ANNUAL REVIEWS

Annual Review of Pharmacology and Toxicology

A Chemical Perspective of Pharmacology and Toxicology

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Annu. Rev. Pharmacol. Toxicol. 2018. 58:1–16

The *Annual Review of Pharmacology and Toxicology* is online at pharmtox.annualreviews.org

https://doi.org/10.1146/annurev-pharmtox-010617-053205

Keywords

dopamine, quinones, amphetamine, electrophiles, prooxidants

Abstract

My chemical training provided a somewhat different perspective of biological problems, in the problem itself and approaches to its solution. I was fortunate to have in my laboratory postdocs and students who shared this perspective and used appropriate tools to address problems in amphetamine pharmacology and air pollution toxicology. These apparently disparate areas of research shared two chemical reactions: prooxidant-based generation of reactive oxygen and formation of covalent bonds between electrophiles and biological nucleophiles. This article is an attempt to summarize that research and to identify those individuals who made the contributions.

INTRODUCTION

I wish to thank the Editorial Committee of the *Annual Review of Pharmacology and Toxicology* for the invitation to write this article and for the honor of serving on the Committee from 1988 to 2007. I want to particularly express my thanks to Paul Insel, Terrence Blaschke, and Horace Loh, who were my colleagues on the Committee, for their friendship and advice; they provided the balance of expertise required in the subdisciplines that constitute this enormous field of scientific inquiry.

Wikipedia defines pharmacology as "[t]he study of the interactions that occur between a living organism and chemicals that affect normal or abnormal biochemical function." The study can take place from the perspective of the living organism or from the perspective of the affecting chemicals. My colleagues and I have maintained the perspective that the properties and reactions of chemicals constitute the basis for their effects on the organism. This article summarizes the work that my laboratory has conducted, based on that underlying research theme. I include my educational background because it represents the intellectual evolution of my research approach and also allows me to acknowledge the many significant contributions from key members of the lab over the years.

PERSONAL SCIENTIFIC TRAINING

I was fortunate to be mentored by outstanding scientists during my formal education and, more importantly, smart enough to listen to them. I started my undergraduate education at the University of California, Berkeley, which, at the time, had an entire college—the College of Chemistry and Chemical Engineering—devoted to chemical education and research, which it performed extremely well. As a chemistry major with a focus on organic chemistry, I was mentored by Henry Rapoport, who was known for his work on the synthesis of natural products. I considered applying for a graduate program in biochemistry but was encouraged by him to study chemistry first and then go into biochemistry. Following his advice, I ended up as a chemistry graduate student at the University of California, Los Angeles (UCLA), where I was mentored by Theodore Geissman, an organic chemist who was studying the biosynthesis of plant pigments. The biosynthetic reactions involved were analogous to the xenobiotic transformations that I was to study subsequently. A major focus of the department of chemistry at UCLA was organic chemical reaction mechanisms, in which the biochemically relevant concepts of neighboring group effects (anchimeric assistance) on reaction centers and host-guest chemistry were being developed. These concepts are the basis for chemical mechanisms of enzyme reactions and provided me with a useful perspective on biological research. My postdoctoral and subsequent career research were based on the teaching and support from Donald Jenden in the department of pharmacology, who was my postdoctoral mentor and later was the chair of that department when I joined it. Don, a graduate of a British medical school with interests in neurophysiology and biomathematics, was a brilliant and innovative scientist. Although not trained in chemistry, he recognized early on the relevance of gas chromatography/mass spectrometry (GC/MS) as a tool for pharmacological research and applied it very successfully to studies on the neurochemistry of acetylcholine, including its unequivocal identification as a neurotransmitter in brain. He established a GC/MS facility in the department, which was key to our research. Don was a very good postdoctoral mentor to me, in part because he emphasized the importance of quantitative analysis—a concept that, to a synthetic organic chemist who worried mostly only about yields, was almost foreign. For my postdoctoral project, Willford Haslett, Don's graduate student, and I worked to identify an active metabolite of tremorine, an acetylenic lactam amine that we named oxotremorine (1). Tremorine had been used as a screening agent for antiparkinsonism agents; it induced tremors in mice that could be antagonized with

antiparkinson agents used during that time. There was some indirect evidence that tremorine's actions were mediated by metabolites, but direct evidence was lacking. The identification studies of oxotremorine as a selective muscarinic agonist were accomplished by brute force, using large quantities of tissue homogenates, isolation of crystalline metabolite, and identification by independent synthesis. Its selectivity for muscarinic receptors suggested cholinergic hyperactivity in the disease process which is now recognized to be a dopamine (DA) deficiency.

After my postdoctoral work at UCLA, I worked for a brief period in industry and then joined the Laboratory of Chemical Pharmacology of the National Heart Institute of the US National Institutes of Health (NIH), whose chief was Bernard B. Brodie. The laboratory was one of the founding sites for modern pharmacology, using chemical tools to investigate biological processes. In the Brodie lab, I was involved in two projects, one that examined the neurochemistry of amphetamine (Amp) (2) and a second that focused on the metabolic formation of electrophiles (3).

In 1970, Don Jenden, then chair, invited me to join the department of pharmacology at UCLA, and I gratefully accepted. The position provided me with an opportunity to establish a research program following my research instincts and scientific background. Additionally, I wanted to train pharmacologically oriented scientists who could use chemical tools in their studies and yet had sufficient biological information to be able to contribute to pharmacology. I decided the laboratory needed both a chemical and biological component and therefore began a research program in drug metabolism and neurochemistry from the lab's inception to the present. I have been very fortunate in having collaborations with outstanding postdoctoral researchers (postdocs) who joined the laboratory and made key contributions to the intellectual environment and technologies employed in our research as well as teaching me (with varying degrees of success). They then continued their careers successfully and, when asked, continued to willingly and enthusiastically collaborate with my laboratory to enhance its productivity and science. Many of the research advancements in the lab were due to the keen interest and dedicated support of these postdocs, especially Björn Lindeke (University of Uppsala), Masayuki Hiramatsu (Meijo University), Yoshito Kumagai (Tsukuba University), and Jon Fukuto (Sonoma State University), who are currently faculty members at the indicated universities. I will be forever grateful to them for contributing their intellect and scientific acumen to our collective research efforts.

BIOTRANSFORMATION OF AMPHETAMINE-RELATED COMPOUNDS

The initial chemical component–research theme in the lab was focused on metabolic transformation studies of Amp and its alpha methyl congener, phentermine, and, as such, represented a continuation of my prior Amp studies at NIH.

N-Oxidation

Björn Lindeke was the first postdoc to join the laboratory. He was a clever, well-trained medicinal chemist from Stockholm University with an interest in applying his skills to chemical mechanisms of biotransformation. He brought knowledge and technology of stable isotope–based synthesis and mass spectral analysis that were initially applied to biotransformation studies of Amp-related compounds (**Figure 1**) (4–7). After these studies, we developed and maintained a research collaboration that persisted for over 10 years and notably included a unique approach to microanalysis using stable isotope–based GC/MS techniques that I believe were a major strength of the laboratory. At the same time, Gerald Miwa and, a little later, Check Sum joined the group as graduate students. Both had undergraduate degrees in chemistry, and together with Björn, they began studies on the metabolism of phentermine (**Figure 1**) (i.e., alpha methyl amphetamine) (5, 7–14).

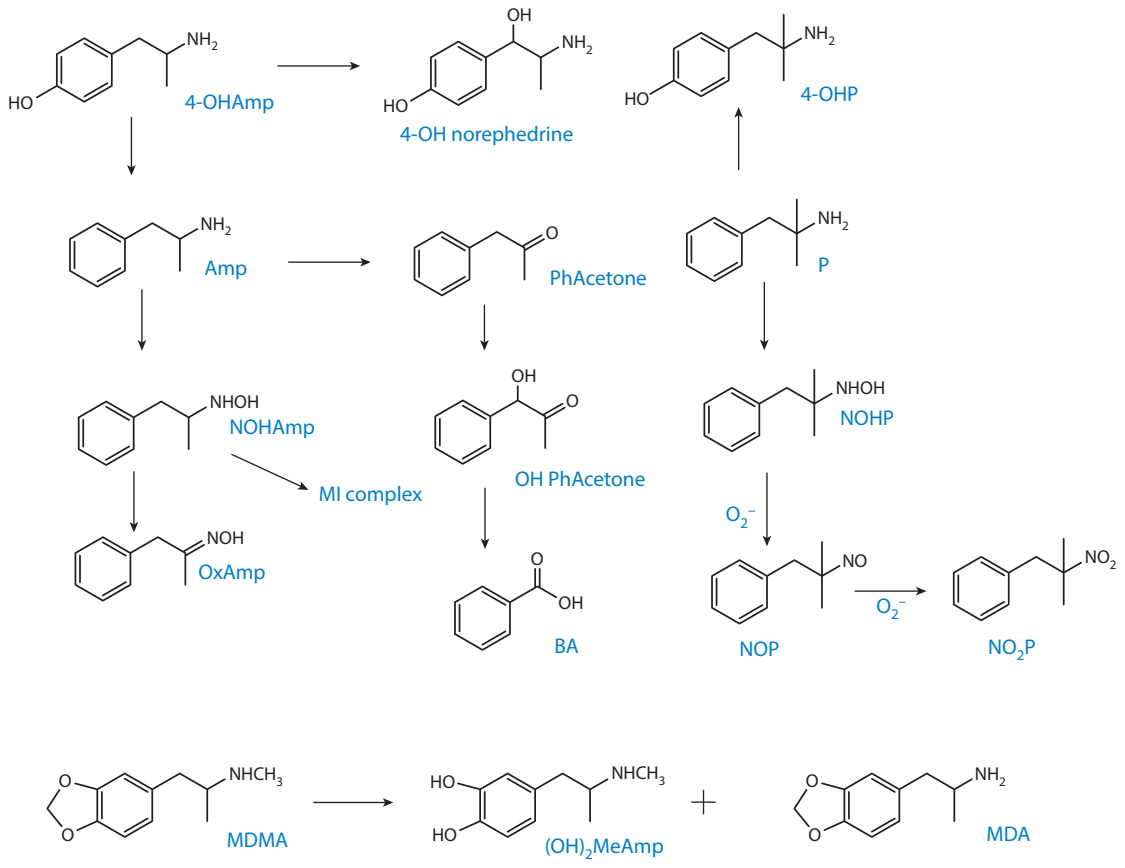

Figure 1

Biotransformation of aryl alkyl amines. Abbreviations: Amp, amphetamine; BA, benzoic acid; MDA, methylenedioxyamphetamine; MDMA, methylenedioxymethamphetamine; MI, metabolic inhibitory; NOHAmp, N-hydroxyamphetamine; NOHP, N-hydroxyphentermine; OHAmp, 4-hydroxy amphetamine; $(OH)_2$MeAmp, 3,4-dihydroxymethamphetamine; OxAmp, phenylacetone oxime; P, phentermine; PhAcetone, phenylacetone.

They demonstrated that the compound underwent N-oxidation to N-hydroxyphentermine (NOHP), a reaction more commonly associated with aromatic amines, which are much weaker bases. At the time, Gerald had proposed and tested the notion that the neutral form of the amino group was the substrate for N-oxidation (15) and, insofar as phentermine was a relatively strong base, we expected N-oxidation to be a minor pathway. John Duncan, a postdoc from the UCLA biochemistry department who began protein purification procedures in the laboratory, subsequently showed that this reaction involves cytochrome P450 (CYP) (16, 17). NOHP was found to undergo further oxidation to the corresponding nitro compound (NO_2P) (18) and N-hydroxyamphetamine (NOHAmp) to phenylacetone oxime (OxAmp) (19) as determined respectively by Michael Maynard and Richard Matsumoto, who were graduate students in the lab.

All these researchers and I continued to communicate with each other when questions arose regarding the details of the interactions of NOHAmp and NOHP with CYP. We had showed that NOHP uncoupled the CYP system to generate hydrogen peroxide, whereas NOHAmp did not. Björn, who returned to Uppsala as a faculty member in 1975, and his group had been studying the

metabolic inhibitory (MI) complexes formed by aryl alkyl amines (e.g., 20) through N-oxidation reactions and concluded that amines such as phentermine with a tertiary alpha carbon did not form these complexes. Further experiments by Gerald Miwa, who was then at Merck, and by our laboratory showed that whereas NOHP indeed uncouples the CYP system with the resulting superoxide available for further oxidation, NOHAmp forms an inhibitory complex and blocks further enzymatic activity. Richard Matsumoto then demonstrated that NOHAmp is converted to OxAmp by a non-CYP enzyme (19). Collectively, we concluded that the presence of the additional alpha methyl group in phentermine sterically prevented formation of the MI complex and uncoupled the system, an unexpected specificity for what was considered to be a nonselective enzyme (21).

Investigations on nitrogen oxidation continued as the laboratory examined the involvement of reactive oxygen species in the hydroxylamine oxidation when Jon Fukuto, an organic chemist (University of California, Berkeley), and Judith Burstyn, an inorganic chemist (UCLA), joined the laboratory as postdocs. Both were interested in the chemical details of CYP-based oxidation. They showed that NOHP uncouples the CYP oxygen reaction to form superoxide, which oxidizes NOHP to the nitroso compound (NOP) and then to the nitro compound, NO_2P, which is the ultimate product. This superoxide-mediated oxidation represents another pathway for N-oxidation by CYP. Jon and Judy, together with John Duncan, showed that superoxide oxidizes NHOP to a nitroxide that disproportionates to NHOP and NOP, which is then oxidized to NO_2P by O_2 or hydroperoxide (16, 17, 21, 22).

Benzoic acid (BA) is a major in vivo metabolite of Amp (23), and its origin was the subject of discussion at several meetings. Although an OxAmp-based pathway had been proposed, we also considered an N-oxidation pathway involving an N-hydroxylated ephedrine that, as shown by Björn and his colleagues, generated benzaldehyde chemically but not enzymatically (24). Chemist postdocs Joseph Gal (University of California, San Francisco) and Craig Kammerer (UCLA) synthesized several deuterium-substituted Amp derivatives that they and John Jonsson used to clarify details of the OxAmp pathway using isotope dilution procedures (25, 26). The results of the study showed that OxAmp is further oxidized to the hydroxyl ketone (OHPhAcetone) and then to benzaldehyde and BA. Thus, although the reaction pathway is somewhat complicated, it appears that alpha carbon oxidation is the metabolic route to BA, with N-oxidation effecting oxidation to the nitro metabolite.

These experiments made extensive use of stable isotope–substituted substrates and GC/MS procedures for identification and quantitation of metabolites. This approach completely changed the nature of metabolic studies, for we could now monitor small volumes of reaction mixtures for intermediates, in contrast to our prior identification of oxotremorine, which was based on isolation and independent synthesis and utilized liter volumes of liver preparations. Stable isotope derivatives were also used to elucidate reaction sequences by monitoring isotope enrichment of intermediates in the formation of BA. The N-oxidation studies showed that the interactions between these metabolites and the CYP system could generate reactive oxygen species capable of reacting nonenzymatically, which could be relevant to some of the long-term pharmacological effects of this group of compounds.

Methylenedioxymethamphetamine (MDMA, ecstasy, adam, molly, mandy). At an international meeting in 1986, I had the opportunity to meet Dr. Takahiko Baba and, through him, Dr. Hidetoshi Yoshimura and members of his Kyushu University laboratory. Dr. Yoshimura and I turned out to have attended middle school in the same region of Kyushu, he in Kurume and I in a small town called Yanagawa, during World War II (1942–1946). Although I was born in Oakland, CA, I had spent the war years in Japan as the result of an inopportune visit to Japan in 1941. This stay did have a positive consequence; I learned to read and speak Japanese, which was of

great help in interacting with numerous colleagues and visitors from Japan when they visited my lab or in research collaborations. Professor Yoshimura was an organic chemist whose laboratory was also investigating the metabolism of Amp, the abuse of which was a major health problem in Japan. It was through him that I met and began a long collaboration with Yoshito Kumagai, who joined the laboratory as a postdoc. This collaboration continues to be intellectually productive, as noted by our recent publications. At the time, we had begun studies of the pharmacology of methylenedioxymethamphetamine (MDMA) (**Figure 1**), whose popularity as a substance of abuse was increasing. We were particularly interested in this compound because of its potential metabolic conversion to a DA analog with attendant neurochemical implications.

When Yoshito arrived at UCLA, he worked with Lena Lin, a graduate student, and with other groups examining the enzymology of the demethylenation reaction {i.e., the conversion of MDMA to 3,4-dihydroxymethamphetamine [$(OH)_2$MeAmp] (**Figure 1**) or alpha methyl N-methyl dopamine (27, 28)}. The reaction was catalyzed by CYP2D1 at low concentrations (1–10 μM) (29) in rat and by CYP3A6 and CYP2B4 in rabbit liver microsomes (30). In humans, the reaction is catalyzed by CYP2D6 (31). These studies also showed CYP2D6 was the enzyme that exhibited substrate inhibition of Amp 4-hydroxylation that we had observed earlier. Yoshito and Jon Fukuto examined the demethylenation reaction in further detail using deuterium-substituted methylenedioxy compounds and found that CYP2B4 exhibited a reverse isotope effect (i.e., the deuterium compound underwent demethylenation faster than its protium analog). Isotope effects typically reflect mass differences, with higher mass isotopes exhibiting slower rate-limiting steps. We thus expected the deuterated MDMA to have a longer half-life that could be used in our pharmacodynamics and pharmacokinetics (PD/PK) studies, but we were clearly disappointed. Jon and Yoshito showed the reverse isotope effect was due to a radical-based pathway in which the reaction catalyzed by CYP2B4 uses a hydroxyl radical generated from hydrogen peroxide (32), again showing a nonenzymatic chemical reaction mediated by CYP. They also wrote a review of the chemistry and biochemistry of the methylenedioxyphenyl function; in addition to forming metabolic intermediate inhibitory complexes, the function is subject to chemical oxidation by biochemically generated reactive oxygen species to generate catechols (33).

To summarize, studies of the biotransformation of these aryl alkyl amines showed their complex interaction with CYP, with nitrogen oxidation reactions forming metabolic inhibitors or uncoupling the system to generate superoxide, hydrogen peroxide, or both. In a more general perspective, these studies identified the reaction capabilities of the different CYP enzymes and the metabolites generated. The N-oxidation products do not have direct neurochemical effects, whereas aromatic ring metabolites of Amp release DA (34) and can be retained in the nerve terminal (2). The catechol metabolite of MDMA [$(OH)_2$MeAmp] (**Figure 1**) is toxic to cells, exhibiting catecholaminergic neuronal properties (35), and forms thiol adducts (36).

PHARMACODYNAMICS

To address the relevance of the Amp and phentermine metabolic pathways to their neurochemistry, the biological component of our laboratory began studies on the catecholaminergic nerve terminal as the target of the Amp class of compounds. Graduate students Glenn Takimoto and Joseph Fischer studied the Amp-based efflux of norepinephrine from preloaded sympathetically innervated tissues (37, 38) and brain synaptosomes (39, 40). Based on the results of these studies, we proposed an exchange diffusion–based model for Amp-induced catecholamine efflux from nerve terminals (34). In this model, Amp interacts with three elements of the presynaptic terminal: the uptake transporter (as a competitive substrate), intraneuronal monoamine oxidase (as an inhibitor), and the vesicular transporter (as an inhibitor). These interactions result in a net

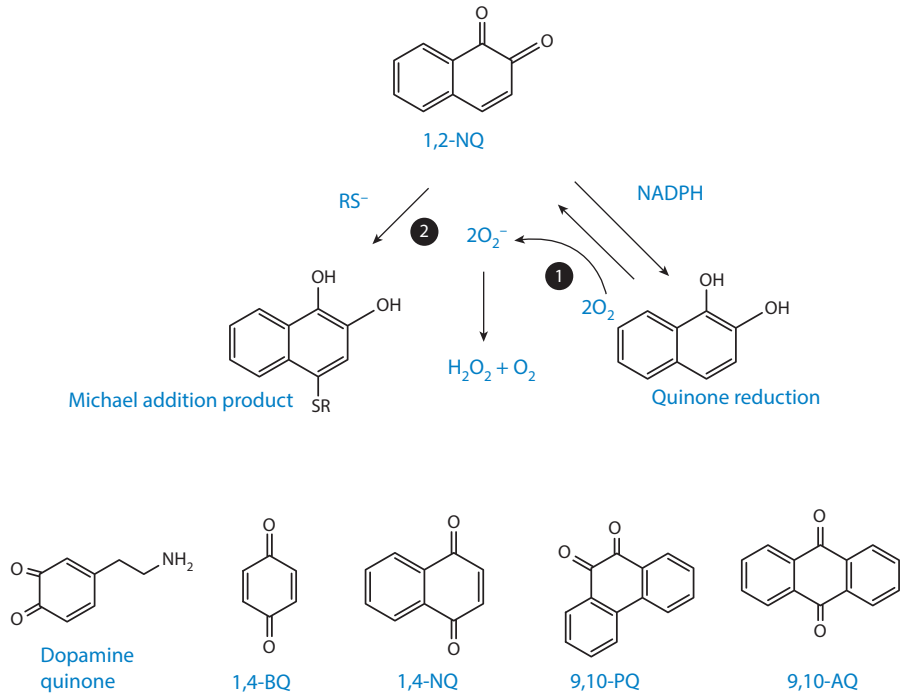

Figure 2

Quinones and their reactions. ❶ Covalent bond formation, ❷ autoxidation. Abbreviations: 1,2-NQ, 1,2-naphthoquinone; 1,4-BQ, 1,4-benzoquinone; 1,4-NQ, 1,4-naphthoquinone; 9,10-AQ, 9,10-anthroquinone; 9,10-PQ, 9,10 phenanthroquinone.

increase of neurotransmitter release from the presynaptic terminal (DA or norepinephrine). Multiple brain studies using microdialysis methods and studies with transporter knockout mice have subsequently confirmed this event (see, for example, 41–45). These analyses indicated micromolar concentrations of DA and 5-hydroxytryptamine (5-HT) can be achieved in extracellular space. These compounds are highly reactive, capable of undergoing autoxidation (oxidation by oxygen) to the corresponding quinone, with superoxide and its disproportionation product hydrogen peroxide as coproducts (see **Figure 2**). Some quinones are also electrophilic; they react with electronegative species such as thiolates (RS^-) and amino side chain nucleophilic functionalities of proteins to form covalent bonds. Hydrogen peroxide also reacts with thiolates to form sulfenic acids (RSOH). It is important to recognize that the anionic forms of thiols are the reactive species, as the pK_a values of protein thiols can vary quite substantially such that, at physiological pH, they can be mostly ionized. The reaction between a thiolate and electrophilic centers, a so-called Michael addition reaction, has been demonstrated for DA (46) and 5-HT (47) and is implicated in neurodegenerative diseases. The second reaction of importance in this context is the reversible quinone-hydroquinone redox couple; the quinone can be reduced to its hydroquinone intracellularly by, among others, an NADPH-dependent quinone reductase. Thus, depending on the availability of NADPH or NADH, the quinone-hydroquinone couple acts as a catalyst or a prooxidant, reducing oxygen to superoxide and peroxide using electrons from these nucleotides. Analogous reactions can be attributed to 5-HT. These reactions are summarized in **Figure 2** for 1,2-naphthoquinone (1,2-NQ).

PHARMACOKINETICS AND PHARMACODYNAMICS

Chemists focus on concentration-based relationships, as do pharmacologists, in the analysis of dose- or concentration-dependent effects. Richard Matsumoto, whose graduate work mostly entailed in vitro metabolic studies (19, 21), suggested that our group needed to devote more effort to relationships between these metabolic studies and PD in intact animals. He suggested that we investigate the relationship between Amp PD/PK using our bioanalytical skills. Although we had in fact performed some PK studies (e.g., 5), we had not used tissue or plasma levels to interpret biological responses. Masayuki Hiramatsu, a postdoc from Toshitaka Nabeshima's laboratory at Meijo University, was ideally suited for this approach and introduced the techniques of in vivo microdialysis and behavior protocols to the laboratory. I had met Dr. Nabeshima at a meeting on NMDA receptor pharmacology, and he raised PK questions that were the basis of a collaborative study (48–51) and suggested that Masayuki could help us.

Amphetamine and Methamphetamine

The amphetamines were particularly amenable to PD/PK analyses. As these compounds act by increasing neurotransmitter concentrations in the synapse through interaction with components of the presynaptic terminal, temporal changes in extracellular concentration of the catecholamines and 5-HT should reflect drug concentrations. In our first set of studies, Masayuki used in vivo microdialysis procedures to measure temporal changes in DA and 5-HT following MDMA administration. The results showed that the temporal pattern of extracellular DA concentration followed the plasma concentration of MDMA ($r^2 = 0.96$) but not that of its primary metabolite methylenedioxyamphetamine, which had similar actions. Extracellular 5-HT also increased, generally following MDMA levels but with a lower correlation (52). This approach was also used in collaborative work with David Segal and Ronald Kuczenski at University of California, San Diego (UCSD) and William Melega at UCLA, who studied the PD of methamphetamine and Amp. The UCSD group was studying the relationship between neurochemistry and behavior, and their careful temporal studies provided an excellent paradigm for PD/PK studies in which our contribution was PK analyses. Thus, in our first study, after Amp administration, increases in microdialysis concentrations of extracellular DA measured unilaterally in striatum paralleled increases in Amp microdialysate concentrations obtained from the contralateral striatum, with correlative r^2 values from 0.81 to 0.98 (53).

These PD/PK studies provided further support for the exchange diffusion model for neurotransmitter release by the amphetamines, instead of a depolarization and neuronal release process for the stimulation of the postsynaptic receptors. The resultant micromolar concentrations of catecholamines and 5-HT present in extracellular space and their ability to generate hydrogen peroxide provided a mechanism by which neuronal damage may occur during a protracted abuse period; its role in a model of DA neurotoxicity has been reviewed recently (54, 55).

AIR POLLUTION TOXICOLOGY

According to the American Lung Association, Los Angeles (LA) currently has the most harmful ozone pollution in the United States, and in 2016 the American Thoracic Society reported that LA air quality is the deadliest in the nation. In 1995, I was invited by John Froines of the UCLA School of Public Health to join the Southern California Particle Center and Supersite, directed by him and supported by the US Environmental Protection Agency (EPA). The Center was investigating chemical mechanisms of air pollution toxicity, and a key hypothesis focused on the contributions of redox-active compounds such as quinones (56). Because of the relevance of that chemistry to

some of our prior studies with Jon and Yoshito on DA and its analogs, I was quite interested in this perspective, and both Yoshito, as an international collaborator, and I joined the Center as investigators. Moreover, Yoshito's laboratory at the National Institute for Environmental Sciences in Tsukuba was also interested in quinone toxicity, and as a result, we have maintained a highly productive research collaboration.

Figure 2 summarizes the quinone reactions of interest (i.e., the Michael addition reaction and the redox reaction, which generate hydrogen peroxide through disproportionation of superoxide). Our first task was to determine whether significant levels of quinones could be found in air pollution mixtures. We applied a now-familiar stable isotope–GC/MS approach to this problem, and Emma Di Stefano and Debra Schmitz, together with Ying Yu, a UCLA chemistry postdoc, and graduate student Chester Rodriquez, set up the procedure and examined samples provided by other members of the Center (57). We determined the concentrations of four quinones—1,2-NQ, 24-naphthoquinone, 9,10-phenanthrenequinone (9,10-PQ), and 9,10-anthraquinone—in a National Institute of Standards and Technology standard reference sample of exhaust, in diesel exhaust particles from Japan, and in ambient air samples collected in the LA Basin and thus unequivocally identified their presence in the three different environmental samples.

Our research plan was to use quinones as surrogates for organic air pollution components and to develop an understanding of their chemical biology. Chester examined the toxicity of five quinones and showed that 1,4-benzoquinone (1,4-BQ) toxicity was not oxygen dependent, whereas 9,10-PQ toxicity increased in the presence of oxygen. This was consistent with the notion that 9,10-PQ toxicity was based on reactive oxygen, whereas BQ was likely electrophilic (58). This notion was further substantiated by Chester and Yoshito's graduate student, Keiko Taguchi, in studies examining the interaction of these quinones with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (58). Yoshito's group also showed that 1,2-NQ, a prooxidant and electrophilic quinone, caused a smooth muscle contraction in a guinea pig tracheal ring preparation with unexpected evidence of an irreversible action (59). This action was then shown to be the result of an electrophile-based irreversible inactivation of protein tyrosine phosphatase 1B (PTP1B), which negatively regulates the epidermal growth factor receptor. Yoshito and members of his group have published an extensive review on the chemical biology of quinones that focuses on features relevant to environmental toxicology (60).

In keeping with a prooxidant- and electrophile-based toxicology mechanism, the Southern California Particle Center and Supersite needed assays to characterize air samples in terms of the two characteristic reactions: (*a*) electron transfer to generate reactive oxygen species or prooxidant content and (*b*) electrophilic reactions forming covalent bonds. These assays would then be applied to multiple samples to test the validity of the toxicity model. However, assays to measure prooxidants [reaction a (**Figure 2**)] and electrophiles [reaction b (**Figure 2**)] would require sufficient sensitivity to be able to examine submilligram levels of analytes. To meet this objective, a dithiothreitol (DTT)-based prooxidant assay was developed that was based on a prior study performed by Yoshito's group (61). Application of the assay to ambient air samples showed the DTT activity to be proportional to cellular adaptive responses (62). In a study examining the relationship between the quinone concentration in particles and their corresponding volatile organic species, we found that the particulate phase containing the metals exhibited most of the redox activity, whereas the vapor phase, which contained most of the quinones we were measuring, exhibited less but still significant activity. To determine quinone contributions to this assay, we then estimated the DTT activity of selected samples whose quinone content had been measured and compared their magnitude to the observed DTT activity. Less than 1% of particle DTT activity was accountable in terms of the measured quinones, and only 33% of the vapor phase DTT activity was accounted for by the measured quinones. Recent reports suggest that, indeed,

there are other quinones (63) as well as other reactive species, such as polyhydroxyl aromatics (64), that may contribute to the DTT activity. We found that the concentrations of naphthoquinones in the volatile phase of air samples from LA Basin communities ranged from 1 to 10 pmol/m^3 but were not detectable in the particle phase, which contained phenanthraquinone (65).

Next, we developed an assay for electrophiles based on the interaction of the electrophilic 1,4-BQ with GAPDH (58, 66). GAPDH has a nucleophilic thiol in its active site that can participate in a Michael addition reaction with electrophiles with widely differing structures.

We applied these chemical assays to diesel exhaust particle samples in a collaborative study with William Linak of the EPA. We found extraction with dichloromethane removed 75% of the electrophiles but only 25% of the prooxidants, consistent with the notion that the electrophiles were mostly organic and the prooxidants more polar and likely to include metals. We further characterized the dichloromethane extract by subjecting it to zinc/acetic acid reduction and acetylation, procedures that convert quinones to their inactive hydroquinone esters (57). This reaction sequence eliminated redox activity completely, which was attributable to quinones, but only 86% of the GAPDH inhibiting– and about 90% of the hemeoxygenase-1–inducing activity, indicating some of the electrophiles were not quinones (67). Nonetheless, these vapor phase studies supported our original hypothesis (i.e., that the organic components of air pollutants exhibit biological properties that can be attributed to quinones and lower-molecular-weight electrophiles).

With this background information, we next examined whether seasonal and location differences in ambient particles of less than 2.5 microns ($PM_{2.5}$) and vapor samples were associated with unique toxicity profiles. We analyzed PM samples from three communities in the LA Basin: commerce in the midtown LA area; Long Beach, near the Port of LA; and San Bernardino in the eastern end of the LA Basin (approximately 60 miles east of LA) (68). Not surprisingly, San Bernardino had the highest levels of prooxidants and electrophiles, reflecting the increases in photochemical and atmospheric chemical transformations that convert aromatic hydrocarbons to quinones with the easterly movement of the air mass, an observation that we had made with phenanthrene, whose quinone concentration increased as the air mass moved from the western end of the LA Basin to the east (69). As we had observed in other ambient air studies, the vapor phase components were mostly electrophilic (80–90%), and the particle phase was mostly prooxidant in character (75–80%). Based on an assay for Fenton reaction capacity in air samples (e.g., 70), we determined that ascorbate, a component of lung lining fluid, can generate hydrogen peroxide upon reaction with prooxidants in particles, thereby exposing lungs continuously to micromolar concentrations of peroxide. Thus, depending on the persistence of the prooxidants, particularly metals, this process could be a major contributor to toxicities associated with air pollution exposure.

We examined the cellular effects of one set of the ambient air samples with a murine macrophage cell line used in previous studies. The results showed the particulate phase to be proinflammatory and the corresponding vapor phase to be protective by activation of the antioxidant/antielectrophile response element. These results suggested a dual exposure could result in an overall reduced inflammatory response, and supportive evidence for that mixed effect was observed when we first exposed macrophages to the vapors and then to the particles [volume constraints precluded a coincubation of the two phases (Y. Shinkai, D.A. Schmitz, E. Di Stefano, A. Eiguren-Fernandez, A.L.N. Guarieiro, E.M. Salinas, J.R. Froines & A.K. Cho, unpublished results)] and found the particle inflammatory response to be attenuated. The protective effects of the vapor phase could be attributed to quinones or other electrophilic substances.

The results of these air pollution toxicology studies are consistent with our initial hypothesis that the actions of air pollutants can be accounted for in terms of two chemical reactions: the generation of reactive oxygen and the formation of covalent bonds. Organic compounds such as quinones, polyhydroxyl aromatics, and reactive olefins, together with reactive metals, participate

in these reactions, and some of our studies have provided evidence for their products. Thus, air pollution contributions to respiratory, cardiovascular, and central nervous system diseases may involve long-term exposure to products of these chemical reactions.

CONCLUSIONS

Much of xenobiotic toxicity has been attributed to the induction of cellular oxidative stress, in which the intracellular concentration of redox active compounds favors their oxidized state (71; see Reference 72 for a quantitative description). For example, the cellular toxicity associated with chronic Amp (73) and chronic exposure to high levels of air pollutants has been attributed to oxidative stress (56). In the case of the amphetamines, the oxidative stress induced by DA quinone and its redox activity has been proposed to be the basis for cellular oxidation (54, 55). Air pollutant effects are also attributed to the components of the particle phase, which we have shown are primarily prooxidant. However, Levonen et al. (74) and others have questioned the validity of the oxidative stress paradigm based on observations that indicate that oxidative protein modifications are low and cellular antioxidant concentrations are abundant in oxidant-dependent pathologies (75). They note that electrophile-based events contribute to the cell signaling events as well and suggest an alternative paradigm should be a better model for these events. Part of the problem is the definition of antioxidants, a term that has been used to describe chemicals that reverse the effects of oxidants but should perhaps be defined chemically as reducing agents. However, there are substances such as dimethyl fumarate, not a chemical reducing agent but an electrophile, that reverse the effects of oxidants indirectly by modulating other cellular responses that mitigate the effects of oxidants. Alternatively, the antioxidants ascorbic acid and DA are both prooxidant in that they can catalyze the reduction of oxygen to its reactive reduction products and are therefore capable of inducing a state of oxidative stress. Thus, the simple terms anti- and prooxidant are not useful in understanding mechanisms.

Perhaps a more productive approach to addressing the cellular events, adverse or adaptive, associated with changes in the redox status of the cell would be to examine the products of the reactions. Many of the events associated with the cellular responses to chemical insults are mediated through thiolate functions on proteins that have been altered by hydrogen peroxide or by electrophiles. Thiols can be oxidized by hydrogen peroxide generated by normal biochemical processes or by the actions of a xenobiotic prooxidant using an available electron source such as ascorbate or NADPH. Electrophiles form covalent bonds with thiols by adding organic electrophiles or metal ions. The question of whether the response to a xenobiotic is adverse or adaptive then becomes one of identifying the target reaction products—do the offending agents differentially alter thiolates such as those of PTP, heat shock proteins, and Keap 1 and thereby dictate the overall cell response? For example, the rates of Michael addition reactions with the relevant thiols by a xenobiotic electrophile could differ in a way that one is favored over the other. Because hydrogen peroxide is a cell signaling molecule, its concentration is tightly regulated, and key to this regulation is its reaction at diffusion rates with peroxiredoxins (76). Accordingly, if its generation by a xenobiotic is involved in a cellular event, formation and reaction must be in close proximity. Elucidation of this aspect of thiol biochemistry will be critical to better understanding pathologies and their amelioration by thiol-modifying agents and will require studies involving concentration versus temporal or kinetic studies. Such studies were common in classical pharmacological research, but unfortunately this approach is not as common in current cell biological studies.

As I look back at the successes enjoyed by our laboratory and its members, I firmly believe that the major contributing factor to that productivity was the close interaction between chemists and biologists all examining the same research topic from the perspective of their scientific training,

with the data being quantitatively analyzed by the application of mathematical tools. We all learned from each other, and the final outcome was clearly synergistic, not additive. I further believe that the future of pharmacology and toxicology will require even more collaboration and interactions of the three disciplines, and I look forward to fostering this development. I am very optimistic that these interactions will occur, for there are now several journals with the term chemical biology in their titles to distinguish them from molecular biological journals (note the irony). These newer journals have papers describing the development of chemical tools to study cellular processes. These advances should also be included in this journal so that future pharmacologists can incorporate them into their research armamentaria.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The research described here was funded by the National Institute of General Medical Sciences, the National Institute of Mental Health, the National Institute on Drug Abuse, the US EPA, the California Air Resources Board, and the South Coast Air Quality Management District. I thank William Melega for his review and suggestions in the preparation of this article. I wish to express my gratitude to all the people who helped and educated me during my research career. Regretfully, several projects and their contributors were not included here; their exclusion was based purely on a need for brevity and continuity, not the value of the project or the contributors. I list the names of the laboratory members below.

In terms of staff, Barbara Hodshon was my first staff research associate (also my first MS graduate student) and came with me to UCLA from NIH; she set up the laboratory and left it, as Mrs. Gerald Miwa, in the capable hands of Cam Nguyen and then Emma Di Stefano and Debra Schmitz, who stayed with me through the thick and thin of funding and student and postdoc turnover. Emma, trained as a chemist, performed the synthetic procedures required for substrates and standards, and Debra, trained as a biologist, performed the biological procedures, including the tissue dissections required for our PK studies. All these staff members developed high-level analytical chemical skills that were the cornerstone of our research productivity. These are the people who provided postdocs and graduate students with an operational laboratory with all instruments in working order and with needed supplies. Critically, they also maintained the institutional memory of the lab, teaching new arrivals the basic procedures developed by their predecessors. Emma and Debra kept the lab operational during the later years of limited funding, performing experiments and maintaining research momentum. Bob Silverman maintained the GC/MS and other instrumentation of the department in outstanding working order and modified instruments for our needs. Ann Chang was a mathematician who taught me how to compute and who translated Don Jenden's differential equations into operational protocols. She is now the chief security information officer for UCLA Health Sciences.

For my graduate students, I tried to apply the lessons I learned from my mentors, which included the concept that students should develop their own research project. These students accepted that challenge enthusiastically, and as a result, they made substantial contributions to the outcome of our research: Lorene Abe-Waggaman, Teresa Y.Y. Chu, Susan D. Cushing, James B. Fischer, Joseph F. Fischer, Verena M. Florence, Barbara J. Hodshon, Sherrell G. Howard,

Andrew A. Lettes, Lena Y. Lin, Richard M. Matsumoto, Michael S. Maynard, Gerald T. Miwa, Nita Patel, Richard W. Ransom, Chester E. Rodriguez, Check Y. Sum, and Glenn S. Takimoto.

These postdocs brought their expertise to the laboratory and established its reputation: John F. Brady, Judith N. Burstyn, Sarmistra Chakrabarti, Mark W. Dudley, John D. Duncan, Arantza Eiguren-Fernandez, Jon M. Fukuto, Joseph Gal, Gosta Hallström, Masayuki Hiramatsu, John A. Jonsson, R. Craig Kammerer, Yoshito Kumagai, Björn Lindeke, James C. Schaeffer, Karen A. Wickham, and Yin Yu.

In the visiting scholars group, I include senior investigators who spent time in the laboratory, participating in the research and teaching: Yoshihiko Funae, Aline Lefol Nani Guarieiro, Akira Hiratsuka, Hiroshi Maruyama, Ettiene A. Mulliez, Erika M. Salinas, Yasuhiro Shinkai, Milan Stefak, Keiko Taguchi, Akihisa Toda, and Jeremy Wright.

Additionally, there were undergraduate students who joined for shorter time periods: William Chang, Joon Dokko, Mari Fujimoto, Marco Ishkandar, Amali Jayasinghe, Marco Mravic, Glen Nagami, Gail Shibata, and Chris H. Takimoto.

Finally, I want to thank my wife, Sachi, who put up with a frequently preoccupied husband, for her support and understanding, and my children, David (Christina Tan) and Nancy (Rick Smith), who provided me with encouragement, joy, and, more recently, a delightful group of grandchildren (Jordan, Garrett, Noelle, and Avery).

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