brain region.

The radial arm maze (Olton and Samuelson, 1976) is a method of measuring memory processes in animals, and because of its advantages, it has become the dominant method of studying animal memory. A radial arm maze consists of a central platform with some variable number of arms (usually 8–12) extending away from the platform. Food pellets are placed in some of these arms, and the animal learns which arms are baited with food, so that it is able to acquire all the pellets with the maximum efficiency. This task is rapidly learned by many species, and since the animal must check a series of arms, it provides a way to measure serial learning. Consequently, the effects of toxicants on memory processes analogous to those studied in humans (e.g., serial position curves or

proactive and retroactive interference) can be measured using this procedure. This behavioral method can be particularly advantageous because it assesses an important brain function (memory), but also because the neurobiology of radial arm maze performance is well-characterized.

C. *In Vitro* Approaches

The tremendous utility of biochemical and molecular methods for understanding neural processes has led to suggestions by several workers that one or more such methods could be used as general "screens" for neurotoxicity. The particular loci that may be useful are manifold and include all aspects of synaptic biochemistry, as well as events in soma and neurites. (Although concern almost always focuses on neuronal function, various glia also can be sites of toxic attack.) In view of the increasingly powerful methods to study these biochemical loci, it is not surprising that they have held some appeal as potential screens for neurotoxicity.

Three strategies that have been espoused rely on (1) study of receptor function using radioligand binding methods, (2) quantification of various proteins as markers of neuronal integrity, and (3) measurement of neurotransmitter and metabolite concentrations as indicators of neuronal activity. Some of the end points include measurements of neurotransmitter concentrations, uptake, synthesis, release, receptor affinity and density, second messenger events, and concentrations of functional and/or structural proteins.

In the ideal, these methods might be of exquisite utility if the brain consisted of a series of homogeneous synapses, each using one neurotransmitter. However, not only is there a plethora of neurotransmitters, but profound functional interactions exist among them. These include the release of various transmitters from one terminal, as well as interactions among transmitters on target cells. Of equal concern is the degree of heterogeneity, even among similar cell types, caused by differences in their efferent and afferent synaptic fields. As noted earlier, it is not surprising that neuronal plasticity is such a complex and exciting area. Because the expression of toxicity can involve so many factors, it seems clear that valuable data will be obtained by focused, hypothesis-directed studies, rather than by random screening. *A priori* selection of a specific neurotransmitter system is unlikely to be productive unless toxicant-induced damage is so severe that gross and widespread damage has occurred.

Thus, although many neurochemical techniques are available for the assessment of neurotoxicity, the ideal use of *in vitro* methods, at least at present, is for mechanistic rather than phenomenological purposes. Detection of neurotoxicity requires a multifaceted strategy using neurobehavioral, neuropathological, and in some cases, pharmacological methods. The weakness in this approach and the inefficiencies inherent in it are obvious. Nonetheless, this may be the most

expeditious way of screening important agents. *In vitro* methods, at present, can best be used to study the neurotoxicity of a particular compound and to provide biochemical and neurochemical mechanistic information. The reader can refer to detailed reviews we have published related to these issues in general (Mailman, 1987; Mailman and Morell, 1982; DeHaven and Mailman, 1983) and to organometals in particular (Morell and Mailman, 1987).

IV. PREDICTING NEUROTOXICITY: ILLUSTRATIVE EXAMPLES

It may seem that much of the material discussed in this chapter is ancillary to the issue of neurotoxicology and food-safety assessment. In fact, it is our contention that this complex field requires a keen awareness of the fundamental disciplines that influence the methods used for assessment. However impressive the power of modern neuroscientific methods, they are most valuable when directed at specific questions. They must be integrated with studies using classical pharmacological and toxicological methods, and great care must be given to the issues of pharmacokinetics and dose–response relationships. A failure to do so may very well produce misleading conclusions, often with significant secondary ramifications. The examples that follow will discuss some of the more interesting controversies that have occurred in this area, many of which are felt to underscore the important, if somewhat ingenuous, philosophy noted here.

A. Diet and Hyperactivity

Causes and treatment of behavioral problems in children have become an important issue, not only socially and politically but also biomedically. In large measure, it might be argued that this is an offshoot of the advances made in treating many previously deadly diseases, as well as the burgeoning knowledge of brain function. The condition sometimes known as hyperactivity has provided a focus for several controversies that are quite illustrative for the purposes of this chapter. Such examples show, at once, the strengths and weaknesses of appropriate use of modern *in vitro* and clinical approaches in studying nutritional neurotoxicity.

Hyperactivity or, as it is now more properly called, *attention deficit disorder* (ADD) is an accepted diagnostic category that occurs in about 1% of all children. Although many developmental insults can cause this condition (encephalitis, hypoxic episodes during birth, drug effects on development, high fever, genetic factors, etc.), in many cases the condition may be idiopathic. Although the range of severity is wide, in most cases ADD results in profound difficulties for the children and their families, particularly during school years. In many (but not all)

children, the use of stimulant drugs such as amphetamine and methylphenidate can be useful. Clearly, however, the available interventions (either psychosocial or pharmacological) leave much to be desired, and the long-term prognosis for many children with ADD is not bright. Thus, effective and safe therapies or preventive measures would be extremely important. Against this backdrop, it is not surprising that the role of diet in this condition became an issue.

1. Erythrosine (FD&C Red # 3)

One of the most controversial hypotheses relating to purported dietary neurotoxicity was espoused in Feingold's book *Why Is Your Child Hyperactive?* (Feingold, 1975). He proposed that diets not containing artificial flavors, colors, or certain natural ingredients could dramatically alleviate symptoms in one-third or more of hyperactive children. When many uncontrolled case reports provided much support for this idea, a series of controlled clinical trials were initiated using challenges with a mixture of the eight food colors then available.

At this time, it was reported that the mixture of food colors being used in the clinical trials could inhibit neuronal uptake of dopamine and other neurotransmitters in rat brain preparations. Soon, this effect was found to be due to only one of the colors, erythrosine (FD&C Red # 3) (Logan and Swanson, 1979; Lafferman and Silbergeld, 1979). Because powerful psychotropic drugs such as cocaine are known to work by this mechanism, this suggested a testable biochemical mechanism, as well as providing the means of designing focused clinical trials.

Following the initial reports on effects of erythrosine on neurotransmitter uptake, various strategies were pursued. Silbergeld and colleagues did a series of studies in which they concluded that erythrosine inhibited the ouabain-binding site on sodium channels, thus inhibiting neuronal uptake by disrupting sodium transport (Silbergeld, 1981). Several independent groups also found that erythrosine had effects on other end points, all of which were essential to proper neuronal functioning (e.g., Levitan, 1977; Augustine and Levitan, 1980; Floyd, 1980; Sako *et al.*, 1980; Columbini and Wu, 1981).

Although these data were exciting, they exemplify a problem in using *in vitro* methods as neurotoxicity screens. Is the effect detected relevant to the target organism? As we have previously noted, this is a general problem in pharmacology and toxicology and requires one to be cognizant of the principles of these disciplines (e.g., dose–effect relationships and pharmacokinetics). In the case of erythrosine, this laboratory was able to provide relevant data based on such basic principles. These can be summarized as follows.

If erythrosine had the wide-ranging biological activity that was being reported, it was perplexing why no clinical toxicity had been reported previously. One conclusion we reached was that erythrosine, by virtue of its lipophilic nature, caused these *in vitro* effects by a nonspecific disordering of biological mem-

branes (Mailman *et al.*, 1980; Mailman and Lewis, 1981; Mailman and DeHaven, 1984), as opposed to specific attack on certain loci (Silbergeld, 1981; Levitan, 1977; Augustine and Levitan, 1980; Sako *et al.*, 1980). Thus, most biological processes depending on the integrity of such membranes would be affected.

A testable hypothesis resulted from these *in vitro* studies. Because of the affinity of erythrosine for biological membranes, even at relatively high dietary doses the color would likely accumulate in the membranes of the gut, liver, and blood cells prior to having access to the brain (Mailman *et al.*, 1980; Mailman and Lewis, 1981; Mailman and DeHaven, 1984). This conclusion was supported by available data and by studies we conducted (Mailman *et al.*, 1980; Niedzwiecki and Mailman, 1981). Thus, even after massive doses of erythrosine, the color does not reach significant concentrations in the brain, making neurotoxicity unlikely at relevant human doses. Consequently, it was not surprising that Swanson, who had reported small but significant clinical effects with the mixture of eight food colors, repeated clinical trials with erythrosine alone and found no effect (Anonymous, 1982).

2. *Sugar and Hyperactivity*

The importance of applying sound methodological principles to the assessment of potential neurotoxicity also is well illustrated in the case of sugar. The ingestion of sugar, or altered glucose metabolism, has been linked to aggressive and hyperactive behavior in both children and adults (Kruesi *et al.*, 1987). "Sugar-responsive" children are believed to exhibit deterioration in behavior after sugar ingestion, and abstinence from sugar is believed to result in behavioral improvement. However, carefully controlled double-blind studies have generated compelling evidence that sugar ingestion does not induce aggressive or hyperactive behavior. These studies, conducted by Rapoport and colleagues used a challenge design whereby children believed to be sugar-responsive were challenged with a bolus of sucrose, glucose, saccharin, or aspartame (Rapoport, 1986). Playroom behavioral measures and observer or parent ratings of hyperactivity showed no effect of the treatment. Moreover, no evidence for increased aggression was observed as a function of sugar ingestion.

This example, as with FD&C Red #3, highlights the importance of rigorous adherence to sound experimental design in the study of the effects of food constituents. This area presents a number of logistical problems, including how to constitute an appropriate placebo condition and maintenance of a double-blind. However, there is no adequate substitute for the use of double-blind, placebo-controlled studies that use instruments of proven validity, reliability, and sensitivity.

B. Aspartame

Aspartame is the generic name for L-aspartyl-L-phenylalanine methyl ester. This dipeptide has a sweetening power approximately 200 times that of sucrose (Mazur *et al.*, 1969) and was first approved for use in numerous dry foods and as a table-top sweetener in 1974 by the Food and Drug Administration (FDA). Some unresolved safety issues slowed its marketing until 1981. Later, it was approved for use in carbonated beverages and is now a commercial success, with sales reported to reach $600 million even by 1983 (Coulombe and Sharma, 1986). Of 231 consumer complaints about aspartame made before June 1984, 67% were for neurological or behavioral symptoms (Bradstock *et al.*, 1986). Because of its relatively recent synthesis, there is a greater body of controlled laboratory investigations and clinical trials than was the case with erythrosine. The available data suggest that neurotoxicity of aspartame would first involve its hydrolysis to aspartate and phenylalanine in the gut mucosa, allowing either of these two amino acids to have potential toxicological actions.

Aspartate is an endogenous excitatory neurotransmitter and might cause neuronal damage through "excitotoxicity" at specific receptors. The neurotoxicity of aspartate resembles that of glutamate in that it produces lesions of the arcuate nucleus, and plasma aspartate concentrations are correlated with neuronal necrosis (Finklestein, 1983). However, the doses of aspartame required to produce neuronal damage in 8-day-old mice exceed by more than thirtyfold the projected daily intake of aspartame for humans (Stegink *et al.*, 1977). Further, humans who have taken excessive doses of aspartame still do not reach plasma concentrations near the threshold needed for producing neurotoxicity, even in the neonatal mouse, a very susceptible animal model. Thus, it seems unlikely that a human could consume sufficient aspartame to induce neuronal necrosis.

An alternative mechanism to link aspartame to behavioral changes results from the metabolism-induced increases in phenylalanine (Pardridge, 1986), an important precursor for many important neurotransmitters. Phenylalanine is readily converted to tyrosine, the precursor for synthesis of the catecholamine neurotransmitters dopamine and norepinephrine. Plasma phenylalanine levels increase significantly in humans administered three sequential doses (10 mg/kg) of aspartame via beverage (Stegink, 1984), and in rats the increased plasma phenylalanine concentration results in elevated brain concentrations of both tyrosine and phenylalanine (Fernstrom *et al.*, 1983; Wurtman *et al.*, 1983). Concomitant administration of dietary carbohydrate doubles the rise in brain phenylalanine relative to aspartame alone (Wurtman *et al.*, 1983). However, changes in brain catecholamine concentrations are seen only at doses on the order of 100 mg/kg or higher. Although there is still debate about whether aspartame increases seizure susceptibility, the present evidence suggests that this compound is not neurotoxic even at quite high doses.

C. Caffeine

Caffeine and the related methylxanthines, theophylline and theobromine, are pharmacologically active compounds consumed by a large majority of the adult population (Graham, 1978; Dews, 1982). Caffeine is an alkaloid found in the coffee bean, tea leaf, and the kola nut and thus occurs in the diet primarily in beverages such as coffee, tea, and cola drinks at concentrations from 3 to 30 mg/fluid ounce. Since consumption of these beverages is often in large volumes, the amount of caffeine ingested can be significant. There have been several excellent reviews of its pharmacology (e.g., Neims and von Borstel, 1983).

Caffeine has CNS stimulant activity that is now accepted as being due to its blockade of adenosine receptors. Although the methylxanthines also inhibit cyclic nucleotide phosphodiesterases, this mechanism is not likely to be important at the doses usually ingested (Burg and Warner, 1975). The ability to reverse effects such as drowsiness and fatigue may account for some of the popularity of caffeine-containing beverages. However, it also can cause adverse effects, including insomnia, restlessness, nervousness, and impaired performance on some tasks requiring high levels of coordination or timing. In addition, numerous peripheral effects occur, including increases in gastrointestinal motility, especially at higher doses.

The issues of the nutritional toxicology of caffeine can be divided into two questions. The first relates to the ingestion of methylxanthines by adults and children. Since the pharmacological properties of caffeine are well known, it is clear that behavioral effects occur acutely. Moreover, animal studies have correlated changes in adenosine binding sites, reticular neuron firing frequency, and the development of behavioral tolerance after 14 days of caffeine administration (Chou *et al.*, 1985). Since humans show great interindividual differences in drug sensitivity for both pharmacokinetic and pharmacodynamic reasons, it is clear that caffeine ingestion is a potential source of nervous system perturbation. An issue less easily defined, but of much greater significance, is the effects of caffeine on nervous system development. It is becoming generally accepted that receptor-specific drugs that can pass the blood–placental barrier have the potential to cause untoward developmental effects. Thus, it is generally advised that ingestion of caffeine-containing foods should be minimized during pregnancy since there are reports of teratogenesis at large doses, and even low doses may cause subtle changes in nervous system function.

D. Monosodium Glutamate

Monosodium glutamate (MSG) is a common flavor enhancer. Glutamate, an endogenous amino acid, is a major excitatory amino acid neurotransmitter in the nervous system. It is known that excessive stimulation of glutamate receptors

(e.g., electrically or with drugs that are glutamate agonists) can lead to neuronal death, via biochemical and molecular mechanisms that have been extensively investigated (Garrattini, 1979; Olney *et al.*, 1986). Under some circumstances, MSG itself can be a potent neurotoxicant. For example, a neurotoxic syndrome has been observed in very young laboratory rodents, in which MSG destroys the arcuate nucleus of the hypothalamus relatively selectively, thus making this a model for use in neuroendocrine studies. Fortunately, a combination of increased accessibility of MSG to periventricular regions and undeveloped ability to metabolize glutamate make only very young animals vulnerable. The critical factor is almost certainly the amount of glutamate available to the brain at possible levels of ingestion. One laboratory has shown that MSG can produce lesions in monkeys, but these results have not been generally replicable. In general, MSG given with food or via drinking water has never been shown to induce neurotoxic effects.

Another issue related to MSG is "Chinese restaurant syndrome," a triad of symptoms that commence shortly after eating Chinese food and include numbness at the back of the neck (gradually radiating to the arms and back), general weakness, and palpitations (cf. Zautcke *et al.*, 1986). Single and double-blind studies in humans, even at very high doses, have provided little support for the theory that MSG is the causative agent in Chinese restaurant syndrome. However, it may be that high doses of MSG, in combination with other ingredients in a Chinese meal, are involved.

E. Neurotoxicity of Cycad Nut

Consumption of flour made from nuts of the cycad plant has been associated with hepatotoxicity, carcinogenicity, teratogenesis, and neurotoxicity. Seen frequently among the Chamorro people on Guam and the neighboring Mariana islands, these disorders have been associated with consumption of the seeds of the cycad, *Cycas circinalis,* as a food and a traditional medicine. Most of the toxicity has been attributed to cycasin, a potent toxin found in the cycad. Cycasin is metabolized to methylazoxymethanol, and the hepatotoxicity, carcinogenicity, and teratogenesis (including microcephaly) associated with ingestion of the cycad by animals or humans are probably due to the potent alkylating properties of this metabolite.

Of special interest are recent observations related to the unusually high prevalence rates in this population of motoneuron and other neural degenerative disorders that resemble amyotrophic lateral sclerosislike disease, Parkinson's disease, and Alzheimer's-like dementia. An unusual nonprotein amino acid found in cycad seeds has recently been implicated as the causative agent in these neural degenerative disorders. Spencer *et al.* (1987) have reported that β-*N*-methylamino-L-alanine (L-BMAA) found in the cycad is an excitotoxin whose effects

can be blocked by *N*-methyl-D-aspartate antagonists. Oral administration of L-BMAA to macaques produces corticomotoneuronal dysfunction, parkinsonian features, and behavioral anomalies, with chromatolytic and degenerative changes of motor neurons in cerebral cortex and spinal cord, that are strikingly similar to the neurological syndrome seen in Guam. Another plant neurotoxin β-*N*-oxalylamino-L-alanine (BOAA) is similar to L-BMAA both structurally and pharmacologically. BOAA is found in the chick pea (*Lathyrus sativa* or khesari dhal), which may be consumed in large amounts during times of famine in India (Davidson *et al.*, 1979). Prolonged ingestion of the chick pea can induce neurolathyrism, an irreversible spastic paralysis of the legs, possibly due to destruction of motoneurons in the pyramidal tract (Spencer *et al.*, 1987). In total, these data have led to a very provocative hypothesis: that many such neurotoxins, present in our food and the environment, may cause or predispose neurological illnesses after low doses over long periods of time. Although the available data are clearly insufficient to support this notion at present, it is a particular concern in terms of nutritional neurotoxicology.

V. CONCLUSIONS

It is irrefutable that the biochemical and molecular neurosciences will be increasingly important in understanding the mechanisms involved in the injury to and response by the nervous system. However, recent neurotoxicological studies have provided both valuable lessons and interesting new hypotheses. The recurring value of fundamental principles of pharmacotoxicodynamics and kinetics are probably the most cogent of these messages. Particularly relevant to this chapter is the need for adherence to these fundamentals in evaluating emerging data. Pharmacotoxicokinetic data should be an integral part of *in vivo* toxicity studies, especially in the area of nutritional toxicity. As important, dose–effect relationships are critical, not only to interspecies extrapolations but also to correlating *in vivo* and *in vitro* findings. Conclusions based on *in vitro* data should be made only after relation to estimated (or better, empirically determined) tissue concentrations. As noted in the introduction of this chapter and in several examples, the lay public is particularly susceptible to accepting premature reports or hypotheses as true. The area of nutritional toxicology has a special responsibility not to perturb the public consciousness unreasonably.

REFERENCES

Amalric, M., and Koob, G. F. (1987). Depletion of dopamine in the caudate nucleus but not in nucleus accumbens impairs reaction time performance in rats. *J. Neurosci.* **7**, 2129–2134.

Annau, Z. (1986). "Neurobehavioral Toxicology." The Johns Hopkins Univ. Press, Baltimore.

Anonymous (1982). Defined diets and childhood hyperactivity. *J. Am. Med. Assoc.* **248,** 290–292.

Augustine, G. J., and Levitan, H. (1980). Neurotransmitter release from a vertebrate neuromuscular synapse affected by a food dye. *Science* **207,** 1489–1490.

Bakir, F., Damuluji, S. F., Amin-Zaki, L., Murtadha, M., Khalidi, A., Al-Rawi, N. Y., Tikriti, S., Dhahir, H., Clarkson, T. W., Smith, J. C., and Doherty, R. A. (1973). Methylmercury poisoning in Iraq. *Science* **181,** 230–241.

Bradstock, M. K., Serdula, M. K., Marks, J. S., Barnard, R. J., Crane, N. T., Remington, P. L., and Trowbridge, F. L. (1986). Evaluation of reactions to food additives: The aspartame experience. *Am. J. Clin. Nutr.* **43,** 464–469.

Burg, A. W., and Warner, E. (1975). Effect of orally administered caffeine and theopylline on tissue concentrations of 3′,5′ cyclic AMP and phosphodiesterase. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **34,** 332 (Abstr.).

Chou, D. T., Khan, S., Forde, J., and Hirsh, K. R. (1985). Caffeine tolerance: Behavioral, electrophysiological, and neurochemical evidence. *Life Sci.* **36,** 2347–2358.

Columbini, M., and Wu, C. Y. (1981). A food dye, erythrosine B, increases membrane permeability to calcium and other ions. *Biochim. Biophys. Acta* **648,** 49–54.

Coulombe, R. A., and Sharma, R. P. (1986). Neurobiochemical alterations induced by the artificial sweetener aspartame (NutraSweet). *Toxicol. Appl. Pharmacol.* **83,** 79–85.

Davidson, S., Passmore, R., Brock, J. F., and Truswell, A. S. (1979). "Human Nutrition and Dietetics." Churchill Livingstone, New York.

DeHaven, D. L., and Mailman, R. B. (1983). The use of radioligand binding techniques in neurotoxicology. *Rev. Biochem. Toxicol.* **5,** 193–238.

Dews, P. B. (1982). Caffeine. *Annu. Rev. Nutr.* **2,** 323–341.

Evans, H., and Daniel, S. (1987). Modification of conditioned responding by exposure to organometals. *In* "Neurotoxicants and Neurobiological Function" (H. Tilson and S. Sparber, eds.) pp. 261–278. Wiley, New York.

Feingold, B. A. (1975). "Why is Your Child Hyperactive?" Random House, New York.

Fernstrom, J. D., Fernstrom, M. H., and Gillis, M. A. (1983). Acute effects of aspartame on large neutral amino acids and monoamines in rat brain. *Life Sci.* **32,** 1651–1658.

Finkelstein, M. W., Daabees, T. T., Stegink, L. D., and Applebaum, A. E. (1983). Correlation of aspartate dose, plasma dicarboxylic amino acid concentration, and neuronal necrosis in infant mice. *Toxicology* **29,** 109–119.

Floyd, R. A. (1980). Erythrosine B (red dye no. 3) mediated oxidation–reduction in brain membranes. *Biochem. Biophys. Res. Commun.* **96,** 1305–1311.

Gallagher, M., King, R. A., and Young, N. B. (1983). Opiate antagonists improve spatial memory. *Science* **221,** 975–976.

Garrattini, S. (1979). "Evaluation of the neurotoxic effects of glutamic acid. *In* "Nutrition and the Brain" (R. Wurtman and J. Wurtman, eds.), Vol. 4, pp. 79–124. Raven, New York.

Graham, D. M. (1978). Caffeine: Its identity, dietary sources, intake and biological effects. *Nutr. Rev.* **36,** 97–102.

Iversen, L. L., Iversen, S. D., and Snyder, S. H. (1977). "Handbook of Psychopharmacology," Vol. 7. Plenum, New York.

Justice, J. B., Jr., Michael, A. C., and Neill, D. B. (1985). In vivo voltammetry. *In* "Neuromethods 2: Amines and Their Metabolites" (A. B. Boulton, G. B. Baker, and J. B. Baker, eds.), pp. 197–266. Humana, Clifton, New Jersey.

Kolbye, A. C., Mahaffey, K. R., Fiorino, J. A., Corneliussen, P. C., and Jelinek, C. F. (1974). Food exposures to lead. *Environ. Health Perspect.* **7,** 65–74.

Kruesi, M. J. P., Rapoport, J. L., Cummings, E. M., Berg, C. J., Ismond, D. R., Flament, M., Yarrow, M., and Zahn-Waxler, C. (1987). Effects of sugar and aspartame on aggression and activity in children. *Am. J. Psychiatry* **144,** 1487–1490.

Lafferman, J. A., and Silbergeld, E. K. (1979). Erythrosin B inhibits dopamine uptake in rat caudate synaptosomes. *Science* **205,** 410–412.

Laties, V. G., and Wood, R. W. (1986). Schedule-controlled behavior in behavioral toxicology. *In* "Neurobehavioral Toxicology" (Z. Annau, ed.), pp. 69–73. The Johns Hopkins Univ. Press, Baltimore.

Levitan, J. (1977). Food, drug and cosmetic dyes: Biological effects related to lipid solubility. *Proc. Natl. Acad. Sci. U.S.A.* **74,** 2914–2918.

Lewin, R. (1987). Environmental hypothesis for brain diseases strengthened by new data. *Science* **237,** 483–484.

Lewis, M. H., Baumeister, A. A., McCorkle, D. L. and Mailman, R. B. (1985). A computer supported method for analyzing behavioral observation: Studies with stereotypy. *Psychopharmacology* **85,** 204–209.

Logan, W. J., and Swanson, J. M. (1979). Erythrosin B inhibition of neurotransmitter accumulation by rat brain homogenates. *Science* **206,** 363–364.

Mackintosh, J. H., Chance, M. R. A., and Silverman, A. P. (1977). The contribution of ethological techniques to the study of drug effects. *In* "Handbook of Psychopharmacology" (L. L. Iversen, S. D. Iversen, and S. H. Snyder, eds.), Vol. 7, pp. 3–35. Plenum, New York.

Mailman, R. B. (1987). Mechanisms of CNS injury in behavioral dysfunction. *Neurotoxicol. Teratol.* **9,** 417–426.

Mailman, R. B., and DeHaven, D. L. (1984). Responses of neurotransmitter systems to toxicant exposure. *In* "Cellular and Molecular Neurotoxicology" (T. Narahashi, ed.), pp. 207–224. Raven, New York.

Mailman, R. B., and Lewis, M. H. (1981). Food additives and developmental disorders: The case of erythrosin (FDC red #3), or guilty until proven innocent. *Appl. Res. Ment. Retard.* **2,** 297–305.

Mailman, R. B. (1987). Mechanisms of CNS injury in behavioral dysfunction. *Neurotoxicol. Teratol.* **9,** 417–426.

Mailman, R. B., Ferris, R. M., Tang, F. L. M., Vogel, R. A., Kilts, C. D., Lipton, M. A., Smith, D. A., Mueller, R. A., and Breese, G. R. (1980). Non-specificity of the biochemical actions of erythrosine (red #3): What relation to behavioral changes? *Science* **207,** 535–536.

Mazur, R. H., Schlatter, J. M., and Goldkamp, A. H. (1969). Structure–taste relationships of some dipeptides. *J. Am. Chem. Soc.* **91,** 2684–2691.

Morell, P., and Mailman, R. B. (1987). Selective and nonselective effects of organometals on brain neurochemistry. *In* "Neurotoxicants and Neurobiological Function: Effects of Organoheavy Metals" (H. Tilson and S. Sparber, eds.), pp. 201–229. Wiley, New York.

Neims, A. H., and von Borstel, R. W. (1983). Caffeine: Metabolism and biochemical mechanisms of action. *In* "Nutrition and the Brain" (R. J. Wurtman and J. J. Wurtman, eds.), Vol. 6, pp. 1–30. Raven, New York.

Niedzwiecki, D. M., and Mailman, R. B. (1981). Measurement of erythrosine (F.D.C. red #3) by reverse phase HPLC with fluorescent detection: Preliminary toxicokinetic results. *Toxicologist* **1,** 152.

Norton, S. (1977). The study of sequences of motor behavior. *In* "Handbook of Psychopharmacology" (L. L. Iverson, S. D. Iversen, and S. H. Snyder, eds.), Vol. 7, pp. 83–105. Plenum, New York.

Norton, S. (1982). Effects of food chemicals on behavior of experimental animals. *In* "Nutritional Toxicology" (J. N. Hathcock, ed.), Vol. 1. Academic Press, New York.

Norton, S. (1986). Observational methods. *In* "Neurobehavioral Toxicology" (Z. Annau, ed.). The Johns Hopkins Univ. Press, Baltimore.

Olney, J. W., Price, M. T., Samson, L., and Labruyere, J. (1986). The role of specific ions in glutamate neurotoxicity, *Neurosci. Lett.* **65,** 65–71.

Olton, D. S., and Samuelson, R. J. (1976). Remembrance of places passed: Spatial memory in rats. *J. Exp. Psychol.: Anim. Behav. Processes* **2,** 97–116.

Palkovits, M., and Brownstein, M. (1988). "Maps and Guide to Microdissection of the Rat Brain." Elsevier, New York.

Pardridge, W. M. (1986). Potential effects of the dipeptide sweetner aspartame on the brain. *In* "Nutrition and the Brain" (R. J. Wurtman and J. J. Wurtman, eds.), Vol. 7, pp. 199–241. Raven, New York.

Pryor, G. T., Uyeno, E. T., Tilson, H. A., and Mitchell, C. L. (1983). Assessment of chemicals using a battery of neurobehavioral tests: A comparative study. *Neurobehav. Toxicol. Teratol.* **5,** 91–117.

Rafales, L. S., Bornschein, R. L., Michaelson, I. A., Loch, R. K., and Barker, G. F. (1979). Drug induced activity in lead-exposed mice. *Pharmacol. Biochem. Behav.* **10,** 95–104.

Rafales, L. S., Lasley, S. M., Greenland, R. D., and Mandybur, T. (1983). Effects of acrylamide on locomotion and central monoamine function in the rat. *Pharmacol. Biochem. Behav.* **19,** 635–644.

Rapoport, J. L. (1986). Diet and hyperactivity. *Nutr. Rev. (Suppl.)* 158–162.

Rothman, S. (1984). Synaptic release of excitatory amino acid neurotransmitter mediates anoxic neuronal death. *J. Neurosci.* **4,** 1884–1891.

Sako, F., Kobayashi, N., Watabe, H., and Taniguchi, N. (1980). Cytotoxicity of food dyes on cultured fetal rat hepatocytes. *Toxicol. Appl. Pharmacol.* **54,** 285–292.

Silbergeld, E. K. (1981). Erythrosin B is a specific inhibitor of high affinity ^{3}H-ouabain binding and ion transport in rat brain. *Neuropharmacology* **20,** 87–90.

Spencer, P. S., Nunn, P. B., Hugon, J., Ludolph, A. C., Ross, S. M., Roy, D. N., and Robertson, R. C. (1987). Guam amyotrophic lateral sclerosis–Parkinsonism–dementia linked to a plant excitant neurotoxin. *Science* **237,** 517–522.

Stegink, L. D. (1984). Aspartame metabolism in humans: Acute dosing studies. *In* "Aspartame Physiology and Biochemistry" (L. D. Stegink and L. J. Filer, eds.), pp. 509–553. Dekker, New York.

Stegink, L. D., Filer, L. J., and Baker, G. L. (1977). Effect of aspartame and aspartate loading upon plasma and erythrocyte free amino acid levels in normal adult volunteers. *J. Nutr.* **107,** 1837–1045.

Tsubaki, T., and Irukayama, K. (1977). "Minimata Disease." Elsevier, Amsterdam.

Westerink, B. H. C., Damsma, G., Rollema, H., De Vries, J. B., and Horn, A. S. (1987). Scope and limitations of in vivo brain dialysis: A comparison of its application to various neurotransmitter systems. *Life Sci.* **41,** 1763–1776.

Wurtman, R. J., Hefti, F., and Melamed, E. (1983). Neurochemical changes following high-dose aspartame with dietary carbohydrates. *N. Engl. J. Med.* **309,** 429–430.

Zautcke, J. L., Schwartz, J. A., and Mueller, E. J. (1986). Chinese restaurant syndrome: A review. *Ann. Emerg. Med.* **15,** 1210–1213.

3

Nutritional and Safety Implications of Oxidized Sulfur-Containing Amino Acids

K. C. Chang

I. INTRODUCTION

The nutritional quality of a food protein is determined by the composition of the essential amino acids, the digestibility of the protein, and the utilizability of the absorbed amino acids. The quantity of available amino acids absorbed, particularly of the first-limiting amino acids, plays a major role in determining protein quality. It is known that several essential amino acids, such as the sulfur-containing amino acids and lysine, can exist either partially or completely in biologically unavailable forms in the protein, either in their native state or in proteins that have undergone food processing (Satterlee and Chang, 1982). Sul-

NUTRITIONAL TOXICOLOGY, VOL. III

fur-containing amino acids are in low supply in many food proteins, particularly in legumes. During the processing and/or storage of food proteins, the sulfur-containing amino acids may be oxidized so that the nutritional quality of the food is reduced. Various amounts of oxidized sulfur-containing amino acids have been found in several food products. The altered amino acid (e.g., lysinoalanine) has been shown to be toxic when it is consumed in large quantities in the free form (Struthers *et al.*, 1979). Unfortunately, there are few reports dealing with the toxicity of oxidized sulfur-containing amino acids. The fact that oxidized sulfur-containing amino acids can exist in several chemical forms and that the methodology of analyses is more tedious than for the other amino acids has slowed the research effort to elucidate the bioavailability and toxicity of oxidized sulfur-containing amino acids in food proteins.

II. CHEMISTRY, OCCURRENCE, AND FORMATION DURING FOOD PROCESSING

The absolute configurations of several oxidized sulfur-containing amino acids that have been studied with respect to biological availability are shown in Fig. 1. Peptide-bound amino acids are in the L forms, synthetic amino acids can exist in the D or L form or in a racemic mixture. Under oxidizing condition, L-cysteine can be converted to L-cysteinesulfenic acid (Fig. 1, i), L-cysteinesulfinic acid (Fig. 1, ii), and L-cysteic acid (Fig. 1, iii). L-Cysteinesulfenic acid is an unstable intermediate, whereas L-cysteinesulfinic acid and L-cysteic are stable compounds. Various oxidized forms of L-cystine studied include L-cystine *S*-oxide (or L-cystine monoxide) (Fig. 1, iv), L-cystine *S,S'*-dioxide (Fig. 1, v), and L-cystine *S,S*-dioxide (Fig. 1, vi). L-Methionine can be oxidized to L-methionine sulfoxide (Fig. 1, vii) and L-methionine sulfone (Fig. 1, viii). Free methionine sulfoxide could exist in four diastereoisomers due to the two asymmetrical centers of the α-carbon and the sulfur atom (Lavine, 1947). Methionine sulfoxide is largely converted to methionine during acid hydrolysis of proteins. Direct determinations of methionine sulfoxide in food proteins can be carried out using chromatographic separation after alkaline hydrolysis (Chang *et al.*, 1982; Todd *et al.*, 1984). Methionine sulfone and cysteic acid in the proteins can be quantitatively determined using acid hydrolysis followed by chromatography (Moore, 1963).

Several oxidized sulfur-containing amino acids, such as methionine sulfoxide, *S*-methylcysteine sulfoxide and cysteic acid are naturally present in legume seeds (Zacharius, 1970; Kasai *et al.*, 1971, 1972; Pusztai and Watt, 1974; Otoul *et al.*, 1975), and in large proportion in the resilium protein of surf clam. In food processing, the application of various oxidizing agents (Lindsay, 1985) for the production of desirable functional properties, for preservation, and detoxication

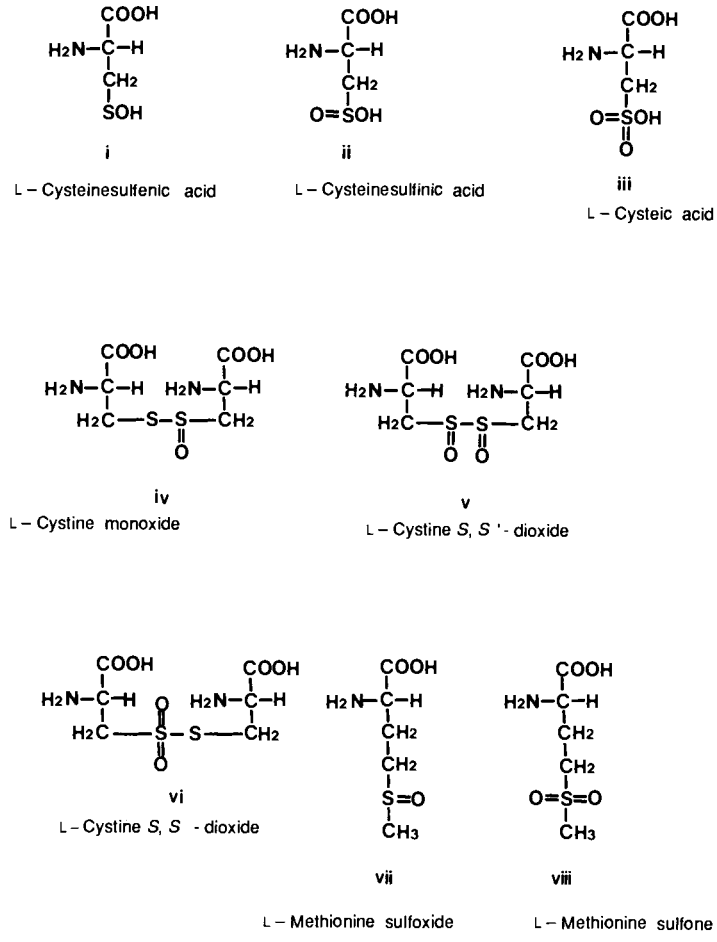

Fig. 1. Absolute configuration of oxidized sulfur-containing amino acids.

may also oxidize the sulfur-containing amino acids (Meyer and Williams, 1977). In addition, lipid hydroperoxide, formed during food storage, may oxidize the sulfur-containing amino acid residues of proteins (Tannenbaum *et al.*, 1969). Commerical food products including soy isolate, milk, or soy-based infant formulas have been found to contain up to 51% of the total methionine as methionine sulfoxide (Marshall *et al.*, 1982; Todd *et al.*, 1984).

The oxidation of the sulfur-containing amino acids in food proteins is affected by several factors, such as pH, temperature, the type and concentration of oxidizing agents, and the type of protein source. In normal food processing, heating without adding oxidizing agents is not likely to oxidize the sulfur-containing

amino acids (Marshall *et al.*, 1982). Under neutral pH or slightly acidic conditions, treatment of casein, soy isolate, and egg white solid with hydrogen peroxide at concentrations up to 1% (w/v) at 40°C causes the formation of methionine sulfoxide but does not cause the production of methionine sulfone. The rates of formation of methionine sulfone at 40°C with concentrations of hydrogen peroxide greater than 1% differ among protein sources (Chang *et al.*, 1982). When the oxidation temperature is raised to 90°C, cysteic acid and methionine sulfone are formed at low concentrations of hydrogen peroxide. Oxidation of casein and soy isolate under mild conditions converts methionine to methionine sulfoxide without affecting cystine/cysteine (Chang *et al.*, 1982) and other amino acids including tryptophan (K. C. Chang, unpublished data). The oxidation of a fish protein concentrate by various levels of hydrogen peroxide at 50°C does not cause appreciable destruction of tryptophan (Rasekh *et al.*, 1972).

The sulfur-containing amino acids react with organic hydroperoxides at a slower rate than with hydrogen peroxide (Caldwell and Tappel, 1964). Methionine sulfoxide is formed in casein during storage in a model system of lipid oxidation (Tannenbaum *et al.*, 1969; Karel *et al.*, 1975; Cuq *et al.*, 1978). In frankfurter processing, the use of high-peroxide fat can lower the methionine content (Strange *et al.*, 1980). Free-methionine oxidation in the storage of methionine-fortified model food systems does not take place unless oxidized lipids are used in the formulation (O'Keefe and Warthesen, 1978; Tufte and Warthesen, 1979). The sterilization of milk with 0.018-*M* hydrogen peroxide at 50°C for 30 min converts 50% of the methionine to methionine sulfoxide (Cuq *et al.*, 1978). Hydrogen peroxide is widely used in food processing—for example, in the pasteurization of milk for cheesemaking (Roundy, 1958), in the improvement of the textural properties of egg white in angel food cakes (Cunningham and Cotterill, 1962), in the treatment of skim milk for sponge bread baking (Guy *et al.*, 1968), and in the bleaching of fish protein concentrate (Rasekh *et al.*, 1972).

III. BIOAVAILABILITY

A. Bioavailability of Oxidized Cystine/Cysteine

Depending on the extent of oxidation, oxidized cystine/cysteine can be biologically used to varying degrees (Table I). L-Cysteinesulfenic acid is chemically unstable and can be readily reconverted to the unoxidized cysteine or oxidized to cysteinesulfinic acid. L-Cysteinesulfenic acid in the form of *S*-(guanylthio)cysteine·2HCl (which hydrolyzes to thiourea and L-cysteinesulfenic acid in water) can partially supplement a 6% casein diet for the growth of young rats (Bennett, 1937). L-Cystine monoxide and L-cystine *S,S'*-dioxide can be used to supple-

ment cystine/cysteine-deficient diets for the growth of young rats and chicks, indicating that these two oxidized forms can be readily reversed to the unoxidized form for the protein synthesis (Bennett, 1937; Crawford *et al.*, 1984). L-Cystine *S,S*-dioxide is only 47% potent when compared with cysteine supplementation for the growth of young chicks (Crawford *et al.*, 1984). In rats, L-cysteinesulfinic acid is metabolized to pyruvate, sulfate, and CO_2 (Stipanuk and Rotter, 1984). Oxidized L-cystine can be utilized by animals when the sulfur atom is in the 4-valence state but cannot be utilized when the sulfur atom is in the 6-valence state. Oxidized cysteine cannot be utilized when the sulfur atom is in either the 4-valence (cysteinesulfinic acid) or the 6-valence state (cysteic acid). In animals, there are no enzyme systems to reduce cysteic acid to cystine. Cysteic acid, however, can be partially converted to taurine, which reacts with cholesterol to form cholic acid in the rat (Stipanuk and Rotter, 1984).

B. Bioavailability of Methionine Sulfone

D-Methionine, DL-methionine sulfoxide, and DL-methionine sulfone cannot be utilized for protein synthesis by *Escherichia coli* K12 (Lemoine *et al.*, 1968). It is unlikely that these amino acids can be utilized directly for the growth of organisms of higher classes. The ability of the subject to utilize these free amino acids for growth therefore depends largely on the extent of absorption, reduction, and/or transformation to the L-methionine. The bioavailability of peptide-bound amino acids is further affected by the digestibility and release rates in the digestive tract. Researches on the bioavailability of free DL- or L-methionine sulfone (Table II) consistently indicate that animals cannot utilize these oxidized forms of methionine for growth (Bennett, 1941; Njaa, 1962; Miller and Samuel, 1968; Anderson *et al.*, 1976; Kuzmicky *et al.*, 1977; Sjoberg and Bostrom, 1977).

C. Bioavailability of Free Methionine Sulfoxide

Unlike methionine sulfone, the bioavailability of methionine sulfoxide varies from one report to another (Table II). The bioavailability relative to that of L-methionine for the free L-methionine sulfoxide varies from 38 to 100%, whereas the bioavailability for free DL-methionine sulfoxide varies from 26 to 100%. The discrepancies in the bioavailability of methionine sulfoxide among various studies may be due to differences in assay methodology.

Similar to unoxidized methionine (Zezulka and Calloway, 1976), the bioavailability of oxidized methionine is also affected by the stereoisomeric configuration. The partially oxidized methionine, methionine sulfoxide, can exist in four diastereoisomers (Toennies and Kolb, 1939). Hydrogen peroxide oxidation of

TABLE I

Bioavailability of Oxidized Cystine/Cysteine

Oxidized form tested[a]	Assay method[b]		Availability (% relative to L-Cys)	Reference
	Basal or control diet	Measurement[c,d]		
Cysteic acid (F)	9% casein	Growth due to supplementation of 0.5% cysteic acid over 8 weeks	Not available (0%)	Lewis and Lewis (1926)
L-Cysteinesulfenic acid (F)	6% casein	Growth due to supplementation of 20 mg/day/rat L-Cys-equivalent *S*-(guanylthio)cysteine over 2 weeks	Partially available	Bennett (1937)
L-Cystine *S*,*S*′-di-oxide (F)	6% casein	Growth due to supplementation of 20 mg/rat/day L-Cys-equivalent oxidized Cys over 2 weeks	Available (100%)	Bennett (1937)
L-Cysteinesulfinic acid (F)	6% casein	Growth due to supplementation of 20 mg/rat/day L-Cys equivalent oxidized Cys over 2 weeks	Not available (0%)	Bennett (1937)
Cysteic acid (F)	10% casein	NPU due to supplementation of 0.1% cysteic acid over 10 days	Not available (0%)	Miller and Samuel (1968)

Cysteic acid (F)	18% amino acid mixture with 0.2% L-methionine	Growth due to supplementation of 0.14–0.42% cysteic acid over 2 weeks	Not available (0%)	Anderson *et al.* (1976)
Cysteic acid (F)	Soybean, 22% protein	Slope ratio due to supplementation of 0.2–0.4% cysteic acid over 14 days	Not available (0%)	Kuzmicky *et al.* (1977)
Cysteic acid (F)	Fish protein, 10%	NPU and BV due to supplementation of 0.1% cysteic acid over 4 days	Not available (0%)	Sjoberg and Bostrom (1977)
L-Cysteine monoxide (F)	Amino acid mixture, 20% containing DL-methionine	Slope ratio due to supplementation of 0.05–0.1% L-Cys equivalent oxidized Cys over 6 days	Available (100%)	Crawford *et al.* (1984)
L-Cystine *S,S'*-dioxide (F)			Partially available (47%)	
L-Cystine *S,S'*-dioxide (F)				
L-Cystinesulfinic acid (F)			Available (93%)	

[a]F in parentheses represents free amino acids.

[b]Young chicks were used in the reports of Kuzmicky *et al.* (1977) and Crawford *et al.* (1984); all other experiments used young rats.

[c]BV and NPU represent biological value and net protein utilization, respectively.

[d]Cys represents cystine/cysteine.

TABLE II

Bioavailability of Oxidized Methionine

Oxidized form tested[a]	Assay method[b]		Availability (% relative to L-methionine)	Reference
	Basal or control diet	Measurement[c]		
DL-MetSO (F)	15% arachin	Growth due to supplementation of 6 mg/rat/day DL-methionine-equivalent DL-MetSO over 16 days	Available (100%)	Bennett (1939)
DL-$MetSO_2$ (F)	15% arachin	Growth due to supplementation of 5 mg/rat/day DL-methionine-equivalent DL-$MetSO_2$ over 16 days	Not available (0%)	Bennett (1941)
DL-MetSO (F)	Soy meal, 8% protein	Urinary nitrogen excretion due to supplementation of 0.12% methionine-equivalent oxidized methionine	Partially available (75%)	Njaa (1962)
L-MetSO (F)			Available (100%)	
D-MetSO (F)			Partially available (50%)	
L-$MetSO_2$ (F)			Not available (0%)	
L-MetSO (P) in oxidized casein	10% casein	NPU of unoxidized and oxidized casein over 10 days	Partially available (82%)	Ellinger and Palmer (1968)
DL-MetSO (F)	10% casein	NPU due to supplementation of 0.1% oxidized methionine	Partially available (26%)	Miller and Samuel (1970)
DL-$MetSO_2$ (F)			Not available (0%)	
L-MetSO (F)	Amino acid mixture, 8%	Growth due to replacement of L-methionine with 0.6% L-MetSO over	Partially available (66%)	Miller *et al.* (1970)
L-MetSO (P) in oxidized casein and fish meal	Unoxidized casein or fish meal, 10% protein	BV and NPU of unoxidized and oxidized protein	Available (100%)	Slump and Schreuder (1973)
L-MetSO (F)	18% casein or	Growth due to replacement of	Partially available	Anderson *et al.* (1975)

L-$MetSO_2$ (F)	amino-acid mixture containing 0.8% L-methionine and 0.34% L-cysteine	methionine with 0.56–1.66% oxidized methionine over 2 weeks	(60%) Not available (0%)	
L-MetSO (F)	14–15% amino acid mixture	Growth due to replacement of methionine with 0.66% L-MetSO over 17 days	Partially available (38%)	Gjoen and Njaa (1977)
L-MetSO (F)	Soybean meal, 14–15% protein	Growth due to supplementation of L-MetSO over 10–20 days	Available (100%)	Gjoen and Njaa (1977)
L-MetSO (P)	Unoxidized fish meal, 14–15% protein	Growth due to oxidized fish meal over 11 days	Available (90%)	Gjoen and Njaa (1977)
L-MetSO (F)	Unoxidized casein or fish meal, 8% protein	Nitrogen balance due to supplementation of 0.15–0.16% L-MetSO over 5 days	Available	Gjoen and Njaa (1977)
DL-MetSO (F) L-MetSO (F) D-MetSO (F) DL-$MetSO_2$ (F)	Soybean, 22% protein	Slope ratio due to supplementation of 0.2–0.4% oxidized methionnine over 14 days	Partially available (59%) Partially available (77%) Partially available (52%) Not available (0%)	Kuzmicky *et al.* (1977)
L-MetSO (P) in oxidized fish protein L-$MetSO_2$ (F)	Fish meal, 10% protein Fish meal, 10% protein	PER (28 days), BV and NPU (4 days) of oxidized protein BV and NPU due to supplementation of L-$MetSO_2$	Available (100%) Not available (0%)	Sjoberg and Bostrom (1977)

(*continued*)

TABLE II (*Continued*)

Oxidized form tested[a]	Assay method[b]		Availability (% relative to L-methionine)	Reference
	Basal or control diet	Measurement[c]		
L-MetSO (P) in oxidized casein	Casein, 10–14%	PER (28 days), NPU (11 days)	Partially available (85–90%)	Cuq *et al.* (1978)
L-MetSO (F)	Amino acid mixture, 14–15% containing 0.8% methionine and 0.32% cysteine	Growth due to replacement of 0.8% methionine with L-MetSO over 25 days	Partially available (75%)	Aksnes (1984)
L-MetSO (P)	Unoxidized casein and soy isolates	PER (28 days) and NPR (14 days) of oxidized proteins containing various levels of L-MetSO	Partially available	Chang *et al.* (1985)

[a]MetSO and $MetSO_2$ represent methionine sulfoxide and methionine sulfone, respectively; F and P in parentheses represent free or peptide-bound amino acids.

[b]Young chicks were used in the report of Kuzmicky *et al.* (1977), both young and mature rats were used in the study of Chang *et al.* (1985), and all other experiments used young rats.

[c]BV, NPU, NPR and PER represent biological value, net protein utilization, net protein ration and protein efficiency ratio, respectively.

L-methionine yields an equal molar mixture of two isomers, that is, L-methionine dl-sulfoxide, which can be separated in the forms of picrates (Lavine, 1947). The bioavailability of purified D-, L-, and DL-methionine sulfoxide have been investigated whereas the isomers due to the asymmetric sulfur atom have not been separately fed to animals. Young rats and chicks seem to utilize L-methionine sulfoxide more efficiently than D-methionine sulfoxide (Miller and Samuel, 1970; Kuzmicky *et al.*, 1977; Njaa and Aksnes, 1982; Iwami *et al.*, 1983).

To compare the results of bioavailability of methionine sulfoxide among various studies is difficult due to differences in methodologies. The availability of the DL-methionine sulfoxide for the growth of rats seems to increase when the amount of proteins in the basal diet is raised (Bennett, 1939; Miller and Samuel, 1970). An increase in protein content can partially overcome the problem due to imbalance of amino acids. Although certain dietary proteins (such as legume proteins) are deficient in the sulfur-containing amino acids, the proteins still contain approximately one-half of the requirement for maximal growth of rats. However, when a free amino acid mixture that is free of the sulfur-containing amino acids is used for the basal diet, an increase in the dietary level of basal amino acids (Gjoen and Njaa, 1977) reduces the ability of L-methionine sulfoxide to support the growth of rats (Miller *et al.*, 1970). The bioavailability of amino acids, assessed by the growth of rats, is also affected by the amounts of the sulfur-containing amino acids in the diet. When rats were fed a higher level of the oxidized sulfur-containing amino acids than that required by the rats (Aksnes, 1984), a greater weight gain was seen than when they were fed with lower levels of the sulfur-containing amino acids (Miller *et al.*, 1970; Gjoen and Njaa, 1977).

Partial replacement of the L-methionine sulfoxide by L-cysteine increases the growth of rats more than when L-methionine sulfoxide is used as the sole source of the sulfur-containing amino acids and approaches that of methionine-fed rats. This phenomenon is logical because when L-methionine sulfoxide is tested as the sole source of the sulfur-containing amino acids, the L-methionine sulfoxide must be converted to L-methionine, which is then partly metabolized to cysteine for protein synthesis. Cystine/cysteine can replace 68% of L-methionine for the maximal growth of rats (Rao *et al.*, 1961). In addition to the assessment of bioavailability by animal-growth [weight gain, protein efficiency ratio (PER), and net protein ratio (NPR)] and nitrogen-balance [biological value (BV) and net protein utilization (NPU)] techniques, L-methionine sulfoxide has been reported to be as effective as L-methionine in nitrogen-sparing action in rats fed protein-free diet (Njaa and Aksnes, 1982). However, using hepatic glutathione synthesis and triglyceride accumulation as the index of bioavailability, L-methionine sulfoxide and DL-methionine sulfoxide have been found to be less effective than L-methionine (Iwami *et al.*, 1983; Yanagita *et al.*, 1984).

D. Bioavailability of Peptide-Bound Methionine Sulfoxide

Since only the L form of amino acids is naturally present in proteins, the oxidized proteins should not be expected to contain D-methionine sulfoxide. However, similar to the free methionine sulfoxide, the bioavailability of peptide-bound methionine varies from one study to another. Generally, peptide-bound L-methionine sulfoxide is more available than free methionine sulfoxide but inferior to L-methionine for the growth of young rats (Ellinger and Palmer, 1968; Slump and Schreuder, 1973; Gjoen and Njaa, 1977; Cuq *et al.*, 1978; Chang *et al.*, 1985). Again, the discrepancy in the bioavailability of peptide-bound methionine may be due to differences in assay methodology used for availability assessment. Age of the animals plays an important role in the utilization of the peptide-bound methionine sulfoxide (Miller *et al.*, 1970; Chang *et al.*, 1985). Mature rats can utilize a soy isolate containing 60% methionyl residue as methionine sulfoxide whereas weanling rats utilize the same soy isolate less efficiently (Chang *et al.*, 1985). In a long-term feeding study (132 days), Miller *et al.* (1970) found that the plasma total "methionine-like" compounds decreased as the rats became older, probably indicating that an adaptation mechanism was developed with aging. Other researchers did not observe this adaptation phenomenon in young rats fed over a 28-day period (Sjoberg and Bostrom, 1977). Hence, the development of an adaptation mechanism for utilization of L-methionine sulfoxide may take place slowly, and it may take longer than 28 days for the plasma content of methionine sulfoxide to be reduced significantly.

E. Digestibility of Oxidized Food Proteins

Mild oxidation of proteins oxidizes the methionyl residue to methionine sulfoxide without the oxidation of cystine/cysteine and the formation of methionine sulfone. The overall *in vitro* digestibility of mildly oxidized casein is not lower than the unoxidized casein. However, methionine in mildly oxidized casein was released by pronase at a slower rate than that of unoxidized casein (Cuq *et al.*, 1973). Although *in vitro* digestibility of proteins is not decreased by mild oxidation (Slump and Schreuder, 1973; Cuq *et al.*, 1978; Sjoberg and Bostrom, 1977; Chang *et al.*, 1985), a slow release rate of methionine/methionine sulfoxide from oxidized proteins may still occur (Cuq *et al.*, 1978).

F. Absorption of L-Methionine Sulfoxide

L-Methionine sulfoxide is absorbed in the intestine, though the absorption rate of the sulfoxide is lower by 20–40% than that of L-methionine (Miller *et al.*, 1970; Anderson *et al.*, 1976; Sjoberg and Bostrom, 1977; Aksnes and Njaa,

1983). However, L-methionine sulfoxide is completely absorbed even if at a slower absorption rate than that for L-methionine (Aksnes and Njaa, 1983). Shortly after ingestion of a diet containing free methionine sulfoxide or oxidized proteins, there is a rise of the methionine sulfoxide level in the plasma. The plasma L-methionine sulfoxide level in rats fed L-methionine sulfoxide is 10- to 20-fold (or more) higher than that of L-methionine fed as a control diet (Miller *et al.*, 1970; Gjoen and Njaa, 1977; Sjoberg and Bostrom, 1977). In rabbits, increasing sodium concentration increases the rate of transport of L-methionine sulfoxide across the intestinal cell membrane (Ganapathy and Leibach, 1982). The absorption process of L-methionine sulfoxide in the intestines of rabbits and rats is inhibited by several neutral amino acids including L-methionine, indicating that L-methionine sulfoxide and L-methionine probably share a common carrier system (Ganapathy and Leibach, 1982; Higuchi *et al.*, 1982). However, a different absorption process for L-methionine sulfoxide has also been proposed (Aksnes and Njaa, 1983). The intestinal absorption of both L-methionine and L-methionine sulfoxide is stimulated by glutathione (Aksnes and Njaa, 1983).

G. Reduction of L-Methionine Sulfoxide

Since mildly oxidized proteins are well digested, and free methionine is completely absorbed, the major determinant of the utilization of L-methionine sulfoxide, therefore, lies in the reduction rate of L-methionine sulfoxide, which must be converted to L-methionine for protein synthesis. L-Methionine sulfoxide-reducing activity occurs in various organs of rabbits, including intestine, kidney, and liver (Ganapathy and Leibach, 1982). The kidney tubular cells possess a greater reducing ability than the intestinal brush border cells. Various reducing agents such as dithiothreitol, glutathione, NADPH, and NADH stimulate the reduction of L-methionine sulfoxide to L-methionine. Smith (1972) injected radioactive L-methionine sulfoxide and L-methionine sulfone into rats fed Purina rat chow and observed that 12% of the methionine sulfoxide injected was excreted in the urine, largely in *N*-acetylated forms. Therefore, absorbed L-methionine sulfoxide could be excreted if not reduced and utilized. Free L-methionine sulfoxide has also been reported to be enzymatically reduced in rat liver and kidney (Aymard *et al.*, 1979; Ejiri *et al.*, 1979). However, Aksnes (1984) had a contradictory finding that various organs from the rats could not reduce L-methionine sulfoxide. Aksnes attributed the reducing activity found by Aymard *et al.* (1973) to the possible contamination of bacteria such as *E. coli,* which could enzymatically reduce L-methionine l-sulfoxide stereospecifically. Free methionine-sulfoxide reductase has not been isolated and purified to homogeneity from rats or other higher vertebrates. The biochemistry and physiological implication of the presence of free methionine-sulfoxide reductase and peptide-bound methionine-sulf-

oxide reductase in various organisms have been reviewed by Brot and Weissbach (1983). L-Methionine sulfoxide may be reduced in the kidney and reabsorbed into the circulation system.

H. Absorption and Excretion of L-Methionine Sulfoxide in Humans

L-Methionine sulfoxide is readily absorbed by adult humans (Marable *et al.*, 1980, 1981; Stegink *et al.*, 1986). Only negligible amounts of methionine sulfoxide and *N*-acetyl-L-methionine sulfoxide were excreted in the urine after a one- or two-dose ingestion of L-methionine sulfoxide by adult humans indicating that a reduction system is also present in humans. The effect of ingestion of L-methionine sulfoxide or oxidized proteins on the growth and/or nitrogen balance in human infants and adults has not been examined.

IV. SAFETY IMPLICATION OF OXIDIZED SULFUR-CONTAINING AMINO ACIDS

Although L-cysteinesulfinic acid and cysteic acid are not biologically available, most short-term feeding studies have not observed any toxicity resulting from supplementing low levels of cysteic acid in the diets of young animals. However, cysteic acid, when supplemented at 0.1% of the diet for young rats, has been shown to slightly decrease the NPU of a 10% casein diet (Miller and Samuel, 1968, 1970). The long-term effect of consuming oxidized cystine/cysteine has not been explored.

Methionine sulfone can depress food intake of rats and can depress the net protein utilization of a 10% casein basal diet (Miller and Samuel, 1968, 1970). Because methionine sulfone is formed under severe oxidation conditions not normally used in the food industry, the toxicity of methionine sulfone in humans is not of a practical concern.

Methionine sulfoxide does not depress food intake of rats fed casein basal diets. The liver composition of young rats on a diet containing free L-methionine sulfoxide for 17 days is not different from that of the control rats (Miller *et al.*, 1970). Liver weight, serum protein content, hair growth, and hemoglobin are not influenced by feeding free L-methionine sulfoxide at the level of 0.5% of the diet for 132 days.

Posttranslational oxidation of methionyl residues of proteins occurs in various organs (Brot and Weissbach, 1983). The microsomes of the rat liver contain a general enzyme-catalyzed oxidation system that can oxidize various α-thiocarboxylic acids to their sulfoxides (Lee *et al.*, 1970). Large quantities of methionine sulfoxide in the urine of an infant with hypermethioninemia implies that formation of methionine sulfoxide and acetylated compounds might be a mecha-

nism for detoxifying excess methionine (Perry *et al.*, 1965). Rats fed excessive amounts of methionine also excreted methionine sulfoxide in the urine (Daniel and Waisman, 1969). From the results of experiments using rats and chicks, methionine sulfoxide does not seem to be toxic. The long-term toxic effect of consuming a high level of methionine sulfoxide, particularly on the physiological functions of the liver and kidney, is not known.

The sulfur-containing amino acids are the first-limiting essential amino acids of legume proteins. The presence of oxidized sulfur amino acids in soy-based infant formula would accentuate the dependence for the sulfur-containing amino acids for optimal growth of infants who consume the soy-based formula as the sole source of food. It was estimated in 1979 that approximately 10% of formula-fed infants were being fed formulas with protein from soy isolate (Fomon and Ziegler, 1979). In addition to the need to investigate the long-term toxicity of L-methionine sulfoxide, research should also be directed to clarify the availability of this oxidized amino acid to human infants or to animals other than rats and chicks.

ACKNOWLEDGMENTS

The author gratefully acknowledges the support and discussions of H. Klosterman and L. Satterlee and the financial support from the N. D. Agricultural Experiment Station for Project #2703 Food Protein Utilization.

REFERENCES

Aksnes, A. (1984). Studies on the *in vivo* utilization and the *in vitro* enzymatic reduction of methionine sulfoxide in rats and rat tissues. *Ann. Nutr. Metab.* **28,** 288–296.

Aksnes, A., and Njaa, L. R. (1983). Absorption of methionine and methionine sulphoxide in rat intestine and the effect of glutathione. *Br. J. Nutr.* **50,** 583–588.

Anderson, G. H., Li, G. S. K., Jones, J. D., and Bender, F. (1975). Effect of hydrogen peroxide treatment on the nutritional quality of rapeseed flour fed to weanling rats. *J. Nutr.* **105,** 317–324.

Anderson, G. H., Ashley, D. V. M., and Jones, J. D. (1976). Utilization of L-methionine sulfoxide, L-methionine sulfone and cysteic acid by the weanling rat. *J. Nutr.* **106,** 1108–1114.

Aymard, C., Seyer, L., and Cheftel, J. C. (1979). Enzymatic reduction of methionine sulfoxide. *In vitro* experiments with rat liver and kidney. *Agric. Biol. Chem.* **43,** 1869–1872.

Bennett, M. A. (1937). Metabolism of sulphur. V. The replaceability of L-cystine in the diets of rats with some partially oxidized derivatives. *Biochem. J.* **31,** 962–965.

Bennett, M. A. (1939). Metabolism of sulphur. The replaceability of dl-methionine in the diet of albino rats with its partially oxidized derivative dl-methionine sulfoxide. *Biochem. J.* **33,** 1794–1797.

Bennett, M. A. (1941). The replaceability of dl-methionine in the diet of the albino rat with

dl-methionine sulfone and dl-methionine methyl sulfonium chloride. *J. Biol. Chem.* **141,** 573–579.

Brot, N., and Weissbach, H. (1983). Biochemistry and physiological role of methionine sulfoxide residues in proteins. *Arch. Biochem. Biophys.* **223,** 271–281.

Caldwell, K. A., and Tappel, A. L. (1964). Reactions of seleno- and sulfoamino acids with hydroperoxides. *Biochemistry* **3,** 1643–1647.

Chang, K. C., Marshall, H. F., and Satterlee, L. D. (1982). Sulfur amino acid stability. Hydrogen peroxide treatment of casein, egg white, and soy isolate. *J. Food Sci.* **47,** 1181–1183.

Chang, K. C., Kendrick, J. G., Marshall, H. F., and Satterlee, L. D. (1985). Effect of partial methionine oxidation on the nutritional quality of soy isolate and casein. *J. Food Sci.* **50,** 849–850.

Crawford, L., Finley, J. W., and Robbins, K. R. (1984). Bioavailability of some oxidized cystine derivatives. *Nutr. Rep. Int.* **29,** 791–795.

Cunningham, F. E., and Cotterill, O. J. (1962). Factors affecting alkaline coagulation of egg white. *Poult. Sci.* **41,** 1453–1461.

Cuq, J. L., Provansal, M., Guilleux, F., and Cheftel, C. (1973). Oxidation of methionine residues of casein by hydrogen peroxide. Effects on *in vitro* digestibility. *J. Food Sci.* **38,** 11–13.

Cuq, J. L., Besancon, P., Chartier, L., and Cheftel, C. (1978). Oxidation of methionine residues of food proteins and nutritional availability of protein-bound methionine sulfoxide. *Food Chem.* **3,** 85–102.

Daniel, R. G., and Waisman, H. A. (1969). Adaptation of the weanling rat to diets containing excess methionine. *J. Nutr.* **98,** 299–305.

Ejiri, S., Weissbach, H., and Brot, N. (1979). The conversion of methionine sulfoxide to methionine in *Escherichia coli* and rat tissue. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **38,** 829.

Ellinger, G. M., and Palmer, R. (1968). The biological availability of methionine sulfoxide. *Proc. Nutr. Soc.* **28,** 42A.

Fomon, S. J., and Ziegler, E. E. (1979). Soy protein isolates in infant feeding. *In* "Soy Protein and Human Nutrition" (H. L. Wilcke, D. T. Hopkins, and D. H. Waggle, eds.), pp. 79–100. Academic Press, New York.

Ganapathy, V., and Leibach, F. H. (1982). Transport and utilization of methionine sulfoxide in the rabbit. *Biochim. Biophys. Acta* **693,** 305–314.

Gjoen, A. U., and Njaa, L. R. (1977). Methionine sulphoxide as a source of sulphur-containing amino acids for the young rat. *Br. J. Nutr.* **37,** 93–105.

Guy, G. J., Vettel, H. E., and Pallansch, M. J. (1968). Effects of hydrogen-peroxide treatment of skim milk for sponge bread baking. *Cereal Sci. Today* **13,** 434–447.

Higuchi, M., Iwami, K., Nakamura, A., Yasumoto, K., and Iwai, K. (1982). *In vitro* and *in situ* absorption of methionine sulfoxide in rat small intestine. *Agric. Biol. Chem.* **46,** 2533–2538.

Iwami, K., Nakamura, A., Higuchi, M., Yasumoto, K., and Iwai, K. (1983). Evaluation of L-methionine sulfoxide bioavailability by sulfur incorporation into liver glutathione and urinary excretion as inorganic sulfate. *Agric. Biol. Chem.* **47,** 2555–2559.

Karel, M., Schaich, K., and Roy, B. R. (1975). Interaction of peroxidizing methyl linoleate with some proteins and amino acids. *J. Agric. Food Chem.* **23,** 158–163.

Kasai, T., Sakamura, S., and Sakamoto, R. (1971). Amino acid composition of green gram (*Phaseolus radiatus h.* var. *typicus* Prain). Part II. Contents of free amino acids, γ-glutamyl peptides and protein amino acids in green gram seeds and seedlings. *Agric. Biol. Chem.* **35,** 1607–1609.

Kasai, T., Sakamura, S., and Sakamoto, R. (1972). Isolation identification of γ-l-glutamyl-L-methionine from green gram seed. *Agric. Biol. Chem.* **36,** 967–969.

Kikuchi, Y., and Tamiya, N. (1981). Methionine sulfoxide in the resilium protein of surf clams. *J. Biochem. (Tokyo)* **89,** 1975–1976.

Kuzmicky, D. D., Kohler, G. O., Walker, H. G., Jr., and Mackey, B. E. (1977). Availability of oxidized sulfur amino acids for the growing chick. *Poult. Sci.* **56,** 1560–1565.

Lavine, T. F. (1947). The formation, resolution, and optical properties of the diastereoisomeric sulfoxides derived from L-methionine. *J. Biol. Chem.* **169,** 477–491.

Lee, Y. C., Hayes, M. G. J., and McCormick, D. B. (1970). Microsomal oxidation of α-thiocarboxylic acids to sulfoxides. *Biochem. Pharmacol.* **19,** 2825–2832.

Lemoine, F., Waller, J. P., and Van Rapenbusch, R. (1968). Studies on methionyl-transfer RNA synthetase. 1. Purification and some properties of methionyl transfer RNA synthetase from *Escherichia coli* K-12. *Eur. J. Biochem.* **4,** 213–221.

Lewis, G. T., and Lewis, H. B. (1926). The metabolism of sulfur. XI. Can taurine replace cystine in the diet of the young white rat? *J. Biol. Chem.* **69,** 589–598.

Lindsay, R. C. (1985). Food additives. *In* "Food Chemistry" (O. R. Fennema, ed.), pp. 663–664. Dekker, New York.

Marable, N. L., Todd, J. M., Korslund, M. K., and Kennedy, B. W. (1980). Human urine and/or plasma levels of methionine, methionine sulfoxide and inorganic sulfate after consumption of a soy isolate. *Qual. Plant.—Plant Foods Hum. Nutr.* **30,** 155–161.

Marable, N. L., Korslund, M. K., Kehrberg, N. L., Kennedy, B. W., Kerste Her, J. E., and Clevidence, B. A. (1981). Human plasma amino acids after consumption of methionine sulfoxide in soy isolate or infant formula. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **40,** 848.

Marshall, H. F., Chang, K. C., Miller, K. S., and Satterlee, L. D. (1982). Sulfur amino acid stability. Effects of processing on legume proteins. *J. Food Sci.* **47,** 1170–1174.

Meyer, E. W., and Williams, L. D. (1977). Chemical modification of soy proteins. *In* "Food Proteins—Improvement through Chemical and Enzymatic Modification" (R. E. Fenney and J. R. Whitaker, eds.), pp. 52–66. American Chemical Society, Washington, D.C.

Miller, D. S., and Samuel, P. (1968). Methionine sparing compounds. *Proc. Nutr. Soc.* **27,** 21A–22A.

Miller, D. S., and Samuel, P. D. (1970). Effects of the addition of sulfur compounds to the diet on utilization of protein in young growing rats. *J. Sci. Food Agric.* **21,** 616–618.

Miller, S. A., Tannenbaum, S. R., and Seitz, A. W. (1970). Utilization of L-methionine sulfoxide by the rat. *J. Nutr.* **100,** 909–915.

Moore, S. (1963). On the determination of cystine as cysteic acid. *J. Biol. Chem.* **238,** 235–237.

Njaa, L. R. (1962). Utilization of methionine sulfoxide and methionine sulfone by the young rats. *Br. J. Nutr.* **16,** 571–577.

Njaa, L. R., and Aksnes, A. (1982). The nitrogen-sparing effect of methionine sulphoxide and some other sulphur-containing amino acids. *Br. J. Nutr.* **48,** 565–570.

O'Keefe, L. S., and Wartheson, J. J. (1978). A high pressure liquid chromatographic method for determining the stability of free methionine in methionine-fortified food systems. *J. Food Sci.* **43,** 1297–1300.

Otoul, E., Marechal, R., Dardenne, G., and Desmedt, F. (1975). Des dipeptides soufres differencient nettement *Vigna radiata* de *Vigna mungo. Phytochemistry* **14,** 173–179.

Perry, T. L., Hardwick, D. F., Dixon, G. H., Dolman, C. L., and Hansen, S. (1965). Hypermethioninemia: A metabolic disorder associated with cirrhosis, islet cell hyperplasia, and renal tubular degeneration. *Pediatrics* **36,** 236–250.

Pusztai, A., and Watt, W. B. (1974). Isolectins of *Phaseolus vulgaris:* A comprehensive study of fractionation. *Biochim. Biophys. Acta* **365,** 57–71.

Rao, P. B. R., Norton, H. W., and Johnson, B. C. (1961). The amino acid composition and nutritive value of proteins. IV. Phenylalanine, tyrosine, methionine and cystine requirements of the growing rat. *J. Nutr.* **73,** 38–42.

Rasekh, J., Stillings, B. R., and Sidwell, V. (1972). Effect of hydrogen peroxide on the color, composition and nutritive quality of fish protein concentrate. *J. Food Sci.* **37,** 423–425.

Roundy, Z. (1958). Treatment of milk for cheese with hydrogen peroxide. *J. Dairy Sci.* **41,** 1460–1465.

Satterlee, L. D., and Chang, K. C. (1982). Nutritional quality of deteriorated proteins. *In* "Food Protein Deterioration—Mechanism and Functionality" (J. Cherry, ed.), ACS Symp. Ser. 206, pp. 409–431. American Chemical Society, Washington, D.C.

Sjoberg, L. B., and Bostrom, S. L. (1977). Studies in rats on the nutritional value of hydrogen peroxide treated fish protein and the utilization of oxidized sulphur amino acids. *Br. J. Nutr.* **38,** 189–205.

Slump, P., and Schreuder, H. A. W. (1973). Oxidation of methionine and cystine in foods treated with hydrogen peroxide. *J. Sci. Food Agric.* **24,** 657–661.

Smith, R. C. (1972). Acetylation of methionine sulfoxide and methionine sulfone by the rat. *Biochim. Biophys. Acta* **261,** 304–309.

Stegink, L. D., Bell, E. F., Filer, L. J., Jr., Ziegler, E. E., Anderson, D. W., and Seligson, F. H. (1986). Effects of equimolar doses of L-methionine, D-methionine and L-methionine-dl-sulfoxide on plasma and urinary amino acid levels in normal adult humans. *J. Nutr.* **116,** 1185–1192.

Stipanuk, M. H., and Rotter, M. A. (1984). Metabolism of cysteine, cysteine sulfinate and cysteine sulfonate in rats fed adequate and excess levels of sulfur-containing amino acids. *J. Nutr.* **114,** 1426–1437.

Strange, E. D., Benedict, R. C., and Miller, A. J. (1980). Effect of processing variables on the methionine of frankfurters. *J. Food Sci.* **45,** 632–637.

Struthers, B. J., Dahlgren, R. R., Hopkins, D. T., and Raymond, M. L. (1979). Lysinoalanine: Biological effects and significance. *In* "Soy Proteins and Human Nutrition" (H. L. Wilcke, D. T. Hopkins, and D. H. Waggle, eds.), pp. 236–260. Academic Press, New York.

Tannenbaum, S. R., Barth, H., and LeRoux, J. P. (1969). Loss of methionine in casein during storage with autoxiding methyl linoleate. *J. Agric. Food Chem.* **17,** 1353–1354.

Todd, J. M., Marable, N. L., and Kehrberg, N. L. (1984). Methionine sulfoxide determination after alkaline hydrolysis of amino acid mixtures, model protein systems, soy products, and infant formulas. *J. Food Sci.* **49,** 1547–1551.

Toennies, G., and Kolb, J. J. (1939). Methionine studies. II. dl-Methionine sulfoxide. *J. Biol. Chem.* **128,** 399–405.

Tufte, M. C., and Warthesen, J. J. (1979). Methionine stability in methionine-fortified model food system as influenced by method of methionine incorporation and by lipid oxidation. *J. Food Sci.* **44,** 1767–1771.

Yanagita, T., Enomoto, N., and Sugano, M. (1984). Hepatic triglyceride accumulation as an index of the bioavailability of oxidized methionine to the growing rat. *Agric. Biol. Chem.* **48,** 815–816.

Zacharius, R. M. (1970). Composition of the nitrogeneuous components of the bush bean seed (*Phaseolus vulgaris*) including isolation of δ-acetylonithine. *Phytochemistry* **9,** 2047–2051.

Zezulka, A. Y., and Calloway, D. H. (1976). Nitrogen retention in men fed isolated soybean protein supplemented with L-methionine, D-methionine, *N*-acetyl-L-methionine or inorganic sulfate. *J. Nutr.* **106,** 1286–1291.

4

Antinutritive Effects of Phytate and Other Phosphorylated Derivatives

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I. CHEMICAL ASPECTS

Phytic acid is the focus of research in several fields related to its chemical reactions with polyvalent cations. Interests include occurrence and functions in plant seeds, nutritional significance, preservative applications in food technology, and potential medical and industrial uses. These topics are treated by Cosgrove (1980) and Graf (1986).

* Deceased.

A. Structure and Properties

Phytic acid is *myo*-inositol hexakisphosphate, which exists in the chair form in dilute acidic solutions. The phosphate groups can be removed hydrolytically by enzymes or heat to yield a large number of homologs and positional isomers ranging from inositol monophosphate (IP) to inositol pentakisphosphate (IP_5). The term *phytin,* which appeared in the early literature, apparently referred to the mixed calcium–magnesium salt.

Phytic acid is relatively unstable but readily complexes with all polyvalent cations to form compounds that are quite stable. This topic, in the context of biological milieus, has been extensively reviewed (Cheryan, 1980; Cosgrove, 1980; Wise, 1983; Kratzer and Vohra, 1986). Many of these complexes are relatively insoluble, which may account for a decrease in intestinal absorption of minerals. Phytic acid can complex a cation within a single phosphate group or between two phosphate groups on either the same or different molecules. If a ring structure forms, it is a chelate.

In foods, the binding of phytate to cations is complicated by binding to proteins. At low pH there is strong electrostatic binding of phytic acid at the terminal α-amino group, the ϵ-amino group of lysine, the imidazole group of histidine, and the guanidyl groups of arginine (reviewed by Cheryan, 1980; Anderson, 1985). The complex is insoluble and redissolves only at about pH 3 or below; however, as the pH is raised to the isoelectric point, the charge on the protein is neutralized, and phytate is released.

The solubility of the protein increases as the pH is raised toward neutrality or higher; and, beginning above pH 6, a ternary cation–phytic acid complex is formed that has increasing stability up to pH 10. Under these conditions, cations (usually calcium, magnesium, and zinc) form a bridge between the carboxyl groups of the protein and phytate. Experimentally these complexes have been disrupted by high ionic strength, high pH (greater than 10), and high concentrations of the chelating agent ethylenediaminetetraacetic acid (EDTA). These generalities may vary with specific proteins and require further study.

The phytate–protein complexes effect changes in protein structure that can decrease solubility and vulnerability to attack by proteolytic enzymes. The composition of a seed and the manufacturing processes that concentrate the protein profoundly affect the final mineral, phytate, and protein composition of the food. These changes can adversely impact mineral bioavailability (Erdman and Forbes, 1981) and protein quality (Anderson, 1985). Tests to evaluate protein quality can be adversely affected by phytate inhibition of trypsin, which has been shown *in vitro* (Singh and Krikorian, 1982).

B. Methods for Separation and Quantitation

Methods for isolating and quantitating phytic acid and the lower inositol phosphates have been extensively reviewed (Cosgrove, 1980; Cheryan, 1980;

Oberleas and Harland, 1986). Briefly, mixtures of inositol phosphates have been separated from other components in an acidic sample extract and from each other (usually by number of phosphate groups only) by paper chromatography, paper electrophoresis, thin-layer chromatography, and ion-exchange chromatography.

Phillippy *et al.* (1987) described an ion-exchange method for preparing several hundred milligrams to several grams of *myo*-inositol bis- (IP_2), tris- (IP_3), tetrakis- (IP_4), and pentakisphosphates from 10 g sodium phytate. Separations were made after partial hydrolysis either by phytase or by autoclaving an acidic solution of sodium phytate. Identification of fractions was made by phosphorus analysis and mass spectrometry.

Quantitative methods for determining phytic acid often employ the addition of a controlled amount of Fe(III) to an acidic sample extract to precipitate the phytic acid. Subsequently, the iron and/or phosphorus in the precipitate or the excess iron are determined. Ion-exchange columns can be used to separate phytic acid from other components of the extract. Other useful techniques include high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR). Hydrolysis by phytase, followed by inositol and phosphorus determinations, can be valuable in quantitating the lower inositol phosphates. An official first action method for phytate analysis has been accepted by the Association of Official Analytical Chemists (Harland and Oberleas, 1986).

II. DISTRIBUTION IN FOODS

A. Natural Occurrence

Phytate occurs in highest concentrations in seeds, typically 1–2% of dry weight (reviewed by Cheryan, 1980; Reddy *et al.*, 1982). Much lower concentrations can be found in some roots, tubers, and fruits. Phytate is often localized in certain seed structures, with wide variations among plant species. For example, most of the phytate in corn is concentrated in the germ whereas that in wheat and rice is in the outer layers of the seed. Phytate in soybeans is not localized. Phytate can be synthesized by bacteria. The metabolism of phytate in plants has been reviewed by Scott and Loewus (1986).

B. Effects of Food Processing

The phytate content of foods can be markedly influenced by processing, such as milling cereal grains. Of contemporary concern is the large food industry involved in the concentration of protein from seeds, particularly from soybeans, to supplement or replace meat products. These processes include adjustments in pH, aqueous washes, drying, and sometimes hot pressure extrusion to give a wide range of products that vary in protein, fiber, phytic acid, and essential minerals. These processes can have profound influences on mineral bioavaila-

bility (reviewed by Smith and Circle, 1978; Wilcke *et al.*, 1979; and Altschule and Wilcke, 1985).

Other food processes cause hydrolysis of phytate to yield either lower inositol phosphates or inorganic phosphate and *myo*-inositol. These processes include fermentation, such as the action of yeast in breadmaking, and heat under aqueous conditions. Although disappearance of phytate has been reported during these processes, there is little information about the formation of other inositol phosphates. Nayini and Markakis (1983a,b) observed increases in P_i and IP through IP_5 as the phytate decreased in bread, following fermentations of up to 3 hr. They also found the lower inositol phosphates in commercial bread.

Phillippy *et al.* (1988) observed that the total inositol phosphates (mmol/kg dry weight) in nine thermally processed or fermented foods ranged from 1.35 in white bread to 23.26 in tofu and 26.05 in soy isolate. Phytic acid accounted for more than 40% on a molar basis of the total inositol phosphates in all of the foods studied. The predominant species after phytic acid was IP_5. IP_2 and IP_3 were quite low in some foods and could not be quantitated accurately.

C. Dietary Phytate Intakes

Oberleas and Harland (1981) estimated daily intakes of 290 and 2575 mg phytate for nonvegetarian and vegetarian menus, respectively. Ellis *et al.* (1982) analyzed 7-day hospital diets (2800 kcal/day) and reported values of 387, 440, and 1144 mg phytic acid/day for regular, ovo-lacto-vegetarian, and soy meat-substitute diets. Female and male nutrition students in Scotland consumed 501 and 842 mg phytate/day, respectively, in self-selected diets (Wise *et al.*, 1987). Bindra *et al.* (1986) found 423 and 788 mg phytate/1000 kcal daily in omnivore and vegetarian diets, respectively. Freeland-Graves *et al.* (1980) designed a lacto-ovo-vegetarian diet for women that supplied 4638 mg phytate/day.

III. BIOLOGICAL EFFECTS

A. Absorption and Metabolism of Dietary Phytate

The absorption of phytate cannot be evaluated accurately by balance techniques due to hydrolysis of phytic acid by enzymes in the gut and to synthesis by intestinal bacteria. Nahapetian and Young (1980) gave either [^{14}C]phytate or [^{14}C]*myo*-inositol to rats that had received either a low-calcium or a high-calcium diet for 6 weeks. The high-calcium diet markedly increased ^{14}C-activity from the labeled phytate in the feces but had no effect on fecal excretion of carbon-14 from the labeled *myo*-inositol. With the low-calcium diet, 60% of the carbon-14 from labeled phytate was oxidized to CO_2. This study leaves unanswered the question of whether phytate *per se* was absorbed.

B. Mineral Bioavailability

The chief adverse effects of dietary phytate are due to decreased bioavailability of essential minerals, principally zinc, calcium, ion, and manganese. These effects have been shown unequivocally with phytate incorporated in purified diets fed to experimental animals; however, the effects of phytate in foods are more difficult to assess due to the presence of other components such as fiber, minerals, and amino acids, which may also affect the bioavailability of the element under study. Table I provides a simplified overview of conditions and factors that can modify phytate–element interactions obtained with typical diets. A modification, either negative or positive, is denoted by a plus; however, the absence of a plus may mean that the condition or factor either had no effect or had not been studied. The data are taken from studies with animals and humans.

Definitions of bioavailability of essential elements usually include evaluation of absorption of the element and its utilization for essential metabolic and structural functions (Fox *et al.*, 1981; O'Dell, 1984). Bioavailability of a toxic element requires evaluation of absorption and production of adverse physiological effects. *In vitro* methods have been used to investigate phenomena in the gut lumen that can affect bioavailability.

TABLE I

Principal Conditions and Factors Modifying Interactions between Phytate and Essential Elements[a]

Modifying conditions and factors	Essential elements						
	Zn	Fe	Mn	Cu	P	Ca	Mg
Dietary components							
Vitamins		+			+	+	
Ca	+	+		+	+		
Other essential elements		+			+		+
Protein/amino acids	+	+		+	+		
Fiber	+	+			+		
Phytase					+		
Food processing	+				+	+	+
Experimental design							
Response index	+						
Duration	+		+				
Other	+						
Physiological factors							
Age	+				+		
Species	+				+		

[a] + indicates modification of a phytate-essential element response reported in one or more papers included in this review. For details and references see text.

1. Zinc

Among the essential minerals, the utilization of zinc appears to be most adversely affected by phytate. Recognition of this effect in humans evolved from extensive studies in the Mideast of conditions in which there was delayed sexual development and growth retardation (Prasad *et al.*, 1963; Sandstead *et al.*, 1967; Ronaghy *et al.*, 1969; Halsted *et al.*, 1972). The main dietary staple was whole grain bread and (in some countries) pulses, both of which supply significant amounts of phytate.

O'Dell and Savage (1960) achieved a significant scientific breakthrough when they showed that phytate could decrease zinc bioavailability in animals. Turnlund *et al.* (1984) reported that adding 2.34 g phytate (sodium phytate)/day to a liquid formula diet decreased zinc absorption of young men from 34.0 to 17.5%.

Davies and Nightingale (1975) and Flanagan (1984) reported evidence that dietary phytate could increase losses of endogenous zinc from the gut. Several dietary components can affect the efficiency of zinc absorption in the presence of phytate, the most important of which is calcium. Complexes of phytate, calcium, and zinc are more insoluble than those of phytate and zinc at the pH of the small intestine (Oberleas *et al.*, 1966). Data from *in vitro* systems show that significant amounts of zinc can be removed from phytate–calcium–zinc complexes by EDTA (Oberleas *et al.*, 1966), cysteine hydrochloride, histidine, and picolinic acid (Wise and Gilburt, 1982a). The effects of synthetic and naturally occurring chelating agents have been shown to increase zinc bioavailability with diets containing added or endogenous phytate and varying dietary calcium levels (reviewed by Kratzer and Vohra, 1986).

From many types of studies, it was logical to suggest that molar ratios of dietary phytate to zinc would have some predictive value in assessing the potential hazard of phytate in the diet (Oberleas, 1975). Oberleas and Harland (1981) published data on phytate, zinc, and phytate : zinc molar ratios in a range of foods. Because calcium so markedly enhances the phytate effect in lowering zinc bioavailability, Davies *et al.* (1985) proposed the use of a (phytate) (calcium)/(zinc) molar ratio [(P)(Ca)/(Zn)] to predict the net effect on zinc utilization.

Tao *et al.* (1986) fed Japanese quail from hatching to 7 days of age a diet containing only one inosital phosphate, either IP_3, IP_4, IP_5, or IP_6. When the diet contained 8.33 mmol of the respective inositol phosphate/kg (i.e., 5.5 g phytic acid/kg), body weight, feather quality, tibia zinc, and pancreas zinc were decreased similarly with IP_5 and IP_6, but were not decreased with IP_3 or IP_4. Inositol and inorganic phosphate had no effect. When the concentration of inositol phosphates was doubled, IP_3 caused increased tibia zinc and IP_4 caused decreased pancreas zinc. Lönnerdal *et al.* (1987) dosed fasted suckling rat pups with zinc-65 and cold zinc at molar ratios of zinc : IP of 4 : 1. After 6 hr, the liver uptake of zinc was 29, 28, 19, 5, and 31% for IP_3, IP_4, IP_5, IP_6, and zinc

chloride with no IP, respectively. There are many possible reasons for the difference in the effects of IP_5 on zinc as determined by the two studies that merit further investigation. Table II summarizes the effects of the inositol phosphates on zinc, manganese, and calcium.

An extensive literature deals with the bioavailability of zinc in foods that contain phytate. Many of the animal studies have been reviewed by Erdman (1979), O'Dell (1979), and Davies (1982). Early studies using Iranian bread focused on the effects of phytate on zinc bioavailability. Two of three young men who received approximately 2.5 g phytic acid/day supplied by whole wheat unleavened Iranian flat bread (tanok) had zinc balances that were more negative than when they received the equivalent amount of sodium phytate in the same diet with white bread (Reinhold *et al.*, 1973). Zinc balances were positive for all subjects during control periods when phytate intake was relatively low. In a subsequent balance study, Reinhold *et al.* (1976b) fed two men 500 g of a sourdough-leavened Iranian bread (bazari) prepared from 80 to 90% extraction wheat flour that supplied 1.4 g phytic acid. The mean total acid-detergent fiber of the control diet (white bread) was 21.9 g/day and of the diet with bazari was 29.7 g/day for one subject and 34.0 g/day for the other. Zinc balances were positive with white bread but negative with bazari. The authors attributed the negative balances to the dietary fiber. Reinhold *et al.* (1976a) summarized their data to support their interpretation of high fecal zinc losses as being due to phytate and at least partially due to fiber as a determinant of zinc deficiency in many Iranians. More recently, Davies (1982) reviewed human and animal studies on the effects of phytate and fiber on zinc bioavailability.

Sandström *et al.* (1980) investigated the effects of foods on absorption of

TABLE II

General Effects of Lower Inositol Phosphates on Interacting Essential Elements in Animals[a]

Interacting element	Adverse effect of inositol phosphate[b]			
	IP_3	IP_4	IP_5	IP_6
Zn	0	+ 0	+	+
Mn	0	0	+	+
Ca	−	−	+	+

[a]Adverse effects varied with dietary levels of interacting element and inositol phosphate. For details and references see text.

[b]+ indicates adverse effect of inositol phosphate to decrease bioavailability or increase requirement of interacting element; 0 indicates no effect; − indicates beneficial effect. Mixed symbols indicate variation in results among studies.

extrinsic zinc-65 by humans consuming meals of white bread and whole wheat bread. Absorption of zinc-65 from white bread alone, which contained only 0.4 mg total zinc, was 38.2%; however, when zinc chloride was added to increase the zinc to 3.6 mg, zinc absorption was 13.2%. With meals of whole wheat bread (1.3 mg endogenous zinc) or whole wheat bread plus zinc chloride (3.5 mg zinc), zinc absorption was 16.6 and 8.2%, respectively. Thus, at a given zinc intake, zinc was absorbed more efficiently from white bread than from whole wheat bread. Other meals contained 3.1–3.5 mg total zinc supplied by foods. Milk and cheese with white bread did not influence zinc absorption. With whole wheat bread, zinc absorption was slightly increased by consuming milk, beef, or egg as well; however, much greater proportions of the absorbed dose occurred with combinations of these foods. For whole wheat bread meals plus one or more foods, there was a significant correlation ($r = 0.85$, $P < 0.001$) between protein in the meal and zinc absorption. The amounts of calcium supplied by the meals that contained milk and/or cheese ranged from 292 to 547 mg whereas the meals of white bread and whole wheat bread contained only 11 and 19 mg calcium, respectively.

Several investigators have studied the effects of phytate in wheat bran and have attempted to separate the effects of phytate and fiber in bran and other foods on zinc bioavailability. Sandberg *et al.* (1982) observed approximately 50% decreases ($P < 0.001$) in zinc absorption using balance techniques when 16 g wheat bran was consumed daily compared with the control low-fiber diet of ileostomy patients. The low-fiber diet, and the wheat bran plus the low-fiber diet, supplied 792 and 2574 mg phytate/day, respectively. Andersson *et al.* (1983) fed white, brown , and wholemeal breads to five healthy subjects. Sodium phytate, zinc sulfate, ferrous sulfate, and calcium carbonate were added to provide constant concentrations of phytate, zinc, iron, and calcium. The breads, which were fed daily at breakfast for 24 days each, supplied about 1.4 g phytate/day. There were no effects of bread type on zinc balance.

In two balance studies, Morris and Ellis (1983, 1985b) fed muffins containing either wheat bran or wheat bran that had been dephytinized through the action of endogenous phytase. In the first study the whole incubation mixture was freeze-dried for use whereas in the second study the water-insoluble fraction after incubation was used. Two bran muffins were fed with each of the three daily meals. In the first study, the daily intakes of zinc were approximately 17 mg for both groups. The intakes of phytic acid were 2 g with the whole bran muffins and 0.2 g with the dephytinized bran muffins, which gave mean phytate/zinc molar ratios of 11.8 and 1.2, respectively. Bran muffin type did not affect zinc balance. Values for apparent zinc absorption with each type of bran muffin were greater for the last 10 days of each period than for the first 5 days. In the second study, six muffins were consumed each day for 15-day periods. All muffins contained dephytinized bran that, with the remaining diet, supplied 0.5 g phytic acid/day.

Sodium phytate was added to other muffins to provide either 1.7 or 2.9 g phytic acid/day. Mean daily intake of zinc was 10.6 mg, and the phytate/zinc molar ratios were 4.7, 16.3, and 17.5. Calcium intake was 750 mg/day. Phytate intake did not affect apparent zinc balance, and phytate-zinc molar ratios did not correlate with the apparent zinc absorption.

Nävert *et al.* (1985) prepared bread with wheat bran subjected to prior yeast leavening to reduce its phytate content. Zinc absorption was based on use of extrinsic zinc-65. Meals consisted of 200 g fermented milk, 10 g butter, 200 g deionized water, and 2 rolls containing 40 g wheat flour and 10, 16, or 30 g wheat bran, which was consumed either raw or baked into the rolls after 15 min, 45 min, 3 hr, or 16 hr of fermentation. Leavening of the bran reduced phytate in a time-dependent manner. With 10 g wheat bran, a 3 hr fermentation was required to reduce phytate enough (by approximately one-half) to permit improved zinc absorption compared with raw bran not incorporated in bread. When 30 g bran leavened for 16 hr was present in bread, the total phytic acid content was similar to that in 10 g unleavened bran, and percent zinc absorption was similar. In the absence of bran in the bread, equivalent phytate (as sodium phytate) and zinc (as the sulfate) gave zinc absorption values that were similar to those with bran.

Sandström *et al.* (1987a) investigated the absorption of zinc-65 extrinsic label in subjects who received a meal of 200 ml milk and 60 g of a test cereal. Rye, barley, oatmeal, triticale, and whole wheat were fed as bread, porridge, or commercially prepared oatmeal flakes. Zinc absorption was negatively correlated ($P < 0.001$) with phytate content of the meal.

Extrusion cooking reduced apparent zinc balance in ileostomy patients who were otherwise normal (Kivistö *et al.*, 1986). Subjects consumed the same low-fiber diet plus 32.4 g wheat starch, 16.2 g wheat bran, and 5.4 g gluten for 4 days. For the second 4-day period, the starch–bran–gluten mixture was cooked under mild extrusion conditions. The concentrations of fiber and phytate were not affected; however, phytase was destroyed (Sandberg *et al.*, 1986). Consumption of the extruded mixture decreased apparent zinc absorption from 2 to 1.2 mg/24 hr ($P < 0.05$).

The effects of phytate in soy on zinc bioavailability have also been studied. Golden and Golden (1981) compared soy-based with cow's milk-based formulas in severely malnourished children who have been partially rehabilitated for 4–12 weeks following hospital admission. The soy formula contained 52 μmol zinc and 1.33 μmol phytic acid/liter. The cow's milk formula contained 69 μmol zinc/liter. Children received their assigned formula until their weight gain slowed as they approached normal rates and was steady for 7 days. Then supplemental zinc (as the acetate) was added to the formula four times per day to supply 25–150 μmol zinc/kg body weight per day. The supplements, which were higher for soy-fed infants, did not increase *ad libitum* formula consumption.

After supplementation, all children showed increases in rate of weight gain, regrowth of the thymus, and activation of the sodium pump. Plasma zinc values were lower with soy than with cow's milk formula before, but not after, zinc supplementation. All values rose after supplemental zinc. The greater the rise in plasma zinc, the greater the increase in the efficiency of weight gain, and the increases were greater in soy-fed children. The children receiving soy formula had to achieve an increment of 2.3 μmol zinc/liter plasma greater than children fed cow's milk formula for a corresponding decrease in energy cost for tissue deposition.

The absorption of extrinsic zinc-65 from various types of milk and infant formulas by healthy adults was studied by Sandström *et al.* (1983). The test foods included pooled pasteurized human milk, pasteurized homogenized cow's milk with 3% fat, humanized cow's milk (casein–whey protein ratio, 40 : 60) with lactose, oatmeal–wheat flour–cow's milk formula (cereal protein–cow's milk protein ratio, 25 : 75) with lactose and starch, and soy isolate formula with dextrin–maltose, glucose, and rice starch. The cereal–cow's milk formula was designed for use by children over 4 months old. The cereal–milk and soy formulas contained 200 mg phytic acid/liter; however, the calcium content of the cereal–cow's milk formula was almost twice that of the soy formula, 1030 vs. 550 mg calcium/liter. The serving size for the cereal–cow's milk formula was 525 ml and 450 ml for all others.

Compared with all other foods, absorption of zinc from cow's milk formula (41%) was higher and that from soy isolate formula (14%) was lower. There were no differences between cow's milk and humanized cow's milk formulas; however, zinc absorption from the cereal–milk formula was lower than that for the humanized cow's milk formula. Phytic acid in the soy and cereal–milk formulas (200 mg/liter) probably contributed to the low zinc absorption with these formulas; however, absorption from the soy formula was lower than that from the cereal–milk formula. The concentration of citrate in human milk, and in cow's milk and cereal–milk formulas was increased by 2 mM by adding aqueous potassium citrate solution just before serving. These increases, which were approximately 2/3, 1/3, and 1/3, respectively, had no effect on zinc absorption.

With the same techniques, Lönnerdal *et al.* (1984) reported that substitution of lactose for dextrin, maltose, glucose, and starch in the soy formula did not affect zinc absorption in healthy young adults. A soy flour formula contained approximately twice as much phytate as the soy isolate formula, and zinc absorption values were 8.2 and 14.0%, respectively. Absorption of zinc from a cow's milk formula was 32.2%; however, when sodium phytate was added to give phytate values approximating those of the soy isolate and soy flour formulas, zinc absorption was similar to that with the soy formulas. More than doubling the calcium content (as $CaCl_2$) did not affect zinc absorption with cow's milk formula but effected a modest increase with soy formula. This result is at variance with a large body of literature showing that increasing calcium in the presence of

phytate decreases zinc absorption. Further investigation is needed. Adding iron (19 vs. 2.2 mg total iron/liter) did not affect zinc absorption with cow's milk formula; however, removing supplemental iron (3.9 vs. 9 mg total iron/liter) from the soy formula decreased zinc absorption to 9.6 from 14.0% ($P < 0.001$). This result is in disagreement with data showing decreased zinc absorption with higher iron intake. Citrate added to the soy formula to increase the concentration by 25 mM did not affect zinc absorption. This study and that of Sandström *et al.* (1983) provide important information; however, there may be some relative differences in zinc absorption between adults and infants with these infant foods.

Sandström and Cederblad (1980) used an extrinsic label of zinc-65 to determine absorption of zinc from mixed meals with protein supplied by chicken, beef, or soybeans. For the meat-containing meals (without defatted soy flour or soybeans) either with or without zinc added to the chicken or beef, zinc intakes ranged from 1.3 to 8.1 mg. There was a significant correlation ($P < 0.001$) between milligrams of zinc in the meal and milligrams of zinc absorbed. Although statistically significant differences were presented only for milligrams of zinc absorbed, it appears, from means ± SD and ranges, that 3.3 mg zinc added to either chicken or beef decreased the percentage of zinc absorbed whereas the milligrams of zinc absorbed increased. This greatly limits interpretation of the data because there were few meals that had the same zinc content.

Of the meals that supplied similar amounts of zinc, the following conclusions can be drawn. There were no significant differences between chicken (1.3 mg zinc) vs. chicken with 25% protein replaced by defatted soy flour (1.6 mg zinc), chicken plus beef (2.4 mg zinc) vs. soybeans (2.5 mg zinc), and chicken plus zinc (4.5 mg zinc) vs. beef (4.6 mg zinc). The results suggest that a small ligand in chicken meat (such as one or more amino acids or peptides formed during digestion) might increase zinc absorption. There was no evidence for a corresponding effect with beef; however, interpretation is complicated by differences in zinc intake. When 125 ml milk replaced an equivalent amount of protein in the soybean meal, zinc absorption was decreased from 0.49 to 0.38 mg. This was probably due to an increase in calcium from 90 to 220 mg, even though phytate decreased from 620 to 480 mg. Depending on a person's diet, this difference is probably of no practical significance. This study demonstrates some of the problems that can occur in assessing the bioavailability of an element in foods.

Vegetarians generally consume diets that are elevated in phytate and fiber. Harland and Peterson (1978) obtained 28-day menus for 16 adult male vegetarians. They calculated the following daily ranges of nutrient intake: zinc 5–18 mg, calcium 1296–5249 mg, phytate 615–5770 mg, phytate/zinc molar ratios 3.39–115, and crude fiber 4–16 g. Three subjects had fasting serum zinc values below 70 μg/dl, generally considered to represent low zinc status. All three of these subjects had low intakes of calcium and zinc, and two of the three had elevated fiber intakes; however, none of their values were extremes for the group.

This incidence of low zinc status is considerably higher than the values reported for United States males in the Second National Health and Nutrition Examination Survey (Pilch and Senti, 1984b). For men 20–44 years of age, 2167 subjects, 0.8% had low zinc status; and for men 45–64 years of age, 1656 subjects, the incidence was 1.4%. The criteria for low zinc status were based on serum zinc values <70 μg/dl for fasting a.m. samples, <65 μg/dl for other a.m. samples, and <60 μg/dl for p.m. samples.

Soy flour, soy concentrate, and soy isolate were used to replace part of the protein in three types of meals by Sandström *et al.* (1987b). At isonitrogenous substitution levels, soy flour supplied the most zinc, soy concentrate was intermediate, and soy isolate supplied one-half that of soy flour. The phytic acid supplied by soy flour and soy protein concentrate was similar; however, that supplied by soy isolate was about one-half that of the other two. When soy products replaced either 30 or 100% of the protein in meat sauce, the percentage of zinc absorbed was reduced with soy flour and soy concentrate but not with soy isolate. Two hundred milliliters of milk with soy flour or soy isolate decreased zinc absorption compared with all-soy proteins. When one-half of the protein in white bread was replaced by soy products, zinc absorption was reduced. Zinc absorption with whole meal bread was less than that with white bread. Replacement of one-half the whole meal bread protein with the soy product protein had no effect on zinc absorption. The percentage of zinc absorbed was related to phytate/zinc molar ratios between 2 and 17. Zinc absorption from the meat sauce meals with phytate/zinc molar ratios above 20 was not further depressed. These results may be of practical importance but are difficult to interpret due to variations in nutrient and phytic acid content of the meals.

Freeland-Graves *et al.* (1980) fed a lacto-ovo-vegetarian diet to nonvegetarian women for 22 days. The menus were designed to meet or exceed recommended dietary allowances (RDAs) of the Food and Nutrition Board (National Research Council, 1980). The daily diet supplied 86 g protein, 7.8 g crude fiber, 1765 mg calcium, 15 mg zinc, and 4638 mg phytic acid. Changes between initial and final measurements that were indicative of impaired zinc status due to the lacto-ovo-vegetarian diet were decreased zinc in saliva sediment (primarily epithelial cells), and increased plasma zinc uptake and increased area under the curve for plasma zinc (0–4 hr) following an oral dose of 50 mg zinc as the sulfate.

High intakes of calcium and phytate were reported for lacto-ovo-vegetarians by Bindra *et al.* (1986). They obtained 3-day dietary records of weighed foods consumed by 59 adult males and 53 adult females who were from 53 Punjabi Sikh families residing near Guelph, Ontario, Canada. Foods from randomly selected daily menus for 15 males and 15 females were prepared in the laboratory by the traditional methods of the study subjects. These and 30 diets from a previous study for female omnivores were analyzed. The vegetarian and omnivore diets supplied (per 1000 kcal) 568 and 422 mg calcium, 4.5 and 5.1 mg

zinc, 788 and 423 mg phytate, and 14.5 and 16.5 g neutral detergent fiber, respectively. The principal source of phytate in the vegetarian diet was chapatis, an unleavened bread prepared from whole wheat flour. Cereals provided most of the dietary zinc, and milk and milk products provided most of the calcium. The mean fasting serum zinc values for the vegetarian subjects were 83.7 and 77.5 μg/dl for the males and females, respectively. Twenty-two females (40%) and 14 males (24%) had serum zinc values below 70 μg/dl, indicating a significant incidence of impaired zinc status. The incidence of impaired zinc status in United States males was described previously (Pilch and Senti, 1984b). For females 20–44 years of age, 1711 subjects, 2.4% had impaired zinc status; and for those 45–64 years of age, 1822 subjects, 2.6% had impaired status. The younger age group excludes pregnant and lactating women and those using oral contraceptives, conditions known to decrease serum zinc values independent of actual zinc status.

Davies and Warrington (1986) reported high concentrations of phytic acid in pulses and high-extraction wheat flours used for preparation of chapatis by Asian immigrants residing in the United Kingdom. After various types of preparation, phytic acid usually remained high in these foods, suggesting that zinc nutriture may be in some jeopardy.

These investigations show conclusively that phytate can interfere with zinc bioavailability. There is, however, some lack of agreement among studies, particularly with respect to specific foods and their components. Calcium clearly augments the adverse effects of phytate on zinc, and numerous other dietary components have lesser effects, both beneficial and adverse. The interpretation of data with extrinsic labels of either stable or radioactive isotopes generally cannot be considered definitive, particularly with processed plant protein foods. The utilization of extrinsic zinc may sometimes be more indicative of zinc bioavailability from other foods in mixed diets than that of the phytate-containing food itself. For human populations that exhibit mild or more severe zinc deficiencies, it appears that phytate is often fairly high in a diet that also contains only small amounts of meat but may contain significant amounts of milk and milk products. Fortunately, such diets may be fairly rich in trace elements such as zinc.

2. *Iron*

Unlike the studies of phytic acid with zinc, a great deal of controversy exists in the literature regarding the effect of phytic acid on iron bioavailability.

Over 40 years ago, McCance *et al.* (1943) demonstrated in nine human adults that iron absorption, as measured by the rise of serum iron after a meal, was reduced by sodium phytate incorporated into the bread of a breakfast meal with ferrous or ferric ammonium sulfate. A large dose of soluble iron salt (8 mg Fe/kg

body weight) was used to produce a significant rise in serum iron concentration, and sodium phytate was added so that the phytate phosphorus was twice that needed to combine all the iron in the same meal.

Sharpe *et al.* (1950) showed that adolescent boys absorbed only one-fifth of a dose of radioiron with a breakfast meal (milk, rolled oats, tomato juice, white bread, and omelet) as iron with water. Phytate (200 mg) added as sodium phytate to milk caused a five times greater decrease in iron absorption than phytate added as oatmeal. In this study all subjects received a daily vitamin supplement containing 50 mg ascorbic acid; however, it is not known whether it was taken with the test meal. In contrast, Walker *et al.* (1948) found that iron retention in humans consuming a 95–100% extraction brown bread meal was virtually the same as that with a 70% extraction white bread. In the study by Sharpe *et al.* (1950), reducing the phytate content of rolled oats to one-half did not produce a proportional increase in iron absorption.

In the rat, Davies and Nightingale (1975) found that phytate decreased iron absorption whereas Ranhotra *et al.* (1974) found no effect. Welch and Van Campen (1975) observed that with intrinsic labeled ^{59}Fe, iron was more available from the mature than the immature soybean seeds, even though the phytic acid content was three times higher in the mature seeds.

Other studies showed that feeding phytate-containing foods or adding sodium phytate to foods caused iron bioavailability to be decreased (Björn-Rasmussen, 1974; Hussain and Patwardhan, 1959; Turnbull *et al.*, 1962; Hallberg, 1987; Hallberg *et al.*, 1987b), not affected (Andersson *et al.*, 1983; Callender and Warner, 1970; Caprez and Fairweather-Tait, 1982; Morris and Ellis, 1980, 1982) or, conversely, increased (Sandberg *et al.*, 1982).

Much of this controversy may be due to the low absorption of iron in general, the levels and sources of food iron and phytic acid used, and the presence of other dietary factors affecting iron absorption. All this is further complicated by fiber components in the diet because they usually coexist in foods with phytic acid.

In a study with six healthy subjects, Andersson *et al.* (1983) provided an iron intake of approximately 14 mg/day with three levels of nonstarch polysaccharides added as bran in white, brown, and wholemeal bread. All subjects were in positive iron balance, and there was no difference in fecal iron excretion; but iron retention tended to decrease with increasing fiber intake. The phytic acid intakes were kept constant at 1.4–1.5 g/day (sodium phytate was added to white and brown bread to equal that of the wholemeal bread). These results suggest that some component(s) in bran other than phytate decreases iron absorption.

Sandberg *et al.* (1982) found that apparent iron absorption was increased in eight ileostomy patients by the addition of 16 g of wheat bran in the diet, despite the fact that the phytate intake was four times higher (3.5 g/day with bran vs. 0.8 g/day without bran).

In a 15-day metabolic balance study, Morris and Ellis (1982) provided 36 g/day of wheat bran in muffins. Mineral balance was compared with that obtained with bran muffins that had been dephytinized by the endogenous phytase but that contained the complete freeze-dried hydrolysis products. The nonvegetarian diet had over 100 mg ascorbic acid and 18.2 mg Fe/day. The mean daily iron balance for the last 10 days was 0.9 mg greater when whole bran (2 g phytic acid/day) was consumed than with the dephytinized bran (0.2 g phytic acid/day) muffins. In another study with 12.9 mg Fe/day and three levels of phytate, iron balances were negative but not affected by the dietary phytate levels (Morris *et al.*, 1986).

Turnbull *et al.* (1962) investigated the effects of sodium phytate, with and without food, on the absorption of either soluble ferrous iron or hemoglobin iron in healthy adults and subjects with iron-deficiency anemia. Absorption of soluble iron was reduced by phytate and by food and was increased by ascorbic acid with food. In contrast, the absorption of hemoglobin iron was not reduced by phytate or by food, and ascorbic acid (80 or 500 mg) had no enhancing effect. In addition, phytate in the presence of food increased hemoglobin iron absorption. Sodium phytate levels used were 4 g in the absence and 10 g in the presence of food. No information on the composition and the amount of food was reported. The enhancing effect of phytate on hemoglobin iron absorption is interesting but remains to be confirmed.

Iron absorption and bioavailability have been extensively reviewed (Hallberg, 1981; Morris, 1983; Finch and Cook, 1984). Food iron generally is classified into two pools, heme and nonheme iron. Heme iron is better absorbed and is little influenced by dietary factors; nonheme iron is less easily absorbed, and its absorption is affected by other dietary factors. Meat is the only food source known to increase both heme and nonheme iron absorption (Hallberg and Rossander, 1984).

As with zinc, it is generally accepted that iron absorption from plant source foods is lower than that from animal source foods (Hallberg, 1981; Layrisse *et al.*, 1969). Phytic acid was identified as the primary responsible factor; however, other factors are involved. Using a radioiron method in a series of studies with parous Indian women, Gillooly *et al.* (1983, 1984) observed poor iron bioavailability from vegetables with high phytate contents (wheat germ, butter beans, and brown and green lentils). A strong inverse correlation ($r = -0.901$) between the polyphenol content, especially the nonhydrolyzable polyphenol, and iron absorption was also observed in 17 vegetables. Reducing the phytic acid and polyphenol contents of sorghum (96 and 92%, respectively) by peeling, significantly increased iron absorption. Hemicellulose and possibly lignin (in cocoa) were found to decrease iron absorption whereas cellulose and apple pectin had no effect.

Cook *et al.* (1981) reported that substituting isolated soy protein for casein or

egg albumin in a semisynthetic meal markedly decreased iron absorption from 2.5% with casein and 2.7% with egg albumin to an average of 0.5% in male volunteers. Full-fat soy flour and textured soy flour also exhibited an inhibiting effect. Studies from another laboratory reported a similar effect with a hamburger meal in which soy flour was substituted for half of the meat protein (Hallberg and Rossander, 1982). In the same study, they further demonstrated that phytic acid was not responsible for this inhibition because removing phytate from soy flour by acid extraction followed by filtration and freeze-drying did not increase the nonheme iron absorption. Total iron absorption, however, was greatly increased by the addition of a small amount of heme iron (1.05 mg). Soy protein was reported to enhance heme iron absorption (Lynch *et al.*, 1985); the factor(s) responsible for this effect was not identified. Thus, the inhibitory effect of soy products on nonheme iron absorption is usually counterbalanced by its high iron content and is further offset by an enhancing effect on heme iron absorption if used as a meat extender.

Iron absorption from a soybean-based infant formula was studied in multiparous Indian women and was found to be lower than that from a cow's milk formula (Derman *et al.*, 1987). Ascorbic acid, a potent enhancer of nonheme iron absorption, had a less marked effect with the soybean-based formula than with the milk-based formula.

The chemical form(s) of iron in soy products is not known. Ellis and Morris (1981) reported that 70% of the soluble iron of defatted soybean eluted in the phytate fraction. Phytic acid from soy products given in a diet to ileostomy subjects was almost completely recovered in the ileostomy contents (Sandström *et al.*, 1986). This limited hydrolysis may also be related to extrusion processing. Although increased iron absorption can be achieved by adding ascorbic acid or meat or by manipulating the processing conditions (Morck *et al.*, 1982), it is important to identify the unknown inhibiting factor(s) in soy. These factors may have effects on other minerals, and their intake may be increasing because soy products are used to a greater extent in human foods.

Wheat bran markedly inhibits iron absorption. Björn-Rasmussen (1974) demonstrated a quantitative dose–response inhibition by bran added to bread. Sodium phytate added to the meal caused a reduction in iron absorption, which indicated that phytate in bran was the responsible factor. However, Simpson *et al.* (1981) found a similar degree of inhibition of iron absorption by bran after its phytate had been destroyed by endogenous phytase. When the dephytinized bran was separated further into a water soluble, phosphate-rich nonphytate fraction and an insoluble, high-fiber fraction, the inhibitory activity was higher in the soluble fraction although none of the dephytinized bran fractions inhibited iron absorption significantly. This finding and that of Reinhold *et al.* (1975) indicated that the inhibition was not caused by phytate but rather by fiber or other components in bran. This was further supported by the findings of Morris and Ellis

(1976) that over 60% of iron in wheat bran is in the form of monoferric phytate, which is water soluble and bioavailable to humans.

Recently, Hallberg and co-workers (Hallberg, 1987; Hallberg *et al.*, 1987b) demonstrated that phytates are the main cause of the inhibitory effect of bran on iron absorption. They showed that dephytinization of bran by endogenous phytase followed by water washing to remove phosphates almost completely removed its inhibitory effect. They pointed out that most of the phytate in bran is bound to cations, with less than 5% present as monoferric phytate. They were able to restore the same inhibition of iron absorption by adding a "physiological" mixture of monoferric and potassium and magnesium phytates as supplied by the bran. In one report, Hallberg (1987) demonstrated a strong semilogarithmic relationship ($r = 0.99$) between the inhibition of iron absorption and the amount of sodium phytate. The inhibitory effect of phytates was counteracted by ascorbic acid and by meat (Hallberg *et al.*, 1986, 1987a; Hallberg, 1987).

Whether phytate or fiber is the major determinant for decreased mineral bioavailability in foods has been the subject of debate and the focus of interest of many researchers for many years. The effects of phytate and fiber on mineral utilization have been reviewed extensively (Kelsay, 1986; Toma and Curtis, 1986; Morris, 1986; Harland and Morris, 1985). Because both phytate and fiber have a high potential binding capacity for minerals including the essential elements and because they generally are present together in many foods, it is very difficult to separate completely the effects of these two in studies with typical human diets. Toma and Curtis (1986) concluded that, in human studies, addition of 15–20 g of fiber from a variety of food sources (wheat bran, neutral detergent fiber, soybean fiber, and cellulose) did not significantly affect iron bioavailability. Dietary fiber has been shown *in vitro* to bind minerals including iron (Reinhold *et al.*, 1975; Platt and Clydesdale, 1984). *In vitro* studies also showed that pH and other minerals such as calcium, magnesium, and zinc, which are commonly found in diets, influenced the iron-binding capacity of wheat bran and phytate as well as their solubility (Rao and Narasinga Rao, 1983; Platt and Clydesdale, 1987). Certain dietary fibers decrease iron bioavailability *in vivo*. Rossander (1987) showed that although pectins and guar gum had no effect on nonheme iron absorption, a slight but significant inhibition was seen with a hemicellulose preparation (ispagula). Phytate appears to be the major contributor to decreased iron absorption in wheat bran, though there is still a residual inhibitor effect of wheat bran, indicating that some other factor(s) also contributes.

3. *Manganese*

Phytic acid can reduce the bioavailability of dietary manganese. Davies and Nightingale (1975) showed that young rats fed a 10 g phytic acid (as sodium phytate)/kg diet for 21 days had markedly reduced whole-body manganese reten-

tion. When inositol phosphates (IP_3, IP_4, IP_5, and IP_6) were fed to Japanese quail from hatching to 7 days of age, IP_5 and IP_6 reduced liver manganese concentrations (Tao *et al.*, 1986). IP_3 and IP_4 had no effects. Morris and Ellis (1985b) fed men dephytinized wheat bran in six muffins per day to supply 0.5 g phytic acid. Sodium phytate was added to other muffins to provide totals of 1.7 and 2.9 g phytic acid/day. Mean manganese intake was 4 mg/day; however, due to different energy needs and diet intakes, manganese intakes ranged from 2.7 to 7.4 mg/day. All men received each level of phytate for 15 days. There was no significant effect of phytate on apparent manganese absorption. In a similar study with dephytinized bran and control bran, supplying 0.2 and 2 g phytic acid/day, respectively, apparent manganese absorption was negative for 5 days but positive for the following 10 days (Morris *et al.*, 1980; Morris and Ellis, 1985b).

Keen *et al.* (1986) found that less of an extrinsic label of manganese-54 was retained with infant soy formula than with human milk, cow's milk, or cow's milk formula. The labeled foods were administered to rat pups and retention was measured 24 hr later. Although the soy formula contained much more manganese than the other formulas, additional manganese(II) chloride did not influence the retention of radioactive manganese from human milk or cow's milk formula.

In animal studies, phytate markedly reduced manganese bioavailability, but additional studies are required to assess the significance for humans. Foods that contain high levels of phytate often contain high levels of manganese (Davies and Reid, 1979; Fox *et al.*, 1985; Davies and Warrington, 1986), which could decrease the effect of phytate.

4. Copper

Relatively few studies have dealt with the effects of phytate on dietary copper utilization. Davies and Nightingale (1975) fed rats a purified diet containing high calcium (13 g/kg) with and without 10 g phytic acid (sodium phytate)/kg. After 21 days whole-body copper accumulation was significantly reduced by phytate.

Turnlund *et al.* (1985) fed four healthy men an adequate liquid formula diet that supplied 2.32 mg copper/day during 15-day balance periods. Apparent copper absorption was based on fecal excretion of the stable isotope, copper-65, and the ratio of copper-63 to copper-65. The former is the more abundant stable isotope. A dietary addition of 2.34 g phytic acid (sodium phytate)/day did not affect copper absorption. There were, however, marked variations among subjects in copper absorption, which averaged 44.1, 33.4, 29.5, and 26.8%.

In two studies on the effect of wheat bran in human subjects consuming normal diets, there were no significant effects on either apparent copper absorption or copper balance (Morris *et al.*, 1980; Morris and Ellis, 1985b). Muffins containing dephytinized wheat bran or nondephytinized bran supplied 0.2 and 2 g phytic acid/day in the first study. Dephytinized bran muffins and the dietary

components supplied 0.5 g phytic acid/day in the second study, and sodium phytate was also added to increase the phytate content to 1.7 and 2.9 g/day. Mean copper intake was 1.4 mg/day.

From these human studies and some animal studies not discussed, it appears that the effect of phytate on copper bioavailability is of less practical importance than it is on some other elements. However, the data of Davies and Nightingale (1975) suggest the potential for a problem in humans. Recent *in vitro* studies have demonstrated that under conditions relevant to the intestinal tract, calcium potentiated copper binding in the presence of phytate (Champagne, 1987). Wise and Gilburt (1982a) obtained data to indicate that amino acids, particularly histidine, are important for removing copper from calcium phytate in the gut. Although the copper intake of many individuals is known to be low, the copper content of phytate-containing foods is high (Davies and Reid, 1979; Fox *et al.*, 1985; Davies and Warrington, 1986).

5. *Phosphorus*

A major portion of the total phosphorus in many plant seeds is present in the form of phytate (Reddy *et al.*, 1982). Phytate phosphorus is generally recognized to be less bioavailable than the usual dietary forms of inorganic phosphorus. The availability of phytate phosphorus varies with species, age, total phytase activity (derived from the food, gut microorganisms, and the host), and other dietary components such as calcium, phosphorus, and vitamin D. Some of these aspects have been reviewed (Reddy *et al.*, 1982; Wise, 1983).

Ruminants were reported to be able to utilize phytate phosphorus quite well. Nelson *et al.* (1976) reported that no phytate phosphorus was found in the feces of steers, and only traces were found in the feces of young calves fed a diet containing mainly soybean meal and sorghum grains. Chicks were reported to utilize phytate phosphorus poorly, but improved utilization of phytate phosphorus as the young matured was also reported for chicks (Nelson, 1967, 1976) and swine (see Reddy *et al.*, 1982). In contrast, Nelson and Kirby (1979) reported that, with a diet based on corn–soybean meal, weanling rats hydrolyzed phytate phosphorus better than mature rats, 71 vs. 39%, respectively. Hamsters were more efficient in hydrolyzing phytate than rats with identical diets (Taylor and Coleman, 1979), possibly due to the hamster's larger ceca.

In humans, McCance and Widdowson (1935) reported that in a study of three adults and one child who consumed 436 and 101 mg phytin phosphorus per day, respectively, the three adults excreted 36–63% of the ingested phytin whereas the child excreted 21–25%. A mean of 80% of the consumed phytate (2 g/day), taken as whole bran muffins, was recovered in the stool of human adults with a dietary calcium intake of 1.1 g/day (Harland and Morris, 1985). In a study of two 10-year-old boys with similar daily intakes of calcium (0.9 gm) and phytate

(1.7 g phytate), Hoff-Jørgensen *et al.* (1946b) reported that 30% of the dietary phytate was recovered in the feces.

The role of intestinal phytase activity or other phosphatases in phytate phosphorus absorption or utilization has been studied. Phytase activity was reported to be present in several animal species including humans (Bitar and Reinhold, 1972). Germ-free chicks (Savage *et al.*, 1964) and rats (Wise and Gilburt, 1982b) were unable to digest dietary phytate whereas conventional rats hydrolyzed phytate (Wise and Gilburt, 1982b). Moore and Veum (1982) found that rats adapted to a low phosphorus diet by increasing their phytate phosphorus digestion and that this adaptation was not related to the activity of phytase or alkaline phosphatase in the duodenal mucosa (Moore and Veum, 1983). They suggested that it may result from enhanced synthesis of microbial alkaline phosphatase or phytase by the intestinal microflora. Using ^{31}P nuclear magnetic resonance spectroscopy, Wise *et al.* (1983) found no phytate hydrolysis products in the diet, stomach, or small intestine of female adult rats fed sodium phytate in a casein-based diet. Phytate hydrolysis products were found, however, in the cecal, colonic, and fecal samples, probably due to the action of cecal bacteria. Because rats are coprophagous, the fecal products would be available for absorption during the next intestinal pass.

The importance of dietary phytase on phytate phosphorus utilization was demonstrated by Pointillart *et al.* (1984, 1987) in pigs fed a low-phosphorus, phytate-rich diet (phytate phosphorus provided two-thirds of total phosphorus). Better phosphorus digestibility and retention were observed in pigs fed either wheat (Pointillart *et al.*, 1984) or triticale (Pointillart *et al.*, 1987), both phytase-rich cereals, compared with the phytase-poor corn. Moreover, mucosal phytase and alkaline phosphatase activities in the small intestine of pigs were not different with wheat or triticale vs. corn. Replacing one-half of the corn by wheat in a diet based on corn–soybean meal significantly increased phytate hydrolysis in chicks and rats (Nelson, 1976; Nelson and Kirby, 1979). Nelson *et al.* (1971) demonstrated that supplementing graded levels of a mold phytase to a diet containing 0.18–0.24% phytate phosphorus supplied primarily by corn and soybean meal increased phytate phosphorus utilization by chicks as indicated by increased bone ash. The amount of phytate phosphorus present in the feces of the chick was reduced in proportion to the amount of phytase added to the diet.

The availability of phytate phosphorus to animals has been reported to be markedly influenced by the calcium and vitamin D contents of the diets. The extent of phytate hydrolysis was shown to be inversely proportional to the calcium intake in rats (Nelson and Kirby, 1979; Nahapetian and Young, 1980; Wise *et al.*, 1983), hamsters (Taylor and Coleman, 1979), and chicks (Waldroup *et al.*, 1964a,b). In a human study of nine subjects that consumed a typical United States diet supplying 1.8 g phytate and three levels of calcium (500, 1050, and 1500 mg) per day, Ellis *et al.* (1986) reported that phytate hydrolysis values were

47, 26, and 16%, respectively. Increased phytate hydrolysis with lower calcium intake in men was reported by Walker *et al.* (1948) and Cruickshank *et al.* (1945).

Several investigators have suggested that vitamin D_3 improved phytate phosphorus utilization based on increased bone ash (or density) or performance in the rat (Boutwell *et al.*, 1946; Roberts and Yudkin, 1961; Pileggi *et al.*, 1955), the chick (Gillis *et al.*, 1949, 1957; McGinnis *et al.*, 1944), and the pig (Fontaine *et al.*, 1985). Dietary vitamin D_3 also increased the level of intestinal phytase activity (Pileggi *et al.*, 1955; Roberts and Yudkin, 1961). However, it is generally agreed that increasing the proportion of inorganic phosphorus in a phytate-rich diet is more effective than increasing the vitamin D level for improved growth and bone mineralization (Pileggi *et al.*, 1955; Gillis *et al.*, 1949).

In a study with four infants, aged 1–12 months, addition of sodium phytate to a milk diet caused an increase in phosphorus absorption although percent phosphorus retention was lower (Hoff-Jørgensen *et al.*, 1946a). In another study with two 10-year-old boys, a phytate-rich diet provided mainly by rye, bran, wheat, and oatmeal increased phosphorus retention but not in relation to total phosphorus intake (Hoff-Jørgensen *et al.*, 1946b). The increased absorption of total phosphorus indicated that some of the phosphorus released from phytate in the intestine must have been absorbed.

McCance and Widdowson (1942a) studied the absorption of phosphorus in five men and five women who consumed a diet in which 40–50% of the calories came first from white and then from brown bread, each for a 3-week period. Absorption of total phosphorus was higher with the brown bread diet than with the white bread diet, but these represented 52 and 69%, respectively, of the total phosphorus intakes. It was also found that percentages of phosphorus absorbed increased when inorganic phosphorus was added to the brown bread diet but were not changed when added to the white bread diet. Adding sodium phytate to the white bread in an amount similar to that in the brown bread increased total phosphorus absorption but reduced the percentage of phosphorus absorbed from 70 to 63%. About 50% of the phosphorus added as sodium phytate was absorbed. Thus, phosphorus in brown bread as well as in sodium phytate is less available than the phosphorus in white bread or inorganic phosphate. This was further supported by the data from three men and three women, which showed that the phosphorus absorptions were increased from 48 to 64% when subjects were given a dephytinized brown bread in which the phytate had been hydrolyzed enzymatically to inorganic phosphates and inositol, and in which everything else remained the same as the brown bread diet (McCance and Widdowson, 1942b).

In a study with two subjects, Reinhold *et al.* (1976b) reported that replacing white bread by a high fiber bazari bread in the diet, which increased phosphorus by one-third, changed the phosphorus balance from markedly positive to nega-

tive. Increased fecal excretion of phosphorus was observed in these subjects. It is not clear, however, whether phytate (1.39 g/day) or fiber (30 and 34 g/day) was responsible for the increased fecal loss of this element.

A study with eight human ileostomy subjects showed that dietary phytate derived from soy flour, soy concentrate, and soy isolate was almost completely recovered in the ileostomy contents and that a 25% replacement of meat protein by soy protein had no obvious effect on the apparent phosphorus absorption (Sandström *et al.*, 1986).

Sandberg *et al.* (1982) studied in ileostomy patients the effect on the absorption of phosphorus of adding 16 g wheat bran/day to a low-fiber diet. An average of 41% of the ingested bran phytate phosphorus was recovered in the ileostomy contents. Although the apparent absorption (intake − ileostomy recovery) of phytate phosphorus increased and that of nonphytate phosphorus remained constant, the relative phytate phosphorus absorption was decreased whereas the nonphytate phosphorus was unchanged. In this study, the bran phytate (3.4 g) contributed 9.4% of the total phosphorus in the diet. In another study, the effects of extrusion cooking were studied with seven ileostomy subjects. The apparent phosphorus absorption was significantly decreased by consuming an extruded high-fiber cereal, 54 g/day of a bran–gluten–starch mixture, compared with absorption with the same product without extrusion (Kivistö *et al.*, 1986). A greater percentage of phytate was recovered in the ileostomy contents with the extruded product than the unextruded product (72 vs. 44%). Extrusion cooking inactivated the phytase that was naturally present in bran (Sandberg *et al.*, 1986), and a phytate complex, which is more resistant to digestion, may have been formed.

Using a HPLC method, the same authors showed in a recent study that an average of 58% of the phytate in the unprocessed bran was hydrolyzed to IP_5, IP_4, and IP_3 in the stomachs and small intestines of these ileostomy patients (Sandberg *et al.*, 1987). In the same study they also showed that when wheat bran was subjected to extrusion cooking, 25% of the inositol hexaphosphate was hydrolyzed to IP_5 and IP_4. There was essentially no digestion of phytate in the extruded products, as indicated by the almost complete recovery of the ingested inositol hexaphosphate in the ileostomy contents and the absence of an increase of IP_3, IP_4, and IP_5.

Schwartz *et al.* (1986) followed phytate excretions of seven male college students who consumed a constant diet containing relatively high fiber and phytate, contributed mainly by wheat bran and whole wheat bread, for the last 4 weeks of a 7-week study. Phytate excretions were quite reproducible in individuals, with an average of 55% of a mean phytate phosphorus intake of 20.4 mmol/day (equivalent to 2.2 g phytic acid) and did not appear to change with time. Data from this study do not suggest an adaptation to phytate ingestion, which is in contrast to conclusions of Walker *et al.* (1948). However, whether there is a long-term adaptation is not known.

Although phytate phosphorus was less available than the inorganic phosphate, it is generally believed that at least a portion of the phosphorus liberated by hydrolysis is absorbed. Phytate had little effect on phosphorus balance unless the available dietary phosphorus level was very low.

No conclusive evidence for absorption of phytate or other IPs is available at present. Most workers measured the degree of phytate hydrolysis and total phosphorus absorption, which obviously does not directly indicate phytate absorption. As mentioned before, the phytate hydrolysis process is a stepwise dephosphorylation reaction. This can occur during food processing or in the intestine while food is being digested.

In United States adults, phosphorus intakes are usually high, and phytate phosphorus represents only a small portion of the total phosphorus. Thus, the impact of phytate on phosphorus availability can be considered of little consequence. On the other hand, adequate phosphorus and calcium are equally important for proper bone development of infants. Therefore, phosphorus bioavailability is an important issue for children consuming soy-based infant formula.

6. *Calcium*

Phytic acid binds calcium strongly and thus markedly decreases its bioavailability. This practical problem for cereal-consuming populations stimulated research in this area more than 40 years ago.

The effects of sodium phytate added to foods have been investigated by several workers. Reduced uptake of calcium-45 was reported by Bronner *et al.* (1954) for adolescent boys who received a low-calcium breakfast of farina (phytate-free) vs. farina plus 277 mg phytic acid (as sodium phytate). Bronner *et al.* (1956) found that with moderate calcium intakes, there was little effect of endogenous phytate in oatmeal on fecal calcium excretion vs. no phytate in a farina breakfast. The conditions of the two studies were otherwise similar. McCance and Widdowson (1942a) found that adding sodium phytate to white bread (in amounts slightly higher than that in brown bread) caused reduced calcium absorption in normal adults when bread supplied 40–50% of the energy needs.

With muffins containing dephytinized wheat bran (total dietary phytate, 0.5 g/day), addition of sodium phytate to provide 1.7 and 2.9 g phytate/day produced apparent absorption values in men of 153, 94, and 23 mg calcium/day, respectively (Morris and Ellis, 1985a). With their calcium intake of 740 mg/day, phytate/calcium molar ratios were 0.04, 0.14, and 0.24. The correlation coefficient for apparent calcium absorption and phytate/calcium molar ratio was 0.62 ($P < 0.01$).

Relatively little is known about the consequences on calcium utilization of consuming inositol phosphates with fewer phosphate groups than phytic acid. Tao *et al.* (1986) fed IP_3, IP_4, IP_5, and IP_6 to newly hatched Japanese quail for

7 days. At a 8.33 mmol/kg diet (equivalent to 0.55% dietary phytic acid), IP_5 and IP_6 decreased tibia ash and caused perosis, a twisting and enlargement of the tibiometatarsal joint. For IP_3 and IP_4, however, tibia weight and ash were increased. Similarly, in other 6-hr studies of calcium-45 absorption by suckling rats, nonabsorbed calcium was 17, 1.4, 0.5, 0.5, and 0.5%, respectively, for IP_6, IP_5, IP_4, IP_3, and control (Lönnerdal *et al.*, 1987). The phytate/calcium molar ratio intubated was 0.08.

The effects of endogenous phytate in cereal foods on calcium metabolism have been compared with those of corresponding foods with phytate decreased either by milling or by the action of phytase. McCance and Widdowson (1942a) observed decreased calcium absorption with brown bread (92% extraction flour) vs. white bread (69% extraction flour) in subjects who received 40–50% of their calories from the breads and calcium intakes ranging from 380 to 720 mg/day. It was considered likely that phytic acid was responsible for the difference, but other components cannot be ruled out. The amount of phytate was almost four times higher in the brown than the white flour. In another study of similar design, marked decreases in calcium balance occurred in three men who consumed high-phytate vs. low-phytate breads (Walker *et al.*, 1948).

Reinhold *et al.* (1973) reported markedly negative calcium balances in three Iranian men consuming 350 g unleavened whole wheat bread (tanok) per day. It supplied 2.6 g phytate daily during the first 22 days of the balance period and 3.4 g phytate during the last 10 days. In a second study, Reinhold *et al.* (1976b) reported negative calcium balances in two men who consumed 500 g of sour-dough-leavened bread (bazari) prepared from 80–90% extraction flour. The bread supplied about 1.4 g phytate/day. These calcium balances were not as negative as those when tanok was consumed.

Two studies with wheat bran in normal ileostomy patients (Sandberg *et al.*, 1982) and in healthy men (Morris and Ellis, 1985a) showed that calcium balance was not affected by the bran. In both studies, calcium intake was high, 1299 mg/day in the ileostomy patients and 1100 mg/day in the men. The corresponding phytate/calcium molar ratios were 0.16 and 0.11. With lower calcium intakes, an adverse effect might have occurred with the same levels of phytate, as indicated by the graded effects of added phytate described above (Morris and Ellis, 1985a). This interpretation agrees with the data of McCance and Widdowson (1942a), which showed improvements in calcium absorption in subjects receiving brown bread (92% extraction flour) when daily calcium intake was increased from about 550 mg/day to about 1200 mg/day. Morris and Ellis (1985a) suggested that persons consuming diets with phytate/calcium molar ratios of greater than 0.2 may be at risk of calcium deficiency unless physiological adjustments could be accomplished to maintain homeostasis.

Osteomalacia and rickets have occurred in humans consuming diets based on large proportions of unleavened wholemeal breads. These problems have been

studied in Asian populations residing in the United Kingdom who retained their traditional food habits (Dunnigan, 1977). The exact roles of phytate in the etiology of the rickets and osteomalacia are not clearly defined; however, it appears that marginal vitamin D status and the adverse effect of phytate on calcium bioavailability are involved. Davies (1982) has reviewed some of the studies of rickets.

As noted in Section III,B,1 on zinc, pulses and breads consumed by the immigrant groups studied contained significant amounts of phytate (Davies and Warrington, 1986). The phytate/calcium molar ratios for the cooked pulses ranged from 0.14 to 1.29. All except the lowest were above 0.27. The chapatis had ratios between 0.22 and 0.29. Phytate/calcium molar ratios in four soy products ranged from 0.24 to 0.55 (Fox *et al.*, 1985).

Collectively, the studies on calcium show that calcium utilization is adversely affected by phytate unless calcium intake is high.

7. *Magnesium*

Phytate has been shown to decrease magnesium bioavailability in several human studies. McCance and Widdowson (1942a) gave normal men and women brown bread and white bread to supply 40–50% of their energy intake. Magnesium intake with the brown bread, as compared to the white bread, was approximately doubled and magnesium absorption as a percentage of intake was reduced almost by one half. Balance, however, was observed with both breads. Addition of sodium phytate to the white bread to supply phytate in an amount slightly higher than that in the brown bread (462 mg phytic acid/100 g bread) caused reduced magnesium absorption; balance did not become negative. In a study of similar design (Walker *et al.*, 1948), magnesium balance was negative in two of three men when the change was made from control diet to consumption of brown bread. After 4 weeks, magnesium balance improved to near zero.

In a study of Reinhold *et al.* (1976b), two men received daily 500 g yeast-leavened bread (bazari) prepared from 80–90% extraction flour. The bread supplied 1.4 g phytate/day. Magnesium balances were negative and significantly different from balances with white bread even though daily magnesium intakes were 650 and 724 mg with bazari and 340 mg with white bread for the two men.

Sandberg *et al.* (1982) gave otherwise normal ileostomy patients a low-fiber diet for 4 days followed by the same diet plus 16 g wheat bran for 4 days. The bran increased magnesium intake by about one-third to 292 mg/day and the phytic acid intake was about 3.4 g/day. Relative absorption of magnesium was significantly decreased by the bran. In another study with ileostomy patients, Kivistö *et al.* (1986) observed a significant reduction in apparent magnesium absorption when 54 g/day of a bran–gluten–starch mixture fed in a low-fiber diet was subjected to mild extrusion processing.

It was shown in young Japanese quail that when sodium phytate (11 g phytic acid/kg diet) was added to a casein–gelatin diet in amounts equal to that in soy flour as the only source of protein, magnesium deficiency occurred in those birds that also received enough zinc (100 mg/kg diet) to prevent zinc deficiency due to the phytate (Fox *et al.*, 1988). It appears that the zinc with the phytate caused reduced absorption of magnesium.

These data show adverse effects of phytate on magnesium absorption, but less marked than those for some other essential minerals. Foods that supply phytate are usually good sources of magnesium unless it has been removed by processing (Fox *et al.*, 1985; Davies and Warrington, 1986). The foods that have been studied above were high not only in phytate but also in fiber; however, phytate added to white bread also adversely affected magnesium bioavailability even though the white bread contains little fiber.

8. Lead

Relatively few studies have investigated factors that modify the interactions between phytate and lead (Table III). Phytate has been found to protect against dietary lead in experimental animals. Wise (1981) reported that 20 g calcium phytate/kg of a purified diet protected against the weight loss and the liver and kidney lead accumulation produced by a 637 mg lead (as the acetate)/kg diet by the age of 7 days in mice. This level of phytate is similar to that in stock diets. Wise (1982) investigated the protection by the same level of calcium phytate against lead in mice fed purified diets containing 1.27, 6.37, or 31.85 mg lead (as the acetate)/kg. There were no effects of diet on body weight. After 3 months, blood lead concentrations were lowered by phytate only with the 31.85 mg lead/kg diet, whereas at 6 months, blood lead was also lowered with the 6.37 mg lead/kg diet.

Rose and Quarterman (1984) fed rats a purified diet containing 200 mg lead/kg

TABLE III

Principal Factors Modifying Interactions between Phytate and the Toxic Minerals Lead and Cadmium[a]

Modifying factors: Dietary components	Toxic minerals	
	Pb	Cd
Ca	+	+
Other essential minerals	+	
Foods		+

[a] + indicates modification of a phytate–toxic mineral response reported in one or more papers included in this review. For details and references see text.

for 4 weeks. Addition of either 6 g calcium (as $CaCO_3$)/kg or 10 g phytate (as sodium phytate)/kg diet reduced lead concentrations in bone, blood, and liver. A greater reduction of femur and blood lead was observed with the combination of calcium and phytate. Body weight was not affected by diet. In another experiment, it was shown that phytate fed for either 2 or 4 weeks after lead was removed from the diet did not affect total lead in the femur or kidney.

Wise and Gilburt (1981) showed *in vitro* that calcium was required for binding lead to phytate at levels relevant to experimental diets. Both calcium and phosphorus can decrease lead absorption in humans (reviewed by Mahaffey, 1985). From these animal studies, it is possible that phytate in the human diet may enhance the effect of calcium in decreasing lead uptake.

9. Cadmium

Little information is available on the effect of phytate on cadmium absorption and toxicity and even less on factors that may modify the known effects (Table III). Rose and Quarterman (1984) fed rats an adequate casein diet containing 5 mg cadmium/kg with supplements of 6 g calcium/kg, or 10 g phytic acid (as sodium phytate)/kg, or calcium plus phytate. After 4 weeks, growth was not affected. Compared with the controls, cadmium concentrations in the liver and kidney were greater with added calcium alone; however, phytate prevented these effects of calcium. In another experiment, phytate fed for either 2 or 4 weeks after cadmium was removed from the diet had no effect on total cadmium in liver and kidney. Wise and Gilburt (1981) showed *in vitro* that at levels similar to those in experimental animal diets, calcium was required to bind cadmium to phytic acid.

Lee *et al.* (1986) reported that the endogenous cadmium of soy isolate was much less bioavailable for Japanese quail than cadmium chloride added to a casein–gelatin diet. It is not known whether the phytate in the soy was involved.

IV. IMPLICATIONS FOR HUMAN NUTRITION

A. Adverse Effects

The data show clear-cut evidence of decreased utilization of several essential minerals due to ingestion of phytate-containing foods. There is much less information on decreased protein digestibility and reduced protein efficiency ratio due to phytate. The phytate intakes in some experimental studies may be higher than amounts actually consumed under many circumstances.

The effects of phytate on mineral bioavailability may be increased by low dietary intakes of most interacting minerals. From large-scale and continuing studies, there is evidence for widespread low intakes of zinc, iron, manganese,

copper, calcium, and magnesium (Anonymous, 1984, 1985; Pennington *et al.*, 1986). Risk groups most frequently included young children and all females. Data from the Second National Health and Nutrition Examination Survey indicated highest prevalences of impaired iron status for children 1–2 years, males 11–14 years, and females 15–44 years of age (Pilch and Senti, 1984a).

The increasing use of mineral supplements could beneficially affect an individual's response to phytate; however, consuming high levels of single elements is undesirable because of possible adverse mineral–mineral interactions, such as zinc antagonism of copper. The typical United States diet supplies phosphorus and protein well in excess of requirements (Anonymous, 1984, 1985). The amino acids released during digestion may increase absorption of some minerals bound to phytate in the gut. There is limited evidence that diversity of protein sources in a meal increases mineral bioavailability in the presence of phytate.

The level of dietary calcium is of particular interest. A low calcium intake is associated with markedly impaired calcium uptake due to phytate, even though the low calcium intake can favor increased phytate hydrolysis in the gut. Elevated dietary calcium protects with respect to calcium but decreases the hydrolysis of phytic acid and greatly enhances the binding of other minerals to phytate, resulting in decreased mineral absorption. The latter is undesirable for essential elements but is desirable for lead. With the lower inositol phosphates, calcium seems to have less effect than with phytic acid. Milk as a source of calcium has produced variable effects, sometimes increasing and sometimes decreasing the adverse effects of phytate on mineral bioavailability.

Seeds that contain large amounts of phytate are also rich in interacting essential elements, except that the amount of calcium is lower than that of other elements with respect to requirement. The commercial processes of refining and concentration of proteins can cause losses and imbalances of essential minerals and produce changes that decrease release of minerals during digestion. Such changes are especially important for infant formulas, even though infants and children may hydrolyze phytate more efficiently than older individuals. Phytate is often associated with fiber in seeds, so the addition of some fiber sources, such as bran, to foods can increase the phytate content.

B. Beneficial Effects

Data from *in vitro* studies showed that the rate of starch digestion was slowed by phytate (Yoon *et al.*, 1983; Desphande and Cheryan, 1984; Thompson *et al.*, 1987). With all IPs through phytic acid, it was found that a decrease in the rate of starch digestibility was directly proportional to the number of phosphate groups on the IPs (Knuckles and Betschart, 1987).

When six healthy adults consumed test breakfasts that included 50 g available

carbohydrate from unleavened breads, 1-hr blood glucose responses were progressively smaller with the following flour sources: white (milled wheat), dephytinized navy bean, dephytinized navy bean plus phytic acid, and undephytinized navy bean (Thompson *et al.*, 1987). The potential benefits of phytate for persons with diabetes and hyperlipidemia have been reviewed by Thompson (1988).

The limited data described in Section III,B,6 on calcium indicate that IP_3 and IP_4 may have beneficial effects on bone mineralization.

The possibility that phytate in foods may reduce lead absorption is important for infants and young children, the primary risk groups. Although the lead contents of foods have been markedly reduced, the intake of environmental lead (e.g., dust and paint) is still oral.

C. Difficulties in Experimental Approaches

The data on effects of phytate on mineral bioavailability show that many compositional factors can affect the net impact of a food on one or more elements. Endogenous and added phytate are not necessarily equal. Dephytinization by hydrolysis may also lead to ambiguous results, particularly if products of hydrolysis are not taken into account. The effects of fiber are not established and technically are virtually impossible to separate clearly from those of phytate. Extrinsic isotope labels have been very useful; however, it cannot be assumed that they always represent equivalent endogenous element.

In the mineral studies with humans, general balance and uptake techniques have been widely used in comparing bioavailability of various mineral species and various food sources. These data are not necessarily indicative of physiological utilization of the available element, which is the critical information that is needed.

D. Research Needs

Models to determine the effects of phytate in human foods and diets need to be used further to assess net effects and to acquire increased understanding of the physicochemical and physiological mechanisms contributing to decreased mineral bioavailability. The major issues to be addressed include (1) responses of the young vs. the adult, (2) adaptation to phytate, (3) relation of dietary components and food processing to phytate effects, (4) production and distribution of smaller inositol phosphates in foods, (5) adverse and beneficial physiological effects of smaller inositol phosphates, (6) effect of phytate-containing foods on lead absorption by infants and children, and (7) methods of removing phytate from foods.

V. SUMMARY AND CONCLUSIONS

Dietary phytate can markedly reduce the bioavailability of zinc, iron, manganese, phosphorus (as phytate), calcium, and magnesium. The most severe effects attributable to phytate in humans have occurred in populations with whole grain bread (sometimes with pulses) as a major dietary component. Marked mineral deficiency syndromes attributable to phytate have not been identified in the United States. The decreased bioavailability of minerals can be counterbalanced to some extent by the large amounts of minerals occurring in most whole seeds. Processing of seeds to prepare protein concentrates and isolates results in mineral loss and sometimes in decreased bioavailability of the remaining minerals. Phytate-containing foods may contribute to marginally low mineral status in some individuals and fortification of some such foods may be needed. Consumption of a varied diet with adequate protein protects against adverse effects of phytate on mineral bioavailability. Additional studies are needed on the potential benefits of dietary phytate and/or lower inositol phosphates on glucose absorption after meals, bone mineralization, and lead absorption.

ACKNOWLEDGMENT

This chapter was written by the authors in their private capacities. No official endorsement by the Food and Drug Administration is intended or should be inferred.

REFERENCES

Altschule, A. M., and Wilcke, H. L. (eds.) (1985). "New Protein Foods." Vol. 5, "Seed Storage Proteins." Academic Press, New York.

Anderson, P. A. (1985). Interactions between proteins and constituents that affect protein quality. *In* "Digestibility and Amino Acid Availability in Cereals and Oilseeds" (J. W. Finley and D. T. Hopkins, eds.), pp. 31–45. American Association of Cereal Chemists, St. Paul, Minnesota.

Andersson, H., Nävert, B., Bingham, S. A., Englyst, H. N., and Cummings, J. H. (1983). The effects of breads containing similar amounts of phytate but different amounts of wheat bran on calcium, zinc and iron balance in man. *Br. J. Nutr.* **50,** 503–510.

Anonymous (1984). "Nutrient Intakes: Individuals in 48 States, Year 1977–78. Nationwide Food Consumption Survey 1977–78," Rep. 1-2. U.S. Department of Agriculture, Hyattsville, Maryland.

Anonymous (1985). "Nationwide Food Consumption Survey. Continuing Survey of Food Intakes by Individuals: Women 19–50 Years and Their Children 1–5 Years, 1 Day." U.S. Department of Agriculture, Hyattsville, Maryland.

Bindra, G. S., Gibson, R. S., and Thompson, L. U. (1986). [Phytate][calcium]/[zinc] ratios in Asian immigrant lacto–ovo vegetarian diets and their relationship to zinc nutriture. *Nutr. Res.* **6,** 475–483.

Bitar, K., and Reinhold, J. G. (1972). Phytase and alkaline phosphatase activities in intestinal mucosae of rat, chicken, calf and man. *Biochim. Biophys. Acta* **268,** 442–452.

Björn-Rasmussen, E. (1974). Iron absorption from wheat bread. Influence of various amounts of bran. *Nutr. Metab.* **16,** 101–110.

Boutwell, R. K., Geyer, R. P., Halverson, A. W., and Hart, E. B. (1946). The availability of wheat bran phosphorus for the rat. *J. Nutr.* **31,** 193–202.

Bronner, F., Harris, R. S., Maletskos, C. J., and Benda, C. E. (1954). Studies in calcium metabolism. Effect of food phytates on calcium-45 uptake in children on low-calcium breakfasts. *J. Nutr.* **54,** 523–542.

Bronner, F., Harris, R. S., Maletskos, C. J., and Benda, C. E. (1956). Studies in calcium metabolism. Effect of food phytates on calcium-45 uptake in boys on a moderate calcium breakfast. *J. Nutr.* **59,** 393–406.

Callender, S. T., and Warner, G. T. (1970). Iron absorption from brown bread. *Lancet* **i,** 546–547.

Caprez, A., and Fairweather-Tait, S. J. (1982). The effect of heat treatment and particle size of bran on mineral absorption in rats. *Br. J. Nutr.* **48,** 467–475.

Champagne, E. T. (1987). Effects of Ca(II) ions on Cu(II) ion–phytic acid interactions. *J. Inorg. Biochem.* **31,** 29–42.

Cheryan, M. (1980). Phytic acid interactions in food systems. *CRC Crit. Rev. Food Sci. Nutr.* **13,** 297–335.

Cook, J. D., Morck, T. A., and Lynch, S. R. (1981). The inhibitory effect of soy products on nonheme iron absorption in man. *Am. J. Clin. Nutr.* **34,** 2622–2629.

Cosgrove, D. J. (1980). "Inositol Phosphates, Their Chemistry, Biochemistry and Physiology." Elsevier, New York.

Cruickshank, W. H., Duckworth, J., Kosterlitz, H. W., and Warnock, G. M. (1945). The digestibility of the phytate-P of oatmeal in adult man. *J. Physiol. (London)* **104,** 41–46.

Davies, N. T. (1982). Effects of phytic acid on mineral availability. *In* "Dietary Fiber in Health and Disease" (G. V. Vahouny and D. Kritchevsky, eds.), pp. 105–116. Plenum, New York.

Davies, N. T., and Nightingale, R. (1975). The effects of phytate on intestinal absorption and secretion of zinc, and whole-body retention of zinc, copper, iron and manganese in rats. *Br. J. Nutr.* **34,** 243–258.

Davies, N. T., and Reid, H. (1979). An evaluation of the phytate, zinc, copper, iron and manganese contents of, and Zn availability from, soya-based textured-vegetable–protein meat-substitutes or meat-extenders. *Br. J. Nutr.* **41,** 579–589.

Davies, N. T., and Warrington, S. (1986). The phytic acid, mineral, trace element, protein and moisture content of UK Asian immigrant foods. *Hum. Nutr.: Appl. Nutr.* **40A,** 49–59.

Davies, N. T., Carswell, A. J. P., and Mills, C. F. (1985). The effect of variation in dietary calcium intake on the phytate–zinc interaction in rats. *In* "Trace Elements in Man and Animals" (C. F. Mills, I. Bremner, and J. K. Chesters, eds.), pp. 456–457. Commonwealth Agricultural Bureaux, Slough, England.

Derman, D. P., Ballot, D., Bothwell, T. H., MacFarlane, B. J., Baynes, R. D., MacPhail, A. P., Gillooly, M., Bothwell, J. E., Bezwoda, W. R., and Mayet, F. (1987). Factors influencing the absorption of iron from soya-bean protein products. *Br. J. Nutr.* **57,** 345–353.

Desphande, S. S., and Cheryan, M. (1984). Effects of phytic acid, divalent cations, and their interactions on α-amylase activity. *J. Food Sci.* **49,** 516–519, 524.

Dunnigan, M. G. (1977). Asian rickets and osteomalacia in Britain. *In* "Proceedings of a Symposium on Child Nutrition in Relation to Mental and Physical Development," pp. 43–70. Kellogg, London.

Ellis, R., and Morris, E. R. (1981). Relation between phytic acid and trace metals in wheat bran and soybean. *Cereal Chem.* **58,** 367–370.

Ellis, R., Morris, E. R., Hill, A. D., and Smith, J. C., Jr. (1982). Phytate : zinc molar ratio, mineral, and fiber content of three hospital diets. *J. Am. Diet. Assoc.* **81,** 26–29.

Ellis, R., Morris, E. R., Hill, A. D., Anderson, R. L., and McCarron, P. B. (1986). Effect of level

of calcium intake on in vivo hydrolysis of dietary phytate. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **45,** 374.

Erdman, J. W. (1979). Oilseed phytates: Nutritional implications. *J. Am. Oil Chem. Soc.* **56,** 736–741.

Erdman, J. W., Jr., and Forbes, R. M. (1981). Effects of soya protein on mineral bioavailability. *J. Am. Oil Chem. Soc.* **58,** 489–493.

Finch, C. A., and Cook, J. D. (1984). Iron deficiency. *Am. J. Clin. Nutr.* **39,** 471–477.

Flanagan, P. R. (1984). A model to produce pure zinc deficiency in rats and its use to demonstrate that dietary phytate increases the excretion of endogenous zinc. *J. Nutr.* **114,** 493–502.

Fontaine, N., Fourdin, A., and Pointillart, A. (1985). Absence d'effet de la vitamine D sur la phytase et la phosphatase alcaline intestinales: relation avec l'absorption du phosphore phytique chez le pore. *Reprod. Nutr. Dev.* **25,** 717–727.

Fox, M. R. S., Jacobs, R. M., Jones, A. O. L., Fry, B. E., Jr., Rakowska, M., Hamilton, R. P., Harland, B. F., Stone, C. L., and Tao, S.-H. (1981). Animal models for assessing bioavailability of essential and toxic elements. *Cereal Chem.* **58,** 6–11.

Fox, M. R. S., Tao, S.-H., and Fry, B. E., Jr. (1985). Atypical zinc deficiency in Japanese quail fed two soy products. *In* "Trace Elements in Man and Animals" (C. F. Mills, I. Bremner, and J. K. Chesters, eds.), Vol. 5, pp. 225–228. Commonwealth Agricultural Bureaux, Slough, England.

Fox, M. R. S., Tao, S.-H., Fry, B. E., Jr., and Lee, Y. H. (1988). Production of Mg deficiency anemia by Zn and phytate in young Japanese quail. *In* "Trace Elements in Man and Animals" (L. S. Hurley, ed.), Vol. 6. Plenum, New York.

Freeland-Graves, J. H., Ebangit, M. L., and Hendrikson, P. J. (1980). Alterations in zinc absorption and salivary sediment zinc after a lacto–ovo–vegetarian diet. *Am. J. Clin. Nutr.* **33,** 1757–1766.

Gillis, M. B., Norris, L. C., and Heuser, G. F. (1949). The effect of phytin on the phosphorus requirement of the chick. *Poult. Sci.* **28,** 283–288.

Gillis, M. B., Keane, K. W., and Collins, R. A. (1957). Comparative metabolism of phytate and inorganic P^{32} by chicks and poults. *J. Nutr.* **62,** 13–26.

Gillooly, M., Bothwell, T. H., Torrance, J. D., MacPhail, A. P., Derman, D. P., Bezwoda, W. R., Mills, W., and Charlton, R. W., (1983). The effects of organic acids, phytates and polyphenols on the absorption of iron from vegetables. *Br. J. Nutr.* **49,** 331–342.

Gillooly, M., Bothwell, T. H., Charlton, R. W., Torrance, J. D., Bezwoda, W. R., MacPhail, A. P., and Derman, D. P. (1984). Factors affecting the absorption of iron from cereals. *Br. J. Nutr.* **51,** 37–46.

Golden, M. H. N., and Golden, B. E. (1981). Effect of zinc supplementation on the dietary intake, rate of weight gain, and energy cost of tissue deposition in children recovering from severe malnutrition. *Am. J. Clin. Nutr.* **34,** 900–908.

Graf, E. (ed.) (1986). "Phytic Acid: Chemistry and Applications." Pilatus, Minneapolis, Minnesota.

Hallberg, L. (1981). Bioavailability of dietary iron in man. *Annu. Rev. Nutr.* **1,** 123–147.

Hallberg, L. (1987). Wheat fiber, phytates and iron absorption. *Scand. J. Gastroenterol.* **22,** 73–79.

Hallberg, L., and Rossander, L. (1982). Effect of soy protein on nonheme iron absorption in man. *Am. J. Clin. Nutr.* **36,** 514–520.

Hallberg, L., and Rossander, L. (1984). Improvement of iron nutrition in developing countries: Comparison of adding meat, soy protein, ascorbic acid, citric acid, and ferrous sulphate on iron absorption from a simple Latin American-type of meal. *Am. J. Clin. Nutr.* **39,** 577–583.

Hallberg, L., Brune, M., and Rossander, L. (1986). Effect of ascorbic acid on iron absorption from different types of meals. Studies with ascorbic-acid-rich foods and synthetic ascorbic acid given in different amounts with different meals. *Hum. Nutr.: Appl. Nutr.* **40A,** 97–113.

Hallberg, L., Brune, M., and Rossander, L. (1987a). Is there a physiological role of vitamin C in iron absorption? *Ann. N.Y. Acad. Sci.* **498,** 324–332.

Hallberg, L., Rossander, L., and Skånberg, A.-B. (1987b). Phytates and the inhibitory effect of bran on iron absorption in man. *Am. J. Clin. Nutr.* **45,** 988–996.

Halsted, J. A., Ronaghy, H. A., Abadi, P., Haghshenass, M., Amirhakemi, G. H., Barakat, R. M., and Reinhold, J. G. (1972). Zinc deficiency in man. The Shiraz experiment. *Am. J. Med.* **53,** 277–284.

Harland, B. F., and Morris, E. R. (1985). Fiber and mineral absorption. *In* "Dietary Fiber: Reviews and Bibliography" (A. R. Leeds and A. Avenell, eds.), Vol. 1, pp. 72–82. Libbey, London.

Harland, B. F., and Oberleas, D. (1986). Anion exchange method for determination of phytate in foods: Collaborative study. *J. Assoc. Off. Anal. Chem.* **69,** 667–670.

Harland, B. F., and Peterson, M. (1978). Nutritional status of lacto–ovo vegetarian Trappist monks. *J. Am. Diet. Assoc.* **72,** 259–264.

Hoff-Jørgensen, E., Andersen, O., Begtrup, H., and Nielsen, G. (1946a). The effect of phytic acid on the absorption of calcium and phosphorus. 2. In infants. *Biochem. J.* **40,** 453–454.

Hoff-Jørgensen, E., Andersen, O., and Nielsen, G. (1946b). The effect of phytic acid on the absorption of calcium and phosphorus. 3. In children. *Biochem. J.* **40,** 555–557.

Hussain, R., and Patwardhan, V. N. (1959). The influence of phytate on the absorption of iron. *Indian J. Med. Res.* **47,** 676–682.

Keen, C. L., Bell, J. G., and Lönnerdal, B. (1986). The effect of age on manganese uptake and retention from milk and infant formulas in rats. *J. Nutr.* **116,** 395–402.

Kelsay, J. L. (1986). Update on fiber and mineral availability. *In* "Dietary Fiber, Basic and Clinical Aspects" (G. Vahouny and D. Kritchevsky, eds.), pp. 361–372. Plenum, New York.

Kivistö, B., Andersson, H., Cederblad, G., Sandberg, A.-S., and Sandström, B. (1986). Extrusion cooking of a high-fibre cereal product. 2. Effects on apparent absorption of zinc, iron, calcium, magnesium and phosphorus in humans. *Br. J. Nutr.* **55,** 255–260.

Knuckles, B. E., and Betschart, A. A. (1987). Effect of phytate and other myo-inositol phosphate esters on α-amylase digestion of starch. *J. Food Sci.* **52,** 719–721.

Kratzer, F. H., and Vohra, P. (1986). "Chelates in Nutrition." CRC Press, Boca Raton, Florida.

Layrisse, M., Cook, J. D., Martinez, C., Roche, M., Kuhn, I. N., Walker, R. B., and Finch, C. A. (1969). Food iron absorption: A comparison of vegetable and animal food. *Blood* **33,** 430–443.

Lee, Y. H., Fox, M. R. S., and Tao, S.-H. (1986). Bioavailability of cadmium from soy isolate. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **45,** 439.

Lönnerdal, B., Cederblad, Å., Davidsson, L., and Sandström, B. (1984). The effect of individual components of soy formula and cows' milk formula on zinc bioavailability. *Am. J. Clin. Nutr.* **40,** 1064–1070.

Lonnerdal, B., Kunz, C., Sandberg, A.-S., and Sandstrom, B. (1987). Inhibitory effects of various inositol phosphates on zinc and calcium absorption. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **46,** 599.

Lynch, S. R., Dassenko, S. A., Morck, T. A., Beard, J. L., and Cook, J. D. (1985). Soy protein products and heme iron absorption in humans. *Am. J. Clin. Nutr.* **41,** 13–20.

McCance, R. A., and Widdowson, E. M. (1935). CCCXX. Phytin in human nutrition. *Biochem. J.* **29,** 2694–2699.

McCance, R. A., and Widdowson, E. M. (1942a). Mineral metabolism of healthy adults on white and brown bread dietaries. *J. Physiol. (London)* **101,** 44–85.

McCance, R. A., and Widdowson, E. M. (1942b). Mineral metabolism on dephytinized bread. *J. Physiol. (London)* **101,** 304–313.

McCance, R. A., Edgecombe, C. N., and Widdowson, E. M. (1943). Phytic acid and iron absorption. *Lancet* **ii,** 126–128.

McGinnis, J., Norris, L. C., and Heuser, G. F. (1944). Poor utilization of phosphorus in cereals and legumes by chicks for bone development. *Poult. Sci.* **23,** 157–159.

Mahaffey, K. R. (1985). Factors modifying susceptibility to lead toxicity. *In* "Dietary and Environmental Lead: Human Health Effects" (K. R. Mahaffey, ed.), pp. 373–419. Elsevier, New York.

Moore, R. J., and Veum, T. L. (1982). Effect of dietary phosphorus and yeast culture level on the utilization of phytate phosphorus by the rat. *Nutr. Rep. Int.* **25,** 221–233.

Moore, R. J., and Veum, T. L. (1983). Adaptive increase in phytate digestibility by phosphorus-deprived rats and the relationship of intestinal phytase (EC 3.1.3.8) and alkaline phosphatase (EC 3.1.3.1) to phytate utilization. *Br. J. Nutr.* **49,** 145–152.

Morck, T. A., Lynch, S. R., and Cook, J. D. (1982). Reduction of the soy-induced inhibition of nonheme iron absorption. *Am. J. Clin. Nutr.* **36,** 219–228.

Morris, E. R. (1983). An overview of current information on bioavailability of dietary iron to humans. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **42,** 1716–1720.

Morris, E. R. (1986). Phytate and dietary mineral bioavailability. *In* "Phytic Acid: Chemistry and Applications" (E. Graf, ed.), pp. 57–76. Pilatus, Minneapolis, Minnesota.

Morris, E. R., and Ellis, R. (1976). Isolation of monoferric phytate from wheat bran and its biological value as an iron source to the rat. *J. Nutr.* **106,** 753–760.

Morris, E. R., and Ellis, R. (1980). Bioavailability to rats of iron and zinc in wheat bran: Response to low-phytate bran and effect of the phytate/zinc molar ratio. *J. Nutr.* **110,** 2000–2010.

Morris, E. R., and Ellis, R. (1982). Phytate, wheat bran, and bioavailability of dietary iron. *In* "Nutritional Bioavailability of Iron" (C. Kies, ed.), ACS Symp. Ser. 203, pp. 121–141. American Chemical Society, Washington, D.C.

Morris, E. R., and Ellis, R. (1983). Dietary phytate/zinc molar ratio and zinc balance in humans. *In* "Nutritional Bioavailability of Zinc" (G. E. Inglett, ed.), pp. 159–172. American Chemical Society, Washington, D.C.

Morris, E. R., and Ellis, R. (1985a). Bioavailability of dietary calcium. Effect of phytate on adult men consuming nonvegetarian diets. *In* "Nutritional Bioavailability of Calcium" (C. Kies, ed.), ACS Symp. Ser. 275, pp. 63–72. American Chemical Society, Washington, D.C.

Morris, E. R., and Ellis, R. (1985b). Trace element nutriture of adult men consuming three levels of phytate. *In* "Trace Elements in Man and Animals" (C. F. Mills, I. Bremner, and J. K. Chesters, eds.), Vol. 5, pp. 443–446. Commonwealth Agricultural Bureaux, Slough, England.

Morris, E. R., Ellis, R., Steele, P., and Moser, P. (1980). Inorganic nutrient balance of humans consuming whole wheat bran vs. dephytinized bran. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **39,** 787.

Morris, E. R., Ellis, R., Hill, A. D., Steele, P., Cottrel, S., Moy, T., and Moser, P. (1986). Apparent zinc and iron balance of adult men consuming three levels of phytate. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **45,** 819.

Nahapetian, A., and Young, V. R. (1980). Metabolism of ^{14}C-phytate in rats: Effect of low and high dietary calcium intakes. *J. Nutr.* **110,** 1458–1472.

National Research Council (1980). "Recommended Dietary Allowances." National Academy of Sciences, Washington, D.C.

Nävert, B., Sandström, B., and Cederblad, Å. (1985). Reduction of the phytate content of bran by leavening in bread and its effect on zinc absorption in man. *Br. J. Nutr.* **53,** 47–53.

Nayini, N. R., and Markakis, P. (1983a). Effect of fermentation time on the inositol phosphates of bread. *J. Food Sci.* **48,** 262–263.

Nayini, N. R., and Markakis, P. (1983b). Effect of milling extraction on the inositol phosphates of wheat flour and bread. *J. Food Sci.* **48,** 1384–1387.

Nelson, T. S. (1967). The utilization of phytate phosphorus by poultry—A review. *Poult. Sci.* **46,** 862–871.

Nelson, T. S. (1976). The hydrolysis of phytate phosphorus by chicks and laying hens. *Poult. Sci.* **55,** 2262–2264.

Nelson, T. S., and Kirby, L. K. (1979). Effect of age and diet composition on the hydrolysis of phytate phosphorus by rats. *Nutr. Rep. Int.* **20,** 729–734.

Nelson, T. S., Shieh, T. R., Wodzinski, R. J., and Ware, J. H. (1971). Effect of supplemental phytase on the utilization of phytate phosphorus by chicks. *J. Nutr.* **101,** 1289–1294.

Nelson, T. S., Daniels, L. B., Hall, J. R., and Shields, L. G. (1976). Hydrolysis of natural phytate phosphorus in the digestive tract of calves. *J. Anim. Sci.* **42,** 1509–1512.

Oberleas, D. (1975). Factors influencing availability of minerals. *In* "Proceedings of the Fourth Western Hemisphere Nutrition Congress" (Council on Foods and Nutrition, ed.), p. 156. American Medical Association, Chicago.

Oberleas, D., and Harland, B. F. (1981). Phytate content of foods: Effect on dietary zinc bioavailability. *J. Am. Diet. Assoc.* **79,** 433–436.

Oberleas, D., and Harland, B. F. (1986). Analytical methods for phytate. *In* "Phytic Acid: Chemistry and Applications" (E. Graf, ed.), pp. 77–100. Pilatus, Minneapolis, Minnesota.

Oberleas, D., Muhrer, M. E., and O'Dell, B. L. (1966). Dietary metal complexing agents and zinc availability in the rat. *J. Nutr.* **90,** 56–62.

O'Dell, B. L. (1979). Effect of soy protein on mineral bioavailability. *In* "Soy Protein and Human Nutrition" (H. L. Wilcke, D. T. Hopkins, and D. H. Waggle, eds.), pp. 187–204. Academic Press, New York.

O'Dell, B. L. (1984). Bioavailability of trace elements. *Nutr. Rev.* **42,** 301–308.

O'Dell, B. L., and Savage, J. E. (1960). Effect of phytic acid on zinc availability. *Proc. Soc. Exp. Biol. Med.* **103,** 304–306.

Pennington, J. A. T., Young, B. E., Wilson, D. B., Johnson, R. D., and Vanderveen, J. E. (1986). Mineral content of foods and total diets: The Selected Minerals in Foods Survey, 1982 to 1984. *J. Am. Diet. Assoc.* **86,** 876–891.

Phillippy, B. Q., White, K. D., Johnston, M. R., Tao, S.-H., and Fox, M. R. S. (1987). Preparation of inositol phosphates from sodium phytate by enzymatic and nonenzymatic hydrolysis. *Anal. Biochem.* **162,** 115–121.

Phillippy, B. Q., Johnston, M. R., Tao, S.-H., and Fox, M. R. S. (1988). Inositol phosphates in processed foods. *J. Food Sci.* **53,** 496–499.

Pilch, S. M., and Senti, F. R. (eds.) (1984a). "Assessment of the Iron Nutritional Status of the U.S. Population Based on Data Collected in the Second Health and Nutrition Examination Survey, 1976–1980." Life Sciences Research Office, Bethesda, Maryland.

Pilch, S. M., and Senti, F. R. (eds.) (1984b). "Assessment of the Zinc Nutritional Status of the U.S. Population Based on Data Collected in the Second National Health and Nutrition Examination Survey, 1976–1980." Life Sciences Research Office, Bethesda, Maryland.

Pileggi, V. J., DeLuca, H. F., and Steenbock, H. (1955). The role of vitamin D and intestinal phytase in the prevention of rickets in rats on cereal diets. *Arch. Biochem. Biophys.* **58,** 194–204.

Platt, S. R., and Clydesdale, F. M. (1984). Binding of iron by cellulose, lignin, sodium phytate and beta-glucan, alone and in combination, under simulated gastrointestinal pH conditions. *J. Food Sci.* **49,** 531–535.

Platt, S. R., and Clydesdale, F. M. (1987). Interactions of iron, alone and in combination with calcium, zinc, and copper, with a phytate-rich, fiber-rich fraction of wheat bran under gastrointestinal pH conditions. *Cereal Chem.* **64,** 102–105.

Pointillart, A., Fontaine, N., and Thomasset, M. (1984). Phytate phosphorus utilization and intestinal phosphatases in pigs fed low phosphorus: Wheat or corn diets. *Nutr. Rep. Int.* **29,** 473–483.

Pointillart, A., Fourdin, A., and Fontaine, N. (1987). Importance of cereal phytase activity for

phytate phosphorus utilization by growing pigs fed diets containing triticale or corn. *J. Nutr.* **117,** 907–913.

Prasad, A. S., Farid, Z., Sandstead, H. H., Schulart, A. R., and Darby, W. J. (1963). Biochemical studies on dwarfism, hypogonadism and anemia. *Arch. Intern. Med.* **111,** 407–423.

Ranhotra, G. S., Loewe, R. J., and Puyat, L. V. (1974). Effect of dietary phytic acid on the availability of iron and phosphorus. *Cereal Chem.* **51,** 323–329.

Rao, K. S., and Narasinga Rao, B. S. (1983). Studies on iron chelation by phytate and the influence of other mineral ions on it. *Nutr. Rep. Int.* **28,** 771–782.

Reddy, N. R., Sathe, S. K., and Salunkhe, D. K. (1982). Phytates in legumes and cereals. *Adv. Food Res.* **28,** 1–92.

Reinhold, J. G., Nasr, K., Lahimgarzadeh, A., and Hedayati, H. (1973). Effects of purified phytate and phytate-rich bread upon metabolism of zinc, calcium, phosphorus, and nitrogen in man. *Lancet* **1,** 283–288.

Reinhold, J. G., Ismail-Beigi, F., and Faradji, B. (1975). Fibre vs. phytate as determinant of the availability of calcium, zinc and iron of breadstuffs. *Nutr. Rep. Int.* **12,** 75–85.

Reinhold, J. G., Faradji, B., Abadi, P., and Ismail-Beigi, F. (1976a). Binding of zinc to fiber and other solids of wholemeal bread. *Trace Elem. Hum. Health Dis.* **1,** 163–180.

Reinhold, J. G., Faradji, B., Abadi, P., and Ismail-Beigi, F. (1976b). Decreased absorption of calcium, magnesium, zinc, and phosphorus by humans due to increased fiber and phosphorus consumption as wheat bread. *J. Nutr.* **106,** 493–503.

Roberts, A. H., and Yudkin, J. (1961). Effect of phytate and other dietary factors on intestinal phytase and bone calcification in the rat. *Br. J. Nutr.* **15,** 457–471.

Ronaghy, H., Fox, M. R. S., Garn, S. M., Israel, H., Harp, A., Moe, P. G., and Halsted, J. A. (1969). Controlled zinc supplementation for malnourished school boys: A pilot experiment. *Am. J. Clin. Nutr.* **22,** 1279–1289.

Rose, H. E., and Quarterman, J. (1984). Effects of dietary phytic acid on lead and cadmium uptake and depletion in rats. *Environ. Res.* **35,** 482–489.

Rossander, L. (1987). Effect of dietary fiber on iron absorption in man. *Scand. J. Gastroenterol.* **22,** 68–72.

Sandberg, A.-S., Hasselblad, C., Hasselblad, K., and Hultén, L. (1982). The effect of wheat bran on the absorption of minerals in the samll intestine. *Br. J. Nutr.* **48,** 185–191.

Sandberg, A.-S., Andersson, H., Kivistö, B., and Sandström, B. (1986). Extrusion cooking of a high-fibre cereal product. 1. Effects on digestibility and absorption of protein, fat, starch, dietary fibre and phytate in the small intestine. *Br. J. Nutr.* **55,** 245–254.

Sandberg, A.-S., Andersson, H., Carlsson, N.-G., and Sandström, B. (1987). Degradation products of bran phytate formed during digestion in the human small intestine: Effect of extrusion cooking on digestibility. *J. Nutr.* **117,** 2061–2065.

Sandstead, H. H., Prasad, A. S., Schulert, A. R., Farid, Z., Miale, A., Jr., Bassily, S., and Darby, W. J. (1967). Human zinc deficiency, endocrine manifestations and response to treatment. *Am. J. Clin. Nutr.* **20,** 422–442.

Sandström, B., and Cederblad, Å. (1980). Zinc absorption from composite meals. II. Influence of the main protein source. *Am. J. Clin. Nutr.* **33,** 1778–1783.

Sandström, B., Arvidsson, B., Cederblad, Å., and Björn-Rasmussen, E. (1980). Zinc absorption from composite meals. I. The significance of wheat extraction rate, zinc, calcium, and protein content in meals based on bread. *Am. J. Clin. Nutr.* **33,** 739–745.

Sandström, B., Cederblad, Å., and Lönnerdal, B. (1983). Zinc absorption from human milk, cow's milk, and infant formulas. *Am. J. Dis. Child.* **137,** 726–729.

Sandström, B., Andersson, H., Kivistö, B., and Sandberg, A.-S. (1986). Apparent small intestinal absorption of nitrogen and minerals from soy and meat-protein-based diets. A study on human ileostomy subjects. *J. Nutr.* **116,** 2209–2218.

Sandström, B., Almgren, A., Kivistö, B., and Cederblad, Å. (1987a). Zinc absorption in humans from meals based on rye, barley, oatmeal, triticale and whole wheat. *J. Nutr.* **117,** 1898–1902.

Sandström, B., Kivistö, B., and Cederblad, Å. (1987b). Absorption of zinc from soy protein meals in humans. *J. Nutr.* **117,** 321–327.

Savage, J. E., Yohe, J. M., Pickett, E. E., and O'Dell, B. L. (1964). Zinc metabolism in the growing chick. Tissue concentration and effect of phytate on absorption. *Poult. Sci.* **43,** 420–426.

Schwartz, R., Apgar, B. J., and Wien, E. M. (1986). Apparent absorption and retention of Ca, Cu, Mg, Mn, and Zn from a diet containing bran. *Am. J. Clin. Nutr.* **43,** 444–455.

Scott, J. J., and Loewus, F. A. (1986). Phytate metabolism in plants. *In* "Phytic Acid: Chemistry and Applications" (E. Graf, ed.), pp. 23–42. Pilatus, Minneapolis, Minnesota.

Sharpe, L. M., Peacock, W. C., Cooke, R., and Harris, R. S. (1950). The effect of phytate and other food factors on iron absorption. *J. Nutr.* **41,** 433–446.

Simpson, K. M., Morris, E. R., and Cook, J. D. (1981). The inhibitory effect of bran on iron absorption in man. *Am. J. Clin. Nutr.* **34,** 1469–1478.

Singh, M., and Krikorian, A. D. (1982). Inhibition of trypsin activity in vitro by phytate. *J. Agric. Food Chem.* **30,** 799–800.

Smith, A. K., and Circle, S. J. (1978). "Soybeans: Chemistry and Technology," Vol. 1. AVI Publ. Westport, Connecticut.

Tao, S.-H., Fox, M. R. S., Phillippy, B. Q., Fry, B. E., Jr., Johnson, M. L., and Johnston, M. R. (1986). Effects of inositol phosphates on mineral utilization. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **45,** 819.

Taylor, T. G., and Coleman, J. W. (1979). A comparative study of the absorption of calcium and the availability of phytate-phosphorus in the golden hamster (*Mesocricetus auratus*) and the laboratory rat. *Br. J. Nutr.* **42,** 113–119.

Thompson, L. U. (1988). Antinutrients and blood glucose. *Food Technol.* **42,** 123–132.

Thompson, L. U., Button, C. L., and Jenkins, D. J. A. (1987). Phytic acid and calcium affect the in vitro rate of navy bean starch digestion and blood glucose response in humans. *Am. J. Clin. Nutr.* **46,** 467–473.

Toma, R. B., and Curtis, D. J. (1986). Dietary fiber: Effect on mineral bioavailability. *Food Technol.* **40,** 111–116.

Turnbull, A., Cleton, F., Finch, C. A., Thompson, L., and Martin, J. (1962). Iron absorption. IV. The absorption of hemoglobin iron. *J. Clin. Invest.* **41,** 1897–1907.

Turnlund, J. R., King, J. C., Keyes, W. R., Gong, B., and Michel, M. C. (1984). A stable isotope study of zinc absorption in young men: Effects of phytate and α-cellulose. *Am. J. Clin. Nutr.* **40,** 1071–1077.

Turnlund, J. R., King, J. C., Gong, B., Keyes, W. R., and Michel, M. C. (1985). A stable isotope study of copper absorption in young men: Effect of phytate and α-cellulose. *Am. J. Clin. Nutr.* **42,** 18–23.

Waldroup, P. W., Ammerman, C. B., and Harms, R. H. (1964a). The availability of phytic acid phosphorus for chicks. 2. Comparison of phytin phosphorus sources. *Poult. Sci.* **43,** 426–432.

Waldroup, P. W., Ammerman, C. B., and Harms, R. H. (1964b). The availability of phytic acid phosphorus for chicks. 3. Effect of calcium and vitamin D_3 levels on the utilization of calcium phytate. *Poult. Sci.* **43,** 926–931.

Walker, A. R. P., Fox, F. W., and Irving, J. T. (1948). Studies in human mineral metabolism. 1. The effect of bread rich in phytate phosphorus on the metabolism of certain mineral salts with special reference to calcium. *Biochem. J.* **42,** 452–462.

Welch, R. M., and Van Campen, D. R. (1975). Iron availability to rats from soybeans. *J. Nutr.* **105,** 253–256.

Wilcke, H. L., Hopkins, D. T., and Waggle, D. H. (1979). "Soy Protein and Human Nutrition." Academic Press, New York.

Wise, A. (1981). Protective action of calcium phytate against acute lead toxicity in mice. *Bull. Environ. Contam. Toxicol.* **27,** 630–633.

Wise, A. (1982). Blood lead levels after chronic feeding to mice of lead acetate with calcium phytate in the diet. *Bull. Environ. Contam. Toxicol.* **29,** 550–553.

Wise, A. (1983). Dietary factors determining the biological activities of phytate. *Nutr. Abstr. Rev.: Clin. Nutr.* **53,** 791–806.

Wise, A., and Gilburt, D. J. (1981). Binding of cadmium and lead to the calcium–phytate complex in vitro. *Toxicol. Lett.* **9,** 45–50.

Wise, A., and Gilburt, D. J. (1982a). In vitro competition between calcium phytate and the soluble fraction of rat small intestine contents for cadmium, copper and zinc. *Toxicol. Lett.* **11,** 49–54.

Wise, A., and Gilburt, D. J. (1982b). Phytate hydrolysis by germfree and conventional rats. *Appl. Environ. Microbiol.* **43,** 753–756.

Wise, A., Richards, C. P., and Trimble, M. L. (1983). Phytate hydrolysis in rat gastrointestinal tracts, as observed by ^{31}P Fourier transform nuclear magnetic resonance spectroscopy. *Appl. Environ. Microbiol.* **45,** 313–314.

Wise, A., Lockie, G. M., and Liddell, J. (1987). Dietary intakes of phytate and its meal distribution pattern amongst staff and students in an institution of higher education. *Br. J. Nutr.* **58,** 337–346.

Yoon, J. H., Thompson, L. U., and Jenkins, D. J. A. (1983). The effect of phytic acid on in vitro rate of starch digestibility and blood glucose response. *Am. J. Clin. Nutr.* **38,** 835–842.

5

Interactions of Vitamin B_6 (Pyridoxine) and Xenobiotics

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I. INTRODUCTION

Vitamin B_6 (pyridoxine) is currently enjoying a new wave of interest among nutrition scientists and the general public. With the development of more specific techniques to define the status of vitamin B_6 and its long history of interaction

NUTRITIONAL TOXICOLOGY, VOL. III

with drugs and environmental toxicants such as ethanol, much concern has arisen over the possibility of subclinical vitamin B_6 deficiency being prevalent in various segments of the population (Serfontein *et al.*, 1984).

Vitamin B_6 is involved in many diverse reactions in the body. As pyridoxal phosphate (PLP), it functions as a coenzyme in the metabolism of amino acids, proteins, carbohydrates, lipids, nucleic acids, and hormones and is involved in decarboxylation, transamination, deamination, racemization, and desulfhydration reactions, to name a few. Therefore, it is not surprising that vitamin B_6 has been reported to react with a number of substances, leading in some instances to biochemical and clinical signs of vitamin deficiency. The following review discusses observations and possible mechanisms concerning the interactions between vitamin B_6 and drugs and other xenobiotics. To better understand these processes, some general principles of vitamin B_6 metabolism are first reviewed.

II. METABOLISM OF VITAMIN B_6

The chemistry, metabolic function, transport, and storage of vitamin B_6 have been extensively studied and reviewed (Fasella, 1967; Holtz and Palm, 1964; Henderson, 1984). In general, vitamin B_6 represents a group of naturally occurring interrelated pyridine derivatives consisting of pyridoxine, pyridoxal, pyridoxamine, and their phosphorylated forms. The B_6 vitamers are phosphorylated by the enzyme pyridoxal kinase (EC 2.7.1.35), whereas pyridoxine 4-oxidase (EC 1.1.3.12) and pyridoxamine-phosphate oxidase (EC 1.4.3.5) catalyze the interconversion of the vitamin B_6 forms. The metabolic relationships among the B_6 vitamers have been described in detail by Henderson (1984).

Unphosphorylated B_6 vitamers are absorbed from the diet and rapidly appear in the liver where they are converted to PLP (Henderson, 1984). Numerous studies have reported that the liver is the primary organ responsible for PLP concentrations in plasma (Merrill *et al.*, 1984; Lumeng *et al.*, 1974; Van den Berg *et al.*, 1982), and as a consequence plasma PLP is often lower in patients with liver disease (Mitchell *et al.*, 1976; Labadarios *et al.*, 1977). Low plasma PLP is a well-known observation in patients with alcoholic liver disease (Bonjour, 1980; Majumdar *et al.*, 1982), but Labadarios *et al.* (1977) showed that liver disease alone was sufficient to induce increased PLP clearance and abnormal vitamin B_6 metabolism. Considering that well over 100 agents are known to induce liver disease (Zimmerman, 1978; Ludwig and Axelsen, 1983), their potential for inducing abnormal vitamin B_6 metabolism is very real, but it has attracted little investigation.

Liver PLP concentrations are regulated by protein binding and enzymatic hydrolysis by a nonspecific plasma membrane-bound alkaline phosphatase (Li *et al.*, 1974; Lumeng and Li, 1974). In plasma, PLP is principally complexed to

albumin through Schiff base formation (Lumeng *et al.*, 1974); and it is believed that plasma PLP represents a dynamic equilibrium among hepatic synthesis, uptake into cells, and/or degradation (Lumeng *et al.*, 1974). Although plasma PLP has often been used as a determinant of vitamin B_6 status, recent reports indicate that its use alone may not be sufficient to assess status under conditions associated with a disturbed plasma PLP/pyridoxal equilibrium such as might occur with pregnancy, fasting, or other conditions (Barnard *et al.*, 1986, 1987).

As a consequence of its role in amino acid metabolism, vitamin B_6 dietary requirements are linked to protein intake and, as has been recently suggested, may also be linked to protein quality (Fisher *et al.*, 1984). Currently, the RDA for adults is about 2 mg but increases to 2.5 mg in pregnant or lactating women. It has been reported that pyridoxal is always present in tissue with concentrations ranging from 70 μmol/kg tissue in brain to 360 μmol/kg tissue in liver (Stanulovic, 1980).

TABLE I

Certain Vitamin B_6-Dependent Enzymes

Enzyme	Function	Product
GABA system		
Glutamate decarboxylase (GAD)	Synthesis of γ-aminobutyric acid (GABA)	GABA
GABA-transaminase (GABA-T, 4-aminobutyrate aminotransferase)	Degradation of GABA	Succinic semialdehyde
Tryptophan metabolism		
5-Hydroxytryptophan decarboxylase (aromatic-L-amino-acid decarboxylase)	Synthesis of serotonin	Serotonin
Kynurenine transaminase (kynurenine–oxoglutarate aminotransferase)	Metabolism of kynurenine and 3-hydroxykynurenine away from tryptophan to niacin pathway	Kynurenic acid and xanthurenic acid
Kynureninase	Metabolism of 3-hydroxy-kynurenine to 3-hydroxyanthranilic acid in tryptophan to niacin pathway	Quinolinic acid
Taurine synthesis		
Cystathionine β-synthase	Convert homocysteine to cystathionine	Cystathionine
Cystathionine γ-lyase	Convert cystathionine to hypotaurine	Cysteine
Cysteic acid decarboxylase	Convert cysteic acid to taurine	Taurine
Other enzymes		
Dopa decarboxylase (aromatic-L-amino-acid decarboxylase)	Convert dopa to dopamine	Dopamine
Ornithine decarboxylase	Convert ornithine to putrescine in polyamine synthesis	Putrescine

Pyridoxine plays a prominent role in the structure and function of the nervous system. PLP has been established as an essential cofactor for enzymes involved in the synthesis and degradation of a number of putative neurotransmitters including γ-aminobutyric acid (GABA), dopamine, norepinephrine, serotonin, taurine, histamine, tyramine, tryptamine, and acetylcholine, indirectly (Ebadi *et al.*, 1982; Dakshinamurti, 1982). In addition, vitamin B_6 is also involved in the synthesis of sphinogolipids in myelin metabolism and in the synthesis of polyamines (Dakshinamurti, 1982). Therefore, vitamin B_6 is often considered in mechanisms associated with drug-induced neurotoxicity or their treatment. Key vitamin B_6-dependent enzymes implicated in mechanisms of neurotoxicity or signs of depletion described in this review are listed in Table I.

III. INTERACTION OF VITAMIN B_6 WITH DRUGS

PLP is chemically a very reactive compound, forming a Schiff base with any substance with an amine group. As a consequence, a number of drugs have been reported to induce either biochemical or clinical signs of vitamin B_6 deficiency in both experimental animals and humans, presumably through Schiff-base formation and a reduction in PLP available as a coenzyme, or by inhibition of PLP synthesis or certain vitamin B_6-dependent enzymes. Rumsby and Shepherd (1980) observed that a variety of drugs could affect tryptophan metabolism, presumably through altered vitamin B_6 metabolism. A few of the agents are discussed below. Table II gives a more extensive listing of drugs known or purported to interfere with vitamin B_6 metabolism.

A. Isoniazid

Isoniazid (isonicotinic acid hydrazide, INH) is probably the most extensively studied drug with respect to effects on vitamin B_6 metabolism. Used in combination therapy, INH is still an important drug for treatment of all types of tuberculosis. Its anti-vitamin B_6 effect was recognized early when it was reported that a peripheral neuritis, similar to that produced by the B_6 antagonist, deoxypyridoxine, could be observed in up to 40% of patients taking 10 mg/kg/day INH (Biehl and Vilter, 1954) and in 19% of patients receiving 6 mg/kg/day (Clark, 1976).

Further investigations into the mechanisms of INH–vitamin B_6 interaction confirmed the formation of a hydrazone (Ebadi *et al.*, 1982). However, it appears that the mechanism for INH-induced PLP depletion is much more complex. It was known that these hydrazones were effective inhibitors of pyridoxal kinase [see Stanulovic (1980) and references cited therein; see also Ebadi *et al.* (1982)], and studies in rats indicated that inhibition of pyridoxal kinase is more important for the antivitamin action of INH than its direct condensation with PLP

TABLE II

Drug Reported to Interact with Vitamin B_6

Drug class	Compound
Antituberculosis	Isoniazid,[a] cycloserine, ethambutol
Antibiotics	Ampicillin, oxytetracycline, nitrofurantoin,[a] nalidixic acid
Nonsteroidal antiinflammatory drug	Indomethacin
Chelating agent	Penicillamine
Anticonvulsants	Phenytoin
Antihypertensive agents	Hydralazine[a]
Antiarrhythmic	Amiodarone
Local anesthetics	Procainamide
MAO inhibitors	Phenelzine,[a] iproniazid,[a] nialamide,[a] Isocarboxazid[a]
Cancer chemotherapeutic agents	Procarbazine[a]
Antiamebic drugs	Metronidazole
Antiparkinsonism drugs	Levodopa
Hormones	Oral contraceptives
Alcohols	Ethanol

[a]Hydrazine and related compounds.

(De Jesus Sevigny *et al.*, 1966). In addition, it was suggested that the hydrazone is more toxic than the parent compound and that, at least *in vitro*, the hydrazone has coenzyme activity due to its slow hydrolysis [see Holtz and Palm (1964) and references cited therein]. Nevertheless, a prophylactic dose of 100 mg pyridoxine/day is usually administered with INH therapy (Nelson *et al.*, 1983). Although clinical signs of INH-induced vitamin B_6 deficiency are considered rare in children, Pellock *et al.* (1985) recently reported that pyridoxine supplementation should also be considered in children receiving more than 10 mg/kg/day INH, especially those who are debilitated or of poor nutritional status.

Another aspect of INH–vitamin B_6 interaction concerns the use of pyridoxine as an antidote to INH poisoning. INH has been reported to be commonly used for suicide attempts among American Indians and Alaskan natives [see Chin *et al.*, (1981) and references cited therein], resulting in convulsions and death. Pyridoxine is the most recommended antidote for INH or acute hydrazine toxicity (Chin *et al.*, 1981). Use of PLP, however, results in convulsions because PLP enhances the uptake of INH into the brain (Holtz and Palm, 1964). In contrast, pyridoxine raised the dose of hydrazines necessary to induce lethal convulsions (Hein and Weber, 1984).

Studies have suggested that convulsions arise from INH inhibition of the B_6-dependent GAD in the brain, resulting in low GABA levels (Wason *et al.*, 1981; Ebadi *et al.*, 1982). However, alterations in GABA turnover may be more critical (Holtz and Palm, 1964, and references cited therein). Although the mechanisms involved are not well understood, numerous studies have shown the

efficacy of short-term pyridoxine therapy (gram doses) to treat INH poisoning (Brown, 1972; Wason *et al.*, 1981; Sievers and Herrier, 1980; Harati and Niakan, 1986).

In a case report, Harati and Niakan (1986) present the first indication of toxicity due to intravenous pyridoxine (10 g) for the treatment of INH poisoning. In an attempt to reduce the likelihood of pyridoxine toxicity, Chin *et al.* (1978, 1981) investigated the combination of central nervous system (CNS) depressants or anticonvulsants with pyridoxine in the treatment of acute INH intoxication in rats and dogs. They observed in dogs, an animal with a sensitivity to INH similar to that of humans, that diazepam, phenytoin, and phenobarbital enhanced the effectiveness of pyridoxine; but the combination of diazepam and pyridoxine was the most effective due to a rapid onset of action and a wide margin of safety (Chin *et al.*, 1981). Sievers *et al.* (1982) have applied this combination therapy to treat INH overdose in humans.

The possible mechanism for this effect postulates that pyridoxine would increase brain GABA levels, and the anticonvulsants and depressants would potentiate the postsynaptic action of GABA with the result that the combined therapy is synergistic against INH-induced convulsions (Ebadi *et al.*, 1982). Since INH toxicity can be enhanced in individuals who are genetically disposed to acetylate the drug more slowly, it is important to realize that pyridoxine therapy is equally effective in these individuals (Hein and Weber, 1984).

An additional aspect of INH–pyridoxine interactions warrants mention. In mice, INH has been reported to be a mutagen and possibly a carcinogen (Kandarkar *et al.*, 1980) similar to other known carcinogenic hydrazines (Toth, 1975), and pyridoxine could prevent the structural changes induced by INH on hepatocytes (Kandarkar *et al.*, 1980). Whether this effect simply reflects INH–pyridoxine hydrazone formation or another mechanism remains to be elucidated.

B. Other Hydrazines

Besides INH, other hydrazines and their derivatives would be expected to react with PLP to form hydrazones. However, these compounds and their interaction with PLP have not been extensively studied. In general, hydrazines, substituted hydrazine derivatives, hydrazones, hydrazides, and related compounds are a group of highly reactive compounds that have been used for many medical and industrial purposes. Examples of hydrazines found in foods and used in medicine are listed in Tables II and III. Hydrazines and their methyl derivatives have also been used in the aerospace industry as rocket fuels, and they have been widely used in the petroleum and metal industry and in chemical and photographic processing. The chemistry, applications, and metabolism of these compounds have been reviewed in detail (Cornish, 1969; Juchau and Horita, 1972).

TABLE III

Hydrazines and Related Compounds Found in Food

Natural hydrazines	Source
N-Methyl-*N*-formylhydrazine	Mushroom (*Gyromitra esculenta*)
4-Hydroxymethylphenylhydrazine	Mushroom (*Agarcius bisporus*)
Agaritine (β-*N*-[γ-L(+)-glutamyl]-4-hydroxymethylphenylhydrazine)	Mushroom (*Agarcius bisporus*)

It has become apparent that these compounds can produce a number of side effects, particularly CNS stimulation, convulsions, and death (Cornish, 1969). It appears that effects on vitamin B_6 metabolism and B_6-dependent enzymes vary with the dose and type of hydrazine examined (Cornish, 1969; Chatterjee and Sengupta, 1980), and these effects are not equally responsive to vitamin B_6 therapy. Pyridoxine prevents convulsive seizures induced by hydrazine, monomethyl, or other hydrazines (Cornish, 1969; Toth and Erickson, 1977; George *et al.*, 1982) in rats and mice, but it is only slightly protective against the toxic effects of *N*-methyl-*N*-formylhydrazine (MFH), found in the wild, edible mushroom, *Gyromitra esculenta* (Toth and Erickson, 1977). The limited effectiveness of PLP or pyridoxal may reflect the intrinsic toxicity of the hydrazones formed as well as other parameters, including the rate of absorption, distribution into the brain, the chemistry of hydrazone formation and the degree of inhibition of B_6-dependent enzymes by these agents (Cornish, 1969).

C. Cycloserine

Cycloserine, a broad-spectrum antibiotic, was introduced as an alternative therapy in the treatment of tuberculosis, particularly in cases resistant to the INH–*p*-aminosalicylic acid–streptomycin combination. Its use was associated with a number of CNS side effects including sedation, epileptic convulsions, and seizures that often appeared within the first 2 weeks of therapy and that were readily ameliorated by pyridoxine [see Holtz and Palm (1964) and references cited therein]. Cycloserine was shown to combine with PLP to form an unstable Schiff base that splits to form a stable pyridoxal–oxime compound (cf. Holtz and Palm, 1964). This oxime inactivates PLP (Bhagavan and Brin, 1983), but other studies have shown that cycloserine could also inhibit a number of B_6-dependent enzymes *in vitro*, including dopa decarboxylase (aromatic-L-amino-acid decarboxylase), GAD, and kynureninase (Holtz and Palm, 1964). These effects could account for abnormal tryptophan metabolism and the seizures observed with cycloserine use. Bhagavan and Brin (1983) reported that these effects could be counteracted by administering 50 mg/day pyridoxine with cycloserine therapy.

D. Penicillamine

Penicillamine (PA, β,β-dimethylcysteine) is a chelating agent used for many years to treat Wilson's disease, cystinuria, heavy metal poisoning, and rheumatoid arthritis. Its side effects are mostly neurological and include sensory and motor neuropathies and epileptic seizures. Because PA has an asymmetrical carbon atom, it exists in both the L and D form, as well as the DL isomer. It was observed early that L-PA was highly toxic and had anti-vitamin B_6 activity (cf. Rumsby and Shepherd, 1981; Holtz and Palm, 1964). Subsequently, the D form was shown to also interact with vitamin B_6, with the DL form being intermediate in toxicity (Holtz and Palm, 1964).

Early investigations into the anti-vitamin-B_6 effect of PA observed that the drug could react with PLP to form a thiazolidine (Holtz and Palm, 1964). However, the antivitamin action of PA appeared more complex than simple chemical interaction. Data indicated that biochemical signs of vitamin B_6 deficiency can occur in the early stages of PA therapy, after which time there appears to be an adaptive response in which B_6 status seems normal, but B_6 metabolism can again be affected as the PA dose is increased (Rumsby and Shepherd, 1981). In addition, all PA isomers were observed to inhibit some B_6-dependent enzymes *in vitro*. Although D-PA competed with the apoenzyme for PLP, L-PA could inhibit the holoenzyme (Holtz and Palm, 1964).

Nevertheless, evidence suggests that the interaction between vitamin B_6 and PA is not clinically significant, particularly because D-PA is the recommended form for therapy. In a study of 79 control and 144 D-PA-treated patients, Rumsby and Shepherd (1981) showed that evidence of vitamin B_6 deficiency could be demonstrated only by *in vitro* assay of erythrocyte aspartate aminotransferase activity because no patient showed clinical symptoms. In our own experience, we have not detected evidence for vitamin B_6 deficiency in rats fed D -PA for six weeks as determined by tyrosine apodecarboxylase activity (M. A. Dubick and C. L. Keen, unpublished results). Generally, a prophylactic dose of 50 mg/day pyridoxine is recommended with D-PA therapy to prevent signs of B_6 deficiency (Sternlieb and Scheinberg, 1964; Bhagavan and Brin, 1983); and in some instances, such as in the treatment of methyl mercury poisoning in rats, pyridoxine given with D-PA reversed the adverse effects of D-PA on body weight and food intake without affecting the binding of D-PA to mercury (Lapin and Carter, 1981).

E. Levodopa

The drug levodopa (L-dopa) has been successfully employed in the treatment of Parkinson's disease for many years, but its use has been associated with neurological and behavioral side effects that were responsive to pyridoxine ad-

ministration (Bhagavan and Brin, 1983). It was reported that L-dopa could also undergo Schiff-base formation with PLP, forming a tetrahydroisoquinoline that was pharmacologically inactive (Ebadi *et al.*, 1982). Other studies indicated that L-dopa or the derivative formed with PLP could inhibit dopa decarboxylase (aromatic-L-amino-acid decarboxylase) and pyridoxal kinase (Ebadi *et al.*, 1982; Bhagavan and Brin, 1983), but the significance of these effects or whether they occur in humans requires further study (Ebadi *et al.*, 1982).

Unlike the cases with the previous drugs, it was found that the condensation of L-dopa with PLP inactivated both agents. Although pyridoxine is necessary for dopa decarboxylase activity, excess vitamin B_6, even in amounts found in over-the-counter multivitamin supplements, could nullify the beneficial effects of L-dopa (Ebadi *et al.*, 1982). Since dopa must be decarboxylated to dopamine before it is effective in the parkinsonian patient, it appears that L-dopa, not being pharmacologically active itself, could account for its diminished therapeutic potential in the presence of pyridoxine. As a consequence, the likelihood of L-dopa inactivation by pyridoxine has been reduced by administering the drug with an inhibitor of peripheral decarboxylase, such as carbidopa. This combination reduces the therapeutic dose of L-dopa so that L-dopa is not readily influenced by the patient's vitamin B_6 intake (Bhagavan and Brin, 1983).

F. Oral Contraceptive

Oral contraceptives (OC) have been widely used for many years. In 1981, Slap reported their use by approximately 40% of American and western European women aged 15–44 years, including one million American adolescents. However, their use was soon associated with adverse nutritional status, especially the vitamins riboflavin, B_6, B_{12}, C, and folic acid. With respect to vitamin B_6, oral contraceptive use was found to increase the urinary excretion of tryptophan metabolites, particularly xanthurenic and kynurenic acids, in 80% of women (cf. Adams *et al.*, 1973; Anonymous, 1979). However, when assays more specific to vitamin B_6 were applied, Adams *et al.* (1973) found that only 20% showed evidence of abnormal B_6 status. In general, most studies have reported abnormal tryptophan metabolism with OC use (Adams *et al.*, 1973; Anonymous, 1979; Bamji *et al.*, 1985) but little if any effect on vitamin B_6 status (Vir and Love, 1980; Bamji *et al.*, 1985).

Studies with experimental animals have found that the estrogen component of these drugs could account for the abnormality in tryptophan metabolism. In rats, Bender *et al.* (1982) reported that estrogen treatment inhibited liver kynureninase activity, resulting in higher urinary excretion of kynurenine with little or no change in plasma, liver, or kidney PLP. This inhibition was noncompetitive with PLP, suggesting that estrogens inhibited the holoenzyme [see Bender *et al.* (1982) and references cited therein].

An important side effect induced by OC is depression. In a review of clinical studies, depression was reported in as many as 56% of OC users, and it was estimated that depression was the reason that as many as 60% of American adolescent users stopped the drug within one year (Slap, 1981). Depression in OC users was found to be responsive to pyridoxine administration (Adams *et al.*, 1973). Oral contraceptives were reported to induce hepatic tryptophan oxygenase activity, decreasing the availability of tryptophan for the synthesis of serotonin in the CNS (Ebadi *et al.*, 1982). By increasing serotonin synthesis, pyridoxine would relieve the depression. London *et al.* (1985) reported that a similar disturbance in tryptophan metabolism accounted for the depression associated with the premenstrual syndrome; consequently, pyridoxine has been proposed to treat this depression, but at much higher doses (Mattes and Martin, 1982; O'Brien, 1982). This area of pyridoxine therapy is highly controversial and beyond the scope of this review.

In general, the mechanisms involved in OC–vitamin B_6 interactions are far from resolved. In addition to the effects cited, OC may also affect hydrocortisone metabolisms. Evidence has suggested that both endogenous and exogenous steroids can compete with PLP for binding with apoenzymes and that these hormones may cause a redistribution of PLP among body pools (Rose, 1978; Stanulovic, 1980; Compton and Cidlowski, 1986). Oral contraceptives may also affect the microsomal drug metabolizing (MFO) system of the liver (Wade *et al.*, 1976). Wade and Evans (1977) further noted that in rats treated with norethindrone, pyridoxine supplementation altered the metabolism of MFO enzymes though the combination did not act as a classical MFO inducer such as phenobarbital or 3-methylcholanthrene. Nevertheless, the data suggest that OC use with or without pyridoxine administration could alter the metabolism of concurrently administered drugs.

At present the vitamin B_6 requirement in OC users remains controversial. Earlier reports suggested that 20–40 mg pyridoxine was needed to correct the biochemical abnormalities seen, but after the estrogen content of these preparations was reduced, others reported that 5 mg/day was sufficient (cf. Anonymous, 1979). Also, Roepke and Kirksey (1981) suggested that over 15 mg/day of pyridoxine is needed by long-time OC users who become pregnant.

G. Ethanol

As with OC agents, the effects of alcohol on nutritional status are not unique to vitamin B_6. Alcoholics generally have inadequate nutrient intakes and also tend to be malnourished and show evidence for intestinal malabsorption, decreased hepatic metabolism, and limited repair mechanisms (Majumdar *et al.*, 1982). Ethanol has been reported to impair the metabolism of the B vitamins, vitamins A, D, and C, as well as magnesium, calcium, zinc, and iron (cf. Bhagavan, 1985).

Numerous studies have reported abnormal vitamin B_6 metabolism in alcoholics (Lumeng and Li, 1974; Baker *et al.*, 1975; Veitch *et al.*, 1975; Bonjour, 1980; Majumdar *et al.*, 1982). Lower than normal plasma PLP levels, independent of the assay method, is a common observation in alcoholics and experimental animals fed ethanol (Lumeng and Li, 1974; Veitch *et al.*, 1975; Parker *et al.*, 1979; Bonjour, 1980). In alcoholics, plasma PLP does not increase as much as in nondrinkers after intravenous infusion of pyridoxine (Labadarios *et al.*, 1977). Liver PLP is also lower in alcoholics and ethanol-fed animals, and this effect is independent of their vitamin B_6 status (Bonjour, 1980; Veitch *et al.*, 1975).

Over the past two decades, studies in humans and experimental animals have reported that ethanol can affect vitamin B_6 metabolism at various levels. Hines (1969) reported that the conversion of pyridoxine to PLP was significantly reduced in four of seven chronic alcoholics consuming alcohol for at least 26 days. Subsequently, other investigators noted impaired phosphorylation of pyridoxine to PLP (Hines and Cowan, 1970; Lumeng and Li, 1974; Kakuma *et al.*, 1976; Parker *et al.*, 1979). Any inhibition of pyridoxal kinase in erythrocytes that might account for this effect seems to be transient and does not fully explain the lower PLP observed (cf. Bonjour, 1980).

Ethanol was also reported to affect vitamin B_6 absorption, but whether ethanol affects overall vitamin B_6 bioavailability has not been satisfactorily answered. Baker *et al.* (1975) first reported that patients with alcoholic liver disease could not use vitamin B_6 from a natural source such as Brewer's yeast, but they could absorb pyridoxine. Recently, Middleton (1986) observed that *in vitro*, ethanol, at concentrations achievable in the upper gastrointestinal tract after oral intake, could inhibit the intestinal hydrolysis of PLP. In addition, 4% ethanol *in vitro* enhanced jejunal uptake of pyridoxine, but no differences in uptake were observed following one year ethanol ingestion in rats (Middleton *et al.*, 1984).

Further investigation into ethanol's action have indicated that many of the effects on PLP levels may be due to its oxidation product, acetaldehyde. Lumeng and Li (1974) observed that acetaldehyde, but not ethanol, impaired the net formation of PLP in erythrocytes from its precursors. It was later suggested that acetaldehyde could form Schiff bases with a number of proteins, including PLP-dependent enzymes with a lower affinity for PLP (Lumeng, 1978; Kakuma *et al.*, 1976; Kakuma *et al.*, 1981). Therefore, acetaldehyde could displace protein-bound PLP, rendering it more susceptible to degradation by alkaline phosphatase. In addition, acetaldehyde is further oxidized to acetate, which has been shown to inhibit the recombination of PLP with GAD (Fonda, 1975). Treating the alcoholic with PLP could protect against acetaldehyde cytotoxicity through Schiff-base formation with available amino groups of proteins, thereby blocking the condensation of acetaldehyde with these groups (Kakuma *et al.*, 1981). *In vitro*, PLP, but not pyridoxine, can reverse acetaldehyde-induced cytotoxicity whereas, *in vivo*, pyridoxine (150 mg/day) is effective (Kakuma *et al.*, 1981). For the normalization of plasma PLP in the alcoholic, intravenous PLP is effec-

tive, but pyridoxine is recommended to correct vitamin B_6 abnormalities overall (Bonjour, 1980). However, the optimum supplementation level of vitamin B_6 in alcoholics remains to be elucidated.

In addition, our understanding of the relationship between ethanol-induced seizures and adverse vitamin B_6 metabolism in the brain is far from complete. The GABA system is known to be an important target site for ethanol in the brain (Kulonen, 1983), and recently Chung *et al.* (1985) observed that withdrawal from chronic ethanol ingestion in the mouse transiently increased cerebrum PLP, possibly leading to a decrease in synoptosomal GABA and rendering the brain hyperexcitable and prone to seizures. This area of ethanol–pyridoxine interactions requires additional study.

H. Anticonvulsants

Interactions between anticonvulsant drugs and folic acid and vitamin D have long been known, but the effects of these drugs on other vitamins are not well documented. In an evaluation of 145 patients (96 male, 49 female) taking anticonvulsants for at least one year, Krause *et al.* (1982) found that the vitamin B_6 status, as determined by erythrocyte aspartate aminotransferase activity, was only impaired in males. However, other determinants of vitamin B_6 status were not made, and no attempts were made to correlate the results with a specific drug. On the other hand, Hansson and Sillanpaa (1976) found that pyridoxine supplementation (80–400 mg/day for as long as 4 months) resulted in lower plasma phenytoin and phenobarbitone concentrations in 12 patients. PLP levels were not measured. It remains to be established if these effects represent direct pyridoxine–drug interactions, impaired absorption, higher tissue uptake, increased excretion, or some other mechanism.

I. Carcinogens and Chemotherapeutic Agents

A number of studies have indicated that vitamin B_6 metabolism is altered in patients with various cancers [see DiPalma and McMichael (1979) and Dickerson (1983) and references cited therein; see also Ramaswamy and Natarajan (1984)], and that vitamin B_6 can affect tumor growth (Wagner *et al.*, 1984). However, this review will focus on the interaction between pyridoxine and chemotherapeutic agents in the treatment of cancer as well as the effects of vitamin B_6 on the action of carcinogens.

Studies have indicated that vitamin B_6 can play a role in the metabolism of certain carcinogens. Azaserine is a carcinogenic amino acid that induces tumors in the pancreas, kidney, and liver (Longnecker and Curphey, 1975). Zurlo *et al.* (1982, 1984) reported that the *in vivo* activation of azaserine to its ultimate carcinogenic form requires vitamin B_6. In addition, 3′-methyl-4-dimethylamino-

azobenzene-induced hepatomas resulted in lower PLP levels in rat liver and altered vitamin B_6 metabolism (Kittler *et al.*, 1986). However, the relationship between these observations and the carcinogenicity of azo dyes remains to be established.

Vitamin B_6 has also been employed to reduce toxicities associated with cancer chemotherapeutic agents, but with mixed results. Mitomycin C (MMC) and 5-fluorouracil (5-FU) are effective chemotherapeutic agents against primary and metastatic hepatic tumors, but their use has been restricted by side effects. Misra *et al.* (1977) reported that coadministration of 100–150 mg/day pyridoxine with MMC reduced its toxicity. However, in a clinical trial, 1.5 mg/day pyridoxine, coadministrated with vincristine for 6 weeks, did not protect against drug-induced neurotoxicity or other side effects (Jackson *et al.*, 1986), though it did offer some protection in mice (Jackson *et al.*, 1984). In addition, pyridoxine has ameliorated neuropathy sometimes associated with the chemotherapeutic agent, hexamethylmelamine (Smith and Rutledge, 1975).

N-Methylformamide (NFM) has antineoplastic activity against certain sarcomas and Ehrlich ascites tumors, but it is hepatotoxic. Although pyridoxal-HCl (30 mg/kg) showed some protection against side effects of 500 mg/kg NFM, it did not enhance the chemotherapeutic effect of this compound, in contrast to vitamins C and K (Osswald *et al.*, 1987).

Misonidazole (MISO) and desmethylmisonidazole (DMM) were introduced into tumor chemotherapy to overcome tumor hypoxia-induced radioresistance, but both drugs can be neurotoxic. Since MISO is metabolized to aminated compounds that could react with PLP, and its neurotoxicity resembles that induced by hydrazine, Eifel *et al.* (1983) investigated the potential of vitamin B_6 to protect against MISO-induced neurotoxicity. Mice receiving intraperitoneal injections of MISO with pyridoxine HCl (220 mg/kg) tolerated higher doses than mice not receiving pyridoxine. They determined that pyridoxine did not affect the pharmacokinetics of MISO, and *in vitro* its metabolite, 2-amino-MISO, formed a Schiff base with pyridoxal-HCl. However, in another study employing 500 mg/day pyridoxine orally or intraperitoneal injection of an unspecified dose of pyridoxal or PLP, Coleman *et al.* (1984) failed to show any protective effect of the vitamin B_6 forms against MISO- or DMM-induced neurotoxicity.

J. Other Drugs

Vitamin B_6 has been employed experimentally and clinically to treat neuropathies and convulsions induced by drugs other than those discussed. For example, pyridoxine has been successfully employed to treat stereotyped behavior and compulsive rituals associated with chronic amphetamine treatment (Frye and Arnold, 1981). In a case report, they found that 10 mg/kg pyridoxine, reduced over the next few weeks to 2 mg/kg, stopped ritual behavior in an 8-year-old

boy. Although these behaviors may reflect an imbalance in the dopamine–serotonin balance in the brain, the actual mechanism related to the action of pyridoxine remains unknown.

An interaction between vitamin B_6 and tricyclic antidepressants was also noted in a case report. A 39-year-old woman developed peripheral neuropathy from amitriptyline therapy that was controlled by oral pyridoxine supplementation, although the dose required was high (500 mg/day) (Meadows *et al.*, 1982). The possible mechanism involved awaits further study.

Vitamin B_6 has also been reported to interact with antiarrhythmic agents. Chalmers *et al.* (1982) reported that the use of amiodarone, a benzofuran compound, could produce photosensitivity in up to 75% of white patients on the drug. Recently, Kaufmann (1984) found that pyridoxine (40–300 mg/day) suppressed amiodarone-induced photosensitivity in three patients, suggesting that the drug inhibited melanin formation in skin via an anti-vitamin-B_6 effect. *In vitro* studies, however, indicted that amiodarone-induced photosensitivity was enhanced by pyridoxine (Guerciolini *et al.*, 1984); in a clinical trial, Mulrow *et al.* (1985) found that pyridoxine supplementation (100 mg/day) did not significantly suppress amiodarone-induced photosensitivity, and one patient on pyridoxine had decreased sun tolerance. This study was complicated, however, by improvement reported in 70% of patients taking placebo.

No discussion of the interactions between vitamin B_6 and drugs would be complete without mention of the controversy surrounding the administration of Bendectin or Debenox for the treatment of nausea associated with pregnancy. Bendectin is an antiemetic containing 10 mg doxylamine and 10 mg pyridoxine. Debenox has a similar formula but also contains 10 mg dicyclamine. Although dicyclamine did not prove to have antiemetic action, the other two agents did (Maling, 1984). However, adverse publicity and law suits concerning teratogenicity of these drugs have resulted in their withdrawal from the market. Both epidemiological and experimental studies have failed to show a link between these agents and childhood cancer or birth defects (McKinney *et al.*, 1985; Hendrickx *et al.*, 1985; Porter *et al.*, 1986). Although high doses of pyridoxine were shown to induce sister chromatid exchange in human lymphocytes in culture (Dozi-Vassiliades *et al.*, 1983), no studies at the dose of pyridoxine in these medications have accounted for any possible mechanism linking either vitamin B_6, doxylamine, or an interaction between these drugs with abnormal fetal development.

IV. VITAMIN B_6 AND ENVIRONMENTAL TOXICANTS

In addition to ethanol, vitamin B_6 has been reported to react with a variety of natural toxicants, pollutants, food additives, pesticides, and other related com-

pounds. Pyridoxine analogs have been synthesized and used experimentally to evaluate the role of vitamin B_6 in metabolic processes (Holtz and Palm, 1964; Mizuno *et al.*, 1980; Nitsch *et al.*, 1983). Some of these compounds are found in foods. Recently, Wada *et al.* (1985) isolated 4-methoxypyridoxine from the seed of the maidenhair tree (*Ginkgobiloba* L.). This compound has potent anti-vitamin B_6 activity and, if consumed, can induce convulsions, presumably through inhibition of GAD (Haug and Nitsch, 1982). Used as a food, gin-nan, in Japan and a drug in China, it has been associated with outbreaks of toxicity (Wada *et al.*, 1985). Hydrazines, whose anti-vitamin B_6 activity have been discussed, also occur in nature (Toth, 1975). Hydrazine occurs in tobacco and tobacco smoke, MFH is found in wild edible mushrooms, and 4-hydroxymethylphenylhydrazine and agaritine occur naturally in the cultivated mushroom *Agaricus bisporus* at concentrations up to 0.05% of fresh tissue weight (Table III). Although cases of mild hydrazine poisoning have been reported (Klosterman, 1974), the role of vitamin B_6 antagonism in the expression of toxicity has not been elucidated.

A. Pollutant Gases

Exposure to carbon monoxide (CO), a component of environmental pollutants and tobacco smoke, has been reported to reduce vitamin B_6 levels in blood, and experimental animals exposed to CO required twice the dosage of vitamin B_6 as controls to maintain normal blood levels [see Mitchell and Schandl (1973) and references cited therein]. In our own studies, we observed that perinatal rat pups reared from vitamin B_6-deficient dams were particularly sensitive to exposure to 0.64 ppm ozone: 65% died compared with fewer than 5% of the *ad libitum* or food-restricted controls (Myers *et al.*, 1986). The mechanisms involved in the effects of these agents on vitamin B_6 metabolism and the relation of these effects to the health of the animal remain to be elucidated.

B. Compounds Used in Foods and the Pharmaceutical Industry

Controversy continues over a possible relationship between food dye consumption and hyperactivity in children. Recently, Kantor *et al.* (1984) examined the possible role of vitamin B_6 in food dye-induced toxicity in rats. Although rats fed the high food dye diet (4%) showed some change in locomotor activity, Kantor *et al.* did not detect significant differences in plasma and brain PLP levels and concluded that any previously reported behavioral abnormalities associated with food dye consumption were probably unrelated to alterations in vitamin B_6 metabolism.

Nevertheless, dyes employed in the pharmaceutical industry for medications have been reported to interact with vitamin B_6. Erythrosine, eosine Y, rose

bengal, mercurochrome, methylene blue, and azure A and B have been shown, *in vitro,* to accelerate the photodecomposition of pyridoxine and pyridoxamine induced by ultraviolet (UV) light (Mizuno *et al.*, 1981). At least for erythrosine, the mechanism appeared to involve singlet-oxygen generation. Other dyes, such as fluorescein, acid red, rhodamine, brilliant blue, and indigo carmine did not induce photodecomposition [see Mizuno *et al.* (1981) and references cited therein]. Yellow light has been shown to prevent significant decomposition of vitamin B_6 (Ang, 1979), but because yellow light can be uncomfortable to work under, Schaltenbrand *et al.* (1987) investigated the effects of low-UV "white" fluorescent lamps on photodecomposition of pyridoxal solutions, *in vitro.* They found that these lamps were not as effective as yellow light.

In addition, adverse interactions between vitamin B_6 and components involved in the storage and processing of food have been well documented and will not be discussed here [see Holtz and Palm (1964) and Tadera *et al.* (1986) and references cited therein]. Recently, Tadera *et al.* (1986) reported that in the presence of ascorbic acid, pyridoxine is hydroxylated to an inactive product, 6-hydroxypyridoxine. The implication of these studies to the potency of pyridoxine in vitamin supplements containing high concentrations of ascorbic acid remains to be elucidated.

C. Pesticides and Rodenticides

Vitamin B_6 has also been explored as a antidote to pesticide and rodenticide poisoning. Acute DDT poisoning is manifested by hyperexcitability, tremors, and convulsions, attributed to its metabolite pp'DDT (Henderson and Woolley, 1970). Since a reduction in the level of striatal GABA was observed, Matin *et al.* (1981) examined the effects of an intraperitoneal injection of pyridoxine (50 mg/kg), 1 hr prior to pp'DDT-induced death in mice. Pyridoxine had no effect on convulsions or striatal GABA concentrations. In contrast, pyridoxal or PLP (25 mg/kg, subcutaneous) was found to alleviate the seizures and related toxicity induced by the rodenticide, Castrix (2-chloro-4-methyl-6 dimethylaminopyrimidine), when injected at the time of or 30 min following injection of Castrix into mice (Karlog and Knudsen, 1963). The mechanisms associated with these different responses to vitamin B_6 remain to be established. Also, in a recent case report, pyridoxine (100 mg) was found to interact with the rodenticide, sodium fluoroacetate, and ethanol in a 29-year-old man (Ramirez, 1986). Ethanol has been used to reverse the neurotoxicity of sodium fluoroacetate, but use of pyridoxine to treat ethanol-induced neurotoxicity resulted in expression of rodenticide toxicity.

D. Other Agents

Hexane and methyl *n*-butyl ketone are solvents used in glues and cleaning fluids in the shoe and fabric industry. Their common metabolite, 2,5-hexane-

dione, produces a marked neuropathy. Misumi *et al.* (1985) injected rats subcutaneously with 10 mg/kg PLP for 18 weeks without any protective action against 2,5-hexanedione-induced neurotoxicity. Whether this relates to improper dosage or chemical form of vitamin B_6 or other factors is unknown.

The failure of vitamin B_6 to treat certain neurotoxicities may be related to elevated PLP in the brain, coupled to changes in brain amino-acid metabolism resulting in higher concentrations of excitatory amino acids or a decrease in GABA (Norris *et al.*, 1985). This hypothesis has been proposed as a mechanism to explain the observation that intraperitoneal injection of PLP lowers the seizure threshold to a number of epileptigenic agents (Norris *et al.*, 1985).

V. VITAMIN B_6 AND NUTRIENTS

Interactions have been reported between vitamin B_6 and amino acids, vitamins, minerals, and carbohydrates. Apart from the link between vitamin B_6 requirements and protein intake, certain amino acids reportedly alter vitamin B_6 metabolism. Sorghum is known to contain high concentrations of leucine, and its use has been linked to endemic pellagra and abnormal tryptophan metabolism in parts of India. Krishnaswamy *et al.* (1976) observed that this defect could be ameliorated by pyridoxine supplementation (100 mg/day). Since high doses of pyridoxine are required, it remains to be established if pyridoxine is inducing B_6-dependent enzymes to correct the defect in tryptophan metabolism or whether leucine itself alters vitamin B_6 bioavailability and requirements.

An interesting interaction has been reported between vitamin B_6 and monosodium glutamate (MSG), a component of Chinese food related to the development of neurological symptoms known as the Chinese restaurant syndrome (CRS). Folkers *et al.* (1981) postulated that persons suffering from CRS have an underlying vitamin B_6 deficiency, since they have reduced erythrocyte aspartate aminotransferase activity. If individuals were given pyridoxine supplements (50 mg/day) for 12 weeks and then were challenged with a dose of MSG, symptoms of CRS did not appear, although aminotransferase activity was not different between MSG responders and nonresponders (Folkers *et al.*, 1981; Anonymous, 1982). Whether MSG unmasks a preexisting subclinical vitamin B_6 deficiency through unknown mechanisms or interferes with normal vitamin B_6 metabolism, leading to vitamin B_6-related neurological symptoms, remains to be elucidated.

Excess methionine and homocysteine have been reported to produce an endotheliemia in rats leading to venous thrombosis (Hladovec, 1979, 1980). Pyridoxine (2.5 or 10 mg/kg) could reverse the effects of some excess doses of these amino acids, but since other agents were more effective, it appeared that these effects were not specifically related to altered vitamin B_6 metabolism (Hladovec, 1980).

Although pyridoxine metabolism is intimately related to that of other B vitamins such as niacin and riboflavin, this discussion will be limited to interactions involving vitamin megadoses. Nadiger (1980a) observed that the increased plasma vitamin E levels following supplementation in children (300 mg/day) was reduced if B-complex vitamins were given concurrently. This effect was reproduced if 30 mg/day pyridoxine was given with the vitamin E (Nadiger, 1980b). Although the mechanism for this interaction is not fully understood, Nadiger *et al.* (1984) attributed this effect to the role of both vitamins in lipid metabolism, in that a higher unsaturated fatty acid content in membranes induced by vitamin B_6 results in higher vitamin E uptake into tissues and a resultant decrease in plasma vitamin E levels. Whether pyridoxine supplementation increases vitamin E requirements is unknown.

Early studies detected a relationship between vitamin B_6 intake and whole blood vitamin C levels (Baker *et al.*, 1964). Despite other reports of a link between vitamins B_6 and C, vitamin B_6 status was not affected following ascorbic acid supplementation up to 1 g/day for 7 days (Shultz and Leklem, 1982). Whether long-term supplementation would have an affect remains to be established. Considering the popularity of vitamin C supplementation and described interactions between these nutrients *in vitro* (Tadera *et al.*, 1986), further studies seem warranted.

Large doses of vitamin B_6 (over 1.5 g/day) have been reported to increase erythrocyte magnesium concentrations (Eisinger and Dagorn, 1986). The mechanisms involved and the effects on magnesium status are unknown, though vitamin B_6 is known to be a chelator of a number of cations (Holtz and Palm, 1964). Whatever the relationship between vitamin B_6 and magnesium, they have been reported to act synergistically when used therapeutically in childhood autism (Martineau *et al.*, 1985).

Xylitol has been used in parenteral nutritional products as a carbohydrate source. Although some abnormalities in oxalate metabolism in xylitol-infused patients were attributed to defects in vitamin B_6 metabolism, a comprehensive study by Hauschildt *et al.* (1976) found no evidence for vitamin B_6 deficiency in patients receiving xylitol.

VI. CONCLUDING REMARKS

This chapter illustrates the wide range of reactivity of vitamin B_6 with a number of drugs and other chemicals encountered in the environment. In many instances these interactions involve a condensation between the xenobiotic and PLP, resulting in Schiff-base formation, but vitamin B_6 absorption, phosphorylation to active coenzyme forms, or specific vitamin B_6-dependent enzymes can also be affected. Although I have attempted to cover major groups of xenobiotics

for which interactions with vitamin B_6 have been described, the list of compounds is far from complete and will increase as methods of assessment of vitamin B_6 status become more refined.

In most cases, the interactions between vitamin B_6 and xenobiotics induce biochemical signs of vitamin deficiency without clinical evidence, suggesting a possible marginal deficiency. Overt vitamin B_6 deficiency would only be expected with chronic exposure to the noxious agent. In the majority of cases where abnormal vitamin B_6 metabolism is detected following exposure to the xenobiotic, large doses of pyridoxine, often 25–50 times or more the RDA, have been effective without compromising the clinical efficacy of a drug or inducing toxicity. However, the effects of long-term exposure to environmental pollutants and other toxicants on vitamin B_6 metabolism and nutritional status, as well as optimum supplementation levels where appropriate, will require further investigation.

REFERENCES

Adams, P. W., Rose, D. P., Fokard, J., Wynn, V., Seed, M., and Stong, R. (1973). Effect of pyridoxine hydrochloride (vitamin B6) upon depression associated with oral contraception. *Lancet* **1,** 897–904.

Ang, C. Y. W. (1979). Stability of three forms of vitamin B6 to laboratory light conditions. *J. Assoc. Off. Anal. Chem.* **62,** 1170–1173.

Anonymous (1979). The vitamin B6 requirement in oral contraceptive users. *Nutr. Rev.* **37,** 344–345.

Anonymous (1982). Possible vitamin B6 deficiency uncovered in persons with the "Chinese restaurant syndrome." *Nutr. Rev.* **40,** 15–16.

Baker, E. M., Canhem, J. E., Nunes, W. T., Sauberlich, H. E., and McDowell, M. E. (1964). Vitamin B6 requirement for adult men. *Am. J. Clin. Nutr.* **15,** 59–66.

Baker, H., Frank, O., Zetterman, R. K., Rajan, K. S., Hove, W., and Leevy, C. M. (1975). Inability of chronic alcoholics with liver disease to use food as a source of folates, thiamin and vitamin B6. *Am. J. Clin. Nutr.* **28,** 1377–1380.

Bamji, M. S., Prema, K., Jacob, C. M., Rani, M., and Samyukta, D. (1985). Vitamin supplements to Indian women using low dosage oral contraceptives. *Contraception* **32,** 405–416.

Barnard, H. C., Vermaak, W. J. H., and Potgieter, G. M. (1986). The effect of acute prolonged starvation on the concentrations of vitamin B6 aldehyde derivatives in whole blood. *Int. J. Vitam. Nutr. Res.* **56,** 351–354.

Barnard, H. C., deCock, J. J., Vermaak, W. J. H., and Potgieter, G. M. (1987). A new perspective in the assessment of vitamin B6 nutritional status during pregnancy in humans. *J. Nutr.* **117,** 1303–1306.

Bender, D. A., Tagoe, C. E., and Vale, J. A. (1982). Effects of oestrogen administration on vitamin B6 and tryptophan metabolism in the rat. *Br. J. Nutr.* **47,** 609–614.

Bhagavan, H. N. (1985). Interaction between vitamin B6 and drugs. *In* "Vitamin B6: Its Role in Health and Disease" (R. D. Reynolds and J. E. Leklem, eds.), pp. 401–415. Liss, New York.

Bhagavan, H. N., and Brin, M. (1983). Drug–vitamin B6 interaction. *Curr. Concepts Nutr.* **12,** 1–12.

Biehl, J. P., and Vilter, R. W. (1954). Effect of isoniazid on vitamin B6 metabolism; its possible significance in producing isoniazid neuritis. *Proc. Soc. Exp. Biol. Med.* **85,** 389–392.

Bonjour, J. P. (1980). Vitamins and alcoholism. *Int. J. Vitam. Nutr. Res.* **50,** 215–230.

Brown, C. V. (1972). Acute isoniazid poisoning. *Am. Rev. Respir. Dis.* **105,** 206–216.

Chalmers, R. J. G., Muston, H. L., Srinivas, V., and Bennett, D. H. (1982). High incidence of amiodarone-induced photosensitivity in north-west England. *Br. Med. J.* **285,** 341.

Chatterjee, A. K., and Sengupta, K. (1980). Regulation of formation in vivo of pyridoxal phosphate in hydrazine-treated rats. *Int. J. Vitam. Nutr. Res.* **50,** 24–28.

Chin, L., Sievers, M. L., Laird, H. E., Herrier, R. N., and Picchioni, A. L. (1978). Evaluation of diazepam and pyridoxine as antidotes to isoniazid intoxication in rats and dogs. *Toxicol. Appl. Pharmacol.* **45,** 713–722.

Chin, L., Sievers, M. L., Herrier, R. N., and Picchioni, A. L. (1981). Potentiation of pyridoxine by depressants and anticonvulsants in the treatment of acute isoniazid intoxication in dogs. *Toxicol. Appl. Pharmacol.* **58,** 504–509.

Chung, S. H., Caidan, S. J., and Norris, D. K. (1985). Enhancement of brain pyridoxal 5′-phosphate level following chronic ingestion of ethanol. *Neurosci. Lett.* **62,** 219–224.

Clark, F. (1976). Drugs and vitamin deficiency. *Adverse Drug React. Bull.* **57,** 196–199.

Coleman, C. N., Hirst, V. K., Brown, D. M., and Halsey, J. (1984). The effect of vitamin B6 on the neurotoxicity and pharmacology of desmethylmisonidazole and misonidazole: Clinical and laboratory studies. *Int. J. Radiat. Oncol. Biol. Phys.* **10,** 1381–1386.

Compton, M. M., and Cidlowski, J. A. (1986). Vitamin B6 and glucocorticoid action. *Endocr. Rev.* **7,** 140–148.

Cornish, H. H. (1969). The role of vitamin B6 in the toxicity of hydrazines. *Ann. N.Y. Acad. Sci.* **166,** 136–145.

Dakshinamurti, K. (1982). Neurobiology of pyridoxine. *Adv. Nutr. Res.* **4,** 143–179.

De Jesus Sevigny, S. S. J., White, S. L., Halsey, M. L., and Johnston, F. A. (1966). Effect of isoniazid on loss of pyridoxal phosphate from, and its distribution in, the body of the rat. *J. Nutr.* **88,** 45–50.

Dickerson, J. W. T. (1983). Nutrition of the cancer patient. *Adv. Nutr. Res.* **5,** 105–131.

DiPalma, J. R., and McMichael, R. (1979). The interaction of vitamins with cancer chemotherapy. *Ca-Cancer J. Clin.* **29,** 280–286.

Dozi-Vassiliades, J., Mourelatos, D., and Myrtsiotis, A. (1983). Induction of sister-chromatid exchange in human lymphocytes by vitamin B6. *Mutat. Res.* **124,** 175–178.

Ebadi, M., Gessert, C. F., and Al-Sayegh, A. (1982). Drug–pyridoxal phosphate interactions. *Q. Rev. Drug Metab. Drug Interact.* **4,** 289–331.

Eifel, P. J., Brown, D. M., Lee, W. W., and Brown, J. M. (1983). Misonidazole neurotoxicity in mice decreased by administration with pyridoxine. *Int. J. Radiat. Oncol. Biol. Phys.* **9,** 1513–1519.

Eisinger, J., and Dagorn, J. (1986). Vitamin B6 and magnesium. *Magnesium* **5,** 27–32.

Fasella, P. (1967). Pyridoxal phosphate. *Annu. Rev. Biochem.* **36,** 185–210.

Fisher, J. W., Willis, R. A., and Haskell, B. E. (1984). Effect of protein quality on vitamin B6 status in the rat. *J. Nutr.* **114,** 786–791.

Folkers, K., Shizukuishi, S., Scudder, S. L., Willis, R., Takemura, K., and Longenecker, J. B. (1981). Biochemical evidence for a deficiency of vitamin B6 in subjects reacting to monosodium L-glutamate by the Chinese restaurant syndrome. *Biochem. Biophys. Res. Commun.* **100,** 972–977.

Fonda, M. L. (1975). The effect of anions on the interaction of pyridoxal phosphate with glutamate apodecarboxylase. *Arch. Biochem. Biophys.* **170,** 690–697.

Frye, P. E., and Arnold, L. E. (1981). Persistent amphetamine-induced compulsive rituals: Response to pyridoxine (B6). *Biol. Psychiatry* **16,** 583–587.

George, M. E., Pinkerton, M. K., and Back, K. C. (1982). Therapeutics of monomethylhydrazine intoxication. *Toxicol. Appl. Pharmacol.* **63,** 201–208.

Guerciolini, R., Del Favero, A., and Cannistraro, S. (1984). Amiodarone-induced photosensitivity and pyridoxine. *Lancet* **1,** 962.

Hansson, O., and Sillanpaa, M. (1976). Pyridoxine and serum concentration of phenytoin and phenobarbitone. *Lancet* **1,** 256.

Harati, Y., and Niakan, E. (1986). Hydrazine toxicity, pyridoxine therapy and peripheral neuropathy. *Ann. Intern. Med.* **104,** 728–729.

Haug, P., and Nitsch, C. (1982). Increase in taurine content before onset of seizures induced by a glutamate decarboxylase inhibitor. *Exp. Brain Res.* **48,** 463–466.

Hauschildt, S., Chalmers, R. A., Lawson, A. M., Schultis, K., and Watts, R. W. E. (1976). Metabolic investigations after xylitol infusion in human subjects. *Am. J. Clin. Nutr.* **29,** 258–273.

Hein, D. W., and Weber, W. W. (1984). Relationship between *N*-acetylator phenotype and susceptibility toward hydrazine-induced lethal central nervous system toxicity in the rabbit. *J. Pharmacol. Exp. Ther.* **228,** 588–592.

Henderson, G. L., and Woolley, D. E. (1970). Mechanism of neurotoxic action of DDT. *J. Pharmacol. Exp. Ther.* **175,** 60–68.

Henderson, L. M. (1984). Vitamin B6. *In* "Present Knowledge in Nutrition," 5th ed., pp. 303–317. Nutrition Foundation, Washington, D.C.

Hendrickx, A. G., Cukierski, M., Prahalada, S., Janos, G., Booher, S., and Nyland, T. (1985). Evaluation of Bendectin embryotoxicity in nonhuman primates: II. Double-blind study in term cynomolgus monkeys. *Teratology* **32,** 191–194.

Hines, J. D. (1969). Altered phosphorylation of vitamin B6 in alcoholic patients induced by oral administration of alcohol. *J. Lab. Clin. Med.* **74,** 882–883.

Hines, J. D., and Cowan, D. H. (1970). Studies on the pathogenesis of alcohol-induced sideroblastic bone-marrow abnormalities. *N. Engl. J. Med.* **283,** 441–446.

Hladovec, J. (1979). Experimental homocystinaemia, endothelial lesion and thrombosis. *Blood Vessels* **16,** 202–205.

Hladovec, J. (1980). Methionine, pyridoxine and endothelial lesion in rats. *Blood Vessels* **17,** 104–109.

Holtz, P., and Palm, D. (1964). The pharmacological aspects of vitamin B6. *Pharmacol. Rev.* **16,** 113–178.

Jackson, D., Long, T., Rich, C., Rardin, D., McMahan, R., Case, D., Wells, B., and Capizzi, R. (1984). Pyridoxine modification of vincristine toxicity. *Proc. Am. Assoc. Cancer Res.* **25,** 313 (Abstr.).

Jackson, D. V., Jr., Pope, E. K., McMahan, R. A., Cooper, M. R., Atkins, J. N., Callahan, R. D., Paschold, E. H., Grimm, R. A., Hopkins, J. O., Muss, H. B., Richards, F., II, Stuart, J. J., White, D. R., Zekan, P. J., Cruz, J. M., Spurr, C. L., and Capizzi, R. L. (1986). Clinical trial of pyridoxine to reduce vincristine neurotoxicity. *J. Neuro-Oncol.* **4,** 37–41.

Juchau, M. R., and Horita, A. (1972). Metabolism of hydrazine derivatives of pharmacologic interest. *Drug. Metab. Rev.* **1,** 71–100.

Kakuma, S., Levinson, R., Baker, H., and Leevy, C. M. (1976). Enhanced acetaldehyde hepatotoxicity in vitamin B6 deficiency. *Gastroenterology* **71,** 914 (Abstr.).

Kakuma, S., Leevy, C. M., Frank, O., and Baker, H. (1981). Protection of pyridoxal 5′-phosphate against toxicity of acetaldehyde to hepatocytes. *Proc. Soc. Exp. Biol. Med.* **168,** 325–329.

Kandarkar, S. V., Maru, G. B., Bhide, S. V., and Sirsat, S. M. (1980). An ultrastructural study of the effect of isoniazid and its antitoxicants on mouse liver. *Indian J. Exp. Biol.* **18,** 157–162.

Kantor, M. A., Trout, J. R., and Lachance, P. A. (1984). Food dyes produce minimal effects on locomotor activity and vitamin B6 levels in postweanling rats. *J. Nutr.* **114,** 1402–1412.

Karlog, O., and Knudsen, E. (1963). Vitamin B6 as an antidote against the rodenticide "Castrix" (2-chloro-4-methyl-6-dimethylaminopyrimidin). *Nature (London)* **200,** 790.

Kaufmann, G. (1984). Pyridoxine against amiodarone-induced photo sensitivity. *Lancet* **1,** 51–52.

Kittler, J. M., Meisler, N. T., and Thanassi, J. W. (1986). Azo dye-induced alterations in vitamin B6 metabolism and in pyridoxal 5′-phosphate-binding proteins in rat liver. *J. Nutr.* **116,** 588–598.

Klosterman, J. (1974). Vitamin B6 antagonists of natural origin. *J. Agric. Food Chem.* **22,** 13–16.

Krause, K.-H., Berlit, P., Bonjour, J. P., Schuidt-Gayk, H., Schellenberg, B., and Gillen, J. (1982). Vitamin status in patients on chronic anticonvulsant therapy. *Int. J. Vitam. Nutr. Res.* **52,** 375–385.

Krishnaswamy, K., Rao, S. B., Raghuram, T. C., and Srikantia, S. G. (1976). Effects of vitamin B6 on leucine-induced changes in human subjects. *Am. J. Clin. Nutr.* **29,** 177–181.

Kulonen, E. (1983). Ethanol and GABA. *Med. Biol.* **61,** 147–167.

Labadarios, D., Rossouw, J. E., McConnell, J. B., Davis, M., and Williams, R. (1977). Vitamin B6 deficiency in chronic liver disease—Evidence for increased degradation of pyridoxal-5′-phosphate. *Gut* **18,** 23–27.

Lapin, C. A., and Carter, D. E. (1981). Early indices of methyl mercury toxicity and their use in treatment evaluation. *J. Toxicol. Environ. Health* **8,** 767–776.

Li, T.-K., Lumeng, L., and Veitch, R. L. (1974). Regulation of pyridoxal 5′-phosphate metabolism in liver. *Biochem. Biophys. Res. Commun.* **61,** 677–684.

London, R. S., Murphy, L., and Kitlowski, K. E. (1985). Treatment of premenstrual syndrome with vitamin B_6: physicians attitudes and perceptions. *In* "Vitamin B_6: Its Role in Health and Disease," pp. 469–477. Alan Liss, New York.

Longnecker, D. S., and Curphey, T. J. (1975). Adenocarcinomia of the pancreas in azaserine-treated rats. *Cancer Res.* **35,** 2249–2257.

Ludwig, J., and Axelsen, R. (1983). Drug effects on the liver. An updated tabular compilation of drugs and drug-related hepatic diseases. *Dig. Dis. Sci.* **28,** 651–666.

Lumeng, L. (1978). The role of acetaldehyde in mediating the deleterious effect of ethanol on pyridoxal 5′-phosphate metabolism. *J. Clin. Invest.* **62,** 286–293.

Lumeng, L., and Li, T.-K. (1974). Vitamin B6 metabolism in chronic alcohol abuse. Pridoxal phosphate levels in plasma and the effects of acetaldehyde on pyridoxal phosphate synthesis and degradation in human erythrocytes. *J. Clin. Invest.* **53,** 693–704.

Lumeng, L., Brashear, R. E., and Li, T.-K. (1974). Pyridoxal 5′-phosphate in plasma: Source, protein binding, and cellular transport. *J. Lab. Clin. Med.* **84,** 334–343.

McKinney, P. A., Cartwright, R. A., Stiller, C. A., Hopton, P. A., Mann, J. R., Birch, J. M., Hartley, A. L., Waterhouse, J. A. H., and Johnston, H. E. (1985). Inter-regional epidemiological study of childhood cancer (IRESCC): Childhood cancer and the consumption of Debendox and related drugs in pregnancy. *Br. J. Cancer* **52,** 923–929.

Majumdar, S. K., Shaw, G. K., Gorman, P. O., Aps, E. J., Offerman, E. L., and Thomson, A. D. (1982). Blood vitamin status (B1, B2, B6, folic acid and B12) in patients with alcoholic liver disease. *Int. J. Vitam. Nutr. Res.* **52,** 266–271.

Maling, T. J. B. (1984). Debendox: A statement from the New Zealand Adverse Drug Reactions Committee. *N.Z. Med. J.* **97,** 203–204.

Martineau, J., Berthelemy, C., Garreau, B., and Lelord, G. (1985). Vitamin B6, magnesium and combined B6–Mg: Therapeutic effects in childhood autism. *Biol. Psychiatry* **20,** 467–478.

Matin, M. A., Jaffery, F. N., and Siddiqui, R. A. (1981). A possible neurochemical basis of the central stimulatory effects of pp′DDT. *J. Neurochem.* **36,** 1000–1005.

Mattes, J. A., and Martin, D. (1982). Pyridoxine in premenstral depression. *Hum. Nutr.: Appl. Nutr.* **36A,** 131–133.

Meadows, G. G., Huff, M. R., and Fredericks, S. (1982). Amitriptyline-related peripheral neuropa-

thy relieved during pyridoxine hydrochloride administration. *Drug Intell. Clin. Pharm.* **16,** 876–877.

Merrill, A. H., Jr., Henderson, J. M., Wang, E., McDonald, B. W., and Millikan, W. J. (1984). Metabolism of vitamin B6 by human liver. *J. Nutr.* **114,** 1664–1674.

Middleton, H. M. (1986). Intestinal hydrolysis of pyridoxal-5′phosphate in vitro and in vivo in the rat: Effect of ethanol. *Am. J. Clin. Nutr.* **43,** 374–381.

Middleton, H. M., Mills, L. R., and Singh, M. (1984). Effect of ethanol on the uptake of pyridoxine–HCl in the rat jejunum. *Am. J. Clin. Nutr.* **39,** 54–61.

Misra, N. C., Jaiswal, M. S. D., Singh, R. V., and Das, B. (1977). Intrahepatic arterial infusion of combination mitomycin-C and 5-fluorouracil in treatment of primary and metastatic liver carcinoma. *Cancer* **39,** 1425–1429.

Misumi, J., Nagano, M., Kaisaku, J., and Hitoshi, T. (1985). Effect of vitamin B12 and B6 on 2,5-hexanedione-induced neuropathy. *Arch. Toxicol.* **56,** 204–206.

Mitchell, D. A., and Schandl, E. K. (1973). Carbon monoxide, vitamin B6 and multiple sclerosis: A theory of interrelationships. *Am. J. Clin. Nutr.* **26,** 890–896.

Mitchell, D., Wagner, C., Stone, W. J., Wilkinson, G. R., and Schenker, S. (1976). Abnormal regulation of plasma pyridoxal 5′-phosphate with liver disease. *Gastroenterology* **71,** 1043–1049.

Mizuno, A., Kamada, Y., Kunita, M., and Matsuda, M. (1980). The effect of antivitamin B6 administration on γ-aminobutyric acid metabolism in retina and electroretinogram. *J. Nutr. Sci. Vitaminol.* **26,** 25–32.

Mizuno, N., Fujiwara, A., and Morita, E. (1981). Effect of dyes on the photodecomposition of pyridoxine and pyridoxamine. *J. Pharm. Pharmacol.* **33,** 373–376.

Mulrow, J. P., Mulrow, C. D., and McKenna, W. J. (1985). Pyridoxine and amiodarone-induced photosensitivity. *Ann. Intern. Med.* **103,** 68–69.

Myers, B. A., Dubick, M. A., Gerriets, J. E., Reiser, K. M., Last, J. A., and Rucker, R. B. (1986). Lung collagen and elastin after ozone exposure in vitamin B6 deficient rats. *Toxicol. Lett.* **30,** 55–61.

Nadiger, H. A. (1980a). Role of vitamin E in the aetiology of phrynoderma (follicular hyperkeratosis) and its interrelationship with B-complex vitamins. *Br. J. Nutr.* **44,** 211–214.

Nadiger, H. A. (1980b). Studies on interrelationship between vitamins E and B-complex. *Nutr. Metab.* **24,** 352–356.

Nadiger, H. A., Rao, A. S., and Sadasivudu, B. (1984). Effect of simultaneous administration of vitamin E and pyridoxine or erythrocyte membrane Na^+K^+ ATPase activity. *Int. J. Vitam. Nutr. Res.* **54,** 307–311.

Nelson, M. V., Bailie, G. R., and Krenzelok, E. P. (1983). Central nervous system stimulation from isoniazid therapy. *Vet. Hum. Toxicol.* **25,** 90–91.

Nitsch, C., Schmude, B., and Haug, P. (1983). Alterations in the content of amino acid neurotransmitters before the onset and during the course of methoxypyridoxine-induced seizures in individual rabbit brain regions. *J. Neurochem.* **40,** 1571–1580.

Norris, D. K., Murphy, R. A., and Chung, S. H. (1985). Alteration of amino acid metabolism in epileptogenic mice by elevation of brain pyridoxal phosphate. *J. Neurochem.* **44,** 1403–1410.

O'Brien, P. M. S. (1982). The premenstrual syndrome: A review of the present status of therapy. *Drugs* **24,** 140–151.

Osswald, H., Herrmann, R., and Youssef, M. (1987). The influence of sodium ascorbate, menadione sodium bisulfite or pyridoxal hydrochloride on the toxic and antineoplastic action of *N*-methylformamide in P388 leukemia or M 5076 sarcoma in mice. *Toxicology* **43,** 183–191.

Parker, T. H., Marshall, J. P., II, Roberts, R. K., Wang, S., Schiff, E. R., Wilkinson, G. R., and Schenker, S., (1979). Effect of acute alcohol ingestion on plasma pyridoxal 5′-phosphate. *Am. J. Clin. Nutr.* **32,** 1246–1252.

Pellock, J. M., Howell, J., Kendig, E. L., Jr., and Baker, H. (1985). Pyridoxine deficiency in children treated with isoniazid. *Chest* **87,** 658–661.

Porter, J. B., Hunter-Mitchell, J., Jick, H., and Walker, A. M. (1986). Drugs and stillbirth. *Am. J. Public Health* **76,** 1428–1431.

Ramaswamy, P. G., and Natarajan, R. (1984). Vitamin B6 status in patients with cancer of the uterine cervix. *Nutr. Cancer* **6,** 176–180.

Ramirez, M. (1986). Inebriation with pyridoxine and fluoracetate: A case report. *Vet. Hum. Toxicol.* **28,** 154.

Roepke, J. L. B., and Kirksey, A. (1981). Effect of vitamin B6 supplementation during pregnancy on the vitamin B6 nutriture of previous long term oral contraceptive users and nonusers. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **40,** 863 (Abstr.).

Rose, D. P. (1978). The interactions between vitamin B6 and hormones. *Vitam. Horm. (N.Y.)* **36,** 53–99.

Rumsby, P. C., and Shepherd, D. M. (1980). The effect of drugs on vitamin B6 function in the rat. *Biochem. Pharmacol.* **29,** 3097–3102.

Rumsby, P. C., and Shepherd, D. M. (1981). The effect of penicillamine on vitamin B6 function in man. *Biochem. Pharmacol.* **30,** 3051–3053.

Schaltenbrand, W. E., Kennedy, M. S., and Coburn, S. P. (1987). Low-ultraviolet "white" fluorescent lamps fail to protect pyridoxal phosphate from photolysis. *Clin. Chem.* **33,** 631–632.

Serfontein, W. J., DeVilliers, L. S., Ubbink, J., and Pitout, M. J. (1984). Vitamin B6 revisited. Evidence of subclinical deficiencies in various segments of the population and possible consequences thereof. *S. Afr. Med. J.* **66,** 437–441.

Shultz, T. D., and Leklem, J. E. (1982). Effect of high dose ascorbic acid on vitamin B6 metabolism. *Am. J. Clin. Nutr.* **35,** 1400–1407.

Sievers, M. L., and Herrier, R. N. (1980). Megavitamin therapy for overdose. *Arch. Intern. Med.* **140,** 1676.

Sievers, M. L., Herrier, R. N., Chin, L., and Picchioni, A. L. (1982). Treatment of isoniazid overdose. *J. Am. Med. Assoc.* **247,** 583–584.

Slap, G. B. (1981). Oral contraceptives and depression: Impact, prevalence and cause. *J. Adolesc. Health Care* **2,** 53–64.

Smith, J. P., and Rutledge, F. N. (1975). Random study of hexamethylmelamine, 5-fluorouracil and melphalan in treatment of advanced carcinoma of the ovary. *Natl. Cancer Inst. Monogr.* **42,** 169–172.

Stanulovic, M. (1980). Metabolic and drug-induced inactivation of vitamin B6. *In* "Vitamin B6 Metabolism and Role in Growth" (G. P. Trylictas, ed.), pp. 113–136. Food & Nutrition Press, Westport, Connecticut.

Sternlieb, I., and Scheinberg, I. H. (1964). Penicillamine therapy for hepatolantricular degeneration. *J. Am. Med. Assoc.* **189,** 748–754.

Tadera, K., Arima, M., Yoshino, S., Yagi, F., and Kobayashi, A. (1986). Conversion of pyridoxine into 6-hydroxypyridoxine by food components, especially ascorbic acid. *J. Nutr. Sci. Vitaminol.* **32,** 267–277.

Toth, B. (1975). Synthetic and naturally occurring hydrazines as possible cancer causative agents. *Cancer Res.* **35,** 3693–3697.

Toth, B., and Erickson, J. (1977). Reversal of toxicity of hydrazine analogues by pyridoxine hydrochloride. *Toxicology* **7,** 31–36.

Van den Berg, H., Bogaards, J. J. P., Sinkeldam, E. J., and Schreurs, W. H. P. (1982). Effect of different levels of vitamin B6 in the diet of rats on the content of pyridoxamine-5′-phosphate and pyridoxal-5′-phosphate in the liver. *Int. J. Vitam. Nutr. Res.* **52,** 407–416.

Veitch, R. L., Lumeng, L., and Li, T.-K. (1975). Vitamin B6 metabolism in chronic alcohol abuse. The effect of ethanol oxidation on hepatic pyridoxal 5′-phosphate metabolism. *J. Clin. Invest.* **55,** 1026–1032.

Vir, S. C., and Love, A. H. G. (1980). Effect of oral contraceptives on vitamin B6 nutriture of young women. *Int. J. Vitam. Nutr. Res.* **50,** 29–34.

Wada, K., Ishigaki, S., Ueda, K., Sakata, M., and Haga, M. (1985). An antivitamin B6, 4′-methoxypyridoxine, from the seed of *Ginkgo biloba* L. *Chem. Pharm. Bull.* **33,** 3555–3557.

Wade, A. E., and Evans, J. S. (1977). Influence of norethindrone on drug-metabolizing enzymes of female rat liver in various B-vitamin deficiency states. *Pharmacology* **15,** 289–301.

Wade, A. E., Evans, J. S., and Seitz, A. (1976). Influence of the oral contraceptive, mestranol, on drug metabolizing enzymes of female rats in thiamin-supplemented and deficiency states. *Pharmacology* **14,** 104–114.

Wagner, R. F., Jr., DiSorbo, D. M., and Nathanson, L. (1984). Nutrition and melanoma. *Int. J. Dermatol.* **23,** 453–457.

Wason, S., Lacouture, P. G., and Lovejoy, F. H., Jr. (1981). Single high-dose pyridoxine treatment for isoniazid overdose. *J. Am. Med. Assoc.* **246,** 1102–1104.

Zimmerman, H. J. (1978). Drug-induced liver disease. *Drugs* **16,** 25–45.

Zurlo, J., Curphey, T. J., Hiley, R., and Longnecker, D. S. (1982). Identification of 7-carboxymethylguanine in DNA from pancreatic acinar cells exposed to azaserine. *Cancer Res.* **42,** 1286–1288.

Zurlo, J., Roebuck, B. D., Rutkowski, J. V., Curphey, T. J., and Longnecker, D. S. (1984). Effect of pyridoxal deficiency on pancreatic DNA damage and nodule induction by azaserine. *Carcinogenesis* **5,** 555–558.

6

Considerations in Designing and Using Standardized Diets in Toxicological Experiments

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I. INTRODUCTION

Currently there is considerable interest in identifying and understanding the mechanisms by which nutritional and dietary factors alter the responses of animals to chemicals such as drugs, food additives, and environmental pollutants. Such considerations are important in evaluating the responses of animals to toxicants because unrecognized or improperly controlled nutritional or dietary variables can significantly alter the outcome or reproducibility of such studies.

NUTRITIONAL TOXICOLOGY, VOL. III

Because of regulatory issues and public interest, potential carcinogens are the most studied of all toxicants.

Long-term animal bioassays are the most widely accepted means of determining the carcinogenic effect of specific substances. Risk-assessment procedures are generally applied to data from whole-animal systems rather than *in vitro* systems. Sequential whole-body physiological phenomena such as absorption, distribution, metabolism, and excretion do not all occur in *in vitro* systems, and these events all contribute to the overall outcome of carcinogen bioassays and other toxicity tests. For extrapolation, *in vitro* studies give qualitative rather than quantitative patterns of metabolic products formed from an administered compound because they do not accurately reflect *in vivo* rates of metabolism.

Many laboratory investigations have been designed to study specific dietary factors that may influence carcinogenesis and other types of toxicity. Individual nutrients and nonnutritive dietary compounds that enhance or inhibit carcinogenesis, for example, have been identified. These include protein, fats, carbohydrates, certain vitamins and trace elements, and lipotropes. Review of all such interactions is beyond the scope of the present chapter. Detailed reviews have been prepared by Clayson (1975), Reddy *et al.* (1980), Conner and Newberne (1984), and Newberne and Rogers (1986), among others.

Although alterations in nutritional status *per se* do not appear to cause tumors directly in experimental animals (possible exceptions are lipotrope and magnesium deficiencies), it is believed that diet or nutritional status can modify spontaneous rates of tumor production and/or the induction and growth of tumors produced by specific carcinogens. For example, specific nutrients appear to have tumor-promoting activity in specific tissues in rodent model systems. These include zinc deficiency and 13-*cis*-retinoic acid (esophagus), vitamin A deficiency and lipotrope deficiency (forestomach), and lipotrope deficiency (liver). In addition, dietary contaminants such as mycotoxins and nitrosamines may also interact with nutritional deficiency, excess, or imbalance to potentiate or diminish the response to a xenobiotic compound.

The mechanisms by which nutritional status modifies responses to xenobiotics are understood in some instances and poorly understood in others. Nutritional status has, for example, been shown to influence microsomal enzyme systems, hormones, the intestinal flora, and the immune system. Reviews of experimental evidence relating effects of nutritional status on responses to xenobiotics are found in *Diet, Nutrition, and Cancer* (National Research Council, 1982) and in reviews by Parke and Ioannides (1981), Conner and Newberne (1984), Guengerich (1984), Belinsky *et al.* (1987), and Meydani (1987).

Many experimental studies on the effects of diet or specific dietary components on tumorigenesis have not been well controlled. Great care is frequently taken with respect to determining the purity and stability of test chemicals with

little attention paid to the composition of the diets fed to the test animals throughout life-span studies. The diets may contain contaminants with tumorigenic activity or naturally occurring carcinogens. In addition, diets used in such studies have ranged from closed-formula commercially available chows of minimally defined composition to highly purified diets containing completely identified amino acid mixtures in place of protein. Individual nutrients may be included at levels ranging from marginally deficient to greatly in excess of known requirements. Some attempts to standardize diets for carcinogenicity tests have been made. The development of the American Institute of Nutrition AIN-76 diet (Bieri *et al.*, 1977; Bieri, 1980) represents a significant step in this direction, although the formulation of this diet may not be appropriate for all strains of rats (Medinsky *et al.*, 1982). The American Institute of Nutrition diet and National Research Council recommendations (1978) should aid investigators in giving greater consideration to overall diet composition.

This review is not intended to detail the numerous reports describing interactions between nutrition or diet and carcinogenesis. Instead, it will identify some known factors that have a significant impact on the outcome of long-term studies and will cite examples of studies in which the use of well-defined conditions have contributed to an increased understanding of nutrient–toxicant interactions.

II. GENERAL CONSIDERATIONS

A. Characteristics and Desirable Nutrient Contents of Diets for Life-span Studies

Nutritional aspects of life-span studies are generally given little consideration. This applies to nutritional needs at both ends of the life cycle (i.e., consideration of requirements of very young animals as well as those of mature and aging animals). The general practice of feeding a single diet throughout life fails to consider the effects on subsequent toxicological challenges of possible nutritional inadequacies during phases of rapid growth and development in very young animals as well as the consequences of overnutrition in mature animals. Changes in nutritional requirements with age have not been adequately studied although maintenance versus growth requirements for protein and amino acids are available for rats (National Research Council, 1978).

The National Research Council recommendations (1978) of 12% protein for growth, pregnancy, and lactation and 4.2% for maintenance are exceeded by virtually all natural-ingredient diets and the AIN-76 diet. The effects of protein content of diets on responses to various toxicants have been well-documented (McLean and Magee, 1970; Campbell, 1979; Meydani, 1987). Increased hepatic

microsomal enzyme activity may play a role in decreasing the toxic effects of some xenobiotics (i.e., pesticides or aflatoxin) (Campbell and Hayes, 1975; Bidlack *et al.*, 1986).

Lack of variation in diet composition (i.e., appropriate reduction in nutrient content in recognition of reduced requirements for maintenance versus those for growth and reproduction) has been a persistent problem in long-term studies. It has been easier to identify specific problems than to provide widely accepted solutions. Among the variables that affect nutrient requirements are age, sex, weight, and body stores (if any). Determination of nutrient requirements is not simple (Baker, 1986). Diets prepared for requirement studies must be well-defined in terms of source and level of protein, source and level of energy, vitamins, minerals, and numerous positive and negative bioavailability factors.

Rates of weight gain during the postweaning period have been used to assess adequate nutrition. Such assessments are based on the assumption that diets providing the most rapid growth rates are superior to those that give lower growth rates. Alternative criteria might include low incidence of spontaneous disease or greatest longevity. Nutritional requirements using these criteria are markedly different from those producing the greatest rates of postweaning growth (Rogers, 1979; Weindruch and Walford, 1982; Cheney *et al.*, 1980, 1983).

Immune competence is another criterion that might be used to assess nutritional adequacy. The immune system is one of the most sensitive physiological systems in terms of nutritional requirements. Williams *et al.* (1978) suggested that, in the case of vitamin requirements, the amounts required for optimum function of the immune system may exceed the amounts required for normal growth. Such considerations are especially important in recognizing the interrelationships of diet and autoimmunity and the role of the latter in longevity. Hansen *et al.* (1982) have reviewed the impacts of nutrition on various immunological functions and the roles of specific nutrients in development of autoimmune diseases and the immunodeficiencies of aging. Since autoimmunity and malignancy generally increase with age, many model systems for immunological senescence involve studies of nutritional factors that lead to premature development of these conditions.

The major functions of the immune system are to provide protection against infectious diseases and to prevent the development of neoplasia. The immune system, like all physiological systems, is subject to environmental influences. Vitale and Broitman (1981) noted that "every nutritional insult that promotes cancer has been shown to suppress some component of cell-mediated immunity." General malnutrition, effects of specific nutrients (protein, vitamins, trace elements), and dietary toxicants all affect the immune system and can contribute to increased sensitivity to infection, autoimmunity, or neoplasia. These conditions *per se* can then lead to further immunological deficits. The roles of specific

nutritional deficiencies of various vitamins and minerals on the immune response have been reviewed by Brandon (1984). Among the vitamins, A, C, E, and the B complex have been studied most extensively with regard to identification of relationships between deficiency and immune dysfunction. Deficiencies of lipotropes (choline, methionine, folic acid, cobalamin) have been reported to depress specific aspects of cellular and humoral immunity (Williams *et al.*, 1978). Effects of deficiencies of iron, zinc, and selenium on the immune system have also been studied extensively.

In addition to specific effects of single vitamins or minerals on immune competence, there are complex immunological responses to overnutrition, to mild or severe malnutrition, and to controlled dietary restriction (Brandon, 1984). Long-term studies have frequently overlooked subtle diet-related changes in the immune system, or such changes have not been evaluated adequately. Miller (1978) has reviewed the use of immunological criteria in assessing nutritional status.

B. Differences in Responses of Animals to Purified and Natural-Ingredient Diets

Natural-ingredient diets consist of processed grains (corn, wheat, oats) and partially or minimally refined components such as soybean or fish meals. Minimal nutrient levels are guaranteed in natural-ingredient open-formula diets, but amounts by which a major or trace nutrient may exceed the minimal levels are not known (Rader *et al.*, 1984). Batch-to-batch variations, seasonal variations, variations in content of naturally occurring compounds such as phytates or protease inhibitors, and variable contamination with pesticide residues or heavy metals must be expected. A description of all possible variables and identification of all specific interactions between nutrients and toxicants are virtually impossible with the use of such diets.

Many differences in responses of animals to purified and natural diets have been reported, and these differences are frequently not fully understood. For example, protective factors present in natural chows may be responsible for the differences in toxicity of 5-fluorouracil in rats fed laboratory chow compared with those fed purified hydrolyzed casein diets (Bounous *et al.*, 1978a,b).

Marked variations in response to 5-fluorouracil were observed when different batches of the same commercial chow were fed, suggesting that the presence of putative protective factors may be highly variable. Natural diets that contain high levels of xenobiotics, which are strong inducers of the mixed-function oxidase systems, can alter the response of animals to chemical carcinogens (Wattenberg *et al.*, 1976).

Wattenberg (1983) and Fiala *et al.* (1985) have reviewed compounds occurring naturally in foods that can protect against carcinogenesis by minimizing

proximate or ultimate carcinogen formation or by inhibiting cellular events at early stages of carcinogenesis. Such compounds include protease inhibitors found in seed foods, soybeans, and soybean preparations (Kennedy and Little, 1981; Troll *et al.*, 1980; Yavelow *et al.*, 1983) as well as selenium, vitamin E, vitamin A, β-carotene, dietary fiber, indoles, and flavones.

Shively *et al.* (1986) reported that components of commercial chow diets induced theobromine metabolism in rats. Because environmental contaminants (such as chlorinated-hydrocarbon pesticide residues and mycotoxins) and the food additive BHA were not found by analysis, the authors proposed that natural ingredients such as indoles or flavones probably were responsible for the inductive effects.

Less is known about dietary influences on extrahepatic metabolism of xenobiotics than about dietary effects on hepatic mixed-function oxidase activity. During the absorptive process, environmental pollutants, drugs, xenobiotics, or food additives can interact with the intestinal mucosal endoplasmic reticulum. Interactions leading to activation or inactivation can alter the overall toxicity of the test compound. Numerous factors have been shown to affect intestinal drug metabolism. These include species differences in intestinal drug-metabolizing enzymes, age, sex, certain hormones, and diet. Dietary iron and the quality and quantity of dietary lipid have been shown to influence intestinal microsomal enzyme activities [see Chhabra and Tredger (1978) for review and additional references].

An area in which natural-ingredient diets and more-purified diets may differ significantly is in their effects on the gut flora. Changes in the metabolic activity of the gut flora can have major influences on the presystemic biotransformations of xenobiotic compounds and naturally occurring compounds (Prins, 1978; Rowland and Wise, 1985). Possible consequences of metabolic activities of the gut flora include: (1) production of toxic metabolites (2) formation of carcinogenic or mutagenic compounds, (3) alterations in host susceptibility to tumor induction, and (4) detoxification of specific compounds. Dietary modifications can significantly alter the capacity of the gastrointestinal microflora to metabolize ingested or endogenously synthesized compounds. In addition, fermentable components of dietary fiber can influence intestinal microbial metabolism of xenobiotics or endogenously synthesized compounds by providing an energy source for microbial growth. Studies by Goldstein *et al.* (1984) showed that alterations in microflora mediate diet-related differences in nitrobenzene-induced methemoglobinemia in rats.

It follows from the preceding comments that valuable information on nutrient–xenobiotic interactions can be obtained by careful analysis of diets and comparison of effects obtained with purified versus natural-ingredient diets. Such comparisons may lead to the development and use of more-sensitive experimental diets.

C. Mechanism of Action of Test Chemicals

Xenobiotics are excreted either unchanged by the body or as metabolites that are generally more polar than the parent compound. The formation of more-polar metabolites usually favors excretion through the kidneys or the gastrointestinal tract. Many xenobiotics are enzymatically oxidized to introduce or unmask a polar functional group. The hepatic microsomal cytochrome *P*-450 monooxygenase system, the major enzyme system catalyzing oxidative metabolism of xenobiotics, plays a pivotal role in determining the fate of these compounds. The metabolite of the oxidative reaction may be conjugated to reduce reactivity and further enhance polarity. Susceptibility to acute or chronic effects of a toxic xenobiotic compound is determined by a balance between pathways for activation and detoxification. The oxidation and conjugation steps generally yield nontoxic derivatives; but in many instances toxic intermediate metabolites are produced (Conney, 1967).

The activities of the mixed-function oxidase system and the conjugating enzymes are affected by dietary factors. Hence, toxic effects of a specific chemical can vary with the diet fed (Conney, 1967; McLean and McLean, 1969). For example, alfalfa and brussels sprouts contain high quantities of indoles, which have been shown to increase oxidative metabolism of various compounds in rat intestine and liver and to increase the rate of metabolism and clearance of antipyrene and phenacetin (Anderson *et al.*, 1982). Microsomal *P*-450 content is enhanced in animals fed high protein diets whereas high carbohydrate diets have the opposite effect.

The teratogenic response to diphenylhydantoin is markedly increased in mice fed purified diet AIN-76 compared with those fed a commercial laboratory chow (McClain and Rohrs, 1985). The differences were ascribed to decreased basal levels of drug-metabolizing enzymes in mice fed the purified diets, associated with a decreased activity in metabolizing diphenylhydantoin to nonteratogenic compounds. The role of dietary fatty acids in these observations was investigated, and the replacement of 5% corn oil in the AIN-76 diet with 5% linseed oil led to a reduction in the teratogenic response to diphenylhydantoin. The basal level of mixed-function oxidase activity was increased by this substitution.

The antioxidant butylated hydroxyanisole (BHA) is added to both plant and animal lipids to reduce storage-related peroxidation. This additive increases glutathionine *S*-transferase activity (Wattenberg, 1983) and NADPH–cytochrome-*c* reductase and aniline hydroxylase activities (Cha *et al.*, 1983). A number of chemicals including benzo[*a*]pyrene, dimethylbenzanthracene, and diethyl-nitrosamine show decreased carcinogenicity in animals fed diets with BHA (Wattenberg, 1978, 1983) (Table I). Induction of the mixed-function oxidase system may not be the only mechanism responsible for anticarcinogenic effects of reducing agents such as BHA, BHT, or propyl gallate (Anonymous, 1982).

TABLE I

Compounds with Decreased Carcinogenicity in Animals Fed Butylated Hydroxyanisole (BHA)[a]

Benzo[*a*]pyrene	4-Nitroquinoline *N*-oxide
7,8-Dihydrodiol	Uracil mustard
Dimethylbenzanthracene	Urethane
Dibenz[*a*,*h*]anthracene	Methylazoxymethanol acetate
Diethylnitrosamine	
trans-5-Amino-3-[2-(5-nitro-2-furyl)vinyl]-1,2,4-oxadiazole	

[a]From Conner and Newberne (1984). Reprinted by permission from Wattenberg (1983).

The observations that toxic effects of specific compounds can vary depending on the composition of the diet indicate that some knowledge of the mechanism of action of a particular test chemical will be of great value in determining the most appropriate composition of a test diet. This also implies that no "standard" diet can be defined that will be useful for all purposes. Emphasis should be placed instead on recognition of and analysis for dietary components that can be expected to affect the response to a particular test chemical.

Because rates of metabolism of many toxic compounds are known to be affected by diet composition, deliberate variations in diet composition can be used to investigate and modify the metabolic pathway(s) of a compound. Studies of this type may lead to a more complete understanding of the relationships among metabolism, toxicity, and/or carcinogenicity for specific compounds.

Recent work by Schrager *et al.* (1986) has demonstrated the utility of carefully controlled nutritional conditions on studies of the mechanisms of carcinogenesis. Zinc deficiency causes hyperplasia in the squamous epithelium of the esophagus (Swenerton and Hurley, 1968). A zinc-deficient diet has been shown to enhance esophageal tumor incidence in rats exposed to methylbenzylnitrosamine (MBN). Studies by Schrager *et al.* (1986) suggest that the enhancement of MBN-induced tumors by zinc deficiency may be due to increased proliferation of the esophageal cells and to an increased vulnerability of the target tissue to the carcinogen.

A number of changes at the level of DNA have been found to be associated with zinc deficiency. For example, Castro *et al.* (1986) showed that the hepatic histone H1° profile is affected by zinc status, with as much as a 50% decrease in one variant subtype (H1°) occurring during zinc deficiency. Although this variant has been implicated in cellular differentiation and in some neoplastic tissue, the amount of the H1° variant has been inversely correlated with the state of morphological differentiation and the proliferative rate of the cells [see Marks *et al.* (1975) and Castro (1987) for additional references].

Magnesium deficiency has been reported to produce generalized medullary bone growth and periosteal tumors of the desmoid type. The bone masses were thought to represent accumulations of cells that are unable to differentiate prop-

erly (Hunt and Belanger, 1972). It is not known whether this specific change, as in the case of zinc deficiency, can affect response to challenge with a xenobiotic.

Magnesium deficiency (as well as deficiencies of protein and zinc) has been reported to increase the resistance of liver chromatin to micrococcal nuclease. The increased chromatin resistance may be suggestive of a greater degree of chromatin condensation, which in turn may decrease available binding sites for polymerases, effector molecules, or genotoxic agents (Castro, 1987). The consequences of such changes are not fully understood, but such observations are important in recognizing that nutritional status *per se* can alter chromatin structure.

D. Nutritional Status and Response to Test Chemicals

Nutritional status *per se* exerts pronounced effects on the ability of an animal to respond to an experimental challenge such as further nutritional challenge or to exposure to an environmental carcinogen or other toxicant. Numerous examples of effects of specific nutrients on responses to xenobiotics and of mechanisms responsible for them are provided in a recent review by Newberne and Conner (1986).

Chemical induction of hepatocarcinoma has been studied extensively in this regard. For example, induction of liver cancer by several classes of carcinogens is enhanced in rats fed diets that are both high in fats and deficient in lipotropes (choline, methionine, folic acid). Compounds whose tumorigenicity is increased in lipotrope-deficient rats include aflatoxin B_1, dibutyl- and diethylnitrosamine, DL-ethionine, and L-azaserine (Conner and Newberne, 1984). Alteration in carcinogen metabolism in tissues of deficient animals is the most likely mechanism by which the dietary imbalance enhances chemical carcinogenesis (Newberne and Rogers, 1976) (Table II). Decreased basal levels of hepatic microsomal oxidases and conjugases can result in decreased clearance of chemical car-

TABLE II

Effect of Lipotrope Deficiency on Hepatic Microsomal Oxidases in Rats[a]

	Diet (units/gm fat-free liver ± SE)	
Enzyme (units)	Lipotrope-deficient	Control
Aminopyrene demethylase (μg antipyrene/hr)	305 ± 23	481 ± 50
p-Nitroanisole demethylase (μg *p*-nitrophenol/hr)	536 ± 116	1,132 ± 74
Benzo [*a*]pyrene hydroxylase (quinine units)	99 ± 11	184 ± 16

[a]Adapted from Rogers (1979).

cinogens. In addition to effects on hepatic enzymes, a lipotrope-deficient diet induces and maintains increased DNA synthesis and mitosis in hepatocytes (Newberne and Rogers, 1976). Decreases in serum very-low-density lipoproteins (VLDL) in lipotrope-deficient animals have also been reported [see Newberne and Rogers (1976)]. Enhanced susceptibility of hepatocytes to carcinogens can result from effects of altered serum VLDL, in conjunction with several hormones, on cell division in the liver (Newberne and Rogers, 1976).

Dietary fiber is also known to have a significant effect on toxicity testing (Wise and Gilburt, 1980). The composition of dietary fibers varies markedly and the broad term includes celluloses, hemicelluloses, pectin, lignin, and other materials. The relative digestibility of dietary fiber may be an important factor in alterations in the microbial flora of the gut. The effects of some specific fibers on the toxicity of specific xenobiotics are listed in Table III.

Protein, riboflavin, and vitamin B_{12} also influence tumor induction in the liver. Relationships between dietary protein and carcinogenesis are complex. In many studies, low protein diets (those with protein near or below requirement) appear to suppress development and growth of spontaneous tumors (National Research Council, 1982, Chap. 6). Chemically induced carcinogenesis appears to be enhanced as protein intake increases to two to three times the normal requirement. Protein deficiency decreases hepatic microsomal oxidase activity. Decreased induction of hepatic tumors by dimethylnitrosamine (DMN) and increased induction of renal tumors in protein-deficient rats were reported by McLean and Magee (1970). DMN is presumably cleared from the blood less rapidly in the protein-deficient animals.

NADPH–cytochrome *P*-450 reductase contains 1 mol each of FMN and FAD. The FMN moiety, but not the FAD moiety, is lost during dietary riboflavin

TABLE III

Dietary Fiber and Toxicity Testing[a]

Toxicant	Source of fiber	Effect[b]
Benzo[*a*]pyrene	Bran	−
Amaranth	Cellulose	0
Amaranth	Alfalfa	−
Dimethylhydrazine	Pectin	+
Dimethylhydrazine	Bran	−
5-Fluorouracil	Stock diet[c]	−
7,12-Dimethylbenz[*a*]anthracene	Stock diet	−
Cadmium chloride	Alfalfa	−
Sodium cyclamate	Wheat bran	−
Sodium cyclamate	Alfalfa	−

[a]From Omaye (1986).

[b](−), reduces toxicity; (0), no effect; (+), increases toxicity.

[c]Stock diet compared with fiber-free purified diet.

depletion. Decreased metabolism of several xenobiotics (e.g., aniline, aminopyrine, and ethylmorphine) is associated with loss of the reductase activity during riboflavin depletion (Bidlack *et al.*, 1986). Riboflavin deficiency decreases hepatic tumor induction by the aminoazobenzenes because it is a cofactor for enzymes that metabolize these compounds. Deficiencies of vitamins C, E, and A also exert marked effects on the hepatic mixed-function oxidase system and hence on xenobiotic metabolism [see Bidlack *et al.* (1986)].

The feeding of both stock diets and antioxidants tends to decrease chemical induction of liver tumors. With antioxidants such as BHT, decreases in toxicity and carcinogenicity are caused mainly by decreased activation and increased detoxification of specific carcinogens (Ulland *et al.*, 1973).

E. Formulation of Diets for Specific Objectives

The field of ultratrace-element research has been particularly susceptible to problems associated with failure to appreciate the importance of diet composition on experimental outcome. Careful planning and description of diet composition are particularly important in studies in which the compound under investigation could be affected by interactions with either a major or a trace element. Nielsen (1984, 1985) has described how variations in diet composition have affected studies on the ultratrace elements vanadium and nickel. In some studies, little attention is given to numerous dietary ingredients that can affect expression of deficiency symptoms of established trace elements such as copper, iron, and zinc. For example, the expression of iron deficiency can be influenced by vitamins (riboflavin, folic acid), minerals (copper), lipids, proteins, and amino acids [see Nielsen (1985) for additional references]; and dietary carbohydrate, ascorbic acid, zinc, and sulfur-containing amino acids can affect expression of copper deficiency. Nielsen (1985) has emphasized that research on the ultratrace elements requires particularly close attention to details of diet composition.

Variations in concentrations of dietary components and nonnutritive components significantly affect the response of experimental animals to trace and ultratrace elements. Rader *et al.* (1984) identified a frequently unrecognized problem of variation in animal diets. Large variations in mineral content were found between batches of a commercially prepared chow diet, with relative concentrations of copper, iron, manganese, and zinc differing markedly. Significant variations in both major and trace mineral levels were measured in tissues of weanling rats nursed by dams fed these diets. The long-term consequences of such variation are not clear, but they can have significant impact on outcome and reproducibility of shorter-term studies.

An example of the use of well-defined diets to achieve specific purposes is the work of Mikol *et al.* (1983). These authors studied the ability of amino acid-defined methyl group-deficient diets to produce liver tumors in rats. Amino acid-

defined diets are needed in investigations of effects of methyl deprivation on hepatocarcinogenesis to overcome problems associated with earlier use of methionine-deficient proteins (from soybean or peanut meals). In some cases, these protein sources were contaminated with aflatoxin, and interpretation of earlier results was complicated by effects caused by the contaminants. The purified diets used in the studies reported by Mikol *et al.* (1983) contained no detectable levels of aflatoxins, and the levels of dimethylnitrosamine (5.3 ppb) and diethylnitrosamine (0.9 ppb) were thought to be too low to account for the observed results. The authors concluded that chronic deprivation of dietary methyl-group donors, methionine and choline, exhibited complete hepatocarcinogenic activity in rats and also markedly enhanced formation of liver tumors in diethylnitrosamine-initiated rats. The authors observed thinning of the hair coat during growth as the only ill effect of feeding the highly purified diets; this may be attributable to the low (40% of NRC recommended levels) zinc in all the diets used.

F. Preparation, Analysis, and Description of Diets

The importance of diet composition in research is frequently underrated although excellent reviews on this topic are available (Greenfield and Briggs, 1971; Newberne, 1975; Wise, 1982).

Preparation of well-defined purified diets for research use is not difficult, though a large number of possible errors can be made. Unfortunately, relatively few reports of toxicological or nutritional research provide sufficient detail to allow assessment of significant nutritional problems. The ideal experimental design uses purified diets that permit specific nutritional changes. A number of improper practices in diet preparation have been identified, several of which are listed below:

1. Addition of nutrients (e.g., fat and fiber) to stock diets and comparison of results obtained with those obtained by feeding the stock diet alone. Such additions dilute the diet, and compensatory changes in food intake by experimental animals result in altered consumption of all nutrients.
2. Failure to recognize changes caused by substitution of one protein source for another. Essential fatty acids, trace minerals, and vitamins are generally present in proteins. Substitution of one protein source for another simultaneously changes the concentration of associated substances. In addition, some proteins contain high levels of phosphorus (i.e., casein); and phytates, which bind trace elements, are associated with proteins of plant origin (e.g., soy). The increased phosphorus content of high casein diets can influence the development of nephrocalcinosis, and the unrecognized presence of phytates can significantly alter trace mineral metabolism.
3. Addition of nutrients from dietary components. Oils used in diets, including oils used as vehicles in gavage studies, may contain vitamins E and A. Use

of oils stripped of these vitamins, however, is costly and results in diets that are highly susceptible to peroxidation. The latter produces problems with respect to diet stability during storage. Also, oil used as a gavage vehicle can contribute substantially to total dietary intake of fat, thereby producing the adverse and procarcinogenic effects of high-fat diets (Anonymous, 1983).

4. Experimental results can vary markedly depending upon the chemical form in which a nutrient is supplied in the diet. Bioavailability cannot be assured simply from analytical confirmation of the desired concentration. Nutrients for which bioavailability may be a problem include biotin, nicotinic acid, lysine, selenium, iron, vitamin A, vitamin E, and vitamin K.
5. In amino acid-defined diets, the use of hydrochloride salts can affect the overall electrolyte balance of the diet. For example, use of hydrochloride salts of amino acids in an amino acid-defined diet can increase the chloride content of the diet manyfold. If arginine-HCl (17% Cl), lysine-HCl (19.8% Cl), and histidine-HCl (18.8% Cl) are included in a purified diet at 1.21%, 1.80%, and 0.45%, respectively, the total calculated chloride contribution from these salts alone is 6467 mg Cl/kg diet. Addition of 1571 mg Cl/kg from NaCl present in a typical mineral mixture (such as that used for the AIN-76 diet) increases the total calculated chloride level to 8038 mg/kg. The ratio of Na : K : Cl in the AIN-76 diet is about 1.0 : 3.5 : 1.5. Using an amino acid mixture such as that described would yield a ratio of Na : K : Cl of 1 : 3.5 : 7.9.

Many other aspects of preparation and use of purified diets are discussed in detail by Greenfield and Briggs (1971), Wise (1982), Rowland and Wise (1985), and Nielsen (1985).

Several major factors in diet composition should be considered in planning, executing, and reporting a toxicological or nutritional experiment:

1. The quality and quantity of dietary components should be examined to assure appropriate levels and to minimize possible interactions. Nielsen (1985) reported that in ultratrace element research, a number of procedures can be used to reduce contamination of dietary components. However, he notes that preoccupation with obtaining an ultralow quantity of one element in the diet may override other aspects such as the quality of protein, chemical forms of mineral supplements, or complexity of carbohydrate (Nielsen, 1985). Such approaches can lead to inconsistent or divergent findings.
2. During long-term studies, nutrient composition should be monitored frequently to assure that composition remains consistent and that levels of possibly interfering nutrients or trace toxicants do not change in a manner that could influence the outcome of the study. The nutritional status of animals should also be monitored by sensitive biochemical tests (Sauberlich *et al.*, 1974), and care should be taken to prevent intake of nutrients or toxicants via drinking water or bedding or via chewing of caging or water bottle stoppers.

3. Attention should be paid to careful reporting of diet composition so that judgments can be made as to whether comparisons between or among studies are appropriate (Nielsen, 1985). Lang and Vesell (1976) reported that only 30% of 4080 reports of studies using animals in nutritional, physiological, biochemical, and psychological research reported the type of food provided and the frequency of feeding. It is important that complete information regarding diet composition be provided. Sources of diet ingredients and procedures used in diet preparation and storage should be clearly described.

III. CONCLUSIONS

Dietary composition is a major factor in determining the toxicity of many compounds. Careful attention to the choice and preparation of the diet used should be as important an aspect of toxicological studies as is the characterization of the test compound. An "ideal" diet must contain all nutrients required for normal physiological function, must be appropriate both for the age and/or breeding (reproductive) status of the animal, must not contain nonnutritive compounds that may interfere with the test nutrient or toxicant, must not contain toxicants or xenobiotic compounds that themselves may alter the outcome of the study, and must be of constant composition so that studies are reproducible. Emphasis should be placed on identification of mechanisms rather than on imprecise demonstrations of toxicity or carcinogenicity. Such an emphasis requires that greater attention be paid to details of diet composition and nutritional status. Carefully designed and executed investigations can be expected to yield valuable information on metabolism and distribution of toxic and carcinogenic compounds.

ACKNOWLEDGMENT

This chapter was written in a private capacity. No official support or endorsement by the Food and Drug Administration is intended or should be inferred.

REFERENCES

Anderson, K. E., Conney, A. H., and Kappas, A. (1982). Nutritional influences on chemical biotransformations in humans. *Nutr. Rev.* **40**, 161–171.

Anonymous (1982). Effects of dietary fat, butylated hydroxytoluene and propyl gallate on microsomal drug metabolism in rats. *Nutr. Rev.* **40**, 189–190.

Anonymous (1983). "Report of the Ad Hoc Working Group on Oil/Gavage in Toxicology." The Nutrition Foundation, Washington, D.C.

Baker, D. H. (1986). Problems and pitfalls in animal experiments designed to establish dietary requirements for essential nutrients. *J. Nutr.* **116,** 2339–2349.

Belinsky, S. A., Kauffman, F. C., and Thurman, R. G. (1987). Effect of nutrition on monooxygenation and conjugation in the liver. *In* "Nutritional Toxicology" (J. N. Hathcock, ed.), Vol. 2, pp. 41–61. Academic Press, Orlando, Florida.

Bidlack, W. R., Brown, R. C., and Mohan, C. (1986). Nutritional parameters that alter hepatic drug metabolism, conjugation, and toxicity. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **45,** 142–148.

Bieri, J. G. (1980). Second report of the Ad Hoc Committee on Standards for Nutritional Studies. *J. Nutr.* **110,** 1726.

Bieri, J. G., Stoewsand, G. S., Briggs, G. M., Phillips, R. W., Woodward, J. C., and Knapka, J. J. (1977). Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. *J. Nutr.* **107,** 1340–1348.

Bounous, G., Pageau, R., and Regoli, D. (1978a). Enhanced 5-F.U. mortality in rats eating defined formula diets. *Int. J. Clin. Pharmacol.* **16,** 265–267.

Bounous, G., Pageau, R. and Regoli, D. (1978b). The role of diet in 5-fluorouracil toxicity. *Int. J. Clin. Pharmacol.* **16,** 519–522.

Brandon, D. L. (1984). Interactions of diet and immunity. *Adv. Exp. Biol. Med.* **177,** 65–90.

Campbell, T. C. (1979). Influence of nutrition on metabolism of carcinogens. *Adv. Nutr. Res.* **2,** 29–55.

Campbell, T. C., and Hayes, J. R. (1975). Role of nutrition in the drug metabolizing enzyme system. *Pharmacol. Rev.* **26,** 171–197.

Castro, C. E. (1987). Nutritional influences on chromatin: Toxicological implications. *In* "Nutritional Toxicology" (J. N. Hathcock, ed.), Vol. 2, pp. 129–155. Academic Press, Orlando, Florida.

Castro, C. E., Alvares, O. F., and Sevall, J. S. (1986). Zinc deficiency alters the proportion of histone H1 molecular subtypes in rat liver. *Nutr. Rep. Int.* **34,** 67–74.

Cha, Y. N., Heine, H. S., and Ansher, S. (1983). Comparative effects of dietary administration of antioxidants and inducers on the activities of several hepatic enzymes in mice. *Drug–Nutr. Interact.* **2,** 35–45.

Chhabra, R. S., and Tredger, J. M. (1978). Interactions of drugs and intestinal mucosal endoplasmic reticulum. *In* "Nutrition and Drug Interactions" (J. N. Hathcock and J. Coon, eds.), pp. 253–299. Academic Press, New York.

Cheney, K. E., Liu, R. K., Smith, G. S., Leung, R. E., Mickey, M. R., and Walford, R. L. (1980). Survival and disease patterns in C57BL/GJ mice subjected to undernutrition. *Exp. Gerontol.* **15,** 237–258.

Cheney, K. E., Liu, R. K., Smith, G. D., Meredith, P. J., Mickey, M. R., and Walford, R. L. (1983). The effect of dietary restriction of varying duration on survival, tumor patterns, immune function, and body temperature in BIOC3F1 female mice. *J. Gerontol.* **38,** 420–430.

Clayson, D. B. (1975). Nutrition and experimental carcinogenesis: A review. *Cancer Res.* **35,** 3292–3300.

Conner, M. W., and Newberne, P. M. (1984). Drug–nutrient interactions and their implications for safety evaluations. *Fund. Appl. Toxicol.* **4,** S341–S356.

Conney, A. H. (1967). Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* **19,** 317–366.

Fiala, E. S., Reddy, B. S., and Weisburger, J. H. (1985). Naturally occurring anticarcinogenic substances in foodstuffs. *Annu. Rev. Nutr.* **5,** 295–321.

Garner, R. C. (1978). Dietary effects on the toxicity of food and environmental contaminants. *World. Rev. Nutr. Diet.* **29,** 178–186.

Goldstein, R. S., Chism, J. P., Sherrill, J., and Hamm, T. E., Jr. (1984). Influence of dietary pectin

on intestinal microfloral metabolism and toxicity of nitrobenzene. *Toxicol. Appl. Pharmacol.* **75,** 547–553.

Greenfield, H., and Briggs, G. M. (1971). Nutritional methodology in metabolic research with rats. *Annu. Rev. Biochem.* **40,** 549–572.

Guengerich, F. P. (1984). Effects of nutritive factors on metabolic processes involving bioactivation and detoxification of chemicals. *Annu. Rev. Nutr.* **4,** 207–231.

Hansen, M. A., Fernandes, G., and Good, R. A. (1982). Nutrition and immunity: The influence of diet on autoimmunity and the role of zinc in the immune response. *Annu. Rev. Nutr.* **2,** 151–177.

Hunt, B. J., and Belanger, L. F. (1972). Localized, multiform, sub-periosteal hyperplasia and generalized osteomyelosclerosis in magnesium-deficient rats. *Calcif. Tissue Res.* **9,** 17–27.

Kennedy, A. R., and Little, J. B. (1981). Effects of protease inhibitors on radiation transformation in vitro. *Cancer Res.* **41,** 2103–2108.

Lang, C. M., and Vesell, E. S. (1976). Environmental and genetic factors affecting laboratory animals: Impact on biomedical research. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **35,** 1123–1124.

McClain, R. M., and Rohrs, J. M. (1985). Potentiation of the teratogenic effects and altered disposition of diphenylhydantoin in mice fed a purified diet. *Toxicol. Appl. Pharmacol.* **77,** 86–93.

McLean, A. E. M., and McLean, E. K. (1969). Diet and toxicity. *Br. Med. Bull.* **25,** 278–281.

McLean, A. E. M., and Magee, P. N. (1970). Increased renal carcinogenesis by dimethylnitrosamine in protein-deficient rats. *Br. J. Exp. Pathol.* **51,** 587–590.

Marks, D. S., Kanefsky, T., Keller, B. J., and Marks, A. D. (1975). The presence of histone H1 in human tissues. *Cancer Res.* **35,** 886–889.

Medinsky, M. A., Popp, J. A., Hamm, T. E., and Dent, J. G. (1982). Development of hepatic lesions in male Fischer-344 rats fed AIN-76A purified diet. *Toxicol. Appl. Pharmacol.* **62,** 111–120.

Meydani, M. (1987). Dietary effects on detoxification processes. *In* "Nutritional Toxicology" (J. N. Hathcock, ed.), Vol. 2, pp. 1–39. Academic Press, Orlando, Florida.

Mikol, Y. B., Hoover, K. L., Creasia, D., and Poirier, L. A. (1983). Hepatocarcinogenesis in rats fed methyl-deficient, amino acid defined diets. *Carcinogenesis* **4,** 1619–1629.

Miller, C. L. (1978). Immunological assays as measures of nutritional status: A review. *JPEN, J. Parenter. Enteral Nutr.* **2,** 554–566.

National Research Council (1978). "Nutritional Requirements of Laboratory Animals," Vol. 10, 3rd rev. ed., pp. 7–37. National Academy of Sciences, Washington, D.C.

National Research Council (1982). "Diet, Nutrition, and Cancer." National Academy Press, Washington, D.C.

Newberne, P. M. (1975). Diet: The neglected experimental variable. *Lab. Anim.* **4,** 20–22 and 44–47.

Newberne, P. M., and Conner, M. W. (1986). Nutrient influences on toxicity and carcinogenicity. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **45,** 149–154.

Newberne, P. M., and Rogers, A. E. (1976). Nutritional modulation of carcinogenesis. *In* "Fundamentals in Cancer Prevention" (P. M. Magee, S. Takayama, T. Sugimura, and T. Matsushima, eds.), pp. 15–40. Univ. Park Press, Baltimore, Maryland.

Newberne, P. M., and Rogers, A. E. (1986). Labile methyl groups and the promotion of cancer. *Annu. Rev. Nutr.* **6,** 407–432.

Nielsen, F. H. (1984). Ultratrace elements in nutrition. *Annu. Rev. Nutr.* **4,** 21–41.

Nielsen, F. H. (1985). The importance of diet composition in ultratrace element research. *J. Nutr.* **115,** 1239–1247.

Omaye, S. T. (1986). Effects of diet on toxicity testing. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **45,** 133–135.

Parke, D. V., and Ioannides, C. (1981). The role of nutrition in toxicology. *Annu. Rev. Nutr.* **1,** 207–234.

Prins, R. A. (1978). Nutritional impact on drug–microbe interactions. *In* "Nutrition and Drug Interactions" (J. N. Hathcock and J. Coon, eds.), pp. 189–251. Academic Press, New York.

Rader, J. I., Wolnik, K. A., Gaston, C. M., Celesk, E. M., Peeler, J. T., Spivey Fox, M. R., and Fricke, F. L. (1984). Trace element studies in weanling rats: Maternal diets and baseline tissue mineral values. *J. Nutr.* **114,** 1946–1954.

Reddy, B. S., Cohen, L. A., McCoy, G. D., Hill, P., Weisburger, J. H., and Wynder, E. L. (1980). Nutrition and its relationship to cancer. *Adv. Cancer Res.* **32,** 237–345.

Rogers, A. E. (1979). Nutrition. *In* "The Laboratory Rat" (H. J. Baker, J. R. Lindsey, and S. H. Washburn, eds.), Vol. 1, pp. 123–152. Academic Press, New York.

Rowland, I. R., and Wise, A. (1985). The effect of diet on the mammalian gut flora and its metabolic activities. *CRC Crit. Rev. Toxicol.* **16,** 31–103.

Sauberlich, H. E., Skala, J. H., and Dowdy, R. P. (1974). "Laboratory Tests for the Assessment of Nutritional Status." CRC Press, Cleveland, Ohio.

Schrager, T. F., Busby, W. F., Jr., Goldman, M. E., and Newberne, P. M. (1986). Enhancement of methylbenzylnitrosamine-induced esophageal carcinogenesis in zinc-deficient rats: Effects on incorporation of [^{3}H]thymidine into DNA of esophageal epithelium and liver. *Carcinogenesis* **7,** 1121–1126.

Shively, C. A., White, D. M., and Tarka, S. M., Jr. (1986). Diet-induced alterations in theobromine disposition and toxicity in the rat. *Toxicol. Appl. Pharmacol.* **84,** 593–598.

Swenerton, H., and Hurley, L. S. (1968). Severe zinc-deficiency in male and female rats. *J. Nutr.* **95,** 1–18.

Troll, W., Wierner, R., Shellabarger, J., Holtzman, S., and Stone, J. P. (1980). Soybean diet lowers breast tumor incidence in irradiated rats. *Carcinogenesis* **1,** 469–472.

Ulland, B. M., Weisburger, J. J., Yamamoto, R. S., and Weisburger, E. K. (1973). Antioxidants and carcinogenesis: Butylated hydroxytoluene, but not diphenyl-*p*-phenylene diamine, inhibits cancer induction by *N*-2-fluorenylacetamide and by *N*-hydroxy-*N*-2-fluorenylacetamide in rats. *Food Cosmet. Toxicol.* **11,** 199–207.

Vitale, J. J., and Broitman, S. A. (1981). Lipids and immune function. *Cancer Res.* **41,** 3706–3710.

Wattenberg, L. W. (1978). Inhibitors of chemical carcinogenesis. *Adv. Cancer Res.* **26,** 197–217.

Wattenberg, L. W. (1983). Inhibition of neoplasia by minor dietary constituents. *Cancer Res. Suppl.* **43,** 2448s–2453s.

Wattenberg, L. W., Loub, W. D., Lam, L. K., and Speier, L. (1976). Dietary constituents altering the responses to chemical carcinogens. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **35,** 1327–1331.

Weindruch, R., and Walford, R. L. (1982). Dietary restriction in mice beginning at 1 year of age: Effect on lifespan and spontaneous cancer incidence. *Science* **215,** 1415–1418.

Williams, E. A. J., Gebhardt, B. M., Yee, H., and Newberne, P. M. (1978). Immunological consequences of choline–vitamin B-12 deprivation. *Nutr. Rep. Int.* **17,** 279–292.

Wise, A. (1982). Interaction of diet and toxicity—The future of purified diet in toxicological research. *Arch. Toxicol.* **50,** 287–299.

Wise, A., and Gilburt, D. J. (1980). The variability of dietary fiber in laboratory animal diets and its relevance to the control of experimental conditions. *Food Cosmet. Toxicol.* **18,** 643–648.

Yavelow, J., Finlag, T. H., Kennedy, A. R., and Troll, W. (1983). Bowman–Birk soybean protease inhibitors as an anticarcinogen. *Cancer Res. Suppl.* **43,** 2454s–2459s.

7

Risk/Benefit Analysis for Vitamin Supplements

John N. Hathcock

I. INTRODUCTION

The desirability of using risk/benefit (R/B) analysis is often stated without indicating how it is done (Ovesen, 1984). Indeed, there seems to be no generally accepted formula for comparing risk and benefit (Cooper, 1984). Nevertheless, there is a continuing public interest in the relative risk and benefit of a wide range of potential influences on health (Russell, 1988), and these, together with the public-health importance, provide impetus for scientific attention to the issue.

One of the major difficulties in developing a system for R/B analysis is the large diversity of types of benefit considered. Risk evaluation on health-related topics is often restricted to health risks whereas benefit evaluation often includes nonhealth benefits such as increased convenience, lower economic cost, and aesthetic factors (Anonymous, 1978a; Darby, 1980). Clearly then, risk and benefit must be defined using similar parameters if objective evaluations are to be

NUTRITIONAL TOXICOLOGY, VOL. III

made. Hall (1981) has provided the following objective definitions: *risk* is "the chance of injury, damage or loss"; *benefit* is "anything contributing to an improvement in condition." For the discussion herein, risk and benefit will be defined similarly as follows:

Risk:	Probability of an adverse effect on health
Benefit:	Probability of an advantageous effect on health

From these definitions, it follows that any action costing time or resources should be taken only when the following inequality is met:

$$P_b \times I_b > P_r \times I_r,$$

Where P is probability; I, impact (if effect occurs); b, benefit; and r, risk. Although this inequality is simple in concept, it is difficult to apply because of two major restrictions. First, the probabilities may not be known or estimatable with acceptable confidence. Second, the impact values are inherently subjective, that is, personal, cultural, or political value judgments may be involved. For example, perhaps there could be general agreement that heart disease and cancer deserve impact values of 1.0, but what agreement could there be on appropriate numerical values for headaches and skin rashes? With the extremely wide range of types of risk and benefits reported for nutrients, as well as other products, it is unlikely that any scientific method for assigning impact values can be developed. Consequently, this discussion will address only the probabilities of risks and benefits because impact values are inherently judgmental in character and, at least for the present and foreseeable future, cannot be arrived at mechanically or quantitatively.

II. STANDARD TYPES OF RISK AND BENEFIT

For vitamins and other nutrients, risk has been traditionally viewed as associated with deficiency. This risk is an alternate way of expressing the major health benefit from nutrients; prevention of deficiency diseases. If the range of intake considered is sufficiently large, however, then two unrelated types of health risk are involved: deficiency and toxicity. For use of vitamin supplements there should be no risk of deficiency. The primary risk of supplementation, if any, is toxicity. Similarly, the primary benefit of supplementation, if any, is prevention of deficiency. For certain nutrients, additional possible benefits are being actively researched.

A. Risk

The basic health risk associated with vitamin supplementation is that of overdose resulting in toxicity; a second type of health risk relates to the possible substitution of supplements for good diet and health care. The types of toxicity produced by excess vitamins are extremely variable. The symptoms of toxicity are influenced by the particular vitamin involved and its dose parameters, and by the sex, age, body size, and numerous other biological characteristics of the individual. For some vitamins, toxicity symptoms and the most common circumstances under which they occur are well described, whereas other vitamins have low orders of toxicity or seem to be nearly innocuous (Hathcock, 1985; Miller and Hayes, 1982; National Nutrition Consortium, 1978).

The definition of toxicity must be clearly delineated and the reliability of its database ascertained before any estimation or evaluation of risk can be performed. Collectively, the clinical literature on vitamin toxicities uses many different terms such as vitamin overdose, hypervitaminosis, megavitamin exposure, and adverse reactions to describe this phenomenon. The specificity of diagnosis and intensity of symptoms vary widely from one reported case to another. Also, within particular syndromes such as hypervitaminosis A, various specific pathological symptoms may occur. It is important to consider all adverse effects on health in assessing risk from excessive supplementation. The impact value of any adverse effect that occurs, however, must be determined by the individual.

Some vitamin toxicity risks widely recognized by many nutritionists, however, may be more apparent than real. For example, "rebound scurvy" is widely discussed as resulting from conditioning in adults who have had large intakes of vitamin C (Alhadeff *et al.*, 1984) and in infants whose mothers took vitamin C supplements during pregnancy (Alhadeff *et al.*, 1984; DiPalma and Ritchie, 1977; Danford and Munro, 1980; Rhead and Schrauzer, 1971). Careful review of the literature does not provide substantiation of this phenomenon. Instead, bibliographic tracing leads to only a few basic sources. Cochrane (1965) questioned whether the infant scurvy he observed might be prenatally conditioned but recognized that the data were inadequate to prove it. Adult subjects conditioned to 2 g of ascorbic acid before a withdrawal period followed by a test dose eliminated the test dose more rapidly than subjects not conditioned to the high intake (Schrauzer and Rhead, 1973). However, none of the subjects conditioned to high vitamin C intakes had their plasma levels fall significantly below 1.0 mg/dl, a level several times higher than ever found in a scurvy patient. Conditioned oral scurvy in an adult has been reported following cessation of a vitamin C supplement of 1 g/day (Siegal *et al.*, 1982), but the lack of plasma ascorbic acid data and the unusually short time after withdrawal before symptoms were noted (7 days) suggest that the symptoms observed were not scurvy (Clark, 1983).

Hence, rebound scurvy resulting from withdrawal after conditioning to a high intake of vitamin C seems to be an attractive hypothesis that is not supported by the available data.

B. Benefit

The basic benefit of nutrient consumption obviously is to meet nutritional needs (i.e., to prevent deficiencies). Accordingly, the most obvious benefit of vitamin supplement intake is to eliminate deficiencies when dietary intake is not sufficient. Benefits of an additional type from supplements are the putative additional, nonnutritional effects of higher than normal intakes.

The adequacy of a particular intake to meet the nutritional need can be defined by a number of approaches. An intake of 1000 RE of vitamin A meets, by definition, the Recommended Dietary Allowance (RDA) for a healthy adult male (National Research Council, 1980). The RDA is set by estimating the mean requirement, increasing the allowance above the mean requirement enough to meet the needs of "nearly all" of the population, increasing the allowance to account for inefficient utilization, and using judgment when information is limited. The mean nutritional requirement is determined from information on nutritional status in individuals with a range of known nutrient intakes from below most individual requirements to above them.

When the database is adequate, "nearly all" is defined as the proportion of the population included up to the mean requirement plus two standard deviations (SD); this includes 97.8% of the population. When there is inadequate knowledge of the variability, judgment must be employed to identify "nearly all" and to set the RDA. Regardless of how it was identified, an intake below the RDA does not necessarily mean that a deficiency will develop in any particular individual. Conversely, an intake that meets or even exceeds the RDA does not guarantee that the individual requirement is met.

An alternative method for defining a recommended intake is through a probability approach (National Research Council, 1986; National Health and Welfare, 1983; World Health Organization, 1985; Lörstad, 1971, 1974; François, 1971). This procedure is fundamentally similar to the RDA method except that no specific probability level is assumed without it being specified, and any inadequacy in knowledge of the variability of the mean requirement becomes an obstacle. The fundamental similarity is that both methods depend on data from the same clinical and biochemical assessments of nutritional status.

From the perspective of benefit, the individual nutritional requirements and the standard dietary allowances that are designed to meet those requirements with an adequate margin of safety or with a high probability, are directed toward the same set of biological functions, that is, providing adequate nutrition to prevent

deficiency states, to provide a margin of safety in doing so, and perhaps to sustain a body nutrient reserve pool.

Although many nutritionists (Anonymous, 1988) are convinced that the benefit of meeting the nutritional requirement is the only benefit produced by nutrient intake from any source, including both food and supplements, there are widespread claims in the popular press of numerous other benefits from intakes well above those recognized as useful by most nutritional scientists. Many claims published in some magazines and newspapers are made by persons with no training in nutrition, medicine, or science, and many of these claims have no scientific plausibility. Others may seem plausible but often are not based on scientifically acceptable data. Some claims of benefits from higher than usual nutrient intakes, however, are based on adequate scientific data or at least are based on scientifically plausible hypotheses. For example, it is well established that niacin intakes in the range of several grams per day have the beneficial pharmacological effect of substantially lowering serum cholesterol (Hoeg *et al.*, 1984; Mallory *et al.*, 1987; Tikkanen and Nikkila, 1987). Such elevated intakes, however, carry a risk of liver toxicity (Patterson *et al.*, 1983) and therefore should be taken only by high-risk heart disease patients under close supervision of a physician.

Scientifically plausible but unproven potential additional benefits of elevated nutrient intakes have been identified for the antioxidant nutrients, namely, ascorbic acid, vitamin E, and selenium (National Research Council, 1982; Pryor, 1987) and also for those nutrients strongly subject to loss when the individual is exposed to certain drugs or environmental chemicals (Brin, 1978). In experimental animals treated with particular chemical carcinogens, the antioxidant nutrients exhibit significant anticarcinogenic activity. Although the effects of these nutrients at intakes above the requirements may be different from those that make these substances essential, the beneficial effects under these circumstances raise questions about whether the RDAs are set high enough (Pryor, 1987; Diplock, 1987; Horwitt, 1988). The hypothesis remains unproven (Draper and Bird, 1987; Bieri, 1987), although it is a reasonable one based on extensive evidence in animals and some as yet equivocal epidemiological data.

Certainly, any discussion of benefit from vitamin supplements requires specification of the type of benefit, the method of its assessment, and the level of evidence that is acceptable.

III. QUANTITATIVE/STATISTICAL ASPECTS

Quantitative evaluation of risks and benefits requires definition and specification of these terms, as previously discussed, and estimation of the probability of

a given risk or benefit at a specified intake. The probability approach to setting recommended intakes has been suggested for estimating the risk of toxicity (National Research Council, 1986). This potential use is based on the concept that, as intake of a nutrient increases, the probability of deficiency decreases and the probability of toxicity increases. From the perspective of safe and adequate intake, this concept can be illustrated as in Fig. 1. This graph is a combination of two sigmoid curves describing the cumulative fraction of a population showing nutritional benefit at first and then toxicity as intake increases. The steepness of the two slopes and the width of the plateau of safety will no doubt vary with the nutrient and specific effects under consideration. For this graph to be derived from real rather than ideal data, the means for the beneficial effect and for the toxic effect must be well separated, and the absolute variance of the two effects must be identical.

Although the mean intakes needed to produce nutritional and toxic effects may be widely different for most nutrients, the absolute variance for an effect (or any biological parameter) with a larger mean can be expected usually to exceed that with a smaller mean, though the percent variance may be similar. Therefore, when the variance of an effect is unknown, the only assumption logically available is that the coefficient of variation (*C.V.*), rather than the variance, may be similar to that of known effects. With this assumption, the (noncumulative)

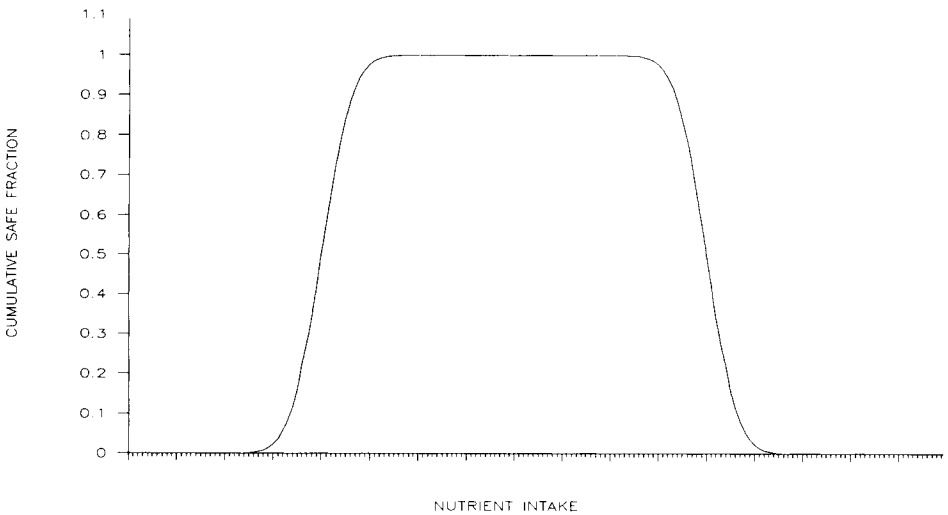

Fig. 1. Nutrient dose–response relationship. The left-hand side of the curve represents an increasing population fraction whose nutrient requirements have been safely met as intake increases; the right-hand side represents a decreasing fraction safe from toxicities. The plateau represents the safe range of intake.

frequency distributions for two effects with very different means but with the same *C.V.* can be visualized as in Fig. 2. The distribution on the left has a mean of 10 with a variance such that 1 SD equals 2, but for the distribution on the right the mean is 100 with 1 SD equaling 20. For both distributions, the *C.V.* is 20%. If the left-hand curve represents a desirable effect and the right-hand curve represents an undesirable effect of equivalent impact value, then *R/B* analysis would suggest an intake above the benefit distribution and below the risk distribution that is an equal number of standard deviations from each mean. In this case, the intake value satisfying this condition is 18.2. That is, in this idealized circumstance with a mean requirement of 10 and a mean toxicity of 100, both of which have 20% *C.V.*, *R/B* equals 1 at an intake of 18.2. This intake is 4.1 SD above the mean requirement and 4.1 SD below the toxicity mean. It should be further noted that 4.1 SD above a requirement mean and 4.1 SD below a toxicity mean provides a very high order of safety; that is, all but 1/13,000 of the population would have the benefit of meeting the nutritional requirement and only 1/13,000 would be at risk of toxicity.

The reliability of the conclusions about risk and benefit reached through this procedure depends on the accuracy of data on both the requirement and its distribution and the toxicity level and its distribution. The type of conclusion reached depends on the characteristics of the data, that is, the magnitude of separation of the means for requirement and toxicity and the widths of the two distributions. If the intake needed to generate a nonnutritional putative benefit is

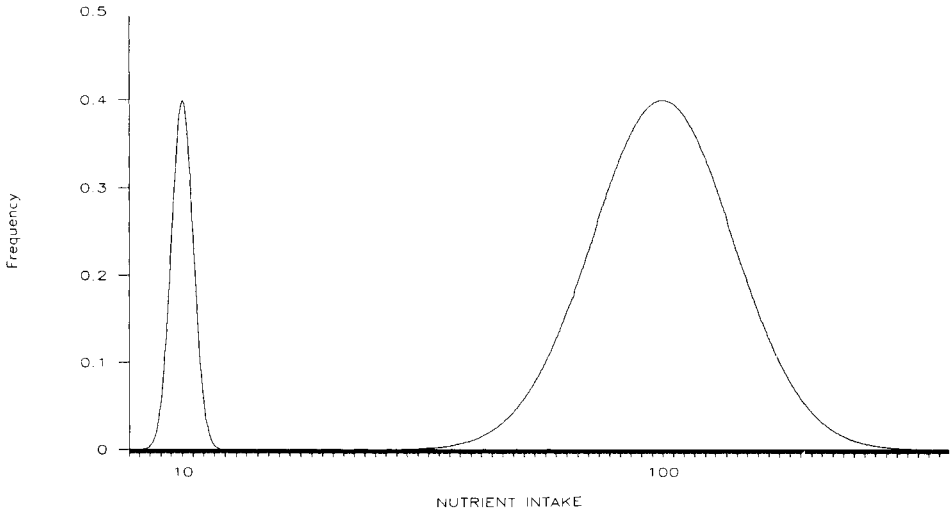

Fig. 2. Idealized distributions of benefit and risk. *Left:* Intake distribution for benefit with mean intake of 10 and 20% *C.V.* *Right:* Intake distribution for risk with mean intake of 100 and 20% *C.V.*

much higher than for meeting the nutritional requirement with an adequate margin of safety, then achieving this benefit will increase the risk of toxicity, and a new *R/B* analysis will be needed.

IV. RISK/BENEFIT EVALUATION OF VITAMIN A INTAKES

Estimation of the probability of risk associated with a particular level of vitamin A intake requires comparison of that intake with the distribution of toxicity, and estimation of the probability of benefit requires comparison of the intake with the distribution of benefit. In this discussion, the benefit considered will be the maintenance of adequate liver stores of vitamin A, as used by Olson (1987) in identifying a recommended dietary intake. The risk considered will include the symptoms most frequently reported for vitamin A overdose, namely, headache, cerebrospinal hypertension, hepatomegaly, and/or cirrhosis, dermatitis, and characteristic birth defects (Bauernfeind, 1980; Miller and Hayes, 1982; Howard and Willhite, 1986; Anonymous, 1987).

The data cited by Olson (1987) support a mean daily requirement for vitamin A by adult men of 506 μg (1685 IU) retinol with 20% *C.V.* This mean and distribution are illustrated by the left-hand curve in Fig. 3. Much less is known about the mean and distribution of toxicities, but an estimate can be made from various reports. The lowest credible reports of vitamin A toxicity have been for consumption of vitamin A supplements of around 25,000 IU. These reports related vitamin A toxicity to intracranial hypertension in nonpregnant adults with acne (Vollbracht and Gilroy, 1976), viral hepatitis (Hatoff *et al.*, 1982), protein–energy malnutrition (Weber *et al.*, 1982), or probable alcohol abuse (Eaton, 1978). Reports of toxicity are somewhat more numerous at intakes of around 40,000–50,000 IU (Bloch, 1955; Pearson *et al.*, 1981; Zafrani *et al.*, 1984; Farris and Erdman, 1982). There are even more reports of vitamin A toxicity at higher doses (Miller and Hayes, 1982; Bauernfeind, 1980; Howard and Willhite, 1986).

The uncertainty about dietary quantities and forms of vitamin A, lack of information about the duration of supplementation in many cases, difficulties in ascertaining accuracy of patient reporting of supplement potency, and probable differences in intensity of symptoms before the patients sought medical attention collectively prohibit determination of the threshold level of susceptibility for vitamin A toxicity. Nevertheless, a set of assumptions that fit the available data will be made in order to examine the most probable *R/B* implications. If the mean toxicity level for vitamin A toxicity is assumed to be 45,000 IU, as is reasonable from the cited reports, and if the percent variability is equal to that for the requirement (20% *C.V.*), then the toxicity distribution is described by the right-hand curve in Fig. 3. This distribution with a mean of 45,000 IU (with 20%

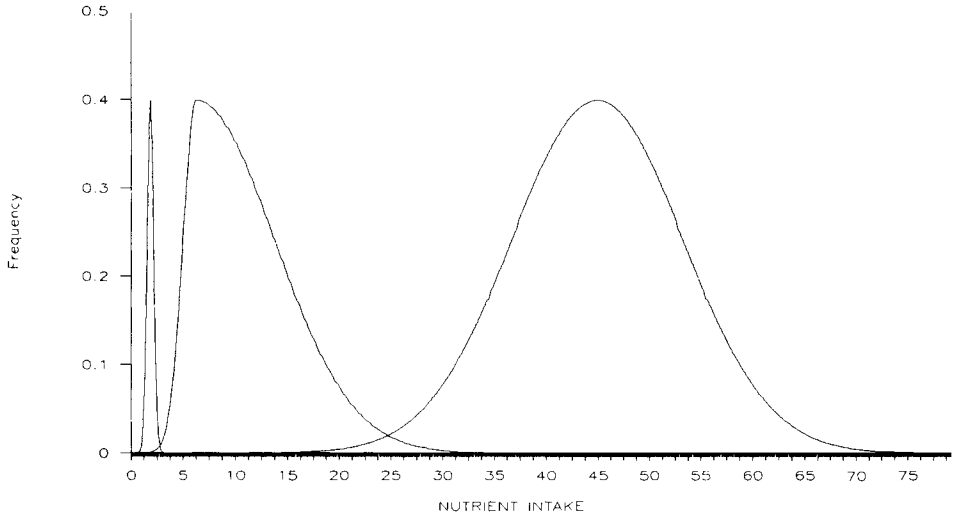

Fig. 3. Vitamin A distributions. *Left:* Requirement distribution with mean of 1685 IU and 20% *C.V. Center:* Intake distribution composite curve from NFCS (1977–1978) (Anonymous, 1978b) mean intake of 6200 IU for adult men and FDA supplement use survey with 95th percentile of 4.3 multiples of the RDA; the left-hand side of this curve has 20% *C.V.* on the mean dietary intake, and the right-hand side is adjusted to locate the 95th percentile at 20,500 IU. *Right:* Toxicity distribution with mean of 45,000 IU and 20% *C.V.*

C.V.) is well separated from the left-hand curve describing requirements with a mean of 1685 IU or 506 μg (with 20% *C.V.*). With these estimates of requirement and toxicity (i.e., of benefit and risk), risk and benefit are equal (and both have very low values) at an intake of 3250 IU (976 μg) vitamin A, a value 4.6 SD above the mean requirement and 4.6 SD below the mean toxicity. A crucial consideration, however, is that both *R* and *B* have values of 1/13,000 or less in the range from 3033 IU to 9000 IU. That is, if these data were graphed as in Fig. 1, the plateau would reach from about 3000 IU to 9000 IU. Furthermore, if these estimates of risk and benefit are valid, the risk of toxicity does not limit a recommended intake to 2 SD above the mean requirement.

If toxicity and requirement are assumed to have equal variability (e.g., 20% *C.V.*), the calculated intake of vitamin A at which risk and benefit are equal is very insensitive to the mean toxic intake (Table I). The calculated intake at which risk equals benefit changes only from 3150 IU to 3350 IU as the mean toxic intake is increased from 25,000 IU to 200,000 IU.

A factor that would have major impact on the results of *R/B* analysis is the mean level of intake for benefit. If putative benefits produced by intakes well above those recognized by most nutritionists and cited by Olson (1987) in calculating a recommended intake are used for *R/B* analysis, the calculated separa-

TABLE I

Calculated Vitamin A Intakes (IU) for Equal Risk and Benefit with Fixed Requirement[a] and Varied Assumptions for Mean Toxic Intake

Assumed mean toxic intake	T − 4 SD (20% *C.V.*)	*R/B* analysis		Requirement (R) + 4 SD (20% *C.V.*)
		$R = B$ intake[b]	SD to R and T	
25,000	5,000	3150	4.37	3033
45,000	9,000	3250	4.60	3033
75,000	15,000	3300	4.78	3033
200,000	40,000	3350	4.91	3033

[a]Requirement for adult men of 506 μg (1680 IU) and variability of 20% *C.V.* as cited by Olson (1987).
[b]Cannot reach 3370 IU (R + 5 SD) because with 20% *C.V.*, T − 5 SD = 0 IU.

tion of risk and benefit would be narrowed; that is, the width of the safety plateau in a graph similar to Fig. 1 would diminish or disappear. The intake at which risk and benefit were equal would be higher; but more importantly, this value would be fewer standard deviations from both the mean for the new benefit effect and the toxicity mean. Obtaining these new, putative benefits by increasing the intake would increase the risk of toxicity.

Estimates of vitamin A intake should account for both diet and supplements. Intake of a particular total level of preformed vitamin A will carry a particular *R/B* ratio regardless of whether the vitamin comes from the diet or from a supplement. In practice, however, a substantial proportion of dietary vitamin A occurs as carotenes, and β-carotene is apparently innocuous (Bendich, 1988) or at least so much less toxic than vitamin A that carotene toxicity usually is not discussed as part of a review of vitamin A toxicity (Olson, 1984; Omaye, 1984). Also, β-carotene does not convert to vitamin A rapidly enough to cause hypervitaminosis A. Any impact of carotenes on vitamin A toxicity through interactions cannot be estimated from available data.

The risk associated with any supplement of vitamin A should be influenced by the amount of dietary vitamin A. The National Food Consumption Survey (NFCS) of 1977–1978 (Anonymous, 1978b) found a mean vitamin A intake of 6200 IU for adult men. Also, a vitamin supplement usage survey by the Food and Drug Administration found that the 95th percentile of supplemental intake for vitamin A by adult men was 4.3 multiples of the RDA, or 14,300 IU. If it is assumed that the users of vitamin A supplements (64.5% of the FDA survey population) consumed the NFCS mean dietary intake of vitamin A, then this combined population has an median intake of 8900 IU total vitamin A and a 95th percentile intake of 20,500 IU total vitamin A. The center curve in Fig. 3 describes this combined dietary and supplemental vitamin A intake distribution. It overlaps slightly with both the requirement distribution and the toxicity dis-

tribution. Thus, R/B analysis for vitamin A supports the conclusion that deficiency is caused by inadequate diet and toxicity is caused by high-potency supplement use.

V. CONCLUSIONS

A probability approach based on frequency distributions can be used to evaluate risk and benefit from supplements. Use of this method depends on knowledge for the mean intake for any risk or benefit under consideration and of the distribution around that mean. Of course, the means will be determined by the effects that are identified as risks and benefits. Each nutrient must be evaluated separately, without assuming that one nutrient is similar to any other in regard to levels of intake that generate risks or benefits.

ACKNOWLEDGMENT

This chapter was written by the author in a private capacity. No official support or endorsement by the United States Food and Drug Administration is intended or should be inferred.

REFERENCES

Alhadeff, L., Gualtieri, C. T., and Lipton, M. (1984). Toxic effects of water-soluble vitamins. *Nutr. Rev.* **42,** 33–40.

Anonymous (1978a). "The Risk/Benefit Concept as Applied to Food." Institute of Food Technologists Expert Panel of Food Safety and Nutrition, Institute of Food Technologists, Chicago.

Anonymous (1978b). "Nutrient Intakes: Individuals in 48 States, Year 1977–78." U.S. Department of Agriculture, Hyattsville, Maryland.

Anonymous (1987). Teratology Society position paper: Recommendations for vitamin A use during pregnancy. *Teratology* **35,** 269–275.

Anonymous (1988). Statement on vitamin and mineral supplements. *Am. J. Clin. Nutr.* **47,** 174.

Bauernfeind, J. C. (1980). "The Safe Use of Vitamin A. A Report of the International Vitamin A Consultative Group." The Nutrition Foundation, Washington, D.C.

Bendich, A. (1988). The safety of β-carotene. *Nutr. Cancer* **11,** 207–214.

Bieri, J. G. (1987). Are the recommended allowances for dietary antixodants adequate? *Free Radical Biol. Med.* **3,** 193–197.

Bloch, H. S. (1955). Chronic hypervitaminosis A. *Minn. Med.* **38,** 627–632.

Brin, M. (1978). Drugs and environmental chemicals in relation to vitamin needs. *In* "Nutrition and Drug Interrelations" (J. N. Hathcock and J. Coon, eds.). Academic Press, New York.

Clark, J. W. (1983). Letter to the editor. *J. Peridontol.* **54,** 182–183.

Cochrane, W. A. (1965). Overnutrition in prenatal and neonatal life: A problem? *Can. Med. Assoc. J.* **93,** 893–899.

Cooper, R. (1984). Polychlorinated biphenyls: Of risks and benefits. *Food Drug Cosmet. Law J.* **39,** 240–267.

Danford, D. E., and Munro, H. N. (1980). Water-soluble vitamins. *In:* "The Pharmacological Basis of Therapeutics" (A. G. Gilman, L. S. Goodman, and A. Gilman, eds.), pp. 1560–1582. Macmillan, New York.

Darby, W. J. (1980). The nature of benefits. *Nutr. Rev.* **38,** 37–44.

DiPalma, J. R., and Ritchie, D. M. (1977). Vitamin toxicity. *Annu. Rev. Pharmacol. Toxicol.* **17,** 133–148.

Diplock, A. T. (1987). Dietary supplementation with antioxidants. Is there a case for exceeding the recommended dietary allowances? *Free Radical Biol. Med.* **3,** 199–201.

Draper, H. H., and Bird, R. P. (1987). Micronutrients and cancer prevention: Are the RDAs adequate? *Free Radical Biol. Med.* **3,** 203–207.

Eaton, M. L. (1978). Chronic hypervitaminosis A. *Am. J. Hosp. Pharm.* **35,** 1099–1102.

Farris, W. A., and Erdman, J. W. (1982). Protracted hypervitaminosis A following long-term, low-level intake. *JAMA, J. Am. Med. Assoc.* **247,** 1317–1318.

François, P. J. (1971). Consumption levels and requirements: General strategies within the framework of a food policy. *F.A.O. Nutr. Newslett.* **9,** 1–8.

Hall, R. L. (1981). Food safety and risk/benefit. *Res. Manage.* **24,** 28–35.

Hathcock, J. N. (1985). Quantitative evaluation of vitamin safety. *Pharm. Times* **51,** 104–113.

Hatoff, D. E., Gertler, S. L., Miyai, K., Parker, B. A., and Weiss, J. B. (1982). Hypervitaminosis A unmasked by acute viral hepatitis. *Gastroenterology* **82,** 124–128.

Hoeg, J. M., Maher, M. B., Bou, E., Zech, L. A., Bailey, K. R., Gregg, R. E., Sprecher, D. L., Susser, J. K., Pikus, A. M., and Brewer, H. B., Jr. (1984). Normalization of plasma lipoprotein concentrations in patients with type II hyerlipoproteinemia by combined use of neomycin and niacin. *Circulation* **70,** 1004–1011.

Horwitt, M. K. (1988). Supplementation with vitamin E. *Am. J. Clin. Nutr.* **47,** 1088–1089.

Howard, W. B., and Willhite, C. C. (1986). Toxicity of retinoids in humans and animals. *J. Toxicol.–Toxin Rev.* **5,** 55–94.

Lörstad, M. (1971). Recommended intake and its relation to nutrient deficiency. *F.A.O. Nutr. Newslett.* **9,** 18–31.

Lörstad, M. (1974). On estimating incidence of undernutrition. *F.A.O. Nutr. Newslett.* **12,** 1–11.

Mallory, M. J., Kand, J. P., Kunistake, S. T., and Tun, P. (1987). Complementarity of colestipol, niacin, and lovastatin in treatment of severe familial hypercholesterolemia. *Ann. Intern. Med.* **107,** 616–623.

Miller, D. R., and Hayes, K. C. (1982). Vitamin excess and toxicity. *In* "Nutritional Toxicology" (J. N. Hathcock, ed.), Vol. 1, pp. 81–133. Academic Press, New York.

National Health and Welfare (1983). "Recommended nutrient intakes for Canadians." Can. Govt. Publ. Centre Supply Serv., Ottawa, Ontario.

National Nutrition Consortium (1978). "Vitamin–mineral safety, toxicity, and misuse." American Dietetic Association, Chicago.

National Research Council (1980). "Recommended Dietary Allowances," 9th Ed. National Academy of Sciences, Washington, D.C.

National Research Council (1982). "Diet, Nutrition, and Cancer." National Academy Press, Washington, D.C.

National Research Council (1986). "Nutrient Adequacy." National Academy Press, Washington, D.C.

Olson, J. A. (1984). Vitamin A. *In:* "Handbook of Vitamins" (L. J. Machlin, ed.), pp. 1–43. Dekker, New York.

Olson, J. A. (1987). Recommended dietary intakes (RDI) of vitamin A in humans. *Am. J. Clin. Nutr.* **45,** 704–716.

Omaye, S. T. (1984). Safety of megavitamin therapy. *Adv. Exp. Med. Biol.* **77,** 169–203.

Ovesen, L. (1984). Vitamin therapy in the absence of obvious deficiency. What is the evidence? *Drugs* **27,** 148–170.

Patterson, D. J., Dew, E. W., Gyorkey, F., and Graham, D. Y. (1983). Niacin hepatitis. *South. Med. J.* **76,** 239–241.

Pearson, M. G., Littlewood, S. M., and Bowden, A. N. (1981). Tetracycline and benign intracranial hypertension. *Br. Med. J.* **282,** 568–569.

Pryor, W. A. (1987). View on the wisdom of using antioxidant vitamin supplements. *Free Radical Biol. Med.* **3,** 189–191.

Rhead, W. J., and Schrauzer, G. N. (1971). Risks of long term ascorbic acid overdose (letter). *Nutr. Rev.* **29,** 262–263.

Russell, C. (1988). Risk *vs.* reality: How the public perceives health hazards. *Washington Post* **24** (June 14), 14–18.

Schrauzer, G. N., and Rhead, W. J. (1973). Ascorbic acid abuse: Effects of long term ingestion of excessive amount on blood levels and urinary excretion. *Int. J. Vitam. Nutr. Res.* **43,** 201–211.

Siegal, C., Barker, B., and Kunstadter, M. (1982). Conditional oral scurvy due to megavitamin C withdrawal. *J. Peridontol.* **53,** 453–455.

Tikkanen, M. J., and Nikkila, E. A. (1987). Current pharmacologic treatment of elevated serum cholesterol. *Circulation* **76,** 529–533.

Vollbracht, R., and Gilroy, J. (1976). Vitamin A induced benign intracranial hypertension. *J. Can. Sci. Neurol.* **3,** 59–61.

Weber, F. L., Mitchell, G. E., Powell, D. E., Reiser, B. J., and Branwell, J. G. (1982). Reversible hepatotoxicity associated with hepatic vitamin A accumulation in a protein-deficient patient. *Gastroenterology* **82,** 118–123.

World Health Organization (1985). Energy and protein requirement. *WHO Tech. Rep. Ser.* **724.**

Zafrani, E. S., Bernuau, D., and Feldmann, G. (1984). Peliosis-like ultrastructural changes of the hepatic sinusoids in human chronic hypervitaminosis A: A report of three cases. *Hum. Pathol.* **15,** 1166–1170.

Index