Biomarker Research in Neurotoxicology: The Role of Mechanistic Studies to Bridge the Gap between the Laboratory and Epidemiological Investigations

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There is an increasing interest in the development and validation of biomarkers for use in biochemical/molecular epidemiological studies. Though the area of neurotoxicology has received much attention in the past several years, it still lags behind with regard to the development of biomarkers, particularly those of health effects and susceptibility. This review discusses several aspects of biomarker research as it relates to neurotoxic compounds and focuses on selected agents (organophosphorus insecticides, styrene, *n*-hexane, carbon disulfide, acrylamide), which have been the subject of a number of investigations in animals and humans. While traditional biomonitoring approaches and novel techniques (e.g., hemoglobin adducts) provide several measurements for monitoring exposure to neurotoxic chemicals, potential markers of genetic susceptibility have been seldom investigated in a neurotoxicology context. Furthermore, the complexity of the nervous system, together with the multiplicity of end points and the limited knowledge of the exact mechanism(s) of action of neurotoxicants, has led to only limited advancements in the development of biomarkers for neurotoxic effects. Significant progress in this area will depend upon an increased understanding of the cellular, biochemical, and molecular targets directly involved in neurotoxicity. — Environ Health Perspect 104(Suppl 1):55–67 (1996)

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Introduction

The field of biomarkers has been the object of increased interest in the past few years, as evidenced by the large number of publications, workshops, and symposia devoted to this topic (*1–8*). The term biomarker may be overused, as it is utilized in reference to different end points of toxicity in animals or humans. For example, the presence of chloracne may be considered a biomarker of exposure to and a health effect of dioxinlike compounds, and behavioral alterations in children exposed to lead may be considered a biomarker of its subtle neurotoxic effects (*9*). Additionally, a number of biochemical and morphological alterations in tissues of animals exposed to toxicants can be considered biomarkers of target organ toxicities. For the purpose of this review, the term biomarker is used to mean biological/biochemical/molecular markers, which can be measured by chemical, biochemical, or molecular biological techniques. Furthermore, the discussion will focus on biomarkers that can be measured in humans and must thus be present in easily and ethically obtainable tissues such as blood or urine.

Biomarkers are usually divided in three categories: biomarkers of exposure, of effect, and of susceptibility (*1*). Additional subdivisions and overlaps between different types of biomarkers should also be considered. For example, within biomarkers of exposure, one could consider either unchanged or metabolized exogenous agents or indicators of biological effective dose (*10,11*). Certain biomarkers of exposure, e.g., DNA adducts, may be also considered as biomarker of effect.

This review will focus on biomarkers for neurotoxicity. Several concepts related to the development, validation, application, and use of biomarkers will be reviewed only briefly, as general considerations reported in several other publications would also apply to biomarkers for neurotoxicity.

Biomarkers in Neurotoxicology

Concern for the acute and long-term effects of chemicals on the nervous system has been growing in the past several years (*12,13*). The discipline of neurotoxicology, which bridges neurosciences and toxicology, plays a very important role in the increasing efforts aimed at understanding how the brain and the nervous system work, how to intervene to prevent damage and restore function, and how environmental factors may play a role in central nervous system disorders. Since the nervous system controls movement, vision, hearing, speech, thought, emotions, heart function, respiration, and many other physiological functions, it is particularly vulnerable to toxic substances, and even minor changes in its structure and function may have profound neurobiological and behavioral consequences.

Neurotoxicity is commonly defined as any permanent or reversible adverse effect on the structure or function of the central and/or peripheral nervous system by a biological, chemical, or physical agent. With regard to biomarkers, the area of neurotoxicity appears to have been progressing more slowly than other fields (*14*). Indeed, this topic has been addressed by only a few reviews in the last decade

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Abbreviations used: ALAD, δ-aminolevulinic acid dehydratase; ZZP, zinc protoporphyrin; GFAP, glial fibrillary acidic protein; CK-BB, creatine kinase isoenzyme BB; CSF, cerebrospinal fluid; MBP, myelin basic protein; CYP, cytochrome P450; GST, glutathione transferase; NAT, *N*-acetyltransferase; MAO B, monoamine oxidase B; AChE, acetylcholinesterase; OPIDP, organophosphate induced delayed polyneuropathy; NTP, neuropathy target esterase; DBH, dopamine-β-hydroxylase; CEC, *S*-(2-carboxyethyl) cysteine; Hb, hemoglobin.

(*13–20*) compared to the large number of publications devoted, for example, to biomarkers related to chemical carcinogenesis. An analysis of the literature, however, suggests that such apparent lack of progress pertains primarily to the area of biomarkers of effects—rather than to biomarkers of exposure—where biological indicators for a large number of neurotoxicants exist. The complexity of the nervous system and its distinctive peculiarities, together with problems associated with the multiplicity of manifestations of neurotoxic effects and the determination of the precise targets for neurotoxicants, are certainly responsible for this limited advancement. With regard to biomarkers of susceptibility, the paucity of available examples may be due to the limited attention that these have received within neurotoxicology. However, as most of such markers relate to enzymes involved in xenobiotic metabolism, it is plausible that they may play a role in susceptibility to several neurotoxicants. In this brief review, I will first analyze biomarkers of exposure, effect, and susceptibility as they may apply to neurotoxic compounds. This will be followed by a more detailed discussion of a selected number of compounds for which research has been active in the past few years as examples of possible research approaches in this area.

Biomarkers of Exposure

It has been stated that "an ideal biomarker of exposure is chemical-specific, detectable in trace quantities, available by noninvasive techniques, inexpensive to assay and quantitatively relatable to prior exposures" (*5*). This general concept certainly applies also to biomarkers of exposure for neurotoxicants. Traditional biomonitoring for exposure to neurotoxic chemicals has relied on chemical measurements of the compound of interest and its metabolites in biological fluids such as blood or urine or in other accessible tissues such as hair or dentine pulp. These measurements are still of great value and, in many instances, are still the best or only valid and reliable exposure markers. Examples of these types of biomarkers abound and can be found in the area of metals, pesticides, and solvents, which represent the three major classes of chemicals that include several neurotoxic compounds. For metals, blood levels or levels in urine are commonly used. In some cases, measurements of metal concentration in dentine pulp (e.g., lead) or hair (e.g., mercury or arsenic) have proven to be useful because they reflect prior and/or cumulative exposure rather than recent exposures (*21*). Exposure to neurotoxic solvents is usually monitored by measuring their concentration in blood or in breath or by measuring levels of metabolites in urine. Urine metabolites are also useful for assessing exposure to pesticides. For compounds that are lipophilic and tend to accumulate in fat tissue (e.g., solvents and organochlorine pesticides), fat biopsy is a way to assess the body burden due to prior or prolonged exposure.

Binding to macromolecules has proven useful in monitoring exposure to toxic and particularly genotoxic compounds because it reflects the dose of a certain agent or its metabolites that escapes detoxification and reaches its target protein or DNA (*22,23*). Recent approaches of this strategy to neurotoxic compounds have occurred with *n*-hexane, carbon disulfide, and acrylamide and are discussed in following sections. As red blood cells are long-lived (approximately 4 months in humans), binding to hemoglobin is considered a good biomarker to measure cumulative internal dose due to repeated exposures. Adducts to albumin can also be measured. Albumin has a shorter lifetime in blood (20 to 25 days), and these measurements will thus reflect more recent exposure than hemoglobin adducts. One advantage of albumin adducts is that potential active metabolites can interact with this protein directly upon their release into the blood stream without having to penetrate a cell membrane (*5*). Thus, albumin adducts may offer a better, more sensitive marker for detecting reactive metabolites in blood. On the other hand, if adducts are meant to reflect the levels of neurotoxicant at the target site, then hemoglobin adducts would be a more precise biomarker of target tissue dose. Ideally, both measurements could be carried out and compared; however, no examples are available for neurotoxic compounds. The limitation of adduct measurements as biomarkers of exposure lies in the fact that their measurement is often difficult and time consuming (e.g., when gas chromatography–mass spectrometry is employed) and that they are limited to compounds or their metabolites capable of forming covalent bonds with proteins. Nevertheless, an additional positive factor of macromolecule adducts measurement is that they may be also considered biomarkers of effect when a similar chemical step is involved in the pathogenesis of neurotoxicity, as shown below for *n*-hexane.

A number of biochemical measurements relate to biological effects of neurotoxic chemicals. However, as they do not directly relate to neurotoxicity, they may be considered biomarkers of exposure rather than biomarkers of effect. In the case of lead, for example, erythrocyte δ-aminolevulinic acid dehydratase (ALAD), which is inhibited by this metal, is a widely used biomarker; however, because of wide interindividual variability, it may not be well suited for lead exposure at or below lead levels of 10 μg/dl, which have been associated with behavioral dysfunction (*24,25*). Elevated zinc protoporphyrin (ZZP) or the ZZP/hemoglobin ratio is also well correlated with blood lead level but only at concentrations higher than 40 μg/dl (*26*). Caution should be exerted when using these biomarkers, as their alterations may be associated with the nutritional state of the organism, e.g., iron deficiency (*27*). The interaction of neurotoxic metals, particularly lead and mercury, with the heme biosynthetic pathway has also been exploited to measure concentrations of porphyrins in urine (*26*). Since different metals inhibit this pathway at different steps, it is possible to fingerprint exposure to a specific metal based on the urinary porphyrin profile (*26,28,29*). These measurements reflect biological effects of metals but are not related to their known mechanisms of neurotoxicity. Nevertheless, they represent additional means to assess exposure to neurotoxic metals. For example, urinary porphyrin changes in dentists exposed to low-level mercury vapors have been found to be a useful measure of cumulative effects of mercury on specific tests of neurobehavioral function (*30,31*).

Biomarkers of Effect

Biomarkers of effect should reflect early biochemical modifications that precede structural or functional damage. Thus, knowledge of the mechanism(s) that lead to ultimate toxicity is necessary or at least extremely important to develop specific and useful biomarkers. Such markers should identify early and reversible biochemical events that may also be predictive of later responses (*20*). Unfortunately, the exact mechanism of action for most neurotoxic chemicals is still unknown, a major factor in the slow progress of biomarker research in this area. Furthermore, finding sensitive and specific surrogate markers for the central and peripheral nervous system in readily accessible tissues can be problematic. Because of the complexity of the nervous system and the diversity of manifestations of neurotoxicity, together with the multiplicity of cellular and biochemical targets

for many chemicals, it is highly unlikely that generic markers for neurotoxicity will be developed. The problem is therefore different from that encountered with genotoxic compounds in which, for example, changes in sister chromatide exchange in circulating lymphocytes may reflect a significant biological effect common to many chemicals. It is therefore a situation not dissimilar from that of the development of *in vitro* assays for neurotoxicants, where it is unlikely that a test such as the Ames test will ever exist. Despite these obvious limitations, different strategies have been employed over the years in the attempt to develop peripheral biochemical indicators of neurotoxicity.

One area of investigation that has received some attention is that of neurotransmission. Several chemicals affect various steps of neurotransmission, including neurotransmitter metabolism, receptor interactions, second messenger systems, or other relevant enzymes (e.g., ATPase) (*14,32,33*). As changes due to neurotoxicant exposure cannot be measured in target tissue, suitable peripheral cell systems must be identified that can mirror identical neurochemical parameters in the nervous system. Cell types that have been used include platelets, erythrocytes, lymphocytes, and fibroblasts (*4,34*), and this strategy has found applications in the field of biological psychiatry and asthma research (*15*). The oldest and probably still the best example of the application of such strategy to neurotoxic compounds is represented by the measurement of red blood cell acetylcholinesterase following exposure to organophosphorus insecticides. This and other examples of this approach for some neurotoxic chemicals are discussed in following sections. The development of such markers should follow the usual steps of characterization and validation. It is necessary to determine whether the particular enzyme or receptor studied in blood cells is indeed the same entity present in the nervous system and whether it is similarly affected *in vitro* by a neurotoxicant. Animal studies should then be carried out to investigate whether a good correlation exists between changes in the peripheral markers and those observed in the nervous system; dose–response and time–course studies should be included to assess sensitivity of measurements, and the duration of the observed changes. Pilot studies in humans should determine interindividual variations as well as sensitivity upon exposure to occupationally and environmentally relevant concentrations. In addition to this background work, one should also consider limitations and pitfalls of this approach. For example, does the change in a neurotransmitter metabolite reflect a primary effect in the central or peripheral nervous system? What other endogenous or exogenous agents (hormones, drugs), pathological conditions, or genetic makeup can affect the measured parameters? Would falsely positive results be obtained for compounds that do not cross the blood–brain barrier?

Additional approaches to develop biomarkers for neurotoxicity have recently been proposed. Animal studies have shown that levels of the astrocyte-specific glial fibrillary acidic protein (GFAP) increase following neuronal damage, and this biochemical measurement has acquired notable importance as an indicator of neurotoxicity (*35*). Though questions still remain on the application of these measurements to humans, it has recently been reported that blood GFAP antibody levels are significantly elevated in workers exposed to lead (*36*). Still, it is unclear whether this finding is related to a damage of the blood–brain barrier rather than a biomarker of neuronal damage. Indeed, levels of another protein, creatine kinase isoenzyme BB (CK-BB) were found to be increased in the blood of boxers and head trauma patients (*37,38*). Since levels of CK-BB are very low in control individuals, this increase may indicate disruption of the blood–brain barrier. The cerebrospinal fluid (CSF) is another compartment that may be accessible in humans. A very recent study has reported the appearance of myelin basic protein (MBP) in CSF of rats following intracerebellar injection of lysolecithin (*39*). As this treatment causes extensive demyelination, the increase of MBP in CSF, which lasted for about 4 days, may represent a useful marker for this effect.

Biomarkers of Susceptibility

Though the importance of genetic factors in the response to neurotoxic chemicals has often been recognized, it has not received much attention in the design of neurotoxicological studies (*40*). Most animal studies use only a single strain of mice or rats so that genetic variations in response are not noted; however, several studies have shown strain differences among laboratory animals in response to a wide range of xenobiotics including neurotoxic agents. For example, strain differences in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in mice have been reported and have been associated with differences in melanin binding or monoamine oxidase activity (*40,41*). Genetic influences in the developmental neurotoxicity of alcohol are also apparent (*42*). In humans, interindividual variations in response to drugs and other xenobiotics are widely observed. The studies of such genetic variability in drug response have been carried out for decades in the field of pharmacogenetics, while applications to environmental chemicals in the field of ecogenetics have been investigated in the past 15 years (*43–45*).

Genetic polymorphisms have been identified for a number of enzymes involved in xenobiotic metabolism, in particular several members of the cytochrome P450 (CYP), glutathione transferase (GST), and *N*-acetyltransferase (NAT) families [see recent reviews (*46,47*)]. A large number of studies have revealed associations between a certain genotype and increased risk for smoking- or other xenobiotic-related cancers. For example, mutants of CYP1A1 and GSTM1 have been associated with an increased incidence of lung cancer, and slow acetylators (NAT 2 mutants) have an increased risk for bladder cancer. Presumably, these individuals have an increased capacity of bioactivating and a reduced capacity of detoxifying polycyclic aromatic hydrocarbons, or they have a decreased ability to detoxify arylamines. Genetic polymorphisms have also been identified for other enzymes involved in xenobiotic metabolism, such as epoxide hydrolase, alcohol and aldehyde dehydrogenases, various esterases, and methyltransferases (*46*).

Organic neurotoxic compounds can be bioactivated or detoxified by these same enzymes, and genetic polymorphism could certainly play a role in differential sensitivity to their effects on the central and peripheral nervous systems. With very few exceptions, biomarkers of susceptibility have not been investigated with regard to neurotoxicants. Yet, these may be of great importance where low exposures leading to subtle behavioral effects are investigated. Furthermore, in addition to their major hepatic localization, several of these enzymes are also expressed in the nervous system (*48*) where they may contribute to *in situ* activation or detoxication of neurotoxicants.

Some research on genetic polymorphism has been carried out in the context of studies on the role of environmental factors in the etiology of neurodegenerative diseases such as Parkinson's disease. The hypothesis underlying these studies is that

genetically determined metabolic differences may contribute to an increased risk for Parkinson's disease as a result of exposure to still unidentified environmental neurotoxic agents. Since MPTP, a toxicant known to cause Parkinson's-like symptoms, needs to be bioactivated by monoamine oxidase B (MAO B), a few studies have sought to determine whether polymorphisms of MAO B (*49*) are involved in Parkinson's disease. Kurth et al. (*50*) identified a single-stranded conformational polymorphism in intron 13 of the MAO B gene and found that one allele occurred with a significantly higher frequency in Parkinson's patients than in controls. This finding, however, was not replicated in another study (*51*). A decade ago it was suggested that impaired debrisoquine hydroxylation (which is due to CYP2D6) was a genetic susceptibility factor for Parkinson's disease (*52*), and this has been confirmed in several, though not all, studies (*53–55*). Though the Parkinson's-causing neurotoxicants that may be metabolized by CP2D6 have not been identified yet, some animal evidence seems to support the hypothesis that debrisoquine-poor metabolizers are at risk for Parkinson's disease. For example, the metabolism of tetraisoquinoline, a possible parkinsonism-inducing substance, is significantly lower in Dark Agouti rats (an animal model of poor debrisoquine metabolizers) than in controls (*56*). Additionally nicotine, which has been consistantly found to be a protective factor in Parkinson's disease, has been shown to selectively induce cytochrome P450s in rat brain without affecting their expression in liver (*57*). Clearly, additonal hypothesis-driven mechanistic investigations will strenghten the observations made in epidemiological studies with regard to the role of these and other polymorphisms in Parkinson's disease and other neurodegenerative disorders.

Genetic polymorphisms in enzymes not associated with xenobiotic metabolism but involved in the metabolism of endogenous neurotransmitters (e.g., catechol-*o*-methyltransferase) may also affect the response to certain neurotoxicants. Similarly, genetic variations in receptors or other enzymes involved in cellular functions, including repair mechanisms, may be relevant to fully predict and assess neurotoxic outcome. Although information in these areas is still scant, it will certainly increase as the knowledge of the human genome progresses and it will certainly offer new tools for investigation.

Organophosphorus Insecticides

Organophosphorus compounds are still among the most widely used insecticides. Organophosphates were developed in the 1940s; their mechanism of action was readily identified as inhibition of acetylcholinesterase (AChE), which leads to accumulation of acetylcholine at cholinergic synapses with an ensuing cholinergic crisis (*58*). Because of the role of acetylcholine as a neurotransmitter in both the central and peripheral nervous systems, AChE is widely distributed throughout the body, including blood cells such as erythrocytes and lymphocytes. However, the physiological role of acetylcholinesterase in these peripheral cells, which are devoid of synaptic contacts, as well as of pseudocholinesterase in plasma, have not been elucidated. Nevertheless, because inhibition of these enzymes in blood also occurs, measurement of their activities has been extensively used as a biomarker of exposure to and effect of organophosphates (*59,60*). When used as a marker of exposure in population studies, the issue of interpersonal variability should be carefully considered. When possible, baseline values should be obtained for each individual, and variations below these activity levels rather than absolute levels should be used to assess exposure. In the absence of preexposure measurements, repeated postexposure measurements should be obtained at different intervals. In this case a significant increase in cholinesterase activity over time would indicate recovery from an initial exposure to an organophosphate (*61*). A much debated issue is whether red blood cell AChE or plasma cholinesterase is a better indicator of exposure to organophosphates. Many organophosphates appear to be better inhibitors of cholinesterase, suggesting that this enzyme may be a more sensitive indicator of exposure; however, this is not true for all organophosphorus compounds. Furthermore, plasma cholinesterase activity displays a higher variability because it can be affected by other exogenous agents (e.g., drugs) or physiological and pathological conditions (e.g., pregnancy or liver damage) (*62*). In addition, genetic variants of human serum cholinesterase exist (*63*). Individuals with atypical cholinesterase, which occurs in homozygous form in 1 out of 3,500 Caucasians and consists of a single aminoacid substitution in position 70 (glycine instead of aspartic acid), have an a abnormal response to the muscle relaxant succinylcholine (*63*). Whether genetic variants of cholinesterase affect the sensitivity to organophosphates remains to be determined.

If blood cholinesterase activity is considered a biomarker of effect of organophosphates, it is important to determine whether this peripheral measurement reflects similar changes occurring in target tissues, i.e., the central nervous system and the muscles, particularly the diaphragm. Surprisingly, very few studies have directly attempted to address this important question, though those that did concluded that blood cholinesterase is a good indicator of target organ enzyme activity (*64–66*). Erythrocyte AChE, in particular, was found to be better correlated with brain or diaphragm activity than plasma cholinesterase. This was observed, for example, in animals exposed to different single doses of the insecticide chlorpyrifos and paraoxon at different times after dosing (*66*). During repeated exposures to the insecticide disulfoton, the strongest correlation was seen between brain and lymphocyte AChE activity (*65*); however, in the recovery period following termination of exposure, red blood cell AChE better reflected brain AChE activity. To strengthen these findings, it is important to conduct additional studies comparing blood and target tissue AChE under various exposure conditions. In particular, several different organophosphates should be tested, possibly at low doses for extended periods of time, to mimic occupational exposure. Nevertheless, measurement of blood AChE activity remains an excellent biomarker for exposure and effect of organophosphate exposure under both acute and chronic conditions.

Upon repeated exposure to organophosphates, tolerance to their toxicity has been shown to develop (*67*). This tolerance is mediated, at least in part, by a homeostatic change in cholinergic receptors whose density decreases to compensate for the prolonged increase in acetylcholine levels (*68,69*). These changes in cholinergic receptors, particularly the muscarinic type, may be seen as a protective mechanism by which the organism normalizes function despite challenge from the external environment. On the other hand, balance of neuronal connections may have been altered, and higher brain functions might be compromised by such receptor alterations. Indeed, in animals repeatedly exposed to the organophosphates disulfoton or diisopropylfluorophosphate, memory deficits have been reported (*70,71*). As such cognitive impairment was also observed in some, but not all, studies of

occupationally exposed workers (*72,73*), a series of experiments were designed to investigate whether a peripheral biomarker of muscarinic receptors could be found that would reflect changes in central nervous system muscarinic receptors. Lymphocytes were considered as possible surrogate tissue because in these cells from rats and humans, muscarinic receptors have been identified at the protein and mRNA levels (*74–76*). Following a 2-week exposure to the organophosphate disulfoton, the density of muscarinic receptors was decreased to a similar degree in hippocampus and cerebral cortex as well as in circulating lymphocytes (*77*). A more detailed time–course study indicated a strong correlation between the levels of muscarinic receptors in brain areas and lymphocytes during the period of exposure (*65*). However, in the recovery period following termination of exposure, such correlation was not present, possibly because of the high turnover of lymphocytes. Thus, measurement of lymphocytic muscarinic receptors should be seen as a useful indicator of central nervous system changes only during prolonged exposure but not afterward.

A number of organophosphates also cause another type of neurotoxicity, characterized as a central–peripheral distal axonopathy (*78,79*). This syndrome, commonly known as organophosphate induced delayed polyneuropathy (OPIDP), is totally independent of inhibition of AChE and is delayed as symptoms appear about 2 to 3 weeks after the initial poisoning when acute cholinergic signs have subsided. The mechanism of initiation of OPIDP involves the phosphorylation of a protein in the nervous system called neuropathy target esterase (NTE) and the aging of the phosphoryl-enzyme complex (*80*). Compounds that age cause OPIDP if the threshold of inhibition of NTE (70–80%) is reached, whereas compounds that do not age (e.g., phosphinates or carbamates) do not cause OPIDP and, when given before a neuropathic compound, actually protect against its delayed neurotoxicity. NTE activity has been found in lymphocytes and platelets (*81,82*). Within 24 hr after acute exposure, there is a good correlation between lymphocyte and brain NTE in the hen, which is the species of choice for OPIDP studies (*83*). Measurement of lymphocyte NTE has been suggested as a potential biomarker to monitor for organophosphate-induced polyneuropathy (*84*). The best example of its application in humans is in a case report of an attempted suicide with the insecticide chlorpyrifos in which, based on 60% inhibition of lymphocyte NTE, it was correctly predicted that a neuropathy would develop well after recovery from acute cholinergic poisoning had occurred (*85*). In a study of workers exposed to the defoliant DEF, inhibition of lymphocyte NTE was observed but was considered a false positive because no clinical or electrophysiological signs of OPIDP were detected in exposed workers (*86*). Recently, compounds that offer protection when given before a neuropathic organophosphate have been found to act as promoters (i.e., to potentiate OPIDP) when given afterward (*87*). These findings have challenged the understanding of the mechanisms of OPIDP and, unfortunately, complicated the use of lymphocyte NTE as a biomarker, as the clinical outcome of a combined exposure to protective/promoter and neuropathic pesticides would be unpredictable (*79*).

The metabolism of organophosphates offers the possibility of investigating possible polymorphisms of metabolic enzymes as biomarkers of susceptibility and of measuring metabolites in the urine as biomarkers of exposure. Most organophosphates are activated to their correspective oxygen analog by an oxidative desulfuration reaction, which is catalyzed by cytochrome P450. Upon phosphorylation of AChE, a portion of the molecule, the leaving group, is released and excreted. Both the parent compound and the oxon can undergo a series of detoxication reactions that are mediated by various A esterases (paraoxonase, carboxyesterase), by P450s, and by glutathione transferases. The leaving group, *p*-nitrophenol in the case of parathion, which is also generated by hydrolytic cleavage, and alkylphosphates are excreted in the urine and can be quantified as an index of organophosphate exposure (*88–90*). In the case of alkylphosphates, studies in humans have indicated that they may represent a sensitive marker of organophosphate exposure because they may be detected even if no significant changes in blood cholinesterase activity can be measured (*91*).

Because of the importance of the activation step in the toxicity of organophosphates, genetically determined differences in cytochrome P450 may play a significant role. Though the need of the conversion to the oxon has been known for almost 40 years (*58*), the specific P450 isozyme(s) responsible for the activation of thioates has not, to my knowledge, been identified. The same is true with regard to glutathione transferases, whose role in the detoxication of organophosphates (particularly the methyl substituted) is still controversial (*92*). Clearly, genetic variations in these enzymes may be important, especially to explain the exacerbated reactions to organophosphate exposure observed in certain individuals. This is certainly an area where some additional research efforts should be addressed.

A metabolic pathway that has been investigated in this context is the hydrolysis of several organophosphates by paraoxonase. This enzyme, which takes its name from its most studied substrate, paraoxon, is capable of hydrolyzing the oxygen analogs of a number of commonly used organophosphorus insecticides such as chlorpyrifos oxon and diazinon oxon. It has been long known that human paraoxonase exhibits a substrate-dependent polymorphism (*93*). One form hydrolyzes paraoxon with a high turnover number, and the other form with a low turnover number (*94,95*). In addition to the observed polymorphism, there is a large variation in enzyme levels (over 10-fold) observed within a genetic class, with a full scale variation of about 60-fold (*94*). Several lines of evidence suggest that high levels of serum paraoxonase are protective against poisoning by organophosphorus insecticides whose active metabolites are substrates of this enzyme. Birds, which have very low levels of serum paraoxonase, are very sensitive to diazinon oxon, pirimiphos oxon, or parathion compared to mammals (*96,97*). Rabbits, which have a very high level of serum paraoxonase, are less sensitive than rats to the acute toxicity of paraoxon (*98*). Furthermore, when rats or mice are injected with paraoxonase purified from rabbit serum to increase the blood hydrolyzing activity, their sensitivity toward the toxicity of paraoxon and chlorpyrifos oxon is significantly decreased (*99–101*). Interestingly, administration of exogenous paraoxonase to mice also offers protection against the toxicity of the parent compound chlorpyrifos (*102*). These animal experiments provide convincing evidence that serum paraoxonase is an important determinant of susceptibility to organophosphate poisoning.

The polymorphism of human paraoxonase has been recently elucidated. One isoform, with arginine at position 192, hydrolyzes paraoxon with a high rate, whereas the other isoform, with glutamine at position 192, hydrolyzes paraoxon at a slower rate (*103*). In population studies,

three genotypes have been observed: individuals homozygous for the low activity allele (48%), individuals homozygous for the high activity allele (9%), and heterozygotes (43%) (*94*). Because not all substrates exhibit polymorphism and because of the large variability of expression, certain considerations should be made about the use of paraoxonase status as a genetic marker for susceptibility to poisoning by organophosphates. For example, an individual who is homozygous for the low-activity allele and also has very low levels of the Gln_{192} protein would be expected to hydrolyze both paraoxon and chlorpyrifos oxon very slowly and would be predicted to be sensitive to both compounds. However, an individual who has very high levels of the Gln_{192} isoform would be able to hydrolyze chlorpyrifos oxon very rapidly but, due to the genetic polymorphism (i.e., Gln instead of Arg at position 192), would still be a relatively low metabolizer of paraoxon.

Styrene

Styrene is a very important solvent used in the manufacture of numerous polymers and copolymers including polystyrene, styrene–acrylonitrile, and styrene–butadiene rubber (*104*). As for other organic solvents, acute exposure to high levels of styrene causes irritation (of both skin and respiratory tract) and central nervous system depression (*105*). Upon chronic exposure, styrene may have carcinogenic and reproductive toxicity (*105,106*). *In vitro* studies suggest that the metabolite styrene oxide may mediate genotoxic and developmental toxic effect of styrene (*107,108*). Styrene exposure has also been reported to cause neurotoxic effects. Indeed, a large number of studies involving occupationally exposed workers have reported signs and symptoms of central nervous system toxicity (*109–114*), though a recent review has challenged several of these findings (*115*).

Because of the widespread use of styrene, its biological monitoring has been extensively investigated (*116*). Styrene is metabolized to styrene oxide by cytochrome P450s; styrene oxide is then detoxified via glutathione transferases and/or epoxide hydrolase, the latter pathway being more significant in humans than in rodents (*117*). Mandelic acid and phenylglyoxylic acid are the most prominent metabolites found in urine (*117*). Styrene in blood, urine, and exhaled air, as well as urinary mandelic acid and phenylglyoxylic acid, have been commonly used for biological monitoring (*116–119*). Threefold differences in the relative urinary excretion of optical enantiomers of mandelic acid have been reported and have been suggested to be related to polymorphisms of cytochrome P450s, leading to R- or S- styrene 7,8-oxide or epoxide hydrolase (*120*). This observation may be relevant with regard to genotoxicity as R-styrene 7,8-oxide has a stronger mutagenic effect than its S enantiomer (*121*). Styrene oxide can form adducts to the *N*-valine residue on hemoglobin (*122–124*), and measurement of these adducts has been used to monitor exposure to styrene in animals and humans (*125–127*). However, styrene oxide is not considered an effective alkylator of hemoglobin compared to other toxicants such as ethylene oxide (*128*). Furthermore, the capacity of humans to form styrene oxide is much lower than rats or mice (*117*); thus, very low levels of styrene oxide (*129*) and styrene oxide–hemoglobin adducts (*125,126*) are found in human blood.

While these measurements can be used as biomarkers of styrene exposure, none appear to be mechanistically linked to the neurotoxicity of styrene. In fact, the specific neurotoxic targets for styrene, its mechanism(s) of neurotoxicity, and the role played by styrene oxide are for the most part still unknown. Exposure of rats to styrene has been shown to decrease levels of brain glutathione (*130,131*), and this effect has been attributed for the most part to detoxication of styrene oxide by glutathione transferases (*132*). Following chronic exposure to styrene, an increase in GFAP was found, possibly reflecting reactive astrogliosis to styrene- or styrene oxide-induced neuronal damage (*133*). An *in vitro* study in PC12 cells has indeed shown that styrene oxide causes depletion of intracellular glutathione and ATP, followed by elevation of free calcium levels and induction of DNA single strand breaks (*107*). A more recent study in primary cultures of murine spinal cord–dorsal root ganglia indicated cytotoxicity of styrene and styrene oxide (the latter being about 10-fold more potent), and oxidation of multiple cellular macromolecules was suggested as a mechanism of toxicity (*134*). A number of animal studies have suggested that styrene can affect the metabolism of dopamine. An increase in dopamine D_2 receptors has been found in rats following adult and developmental exposure to styrene (*135,136*). This increase may be a compensatory reaction to the observed decrease in dopamine levels (*137*), which has been ascribed to condensation of dopamine with the styrene metabolite phenylglyoxylic acid (*138*). These findings were mechanistically correlated with an increase in prolactin levels found in the blood of styrene-exposed workers, as prolactin release from the anterior pituitary gland is chronically inhibited by dopamine (*139–141*). Although of interest, this hypothesis should be viewed with caution, and further evidence should be provided in its support. A decrease in MAO B was found in the brain of rats exposed to styrene (*142*). A dose-related decrease of MAO B activity has also been found in three separate studies of platelets from workers exposed to styrene (*114,141,142*). The effect appeared to be specific for styrene, as it was not observed in platelets of workers exposed to the solvents perchloroethylene and toluene (*143,144*). On the other hand, a decrease in serum dopamine-β-hydroxylase (DBH) activity was found in workers exposed to both styrene and toluene (*141,144*), possibly because of inhibition of its activity by phenol/cresol metabolites of these solvents, but not following exposure to trichloroethylene (*145*). Overall, these observations are of interest because they may lead to effect-related biomarkers for styrene. However, a better understanding of the mechanism of styrene (and/or styrene oxide) neurotoxicity is necessary before these and other biomarkers can be used with confidence.

n-Hexane

The neurotoxicity of *n*-hexane was first identified in humans and then confirmed by animal studies. Symptoms of *n*-hexane neurotoxicity are numbness of the extremities followed by weakness of the intrinsic muscles of the hands and feet and, with continuous exposure, progressive loss of sensory and motor functions. Observations in humans and investigations in animals have characterized this as a distal sensorimotor neuropathy, specifically a distal axonopathy (dying-back) type (*146*). Another solvent, methyl *n*-butyl ketone, also causes a sensorimotor neuropathy, and the formation of the same toxic metabolite, 2,5-hexanedione, from both *n*-hexane and methyl *n*-butyl ketone has been firmly demonstrated (*147*). The pathogenesis of *n*-hexane neuropathy has been recently reviewed (*148*). The reaction of 2,5-hexanedione (and other γ-diketones, but not diketones with other than γ-spacing) with the lysyl groups of proteins leads via a series of intermediate steps to the formation of

chemical adducts characterized as pyrroles (*149,150*). Experiments with diastereoisomers of 3,4-dimethyl-2,5-hexanedione have indicated that the formation of the pyrrole is a critical step in the sequence of events that result in the axonopathy (*151*). Further experiments have also shown that, though formation of pyrrole is a necessary step in neurotoxicity, it is not sufficient because pyrrole oxidation must also occur, leading to neurofilament cross-linking (*152*).

Exposure to *n*-hexane in occupationally exposed workers has been mostly assessed by determination of urinary levels of the toxic metabolite 2,5-hexanedione (*153,154*). However, this measurement reflects only recent exposure, in the order of a few hours to a few days. On the other hand, the mechanistic studies summarized above offer the possibility of using measurement of pyrrole adducts to macromolecules as a biomarker of cumulative exposure to *n*-hexane. Experiments in rats exposed to γ-diketones have shown that formation of hemoglobin adducts is proportional to both time and dose (*155*). As the formation of adducts has been shown to be causally related to the development of neuropathy, hemoglobin may be seen as a surrogate for neurofilaments during *in vivo* exposure (*148*). As such, measurements of hemoglobin pyrrole adducts in the case of *n*-hexane are more than a biomarker of exposure and can be seen as a biomarker of effect. In this regard, more detailed dose–response studies in animals, as well as studies in humans, may be useful to arrive at a better quantitative assessment of neurotoxic risk linked to exposure to *n*-hexane or other γ-diketone precursors.

As the cross-linking of neurofilaments is essential for neurofilament-filled axonal swellings (*148*), the ability to measure protein cross-linking in an accessible tissue would provide an additional means of assessing exposure to and health effects of *n*-hexane. A protein present in erythrocytes, spectrin, has proved to be useful in this regard. The α and β subunits of spectrin are closely associated on the cytoplasmic side of the red blood cell membrane. Cross-linking compounds yield α, β-heterodimers that can be identified in SDS polyacrylamide gels (*148*). Neurotoxic γ-diketones have been indeed shown to cause spectrin dimerization, while administration of nontoxic diketones does not cause spectrin cross-linking (*151,152,156*). This biochemical change, which also has been observed following exposure to carbon disulfide, may serve even more than pyrrole adducts as a biomarker directly related to pathological changes in the axon. Its investigation in animals and humans exposed to other peripheral neurotoxicants is certainly warranted as long as the underlying mechanisms are carefully considered.

Carbon Disulfide

Exposure to carbon disulfide (CS_2) also results in neurotoxicity. The most common effect is a distal sensorimotor neuropathy characterized as a neurofilamentous axonopathy (*157*). Despite the chemical dissimilarity between CS_2 and *n*-hexane, both compounds cause identical neuropathies (*148*). Differently from *n*-hexane, chronic exposure to CS_2 has also been associated with the development of an encephalopathy (*158*) whose mechanism has not been elucidated. The pathogenesis of CS_2 peripheral neuropathy has also been recently reviewed (*148*). Though CS_2 does not form pyrrole adducts, through a series of interactions with amino groups of proteins, it ultimately causes neurofilament cross-linking. CS_2 combines with primary and secondary amines to generate dithiocarbamates, which are converted to isothiocyanate adducts. The latter may undergo nucleophilic addition of sulfydryl or amine groups, creating dithiocarbamate ester and thiourea covalent bridges, respectively (*148*), and the thiourea cross-link appears to be irreversible. These mechanistic studies, and others that have shown the ability of CS_2 to cause cross-linking of proteins (*159*), have led to another potential biomarker that may be useful in assessing the peripheral neurotoxicity of CS_2. As for *n*-hexane, erythrocyte spectrin is cross-linked by CS_2, and the accumulation of spectrin dimers in rats is proportional to both time and dose (*159*). Even more interestingly, spectrin dimers could be detected before clinical or morphological evidence for neurotoxicity (*160*). It has also been shown that carbonyl sulfide, which derives from oxidative metabolism of CS_2 by yet unidentified cytochrome P450(s), can also participate in cross-linking reactions via a protein-bound isocyanate intermediate (*148*). The contribution of carbonyl sulfide in the peripheral neurotoxicity of CS_2 is not known, but in light of possible polymorphisms of the P450(s) involved in the oxidation of CS_2, it would be of interest to define the relative roles of the parent compound and this metabolite.

Concentration of CS_2 in expired air has been used in the occupational setting as a marker of exposure (*161*); however, this measurement only reflects very recent exposure. The reaction of CS_2 with amines to yield dithiocarbamates has led to the monitoring of the latter in blood as a method to assess occupational exposure (*162*). Furthermore, 2-thiothiazolidine-4-carboxylic acid, a metabolite derived either from dithiocarbamate or from trithiocarbonate, has been identified in urine from workers exposed to CS_2 and has been used to monitor exposure (*163,164*); the current biological exposure index is based on such measurements (*165*).

As mentioned earlier, encephalopathy is another manifestation of the neurotoxicity of CS_2 neurotoxicity. Animal studies have shown that, in rats, brain dopamine levels are increased following exposure to CS_2 (*166*), suggesting that, among various possibilities, CS_2 may inhibit DBH. This biochemical effect has indeed been observed *in vitro* (*167*), and *in vivo* in rat adrenals following acute, but not chronic, exposure (*168,169*). On the other hand, in a group of female viscose rayon workers exposed to low levels of CS_2, a decrease in serum DBH was observed only after prolonged exposure (more than 1 year) (*170*). There are no studies, to my knowledge, that have pursued the involvement of alterations of dopamine metabolism in the central neurotoxicity of CS_2; thus, the significance of these observations with regard to possible use as biomarker is unknown. Furthermore, the effects on peripheral dopamine metabolism are probably impossible to discern from those on the central nervous system.

Acrylamide

Acrylamide is an important chemical used in the synthesis of polyacrylamides, which have a variety of industrial applications. Though various toxic effects of acrylamide, (e.g., reproductive toxicity and possibly carcinogenicity) have been suggested by animal studies (*171*), the most relevant effect identified in humans is neurotoxicity, most notably a distal axonopathy (*172–174*).

Earlier studies on the distribution of ^{14}C-acrylamide in rats had indicated that a high level of radioactivity was associated with blood erythrocytes (*175,176*). Hashimoto and Aldridge (*175*) also observed *in vitro* studies in which acrylamide was covalently bound to cysteine residues in brain proteins and hemoglobin, and that, on acid hydrolysis, the formed adduct yielded a compound identified as *S*-(2-carboxyethyl) cysteine (CEC). Based on this information, Bailey et al. (*177*)

developed a gas chromatography–mass spectrometry method to measure CEC in hydrolyzed globin from acrylamide-treated rats. These investigators reported a dose-dependent increase in CEC for intravenous doses of acrylamide of 0.1 to 5 mg/kg and also noted the presence of background levels of CEC in untreated animals. In the past few years, a series of studies have extended these results to both animals and exposed human populations. An initial finding was the identification of a novel adduct [*S*-2-(carboxy-2-hydroxyethyl)-cysteine] in hydrolyzed hemoglobin samples from rats treated with acrylamide *in vivo* and in microsomal suspensions of acrylamide with cysteine *in vitro* (*178*). The presence of this adduct indicated the conversion of acrylamide to glycidamide, a reactive epoxide metabolite. In rats injected with increasing doses (5–100 mg/kg) of acrylamide or glycidamide, hemoglobin adducts increased linearly (*179*), and on a mole per kilogram basis, glycidamide adducts were 3.2 times lower than acrylamide adducts. Following administration of acrylamide, formation of glycidamide adduct generated a concave curve, presumably reflecting the Michaelis-Menten kinetics of its formation, and the percentage of acrylamide converted to glycidamide was inversely proportional to the dose (*179*). Subchronic treatments of rats with acrylamide confirmed that the conversion rate of acrylamide to glycidamide, as determined from hemoglobin adduct formation, is higher at low administered doses (*179*). Glycidamide may be involved in the reproductive toxicity of acrylamide (*180*) and in its potential carcinogenicity because this metabolite, but not the parent compound, is mutagenic in the Ames test (*181*). Uncertainties still exist, however, on the role of glycidamide in the pathogenesis of acrylamide-induced peripheral neuropathy, since contrasting results have been reported (*182–185*).

A method was also developed to determine acrylamide and glycidamide adducts to N-terminal valine following total hydrolysis and by means of the modified Edman degradation procedure, which was used to assess exposure to acrylamide in a group of factory workers (*186*). Adducts were detected in all exposed workers (0.3–34 nmol/g Hb) and in only 1 out of 10 controls (0.01 nmol/g Hb). Glycidamide adducts were determined in a small subset of exposed workers (1.6–32 nmol/g Hb) and were highly correlated with acrylamide adducts. Based on their roles and locations in the production process, workers were divided into four groups (*186*), and the highest adduct levels (14.7 nmol/g Hb) were found in workers involved in the synthesis of acrylamide from acrylonitrile and in the transfer of 35% acrylamide solutions into barrels. Based on extrapolations from air concentrations of acrylamide, it was calculated that only a fraction of adducts (0.44 nmol/g Hb) would derive from inhalation of acrylamide, indicating dermal absorption as a primary route of exposure (*187*). Among the exposed workers, signs and symptoms indicating peripheral neuropathy were found with statistically significant increased frequencies compared to controls. Based on the result of a questionnaire, a neurological examination, and vibration thresholds and electroneuromyographic measurements, a neurotoxicity index for acrylamide-induced peripheral neuropathy was developed (*187*). The neurotoxicity index, which adequately predicted the clinical diagnosis of peripheral neuropathy, was significantly correlated with hemoglobin adducts of acrylamide and with an accumulated *in vivo* dose of acrylamide but not with the concentration of acrylamide in the air or in plasma of exposed workers. A good correlation was also found between the neurotoxicity index and hemoglobin adducts of acrylonitrile (which is explained by a correlation between acrylamide and acrylonitrile exposure) and by mercapturic acid in the urine (which could result from both acrylamide and acrylonitrile) (*187*). Overall, these studies indicate that measurement of hemoglobin adducts of acrylamide is a good biomarker of exposure and may be useful as an indicator of acrylamide-induced peripheral neuropathy. Though the mechanism of the peripheral neurotoxicity of acrylamide has not been fully elucidated, these studies suggest that a covalent bond to axonal proteins may present a relevant step in the pathogenesis of its axonopathy. Further studies may also lead to a better definition of the dose–response relationship of hemoglobin adduct formation and their use as predictors of peripheral neurotoxicity.

Conclusions

In this brief review, I have discussed some aspects of biomarker research as they relates to the assessment of exposure and susceptibility to and the effects of neurotoxic chemicals. By far, the best available tools are in the area of biomarkers of exposure; traditional measurements of neurotoxic chemicals and their metabolites in biological fluids provide useful and reliable indicators of exposure. Novel approaches such as measurements of hemoglobin adducts may be useful for assessing prior and repeated exposures to electrophilic compounds. For these chemicals, further mechanistic studies should be carried out, as the formation of adducts to proteins in the target tissue may represent an essential step in the pathogenesis of neurotoxicity. If this were the case (as discussed for *n*-hexane), measurements of adducts in blood could also used as biomarkers of effect. As several neurotoxicants undergo metabolic activation or detoxification by enzymes that are known to exhibit polymorphisms in humans, it is surprising that little attention has been devoted to this topic within neurotoxicology. The examples and suggestions provided may serve to stimulate more research in this area, which may be relevant in light of the lower levels of exposure normally encountered in developed countries. As techniques for genotyping are becoming more accessible, epidemiological studies on occupationally exposed workers may include such measurements when proper hypothesis can be formulated.

With regard to biomarkers of effect, it is clear that new opportunities will derive only from a better understanding of the cellular and molecular targets for neurotoxicants. Although these efforts may not ultimately lead to useful reliable markers for use in epidemiological studies, they will certainly allow the testing of mechanism-driven hypotheses. In the few cases where this has occurred (e.g., organophosphates), useful biomarkers have indeed been developed. Clearly, neurotoxicity can be manifested in a wide variety of ways, each one characterized by different cellular and biochemical substrates. Thus, the development of one or more markers for neurotoxic effects does not appear as a feasible option. Rather, biomarkers would be specific for a class of chemicals or a target cellular process (e.g., myelination). Still, by understanding the chain of events that ultimately lead to neurotoxicity, it is possible to develop biomarkers that would be indicative of early biochemical alterations preceding irreversible damage. As neurotoxicology, possibly more than other areas of toxicology, requires a multidisciplinary approach, the best use of biomarkers would ultimately be in conjunction with electrophysiological and behavioral assessments.

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