Methods for Assessing the Biological Effects of Specific Plant Components

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Until very recently, phytonutrient research was the province of natural product chemists and consisted of primarily anecdotal clinical references. In recent years, an extensive set of qualitative and semi-quantitative dietary epidemiologic data has been developed. This developing base of epidemiologic data is now being supplemented by biochemical, mechanistic, and genetic epidemiology of a more quantitative nature. As we seek to understand the mechanisms that explain a large body of epidemiologic evidence, newer laboratory methods continue to be developed. Though there is a continuing need for even more discriminating nutrition epidemiology to drive the basic research in this area forward, the focus of in vitro, animal and clinical (human) studies must continue to be refined, and appropriate biomarkers for chronic and acute (death) disease end-points must be developed.

Current Status of the Scientific Knowledge and Additional Knowledge Needed

The methods and databases used for determining modes of action and interaction of phytonutrients and for measuring amounts of phytonutrients within plant or animal tissue are summarized in Table 1.[1–25] Improvements in measurement sensitivity have been made through, for example, improved electrochemical detection methodologies that afford greater sensitivity than was heretofore available.[26] Additionally, the development of benchtop HPLC-MS systems that are less costly than previously available models (typically less than $100,000) now permits the rapid resolution of complex phytochemical mixtures on both a quantitative and a qualitative basis. These recent developments have facilitated screening for biologic activity in a manner akin to the way that drugs are screened and are essential to moving from descriptive findings to elucidating mechanisms of action and interaction of phytonutrients. New models for research using animal models specific to selected biological mechanisms have also advanced substantially, and many of these are cited in this review.

Various phytonutrients are known to be good antioxidants in vitro, and are thought to have strong antioxidant properties in vivo and to exert a protective effect against oxidative damage to macromolecules (Table 2[27–49]). Although the antioxidant role of carotenoids is perhaps one of the most robust of the theses addressing phytochemical function, even this hypothesis has been recently questioned.[50] The phytonutrients with antioxidant activity are a highly diverse collection of compounds. It is widely believed that as antioxidants, phytonutrients can inhibit the propagation of free radical reactions that may ultimately lead to the development of degenerative diseases including cancer, cardiovascular disease, age-related macular degeneration, neurologic diseases, and rheumatoid, joint, or connective tissue disorders. Additionally, phytonutrients appear to protect human health by mechanisms that are independent, or largely independent, of their antioxidant properties. Some examples of the reported involvement of certain phytonutrients are provided in Table 3 and include: 1) limiting cell growth by enhancing cell to cell communication, 2) causing cancer cells to die (apoptosis), 3) arresting cell cycle progression, 4) altering steroidal hormone metabolism, 5) enhancing immune response, and 6) up-regulating the activity of enzymes that detoxify carcinogens.[51–63]

An important barrier to phytonutrient research is the

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Table 1. Phytonutrients: Status of Methods and Data Availability

Class	Analytical Methods: Food Analysis	Analytical Methods: Body Compartments	Database Available?	Selected Analytical Reference(s)[a]
Carotenoids	Y	Y	Y[b]	1–2
Dietary Fiber	Y	Y(S)	Y[c]	3–4
Glucosinolates/Isothiocyanates	Y	Y(U)	N	5–7
Indoles	Y	N	N	8–9
Inositol Phosphates	Y	Y(S)	N	10–11
Phenols and cyclic compounds	Y	N	N	12
Phytoestrogens				
Isoflavones	Y	Y(U,S,P)	Y[d]	13–14
Lignans	Y	Y	N	13–14
Polyphenols				
Flavonoids	Y	N	N[e]	15–17
Non-Flavonoids	Y	N	N	18–19
Protease Inhibitors	Y	N	N	20
Saponins	Y	N	N	21
Sterols, plant	Y	N	N	22–23
Sulfides and thiols	Y	N	N	24–25

U=urine; S=stool; P=plasma.

[a]Selected primary references, or reviews where enough of a consensus exists.

[b]USDA - Nutrition Coordination Center Carotenoid Database for U.S. Foods; available at www.nal.usda.gov/fnic/foodcomp.

[c]USDA Nutrient Database for Standard Reference; available at www.nal.usda.gov/fnic/foodcomp.

[d]USDA - Iowa State University Flavonoid Database.

[e]Under development; will be available at www.nal.usda.gov/fnic/foodcomp upon completion.

lack of good animal models that comprehensively mimic human absorption, storage, and metabolism of phytonutrients, and ultimately, the outcomes of human health. Although animal models have been utilized to study the effects of specific phytonutrients on various disease process, they must be applied with great care and caution.[64] In addition to numerous well-known rat, mouse, and hamster carcinogenesis models,[65] other animal models recently

Table 2. Assays for Antioxidant Effects of Plant Components

Total Antioxidant Activity

- Oxygen Radical Absorbance Capacity (ORAC) assay[27] used to measure total antioxidant activity of:
  - fruits[28]
  - tea and vegetables[29]
  - human plasma[30]

Prevention of Oxidative Damage to DNA

- Single cell microgel electrophoresis (COMET) assay[31] used for:
  - detection of DNA strand breaks[32]
  - detection of DNA oxidation[33,34]
  - assessment of DNA damage in lymphocytes following vegetable consumption[35]
- DNA adduct repair[36,37] [8-oxy-7,8-dihydro-2"deoxyguanosine (8-oxodG) is repaired by excision and 8-hydroxy-2'-deoxyguanosine (8-OHdG) is excreted in urine]; used to monitor:
  - reduced oxidative damage in subjects consuming brussels sprouts[38]

Prevention of Oxidative Damage to Protein

- Carbonyl assay[39–41]

Prevention of Oxidative Damage to Lipids

- F2-isoprostanes (prostaglandin-like) lipid peroxidation assay[42]
  - A reduction (non-significant) in urinary 8-iso-prostaglandin F2 followed consumption of high levels of tomato products[43]
- Breath-ethane and -pentane lipid peroxidation assay[44] for detection of end-products of n-3 and n-6 fatty acid peroxidation:
  - high fruit and vegetable consumption led to reduced levels of exhaled ethane[45]
- spectrophotometric, fluorometric, and HPLC methods for measurement of lipid oxidation products[46]
- ferrous ion oxidation assay (FOX) for lipid hydroperoxides[47]
- conjugated diene detection for assessment of lipid peroxidation; popular for monitoring LDL oxidation in vitro[48]
- oxidative modification of LDL[49]

Table 3. Selected Biological Effects of Plant Components

Enzyme Induction
- Phase 2 enzyme induction[51] by:
  - isothiocyanates and glucosinolates[5,52,53]
  - carotenoids[54]
  - resveratrol[55]
  - withantholides[56]
  - proanthocyanidins[57]
  - polyphenols[15]

Altered Cell Growth, Viability, or Communication
- Regulation of gap junctional communication (GJC)[58] potentially leading (with carotenoids) to inhibition of transformation and chemoprotection against cancer[55,59]
- Apoptosis induction[60]
- Cell proliferation[61]
- Cell cycle arrest (non-ER; CDK6-dependent)[62]

Hormone-like
- Phytoestrogens (which may alter hormone production, metabolism, or action at the cellular level)
  - lignans and isoflavanoids[13]
  - indole-3-carbinol[63]

have been successfully utilized to study the effects of dietary components on carcinogenesis and include the house musk shrew[66] and fish models such as the rainbow trout fingerling (*Salmo* sp.)[8,67] and the Japanese Medaka (*Oryzias latipes*).[68] Both gerbil *(Meriones unguiculatus)* and ferrret *(Mustela putorius furo)* models—which are more appropriate animal models for studying the intestinal absorption of β-carotene and its storage and metabolism in body tissues—have been used to study the effects of carotenoids.[69,70] A canine model that uses mechanically stenosed coronary arteries and intimal damage[71] has been used to demonstrate that wine and grape juice[72] as well as tea[73] inhibit in vivo platelet activity and thrombosis. Cao et al. have shown that supplementation of the rat diet with blueberry extracts (but not with an equal amount of antioxidants from spinach) can block hyperoxia-induced movements of proteins and low MW antioxidants from lung to serum.[74]

Technical Barriers for Biological Effect Assessment

Assessment of the biologic effects of specific plant components is presently confounded by numerous technical barriers. Foremost among them is the need to develop appropriate biomarkers and in particular, to develop non-invasive tests for measurement of those biologic effects. Methods must take into account the need for high sensitivity measurements of small changes in end points. For example, a very slight (<5%) induction of mammalian detoxication (Phase 2) enzymes over the course of 20–30 years is likely to be important in reducing the risk of various cancers, but with current techniques, in vivo detection of such a small change may not be possible.

Ideally, methods for assessing the biologic effects of plant components would be unquestionably relevant to human disease processes. Currently, however, it is not clear that information from in vitro assays can be directly translated to in vivo processes with a high level of confidence. Development of additional broad-spectrum and high-throughput bioassays (e.g., Oxygen Reduction Absorbance Capacity [ORAC], Comet assay for DNA damage, Phase 2 enzyme induction) will allow researchers to screen for large numbers of compounds for a desired activity. Additionally, broad-spectrum bioassays will allow the simultaneous study of groups of compounds and the possible synergistic effects of these compounds. Phytochemicals with putative nutrient or disease-ameliorating effects that are highlighted by these screens must then be examined using appropriate cell culture systems and animal models for studying specific classes of compounds; these approaches are critical to identifying tissue specificity of the various phytonutrients. Cell cultures such as the murine hepatoma system used by Prochaska et al.[51] to monitor Phase 2 enzyme induction by chemoprotective agents have been widely used. However, the use of primary human cell cultures and of animal organ cultures has been much slower in gaining widespread acceptance, due to technical problems. Although Prochaska and Fernandes were successful in demonstrating Phase 2 enzyme induction by the drug oltipraz in the serum of mice, they were not able to repeat this observation in human serum.[75] More recently, Morel and co-workers claim to be able to successfully use human hepatocyte primary cultures to achieve this end.[76]

In addition to these methodologic barriers, there are at least three experimental barriers that require further attention in order to facilitate the study of phytonutrient effects on health. The first is the lack of availability of standard test materials. For most of the classes of phytonutrients enumerated above, no good source of standard test materials exists. For certain of these classes, only one or a few representative compounds are available commercially. For other classes, only one or a few laboratories worldwide have isolated, purified, and characterized some of the representative compounds from plants, and in only a few cases have synthetic protocols been developed. Even where small quantities of pure compound exist, one cannot expect the researchers responsible for developing these sources to dispense samples to everyone who requests them.

Even more critical is the lack of availability of isotopically labeled plants and plant components. Although there has been a rapid upsurge in research on the mode of action of these compounds, only a few laboratories are producing phytonutrients that are radiolabeled and stable-

isotope-labeled. Some of these compounds (e.g. ^{13}C-glucose) can be generated in small quantities in tissue culture and in particular, in root cultures—cultures either initiated by exogenous hormone manipulation or by *Agrobacterium rhizogenes*. A disadvantage of ^{13}C-labeling is its high cost (e.g. $5,000/lb for 100%-$^{13}CO_2$ labeled kale as cited by Steve Britz, USDA). Although less expensive, labeling with ^2H_2O has other problems including discrimination against ^{2}H by the plant, production of multiple isotopomers, and loss of label via transpirational loss of water. The need to use such labeled compounds is becoming more acute since they will play a critical role in experiments that can be designed to support very exciting—but highly speculative—epidemiologic and animal metabolism studies. Availability of plant materials intrinsically labeled with stable isotopes would permit great advances in the understanding of the absorption of these compounds by humans.[77] Availability of radiolabeled as well as stable isotopically labeled material would also permit great advances in the study of the pharmacokinetics and pharmacodynamics of these phytonutrients in a manner that is already used successfully with mineral nutrients such as calcium.[78,79]

A final technical barrier identified by the workshop participants is the almost complete lack of availability of phytonutrient databases. Although a carotenoid database for fruits and vegetables has been compiled[80] and has been used to analyze food frequency questionnaires of cohorts from the Nurses Health Study and the Health Professionals Follow-Up Study,[81] to our knowledge this is the only extant database that can be used to assess the content of any of the phytonutrient classes outlined in Table 1. The most recent version of the database for carotenoids (USDA- Nutrition Coordination Center Carotenoids Database for U.S. Foods) can be downloaded from www.nal.usda.gov/fnic/foodcomp, as can the recently released food database on isoflavones (USDA - Iowa State University Database on Isoflavonoid Content of Foods). A food database for flavonoids is currently under development and will be released at the web site listed above.

Design Barriers for Biologic Effect Assessment

In order to facilitate dramatic progress in understanding the biologic effects of phytonutrients, certain design barriers must be addressed. Chronic exposure studies are extremely expensive, so targets and resources must be chosen and allocated carefully. Although chronic and acute endpoints are in large part known, the route to addressing these endpoints is littered with obstacles that can easily compromise the costly clinical studies designed to develop an understanding of the effect of "non-essential" nutrients on the disease process. Appropriate development of experimental designs must include carefully considered decisions as to whether to administer whole foods, specific nutrients, or classes of nutrients.

All of these approaches have value and are expected to help advance the knowledge base in phytonutrient research. There are, however, cautions about administering high levels of single phytonutrients to humans. While feeding phytonutrient-rich foods has not been associated with harmful effects in humans, this has not been the case with some studies of supplements (e.g. β-carotene).[50] Identification of appropriate stressors such as alcohol, smoking and exercise will also be key to the development of high-quality results from these clinical studies. Additionally, administering single phytonutrients may affect absorption or utilization of other phytonutrients. It is recognized that a large and increasing percentage of the U.S. population consumes dietary supplements, so current as well as recent supplement use may confound outcome variables or markers used to assess biologic effects.

Priority Research Areas

A number of priority research areas focus on phytonutrients. Despite all the media attention in the past few years, the science of phytonutrients is still an embryonic discipline. Numerous questions must be addressed before recommendations can be made for dietary changes that would radically deviate from the 5-A-Day Guidelines.[2] The development of methods that can functionally quantitate biologic activity in animal models and in humans, as well as the identification of both the compounds and the animal models is of paramount importance. Continued development of high throughput screens (e.g., bioactivity-guided fractionation)[83] will be an intimate part of this process and will highlight compounds for isotopic labeling studies so that their mode of action and pharmacokinetics can be investigated. Thus, the basic mechanisms of action of these phytonutrients must be elucidated, along with an understanding of how the activity of these compounds in isolation differs from their activity when delivered in their natural (plant) milieu and how this activity can be modified by the effects of other phytonutrients.

Role of the Federal Government

It was the consensus of the workshop participants that a greater emphasis be placed on:

- developing standard test materials;
- improving high throughput screening and isotopic labeling technologies;
- emphasizing multidisciplinary research so that scientists in disciplines as diverse as plant science and cancer research can work cooperatively through joint funding. (Many of these projects traditionally would not be funded by either the United States Department of Agriculture [USDA], Health and Human Services, or other federal funding agencies.);
- supporting the development and use of nutrient/

phytonutrient databases to increase meaningful prospective epidemiologic studies on phytonutrients consumption — currently not possible due to a lack of a reference phytonutrient food composition database within the USDA Nutrient Database for Standard Reference; and

- developing a website to provide a central communication forum for those engaged in phytonutrient research. Such a website could be set up for a nominal cost by USDA.

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