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BIOACTIVE COMPOUNDS IN NUTRITION AND HEALTH-RESEARCH METHODOLOGIES FOR ESTABLISHING BIOLOGICAL FUNCTION: The Antioxidant and Anti-inflammatory Effects of Flavonoids on Atherosclerosis

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■ **Abstract** Identifying bioactive compounds and establishing their health effects are active areas of scientific inquiry. There are exciting prospects that select bioactive compounds will reduce the risk of many diseases, including chronic diseases such as cardiovascular disease. Recent findings have established that cardiovascular disease is a disease of inflammation, and consequently is amenable to intervention via molecules that have anti-inflammatory effects. In addition, research demonstrating adverse effects of oxidants on atherogenesis raises the possibility that antioxidants can confer cardioprotective effects. This review provides an overview of research approaches that can be used to unravel the biology and health effects of bioactive compounds. Because of the number of bioactive compounds and the diversity of likely biological effects, numerous and diverse experimental approaches must be taken to increase our understanding of the biology of bioactive compounds. Recognizing the complexity of this biology, sophisticated experimental designs and analytical methodologies must be employed to advance the field. The discovery of novel health effects of bioactive compounds will provide the scientific basis for future efforts to use biotechnology to modify/fortify foods and food components as a means to improve public health.

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INTRODUCTION

Nutrition is transiting an era that is defining the role of bioactive compounds in foods. These compounds are defined as components of foods that influence physiological or cellular activities resulting in a beneficial health effect (see Table 1 for chemical classes). This definition distinguishes these compounds from many others that are bioactive, but have detrimental effects and are considered carcinogens or toxins. It is important to appreciate that bioactive compounds are not nutrients. That is, they are not essential for life, a fundamental criterion for a nutrient

TABLE 1 Bioactive compounds: food sources and tabulated data on food content

Bioactive compound family	Primary food sources	Database/tabulated source[a]
Carotenoids	Green, orange, red, yellow fruits & vegetables	(71)
Flavonoids & proanthocyanidins (polymeric flavonoids)	Fruits & vegetables, soy foods & legumes, tea, cocoa	(28, 56, 69, 70) (22, 25)
Glucosinolates & isothiocyanates	Cruciferous vegetables, e.g., broccoli, watercress	(49)
Lignans	Flax seed, flaxseed oil, rye	(28, 50)
Monophenolic alcohols[b]	Olive oil, wines	ND
Monoterpenes[c]	Essential oils of citrus, cherries, mint, & herbs	ND
Organosulfur compounds[d]	Garlic, leek, onion	ND
Phenolic acids[e]	Cereals, coffee, fruits & vegetables	ND
Plant sterols[f]	Rice oil, soybean oil, tall oil	(56)
Saponins	Soy foods	ND
Stilbenes[g]	Grapes, red wine, peanuts	ND
Tannins, hydrolysable[h]	Fruits & vegetables	ND

[a]ND, Database or tabulated source not developed as of late 2003.

[b]Includes tyrosol and hydroxytyrosol.

[c]Includes D-limonene and perillic acid.

[d]Examples include allicin, alliin, diallyl disulphide, and S-allylcysteine.

[e]Examples include caffeic acid, chlorogenic acid, *p*-coumaric acid, and ferrulic acid.

[f]Examples include campesterol, β-sitosterol, and stigmasterol.

[g]Includes resveratrol.

[h]Subclasses include ellago-tannins and gallo-tannins.

classification. This distinction affects the types of experiments that should be designed to study bioactive compounds.

Bioactive compounds typically occur in small amounts in foods (41). Inherently, bioactive compounds have more subtle effects than nutrients. For example, bioactive compounds influence cellular activities that modify the risk of disease, rather than prevent deficiency diseases. One example of how bioactive compounds might modify disease risk is illustrated by the large difference in absolute coronary disease mortality rates at a given total cholesterol level observed in the 25-year follow-up of the Seven Countries Study (72). Specifically, coronary heart disease mortality rates at a similar total cholesterol level (about 5.5 mmole/L or 210 mg/dl) varied from 4% to 5% in Japan and Mediterranean Southern Europe to approximately 15% in Northern Europe. Because of the current focus on reducing risk of chronic diseases, and the potential beneficial role of bioactive compounds, there is

keen interest in studying the health effects of bioactive compounds and unraveling the mechanisms that mediate their effects.

The primary challenge in this area is to identify bioactive compounds and their associated health effects as well as their underlying biological mechanism of action. An impressive and growing number of bioactive compounds have been identified that have potentially important health benefits. These compounds can act as antioxidants, enzyme inhibitors and inducers, inhibitors of receptor activities, and inducers and inhibitors of gene expression, among other actions. Demonstration of these activities alone is not sufficient for a compound to be defined as a bioactive compound; it also must have an associated beneficial health effect. Another important distinction of bioactive compounds is that unlike nutrients, which generally have very specific functions such as being an enzyme cofactor, bioactive compounds also may have overlapping functions or activities such as antioxidant activities wherein multiple compounds may perform the same function or have similar activities.

The number and diversity of bioactive compounds adds much complexity to understanding their effects on health. Consequently, there is a need for more sophisticated experimental designs to demonstrate health effects, such as the use of indexes and global activity measurements. Furthermore, at the bioactive compound level, biological potency is affected by a myriad of factors, such as food source, quantity consumed, how the bioactive compounds are consumed (i.e., alone or with other foods, frequency, etc.), how processing affects the potency of compounds, and other variables, such as genetic variation within a species, and environment (including soil type, rainfall, and temperature). In addition, the biological response (and associated health effect) observed by individuals consuming bioactive compounds will be affected by their genetic variability in diet response, demographic profile, and clinical characteristics.

It is evident that much research needs to done to identify the biology of each important bioactive compound and its effect on health. With the advent of contemporary and powerful methodologies for conducting applied and basic science, rapid progress can be made in understanding the functional importance of each bioactive compound. The purpose of this review is to provide an overview of the research approaches necessary to unravel the biological and health effects of bioactive compounds. This will necessitate an approach that differs from studying nutrient requirements where the amount of a nutrient needed to prevent a nutrient deficiency is defined. Determining the biological effects of bioactive compounds requires in vitro or in vivo experiments that must be correlated to a health outcome. Acute and chronic effects, and direct and indirect effects, need to be studied and mechanisms of action must be delineated. Moreover, it will be important to assess possible interactive effects with other dietary nutrients/dietary constituents that may potentiate or antagonize functions of bioactive compounds.

The flavonoids are one of the groups of bioactive compounds that have been studied extensively. These compounds are used as a case study in this review to scrutinize the methodological issues that must be addressed to elucidate biological

function and health effects, with emphasis on atherosclerosis, the leading cause of morbidity and mortality in developed countries. Once the important bioactive compounds have been identified, biotechnology provides a means to "fortify" foods with the desired molecule(s) that confer specific health effects. This will have a marked impact on food production systems, dietary recommendations, and public health. Bioactive compounds have great potential to be the next generation of dietary factors that confer health effects beyond those seen with presently recommended dietary patterns.

EVIDENCE OF HEALTH EFFECTS OF BIOACTIVE COMPOUNDS

Evidence for the existence of bioactive compounds is based primarily on observational studies that demonstrate the beneficial effects of certain dietary patterns that include vegetarianism (19, 48), high whole-grain consumption (30–32), the "prudent" diet (29, 66), the Mediterranean diet (11), and the traditional Japanese diet (36). The traditional Japanese diet has a high content of soybean products and vegetables. The Mediterranean diet has a high content of olive oil, fruits and vegetables, and whole-grain breads. The "prudent" diet is characterized by high intakes of fruits and vegetables, fish, poultry, whole-grain products, and legumes. A high intake of whole grains includes whole-grain breads, lightly pearled barley, oatmeal, some ready-to-eat cereals, wild rice, brown rice, and whole-grain pastas. Each of these dietary patterns has been associated with a significantly reduced risk of coronary heart disease and other chronic diseases. Some of the studies also have shown that the relationship between the dietary patterns and a low risk of chronic diseases was not accounted for by known nutrient intakes or associated healthy lifestyles (30). Thus, it can be hypothesized that various compounds in foods and possibly other characteristics of foods (such as glycemic index) account for the beneficial health effects. However, observational studies do not prove causality, which must be established in randomized controlled clinical studies/trials. Observational studies have inherent limitations that include variable measurements of dietary intakes, which generally are based on self-report and utilize various food frequency questionnaires, dietary recalls, or diet histories (6, 75–77). A lack of accurate and extensive food composition databases affects dietary intake estimates of nutrients and phytochemicals.

ANTIOXIDANTS AND ATHEROSCLEROSIS

An antioxidant is a chemical that is defined as a substance that delays or prevents oxidation of a substrate (23). The production of oxidants is a typical event associated with aerobic metabolism. Antioxidants function to inhibit oxidant formation,

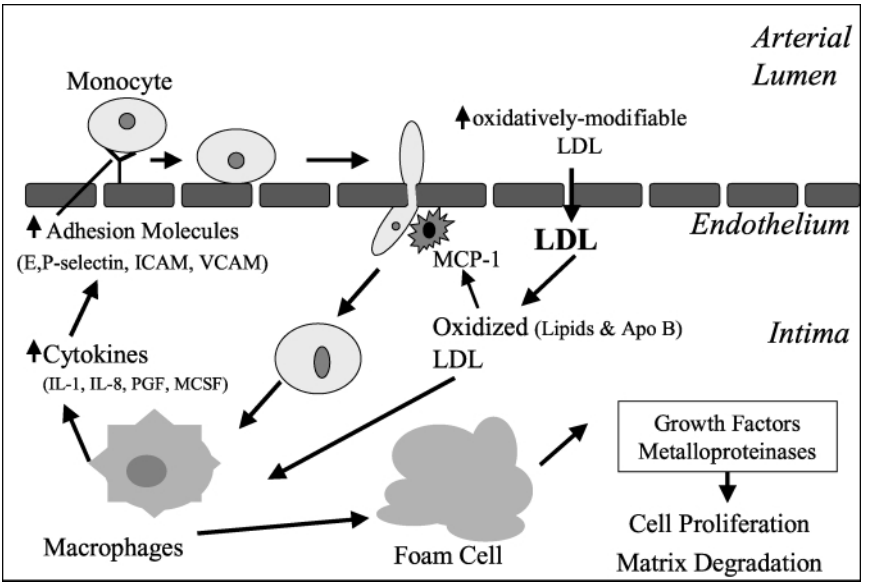

Figure 1 Role of oxidized LDL in atherogenesis.

intercept oxidants, and repair oxidant-induced injury (68). Thus, inadequate antioxidant defenses predispose to oxidant-mediated diseases. With respect to the development of atherosclerosis, oxidative modification of low-density lipoprotein (LDL) is thought to be an important initiating event. LDL accumulates in the extracellular subendothelial space of the arteries and undergoes oxidative modification. Oxidized LDL triggers a cascade of proatherogenic events (Figure 1). Local vascular cells are stimulated by oxidized LDL to produce monocyte chemotactic protein 1 (MCP-1) and granulocyte and macrophage colony-stimulating factors that promote recruitment of monocytes to the endothelium and uptake, followed by conversion to macrophages in the arterial wall (52). In conjunction with this, a cytokine-induced [tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1)] expression of endothelial cell surface adhesion molecules stimulates recruitment of blood mononuclear cells to the endothelium. LDL oxidation is further stimulated by accumulating monocytes and macrophages (as reviewed in Reference 13). This causes modification of the apolipoprotein B component of LDL, thereby increasing recognition by scavenger receptors on macrophages and LDL uptake, resulting in foam cell formation (24). This sequela results in maturation of the atherosclerotic lesion and precipitation of numerous events that stimulate lesion progression including intimal proliferation, fibrosis, calcification, endothelial dysfunction, matrix degradation, vasoreactivity, plaque rupture, and thrombosis (as reviewed in 60, 68).

ANTIOXIDANTS AND INFLAMMATORY DISEASES

Bioactive compounds from a variety of foods may prevent or mitigate numerous chronic diseases including atherosclerosis. Many of these compounds, including the flavonoids, are believed to function as antioxidants and/or possess anti-inflammatory properties. Suppression of the inflammatory response in chronic diseases may beneficially affect disease outcome. A better understanding of the role of antioxidants that have anti-inflammatory properties may aid in reducing the risk of atherosclerosis and other chronic diseases.

ATHEROSCLEROSIS IS AN INFLAMMATORY DISEASE

Atherosclerosis is a disease of inflammation (58, 60). An initiating event in atherosclerosis is believed to be the development of endothelial dysfunction. Potential causes of endothelial dysfunction include elevated levels of oxidatively modified LDL, generation of free radicals (e.g., from smoking), hypertension, diabetes, and elevated levels of homocysteine. The injured endothelium responds to these various insults by developing procoagulant instead of anticoagulant properties, upregulating adhesion molecules (E-selectin, P-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1), and by secreting a number of cytokines and growth factors (MCP-1, interleukin-8, platelet-derived growth factor, and macrophage colony-stimulating factor). The release of these factors leads to the sequestration and accumulation of lymphocytes and macrophages from the blood and the migration and proliferation of underlying smooth muscle cells.

In addition to the well-established coronary vascular disease risk factors (including elevated lipids and lipoproteins, hemostasis factors, blood pressure, and homocysteine), elevated levels of C-reactive protein, an acute-phase protein and sensitive marker of inflammation, is predictive of cardiovascular disease events (57, 58). The increased hepatic synthesis of C-reactive protein is likely mediated by IL-6, which is in turn regulated by the proinflammatory cytokines TNF-α and IL-1 (59).

ANTI-INFLAMMATORY AND ANTIOXIDANT PROPERTIES OF FLAVONOIDS

More than 4000 flavonoids have been identified in plants. The common structure of flavonoids involves two aromatic rings linked through three carbons that typically form an oxygenated heterocycle. Based on their chemical structure, flavonoids are divided into several subclasses (see Table 2 for subclasses, specific compounds, and major food sources), of which flavonols are the most abundant in the plant

TABLE 2 Flavonoids in foods: flavonoid subclasses, compounds, and food sources

Subclass	Compounds	Primary food sources
Flavonols	Quercetin, kaempferol, myricetin, isorhamnetin	Onions, apples, teas, berries, olives, bananas, lettuce, plums, red wine
Flavones	Luteolin, apigenin	Apples, celery, celeriac, lemons, parsley, oregano, lettuce, beets
Isoflavones	Genistein, diadzein	Soybeans, legumes
Flavanones	Hesperetin, naringenin, eriodictyol	Oranges, grapefruits, lemons
Anthocyanidins	Cyandin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	Blueberries, raspberries, strawberries, cranberries
Flavan-3-ols	Catechin, gallocatechin, epicatechin, epigallocatechin, epicatechingallate, epigallocatechingallate, theaflavin, theaflavingallate, theaflavindigallate, thearubigins	Green tea, black tea, plums, apples, cranberries
Procyanidins	Polymeric catechins and epicatechins	Cocoa, chocolate, cinnamon, cranberries, pinto beans, kidney beans, hazelnuts, pecans

kingdom (7). Flavonoids act as antioxidants by chelating redox-active metals and by scavenging free radicals. Chelation of both iron and copper by the carbonyl and hydroxyl groups of flavonoids (7) prevents peroxyl radical (8) and lipid peroxidation (1, 65). Flavonoids also function as terminators of free radicals by donation of electrons to form stable products. Flavonoids are very effective scavengers of hydroxyl and peroxyl radicals (7) as well as quenching superoxide radicals and singlet oxygen (34, 63).

Some flavonoids have anti-inflammatory properties. Quercetin markedly inhibits the production of TNF-α and nitric oxide by lipopolysaccharide-activated macrophages (45). TNF-α inhibition may occur posttranscriptionally whereas inducible nitric oxide synthase inhibition may occur at the transcriptional level (73). In lipopolysaccharide-activated Kupffer cells, quercetin also strongly inhibited nitric oxide production and TNF-α expression (37), possibly through a posttranscriptional process. Quercetin was reported to suppress TNF-α-induced expression of IL-8 and monocyte chemoattractant protein (MCP)-1 (62) due, at least in part, to its ability to inhibit the activation of NF-κB. Similarly, apigenin, one of the most potent flavones, inhibits prostaglandin synthesis induced by IL-1α (21). Apigenin also inhibits the production of IL-6 and IL-8 in human endothelial cells activated by TNF-α.

Intercellular adhesion molecule (ICAM)-1 plays an important role in the inflammatory responses during atherogenesis. Expression of ICAM-1 is induced

by cytokines, such as IL-1, TNF-α, or INF-γ, on endothelial cell surface during inflammation. Quercetin inhibited ICAM-1 expression induced by phorbol 12-myristate 13-acetate and TNF-α in ECV304 human endothelial cell in a dose-dependent manner (39). This inhibition was due to downregulation of activator protein-1 that is associated with inhibition of c-Jun NH2-terminal kinase pathway. In another study, apigenin exhibited a dose- and time-dependent inhibition of cytokine-induced ICAM-1, VCAM-1, and E-selectin expression (21). In this study, apigenin did not inhibit nuclear translocation of NF-κB, but did inhibit reporter gene expression driven by NF-κB.

EVIDENCE FROM ANIMAL STUDIES FOR A ROLE OF FLAVONOIDS IN THE INHIBITION OF ATHEROSCLEROSIS

The apoE$^{-/-}$ mouse has been extensively used as a model to study factors influencing the development of atherosclerosis, including studies on the effects of antioxidant phytochemicals. The flavonoids present in red wine, licorice, and pomegranates, and the purified flavonoids catechin, quercetin, and glabridin have been shown to inhibit fatty streak formation in short-term studies. Although some of this inhibitory effect is thought to be due to the ability of these phytochemicals to inhibit LDL oxidation, other mechanisms cannot be ruled out.

APPROACHES FOR THE IDENTIFICATION OF BIOACTIVE COMPOUNDS, AND THEIR LIMITATIONS

Observational studies can be utilized as a guide for a top-down approach that sequentially studies dietary patterns, food groups, foods, parts of foods, and ultimately single chemicals. Generally, the study of dietary patterns and food groups involves observational and human feeding studies. The study of food components involves human and animal feeding studies. Single potentially bioactive chemicals are studied using in vitro methods, feeding studies, and ultimately clinical trials (see next section). While this approach, or parts of it, has been utilized successfully in studies of diet (4, 18), it has several limitations. Observational studies have the same limitations as described above (see Evidence of Health Effects of Bioactive Compounds section) and when used as a guide for the selection of diets may miss many bioactive compounds. The top-down approach assumes availability of disease-associated endpoints and intermediary markers for feeding studies, and identification of appropriate analytical endpoints for in vitro methods. In many instances, appropriate markers of disease are not available or the marker has not been sufficiently validated as a reliable indicator of disease risk. Only a few intermediary markers have been validated, and interpretation of results often is difficult for other markers. For instance, a bioactive compound may act through the induction

of particular enzymes such as certain detoxification enzymes that cannot be measured in blood samples and feeding studies, and for which appropriate in vitro systems are not available. Another example is the measurement of cell proliferation in studies of colon cancer. While an increase in cell proliferation is demonstrated by in vitro studies and is theoretically involved in colon cancer, the demonstration of an increased risk of colon cancer with an increased level of cell proliferation has been difficult to validate in humans (74). The top-down approach also assumes that single foods will have sufficient amounts of the compound of interest to elicit a measurable and meaningful biological response in feeding studies. It ultimately assumes that a single compound contains the bioactivity of interest, the activity occurs at a cellular level (in vitro studies), and it acts through a single endpoint effect rather than through a combination of several effects. Synergy would be recognized only if biological activity of the single compound occurred in the presence of other necessary potentiator compounds. A large number of candidate compounds may be possible based on the food and/or parts of foods. Feeding studies and selection of these compounds (as well as any potentiator compounds) for further study may be difficult. Thus, this approach may readily identify some single potent bioactive compounds and synergy in some cases, but it may miss many other bioactive compounds or combinations of compounds. Nonetheless, this approach has been successful in the identification of some bioactive compounds (4).

The top-down approach can be complemented by a bottom-up approach, which involves the isolation and identification of compounds, in vitro testing for activity, animal feeding experiments, human feeding experiments, observational studies, and then clinical trials. This approach is described in detail below and reflects approaches often used in drug development. The bottom-up approach has some of the same limitations described for the top-down approach, including a lack of intermediary markers and validation of appropriate endpoints. There is an assumption that the bioactivity of interest resides in a single compound. The bottom-up approach immediately faces the hurdle of selecting compounds from a wide range of candidates and is unlikely to identify synergy between compounds. Nonetheless, the bottom-up approach has been successful in identification of bioactive compounds such as resveratrol (5, 53).

MAKING A CASE FOR STUDYING HEALTH EFFECTS OF BIOACTIVE COMPOUNDS

As previously discussed, atherosclerosis is a complex disease. The recognition that inflammation plays a key role in progression of the disease provides a scientific basis for anti-inflammatory interventions. In addition, understanding that oxidants play a role in the initiation and progression of atherosclerosis provides another target for intervention efforts. That flavonoids confer both anti-inflammatory and antioxidant effects offers promise that they may play an important therapeutic role in protecting against cardiovascular disease, most likely by multiple mechanisms.

To clarify this, it is important to conduct a variety of different studies that employ state-of-the-art methods and endpoint assays. Flavonoids are but one class of bioactive compounds; however, other classes have been identified, and in the future still others will be defined and will require in-depth study. The scientific approach for establishing the biological effects of different bioactive compounds will be quite similar. The following template is a proposed means to study bioactive compounds and their effects on disease markers.

RESEARCH METHODOLOGIES FOR STUDYING THE BIOLOGICAL EFFECTS OF BIOACTIVE COMPOUNDS

Numerous methodological approaches are necessary to understand the biological effects and mechanisms of action of bioactive compounds. As shown in Figure 2, various experimental approaches are used, such as animal or human studies, as well as in vitro studies. These studies provide the requisite means to generate samples for endpoint analyses. Endpoint analyses may be targeted, i.e., a specific disease biomarker such as platelet function, plasma lipids/lipoproteins, selected cell metabolites, mRNA, or proteins. In addition, the recent advent of gene array, proteomic, or metabolomic methodologies permits a global assessment of a treatment effect on a large number of mRNA transcripts, proteins, or metabolites (metabolomics). No longer are we limited to studying the effects of diet on one or two carefully selected candidate endpoints or pathways. The availability of cDNA micro and macro arrays, modern proteomic techniques, and comprehensive cell signaling analysis allows for thorough and simultaneous examination of many

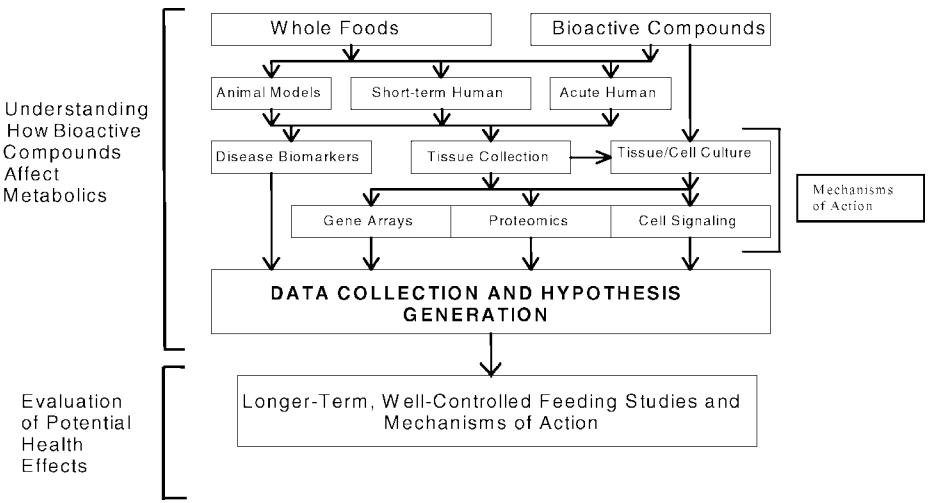

Figure 2 Experimental approach for assessing biological effects of bioactive compounds.

potentially affected metabolic pathways in a single experiment. This global view to investigating the effects of foods and their components on metabolism will be the cornerstone of our approach to identifying future health benefits of the selected foods or bioactive compounds.

An essential first step is to develop a basis for concluding that a compound has a bioactive effect that is important. Typically, this is assessed using in vitro "screens" with cell lines or tissue culture and predetermined endpoint assays. The rationale for this is the cost of isolating or synthesizing large quantities of the compound necessary for feeding studies and the importance of acquiring an understanding of the biology in order to assess biological effects in animals and humans. The screen approach also reduces confounding variables that can be present in feeding studies with animals or human subjects. Establishing a biological effect(s) of a bioactive compound in vitro provides the requisite basis for conducting well-controlled feeding studies, both acute and chronic, in human subjects. Valuable information can be collected from dose-response studies and time-course studies. The dose-response studies are important to provide data about the nature of the dose-response curve. The time-course studies are important to establish whether the effects observed are transient or sustained over the long term, and may provide insight into whether the effects are mediated directly or indirectly. To address this latter issue, thoughtful experimental designs are needed to establish if the effects are direct or mediated by some other signal pathway. The culmination of the experimental approach designed to evaluate the efficacy of a bioactive compound is a major intervention trial that assesses hard endpoints such as clinical events or mortality. A considerable database is necessary to justify a major intervention trial. Thus, various experimental approaches are necessary in building the database about the biology and efficacy of the compound of interest.

MODEL SYSTEMS

Tissue and Cell Culture

Tissue and cell culture provide the simplest system to study the effects of specific bioactive compounds on cell function. The starting material for these studies must include the bioactive compound isolated from food, or synthesized, which is added to the culture medium and conditioned serum. In some cell culture systems it is possible to use defined media (i.e., serum-free) for the study. The advantage of a serum-free culture medium is that cells are only exposed to known constituents. Both short-term (2–4 hour) and long-term (24–48 hour) cultures can be used. This is important to determine the temporal profile of the biological effect of interest. Abundant evidence shows that effects involving changes in gene transcription and protein levels may not be seen in short-term, acute culture periods. Moreover, acute studies are necessary to establish whether short-term, transient responses occur.

Some cell lines that can be considered for use in these studies include models of hepatocytes (HepG2), macrophage/monocytes (THP-1, J774, RAW), fibroblasts

(GM3468), colonocytes (Caco2), adipocytes (3T3-L1), endothelial (HAEC), and skeletal muscle (L6). The cell line to be used depends on the type of endpoint measurements needed. For example, to assess effects on endothelium function, it would be preferable to select an endothelial cell line. In addition, cells can be isolated from tissue collected from animals or humans with defined characteristics (e.g., obesity) or after an intervention (a feeding study with the bioactive compound of interest), and used for primary cell culture. Following culture, cells and media are harvested. Cells can be processed to quantify gene expression, protein abundance, and cell signaling endpoints to establish mechanisms of action. Media from the cell culture can be used for analysis of secreted proteins including hormones and cytokines.

Animal Feeding Studies

Functional whole foods, food fractions, or purified bioactive compounds can be incorporated into semi-purified diets or fed as a supplement at levels intended to provide a maximal response. It is essential that control diets be matched to the test diets in every way, with the exception of the bioactive compound. Control and test diets must be nutritionally adequate. Ideally, the animal model selected should be a good model for human biology. It also is important to understand that there may be strain or breed differences to the diet which necessitate using at least two different strains known to have divergent metabolic, dietary responses. Diets should be fed for an adequate period of time. Pilot studies are necessary to establish the optimal length of the feeding period. Animals are invaluable for mechanistic studies because tissues can be obtained and used to assess relevant endpoint measurements.

Human Feeding Studies: Acute

Functional whole foods, food fractions, or a bioactive compound can be incorporated into a nutritionally adequate meal (i.e., breakfast) or provided as a supplement. By virtue of the acute study design, frequent blood or other samples must be obtained for a period of up to about eight hours. Typically serum/plasma are collected to assay biomarkers of interest, as well as to be used for tissue culture studies. Monocytes can be isolated and subjected to different endpoint analyses. Additional physiological assessments (e.g., endothelial reactivity and blood pressure) may be obtained.

Human Feeding Studies: Chronic

Functional whole foods, food fractions, or a bioactive compound can be incorporated into a nutritionally adequate diet and fed to human subjects. Control diets must be matched to the test diets in every respect, with the exception of the bioactive compound. To the extent possible, when foods or food fractions are used in the test diet, a similar food/food fraction must be replaced in the control diet

(e.g., safflower oil for rice bran oil). Diets should be fed for a sufficient duration to assure that a biological effect is observed, and if so, whether it is sustained. Samples should be obtained, possibly during and clearly at the end of each diet period. Serum/plasma is the most common biological sample obtained, and can be used for biomarker analysis as well as in tissue/cell culture studies. Other biological samples that could be collected include monocyte, muscle, and adipose tissue biopsies. Many other endpoints can be measured, including blood pressure, endothelial health (flow-mediated dilation), heart rate variability, and adipose tissue distribution (by dual energy X-ray absorptiometry).

APPROACHES FOR ENDPOINT ANALYSES

A multitude of options for endpoint analyses are available for assessing the effects of a bioactive compound for in vivo or in vitro experimental approaches. It is evident that the endpoints selected are determined in large part by the hypotheses being tested. Irrespective of the study design, it is prudent to obtain sufficient quantities of a biological sample to be used for the current study as well as for studies that might be done in the future. For example, once C-reactive protein was identified as a strong risk factor for cardiovascular disease and a marker of inflammation (58), many scientists quickly assayed archived samples from previous studies. Given the growing interest in learning more about mechanisms of action, a point of consideration is whether to collect cells (and what type) from animal and human studies that can be used for subsequent genomic and proteomic analyses.

BIOMARKERS ANALYSIS

Following completion of the feeding studies, assessments should be made of disease biomarkers. In animal studies, cumulative food intake, weight gain, adiposity, and organ weights could be obtained. In addition, in vivo endpoints (e.g., flow-mediated dilation, blood pressure, and heart rate variability, turnover/clearance/kinetic studies, isotopic imaging studies, and serum/plasma/tissue analyses) can be quantified. Similar endpoint measurements can be taken in human studies.

Biochemical Analyses

Complementary biochemical and molecular biological approaches are essential to identify metabolic pathways affected by foods and their components. As previously discussed, there are targeted and global approaches for assessing biological effects. The global approach is not restricted to quantifying any single metabolic system or disease endpoint. This approach provides great flexibility and increased likelihood of identifying previously unknown functional properties and functional constituents in the tested foods and their components.

GENE EXPRESSION ANALYSIS The recent introduction of high-density gene expression arrays allows for the simultaneous determination of the expression levels of thousands of genes, including both genes with known functions (named genes) and genes of unknown function (expressed sequence tags, ESTs). For gene expression analysis in animal studies, tissue samples from three to five animals can be pooled for each expression analysis. For an extensive analysis with animal models, different tissues/cells could be collected (e.g., liver, skeletal muscle, adipose, peritoneal macrophages, and intestinal mucosa). This is important to determine what tissue-specific differences exist in expression. For chronic and acute human feeding studies, tissue samples (monocytes, skeletal muscle, adipose) should be run either individually or collectively as pools prepared by combining tissues from two subjects. Expression arrays should be chosen to represent a broad spectrum of genes covering multiple metabolic pathways and diseases. Genes that are significantly overexpressed or underexpressed in response to a treatment can be identified.

PROTEIN ABUNDANCE ANALYSIS In addition to gene expression analysis, it is important to evaluate protein abundance using two-dimensional gel electrophoresis. This is because protein abundance does not always correspond to mRNA levels. Presently, it is possible to reliably quantify the levels of between 1000 and 3000 individual protein spots, depending upon the type of cell or tissue. The relatively low cost of this method allows a greater number of replicate analyses, thereby increasing the power to detect smaller changes in protein levels (differences as small as 1.2- to 1.5-fold).

For protein abundance analysis, a minimum of 10 individual tissue samples should be appropriately extracted and run in duplicate. If necessary, follow-up analyses are conducted with narrower pH ranges and different acrylamide percentages to more closely focus on the proteins of potential interest. Following electrophoresis, gels are silver stained and the individual spots quantified with appropriate software (e.g., PDQuest). Protein spots that differ in abundance can be identified initially by spot matching followed by confirmation with Western blotting with specific antibodies. If the protein remains unidentifiable, preparative two-dimensional gels can be run and the protein spots of interest excised. Following in-gel enzymatic digestion, the peptide mass fingerprints can be determined by matrix-assisted laser desorption time-of-flight mass spectroscopy. The peptide mass fingerprints along with the estimated molecular weight and isoelectric points and obtained partial peptide sequences can be used to interrogate available databases to unequivocally identify known proteins.

CELL SIGNALING ANALYSIS It is evident that metabolic pathways can be profoundly affected by alterations in intracellular signaling achieved through changes in phosphorylation or dephosphorylation of proteins. Previous studies have demonstrated the ability of food components (e.g., the isoflavone genistein) to specifically affect the intracellular signal transduction process. Thus, to obtain a complete picture of

the interaction between food components and metabolic pathways, it is important to analyze changes in the phosphorylation state of cell signaling proteins.

CHARACTERISTICS OF BIOACTIVE COMPOUNDS THAT MAY PREVENT THEIR DETECTION

The effects of bioactive compounds may arise in several ways that may not be identified easily by currently suggested approaches. A false negative result may arise because of the possible dependence of health effects on the simultaneous interaction of multiple components or physiological and cellular effects.

The effect of bioactive compound intakes may not be the result of a single bioactive compound, but may arise from synergy between compounds. In this case, studies of individual compounds would prevent this synergy, mitigate the effect, and possibly prevent identification of the compounds. The health effect would be seen only with consumption of the complete diet or possibly with individual foods. Synergy between components has been suggested for some diets with garlic and other dietary components (2, 3), soybean protein and isoflavones (4), and components of whole grains (33).

Bioactive compounds may have pleiotropic effects that in combination reduce the risk of chronic disease. For instance, a bioactive compound may cause a small reduction in blood cholesterol, blood pressure, arterial dimensions, and oxidative stress (4). The combined effect is a reduction in the risk of coronary heart disease, but examination of any individual endpoint may not produce a significant reduction in risk. Identification of the health effect may necessitate the use of multiple endpoints and new statistical approaches for the analysis of multicomponent data.

Bioactive compounds may influence global effects of diet, such as its effect on blood glucose levels in diabetics and hypertension (35, 43, 61, 61a). In this case, the overall characteristics of the diet are important, such as its glycemic load and antioxidant content. This background effect may influence whether an effect is seen for the bioactive compound. For example, these characteristics may create a stress that is mitigated by the bioactive compound. Another possibility is that an understanding of a bioactive compound's absorption and metabolism may be critical. It is possible that the bioactive component of a diet is not the compound in the diet, but a metabolite of the compound, and diet-induced changes in absorption or metabolism may influence dramatically the formation of the metabolite and the effect of the diet. Thus, a comprehensive approach may need the integration of information on the absorption and metabolism of compounds in the diet as well as identification of parent compounds and their metabolites.

The effects of bioactive agents may be evident only in certain populations. One example in which this may occur is in studies of the microvascular complications of diabetes. Bioactive agents may influence the occurrence of these complications, which include retinopathy and nephropathy. However, these complications can be largely avoided by persons with diabetes with the maintenance of normal fasting

blood glucose levels of 99 mg/dl or lower (50a). Thus, studies of persons with diabetes with well-controlled blood glucose are unlikely to find any effect of the bioactive agents. The stress of an elevated blood glucose level may be essential for identification of the effect of the bioactive compound. Testing of bioactive compounds in this setting would require study designs that evaluate the control of blood glucose levels. The study design for such evaluations would need to address various ethical considerations.

Some effects may require long periods of exposure to bioactive compounds. Chronic diseases develop over many years and are the result of numerous pathogenic processes. Reduction, inhibition, or reversal of these processes can take long periods. Two examples are the reversal of renal basement membrane thickening and atherosclerotic plaque. The control of blood glucose concentration by pancreas transplantation can result in the reversal of basement membrane thickening, but it requires 10 years of essentially normal blood glucose levels (16, 17). In another example, atherosclerotic plaque regressed with intervention by apheresis over a one-year period (46) and garlic over a four-year period (40). Given these possibilities, innovative experimental designs and analysis procedures may be necessary for the identification of certain bioactive compounds.

HYPOTHESIS GENERATION AND EFFICACY TESTING

Sufficient information is important to generate a testable hypothesis. As shown in Figure 2, much information is needed from animal, human, and in vitro studies before large-scale efficacy trials can be conducted. In designing a large-scale efficacy trial, the following considerations are important: (*a*) the amount of food or component required to detect the hypothesized benefit, (*b*) the length of time required to achieve the predicted effect, (*c*) the selection of the most appropriate control food or substance, (*d*) the number of subjects required (i.e., statistical power) to observe statistically significant effects, (*e*) the potential for any carryover effects dictating either a crossover or parallel arm design, (*f*) the sampling frequency to obtain stable endpoint values, and (*g*) the types of samples/measurements required to test the hypothesis.

LINKING BIOACTIVE COMPOUNDS TO BIOMARKERS FOR DISEASE

An important area of development is the identification of intermediary markers for chronic diseases. Chronic disease and the associated clinical events progress over many years. Thus, studies using these "hard" endpoints must involve large numbers of individuals or very long periods, neither of which is suitable for studies assessing causal effects of bioactive compounds. Thus, early indicators of disease are essential for reductions in the timeframe of experiments and number of

subjects necessary for evaluation of the associations between bioactive components and disease. For bioactive compounds to be valuable biomarkers they should be predictive of the disease and its course over time. Implicit is the need for sensitive and reliable assays of the bioactive compounds and new disease biomarkers. As discussed previously, identification of bioactive compounds involves detailed examination of changes in blood composition and gene expression, the cellular environment, following exposure to specific dietary patterns and foods consumed by humans. These studies would capture many of the effects of diet and provide a benchmark for associations between diet and disease.

A total diet approach targeting a food or foods of interest combined with detailed studies of metabolism is one effective means to identify possible bioactive compounds. For example, one health effect of fruit and vegetable intake is a lower risk of coronary heart disease (CHD). Fruits and vegetables contain numerous phytochemicals that may influence the risk of CHD through a wide variety of mechanisms. A study of flavonoid metabolites found that several were increased in subjects given high intakes of fruits and vegetables. These included 3,4-dihydroxyphenylacetic acid, metahydroxyphenylacetic acid, and hippuric and homovanillic acid. These metabolites were screened for an effect on platelet activity, which is associated with the risk of CHD. One of the metabolites, 3,4-dihydroxyphenylacetic acid, significantly reduced platelet activity as shown on Figure 3. These finding provide a basis for the identification of components of a fruit and vegetable diet that

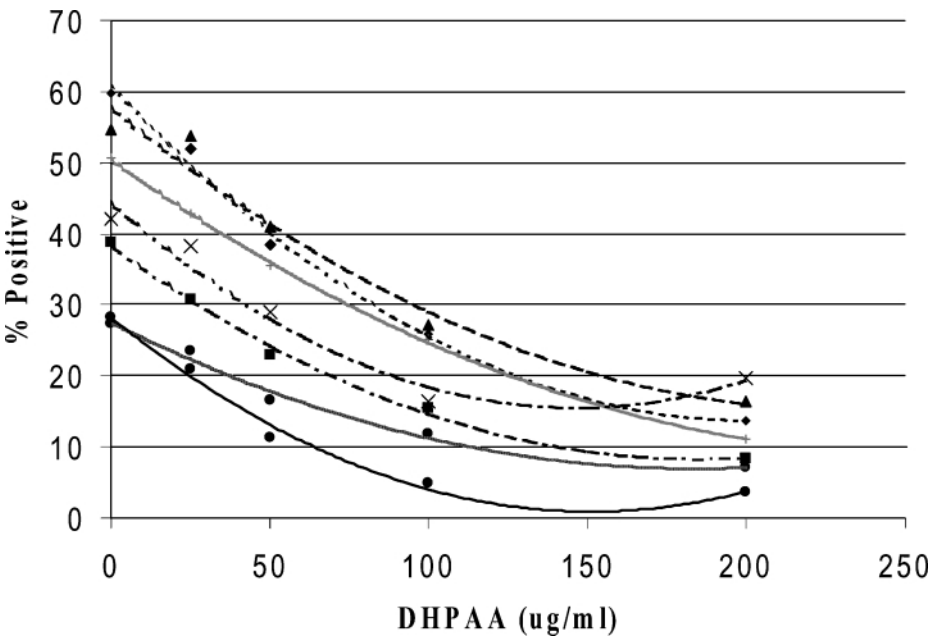

Figure 3 P-Selectin expression with 3,4-dihydroxyphenylacetic acid incubation (n = 7).

contribute to the formation of 3,4-dihydroxyphenylacetic acid and for further studies on the health effects of these foods and levels of 3,4-dihydroxyphenylacetic acid.

DEVELOPMENT AND VALIDATION OF DIET COMPOSITION

The development of menus containing a specific amount of a bioactive compound(s) is critical to the outcome of any study and poses several challenges beyond those associated with planning diets to assess nutrient effects. Excellent detailed discussions of the many aspects of designing research diets, their validation, and the complexities of the multicenter approach have been published (9, 54, 78). This section highlights the unique challenges of working with dietary bioactive compounds beyond the well-recognized nutrients.

CALCULATION OF MENU CONTENT The development of research diets is dependent on the design of the study. Each menu must first be designed, calculated, prepared, and then chemically analyzed to ensure that the study design goals are met relative to the levels of the bioactive compound(s) of interest and that intake is adequate or at the desired level for all nutrients (11). Several commercial databases are available and many research facilities have developed in-house databases for the calculation of nutrient content of menus and diets. All nutrient databases developed for use in the United States derive from information maintained in the National Nutrient Data Bank by the Nutrient Data Laboratory of the Agricultural Research Service, U.S. Department of Agriculture (USDA), which is available on a variety of media (26). However, data regarding the nutrients and bioactive compounds for specialty foods may be added at the local level to make the database more complete for detailed, focused investigations.

Calculation of the dietary content of bioactive component(s) becomes problematic in that USDA databases contain food content data for only a few families of these compounds (Table 1). To meet the need for these values in centralized databases, data for a few families of bioactive compounds have been assembled by research groups (Table 1). For many bioactive compound groups, e.g., phenolic acids (47), centralized or research databases have not been developed and investigators and their staffs must turn to the literature and/or the laboratory for values. It should be noted that due to the paucity of data on the food content of bioactive compounds, values are incorporated into databases from a wide variety of sources (27). Often these sources are from countries in which the foods are grown more widely than in the United States. As a result, the content of bioactive compounds of foods in a research menu may be quite different from the levels indicated in a database or research paper. Thus, calculation of the menu content of a bioactive compound(s) should be considered preliminary and must be chemically analyzed to verify accurate levels.

CHEMICAL ANALYSIS OF FOODS AND MENUS Phillips & Stewart have discussed important concepts and details of validating diet composition by chemical analysis (54). It must be emphasized that chemical analyses of menus are necessary for their initial validation as well as for analyses during the study to detect drift in nutrient or bioactive component content.

Analysis of food components other than "traditional" nutrients again presents unique challenges. Primary among these is identification of a laboratory that has the capability to analyze foods and menus for the bioactive component(s) of interest. Most commercial laboratories are set up to routinely analyze only those nutrients for which values are required on the food label. Thus, of the families of bioactive compounds tabulated in Table 1, only those carotenoids that have vitamin A activity might be candidates for analysis at most commercial laboratories. However, some laboratories, particularly those that are more research-oriented, have become interested in nonnutrient, bioactive food components and have developed relevant analytical systems to quantify a few. Each has its own specialties, and investigators must evaluate laboratories to assess their assay capabilities, including precision and variability.

Another excellent source of expertise for menu analysis is government, research, and food company laboratories whose mission or focus is the development of analytical procedures for specific bioactive compounds and the subsequent analysis of foods. The analytical expertise of each laboratory is best obtained through review of recent scientific publications and discussions with respective scientists. While these laboratories usually are not in the business of analyzing large numbers of foods and menus beyond their own projects, like most research situations collaborations and partnering arrangements often can be developed. Short of developing a working relationship with an analytical laboratory, the investigator must establish the required analytical capability in-house.

ANALYTICAL QUALITY The analysis of bioactive compounds presents interesting analytical challenges due to their similarity of structures within, and often between, families, conjugation with a wide variety of components, generally low concentrations, and "interfering backgrounds" in extracts of many foods. A form of chromatography is usually employed that has adequate resolution and other characteristics to separate the bioactive compound(s) of interest from each other and from interfering compounds of similar structure. With such systems, individual molecules usually are identified by retention time based on the chromatography of pure standards. However, even in the case of traditional nutrients, individual forms have been misidentified using only retention time, which has led the concept of positive identification (14). A similar approach should be used for the analysis of all bioactive compounds, which takes advantage of unique molecular characteristics of the molecule. Typical qualitative data used for such identification include ultraviolet, visible, fluorescent, or mass spectra as well as oxidation/reduction profiles of the type obtained with modern series coulometric detectors. Examination of qualitative data in combination with retention time from a stable chromatographic

system provides the assurance required for positive identification of the bioactive compound of interest.

The requirement for qualitative analytical information often conflicts with accurate and sensitive analyses, a condition required especially for menus and mixed diets (due to dilution with liquids and foods containing low concentrations of bioactive compounds). Currently chromatographic instrumentation is less sensitive when absorption or fluorescent spectra are recorded than when data for a single wavelength, be it absorption or fluorescence, are collected (14). Thus, for each bioactive compound, the analyst must be aware of the general sensitivity of the instrumentation and the level in the food or menu so that the appropriate aliquot can be taken at the outset of the analysis to provide reliable data. In the case of mass spectra, although these outputs provide a large amount of qualitative data about the analyte, the accuracy of the data is often poor. This is due primarily to the variable interference of the matrix (all else that is extracted from the food along with the bioactive compound) from food to food with the ionization process of the analyte. There are two ways to circumvent this problem: (*a*) install an additional, nondestructive quantitative detector (ultraviolet, visible, or fluorescent spectrophotometer) between the chromatographic column and the mass spectrometer, or (*b*) employ isotope dilution techniques with the mass spectrometer as quantitative detector. The latter requires the availability of heavy isotope labeled molecules, e.g., ^{13}C, identical to the bioactive compound(s) of interest (67). Investigators should request as much qualitative information as possible from the analytical laboratory to provide assurance of accurate identification of the compound(s) of interest in the investigation.

VALIDATION OF ANALYTICAL PROCEDURES Validating a procedure means confirming that the entire assay process (extraction, sample purification, and analyte quantification) measures the concentration of the bioactive compound with acceptable accuracy and precision, in the specific foods and menus to be tested at a given laboratory (54). Simply following a standard written method does not guarantee that a laboratory or analyst will produce acceptable results. Accuracy or bias is tested against an appropriate reference material that has been certified by a metrology agency of a country or against a similar material for which reliable analytical data are available. Precision is determined from a series of analyses of the same food or menu over several days. From these data, within-day variability as well as between-day variability can be calculated and evaluated. Regardless of whether a commercial or research laboratory is engaged as the analytical facility for bioactive compounds, appropriate validation data should be requested to assure the investigator that appropriate analytical procedures are in place.

ANALYTICAL QUALITY CONTROL Analytical quality control is the implementation of a system to ensure that the accuracy and precision of chemical measurements meet the requirements of the end use of the data (54). This step is particularly important if analyses of foods, menus, and diets are conducted over a relatively

long period, i.e., several weeks, months, or even years. Even if assays are conducted "out-of-house," the investigator must implement quality control procedures, and in a sense they are more important without knowledge of the entire analysis system.

The four basic components of an analytical quality control system are: appropriate control samples, quality control charts, comprehensive documentation, and selection of a laboratory that practices Good Laboratory Practice Standards (20), although many research laboratories are not required to follow these standards to the letter of the requirement. All of these components have been discussed in detail (see 54 and references therein). A quality control material (QCM) is employed to ensure the absence of deviations in the routine measurement process. Briefly, an appropriate QCM is a homogeneous composite of food(s) or menus similar in type and having bioactive component concentrations comparable to those in samples to be assayed. Additionally, the bioactive components in the QCM should be well characterized and known to be stable for the duration of the study (55).

An important visual document of the quality control process is the quality control chart (QC chart), which is a plot of the analytical values for QCM samples versus assay date. Reference data (lines) added to a QC chart include the mean value of the QCM obtained from early analyses of at least 10 samples using the same analytical procedure as well as control limits (usually ± 3 standard deviation units from the mean value). Data from QCM samples embedded in the analytical sample set (several small groups of QCMs or 1 QCM per 10–15 analytical samples) are then used to populate the QC chart and evaluate analytical quality. If all data from QCM samples fall within the control limits and appear to be normally distributed around the mean, then the investigator can have reasonable assurance that the analyses are well within control. However, if data from one or more QCM samples is outside the limits of the QC chart and reanalysis yields similar results, analyses should be stopped until the reason for the deviation can be ascertained. Phillips & Stewart (54) have discussed additional benefits of QC charts.

Extensive documentation of food and menu samples is essential to provide an audit trail linking reported analytical values for a sample to details such as sample description, preparation and shipment, assay methodology, and associated control sample data. Often careful examination of these observations leads to the cause of an aberrant analytical value.

Multicenter Studies

Occasionally dietary studies are conducted simultaneously in several centers so that the size and heterogeneity of the study population can be increased. Examples of such investigations are the Dietary Approaches to Stop Hypertension (DASH) and Dietary Effects on Lipoproteins and Thrombogenic Activity (DELTA) studies (12, 51). These types of studies greatly increase the complexity of menu design and validation as well as ongoing menu monitoring, and require considerable resources to assure that experimental diets meet the objectives of the study at all research centers over the duration of the study (12).

MODIFYING NUTRIENT PROFILE OF FOOD THROUGH BIOTECHNOLOGY: IMPACT ON ASSESSING BIOLOGICAL EFFECTS OF BIOACTIVE COMPOUNDS

Advances in biotechnology have lead to the development of many new processes and products that have benefited agriculture and society. The benefits of using these new biotechnologies to genetically enhance crops and animals used for food consumption or for health benefits have been reviewed extensively (10, 15, 38, 42). Biotechnologies that enhance productivity and productive efficiency of plants and animals have been developed and approved for commercial use in many countries. With the advent of plant biotechnologies, it is possible to selectively regulate the production of proteins, carbohydrates, lipids, or micronutrients in many species of agronomic interest (25, 64). In some instances the objective is to increase a nutrient of interest, and in others, the objective is to reduce specific nutrients (i.e., saturated and trans fatty acids). The advances that have been made through biotechnology research have resulted in the development of differentiated food crops that have more desirable nutrient profiles for human health.

The emergence of the modern era of biotechnology has provided unprecedented ways to fortify foods with targeted nutrients. For scientists conducting well-controlled feeding studies the increase in genetically modified food crops that have an altered nutrient composition poses challenges. One problem relates to the introduction of the genetically modified food into the food chain before information about nutrient content of the food is added to the benchmark nutrient databases. In addition, co-mingling of foods in the food system occurs, making it difficult to determine whether the food consumed is the same genotype or a mix of different genotypes (and nutrient profiles). These potential concerns are resolved by chemical analysis of the control and test diets before the feeding study is conducted. A chemical analysis of the test diets/food(s) provides information only about the analytes assessed, and the genetically modified food may be altered in ways that are not quantified due to a lack of awareness of these potentially bioactive compounds.

SUMMARY

Research identifying bioactive compounds and then establishing whether they have a beneficial health effect is gaining momentum. It is not unreasonable to speculate that important findings will be made that lead to new ways to exploit bioactive compound chemistry for the betterment of human health. To do this will require research that integrates various scientific disciplines, culminating in well-designed large intervention trials with the compound(s) of interest. As discussed herein, there are model systems that can be used for discovery of bioactive compounds of potential interest. These include assessing bioactive compounds, foods and food components, and whole diets in tissue/cell culture, animal feeding studies,

or human feeding studies, both acute and chronic. In conjunction with these model systems, important information can be gleaned from a combination of top-down approaches (i.e., epidemiologic and observational studies) and bottom-up approaches (in vitro experiments) that provide clarity about the biological effects of the multitude of potential bioactive compounds that likely exist in food. The achievable but daunting task is to identify a bioactive compound that confers beneficial health effects and to gain an understanding of its mechanisms of action. With respect to the latter point, there will be compounds identified for which full expression of biological activity will be dependent on another molecule(s) or metabolic pathway (both dependent and independent of the bioactive compound of interest). Collectively, how molecules and pathways interact will determine whether there is any synergistic effect observed. Identification of important bioactive compounds will result in the use of contemporary biotechnology to modify foodstuffs to be fortified in the compound(s) of interest. The resulting outcome will be the development of foods and food components that will have marked beneficial effects on human health.

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