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# 1 Health Effects of Low-Level Exposure to Nerve Agents\*

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## I. INTRODUCTION

Nerve agents are highly toxic organophosphorous (OP) compounds that are chemically related to some insecticides (parathion, malathion). The four most common nerve agents are tabun (o-ethyl N,N-dimethyl phosphoramidocyanidate; military designation, GA), sarin (isopropyl methyl phosphonofluoridate; military designation, GB), soman (pinacolyl methyl phosphonofluoridate; military designation, GD), and VX (o-ethyl S-2-N,N-diisopropylaminoethyl methyl phosphonofluoridate). These compounds exist as colorless and relatively odorless liquids and are meant for use in weapon systems (shells, rockets, bombs) that are designed to deliver them as aerosols or fine sprays. They exert their toxic effects by inhibiting the cholinesterase (ChE) family of enzymes to include acetylcholinesterase (AChE; E.C.3.1.1.7), a critically important central nervous system (CNS) and peripheral nervous system (PNS) enzyme that hydrolyzes the neurotransmitter acetylcholine (ACh). Although the nerve agents can inhibit other esterases, their potency and specificity for inhibiting AChE account for their exceptionally high toxicity. For example, the rate constants for inhibition of AChE by soman, sarin, tabun, or VX are two to three orders of magnitude greater than for the more commonly known OP compounds such as DFP, paraoxon, or methylparaoxon.<sup>1</sup> Likewise, the rate constants for inhibition of AChE by

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\* The opinions or assertions contained herein are the private views of the authors, and are not to be construed as reflecting the view of the Department of the Army or the Department of Defense.

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the nerve agents are also two to five times greater than for trypsin (E.C.3.4.21.4), chymotrypsin (E.C.3.4.21.1), or carboxylesterase (E.C.3.1.1.1),<sup>2</sup> indicative of selective inhibition of this enzyme.

Nerve agents bind to the active site of the AChE enzymes, thus preventing them from hydrolyzing ACh. The enzyme is inhibited irreversibly, and the return of esterase activity depends on the synthesis of new enzyme (~1–3% per day in humans). All agents are highly lipophilic and readily penetrate the CNS. Acetylcholine is the neurotransmitter at the neuromuscular junction of skeletal muscle, the preganglionic nerves of the autonomic nervous system, the postganglionic parasympathetic nerves, as well as muscarinic and nicotinic cholinergic synapses within the CNS. Following nerve agent exposure and the inhibition of  $\geq 60\%$  of the AChE enzyme pool, levels of ACh rapidly increase at the various effector sites resulting in continuous overstimulation. It is this hyperstimulation of the cholinergic system at central and peripheral sites that leads to the toxic signs of poisoning with these compounds. The signs of poisoning include miosis (constriction of the pupils), increased tracheobronchial secretions, bronchial constriction, increased sweating, urinary and fecal incontinence, muscle fasciculations, tremor, and convulsions/seizures of CNS origin and loss of respiratory drive from the CNS. The relative prominence and severity of a given sign are highly dependent on the route and degree of exposure. Ocular and respiratory effects occur rapidly and are most prominent following vapor exposure, while localized sweating, muscle fasciculations, weakness, paralysis, and gastrointestinal disturbances are the predominant signs following percutaneous exposures and usually develop in a more protracted fashion. The acute lethal effects of the nerve agents are generally attributed to respiratory failure caused by a combination of effects at both central and peripheral levels and are further complicated by copious secretions, muscle fasciculations, and convulsions. There are several excellent reference sources that provide more detailed discussions of the history, chemistry, physiochemical properties, pharmacology and toxicology of nerve agents.<sup>3–7</sup>

Human estimates of nerve agent toxicity have been derived from animal studies. They range from 7  $\mu\text{g/kg}$  (VX) to 80  $\mu\text{g/kg}$  (tabun) as the  $\text{LD}_{50}$  for the i.v. route of administration,<sup>7</sup> while the percutaneous  $\text{LD}_{50}$  for tabun is estimated at 1000 mg, 1700 mg for sarin, 100 mg for soman, and 10 mg for VX, for a 70 kg person, respectively.<sup>4</sup> The rapid onset of effects and extreme toxicity have made these compounds eminently suitable for use as chemical warfare (CW) agents, and in some cases, many thousands of tons of these agents have been synthesized for military use. Exposure to lethal levels of nerve agents will produce toxicities that are precipitate in onset and catastrophic in effect.<sup>8</sup> For these reasons, major medical research efforts since the 1940s have focused on developing the best possible lifesaving therapeutic interventions, pretreatments, or, more recently, prevention of long-term changes in CNS function following a moderate to severe intoxication, using anticonvulsant drugs.<sup>9</sup>

Due to the focus on lifesaving interventions, it was not until the early 1980s that the question of chronic health effects of low-level exposure to nerve agents was

subjected to its first major review. The Committee on Toxicology, National Academy of Sciences, studied the available literature reports from the soldier-volunteer test program of the former Army Chemical Center at the then Edgewood Arsenal, now a part of Aberdeen Proving Ground, MD.<sup>10</sup> Soldier-volunteers participated in this test program from 1958 to 1975. There were 15 anticholinesterases (anti-ChE) tested on approximately 1400 subjects during this timeframe, with the great majority of anti-ChE agents being tested during the 1950s and 1960s.

The National Academy of Sciences review found that mortality data compiled in 1981 did not indicate increased deaths among soldier-volunteers when compared to comparable soldiers outside the testing program. There was no clear-cut indication of long-lasting CNS effects and no evidence for mutagenicity, carcinogenicity, male reproductive, or cataractogenic effects.<sup>10</sup> The National Academy of Sciences review committee also reported confidence that its analyses would have had the power to detect any major health effects, had they been present. In general, that viewpoint was considered to be “state-of-the-art,” with very little contention until the appearance of Persian Gulf War Illness in the early 1990s.

As a result of concern regarding a high incidence of undiagnosed illness among veterans of Operation Desert Shield/Storm, a Presidential Advisory Committee was formed to analyze the full range of the Federal Government’s outreach, medical care, research, and coordinating activities pertinent to Gulf War Veterans’ Illness (GWI). The Presidential Advisory Committee also looked at short- and long-term health effects of selected Gulf War risk factors, e.g., chemical/biological weapons, depleted uranium, infectious diseases, vaccines against potential biological warfare agents, pyridostigmine bromide, etc. The Presidential Advisory Committee gave specific and serious attention to the question of health effects of low-level exposure to nerve agents. Their conclusions could be summarized as follows:

1. Available scientific evidence does not indicate that long-term, subtle, neuropsychological and neurophysiological effects could occur in humans following low-level (asymptomatic) exposure.
2. The amount of data from either human or animal research on low-level exposures is minimal.
3. To close this gap in the current knowledge base, the Department of Defense was urged to support additional research on the long-term health effects of low-level exposures to CW agents, the nerve agents in particular.<sup>11</sup> Such an increased level of research has already been initiated, and some elements of it are discussed throughout this chapter.

Because of the great national interest, and perhaps because of technological advances in allowing public access to data, the current status of the federal portfolio of research in this area is readily available through the Internet. The current, annually updated summaries of progress in this research area of vital national interest can be found at <http://www.va.gov/resdev/>. This website is the Internet-based version of the Department of Veterans’ Affairs Annual Report to Congress on Federally Sponsored

Research on Gulf War Veterans' Illness for 1998. At this site, one can find an "Overview of the Federal Research Program" (see Appendix D). Among the long-term research recommendations of that overview are the following:

- Development of exposure biomarkers for CW agents
- Development of a strategic research plan for investigating the long-term health effects of exposure to low concentrations of CW agents. The author of the overview notes that these recommendations have been guiding the selection of new research projects since November 1996. The Annual Report to Congress for 1998 lists 10 research projects whose primary focus relates to CW agent exposure and health effects.<sup>12</sup>

Another impetus for renewed investigations in this area is based on the question, "Is the current United States military medical treatment doctrine, as well as physical protective measures (protective masks, clothing, and support systems), adequate to protect soldiers in future deployments from effects of exposure to low levels of CW agents?"<sup>13</sup>

Two recent reviews of the literature on potential long-term health effects from low-level exposure to nerve CW agents have presented slightly different analyses of this issue and, not surprisingly, they have reached slightly different conclusions. Brown and Brix<sup>14</sup> argued that for nearly all accidental or wartime exposures to nerve agents or OP compounds, it is difficult to obtain reliable exposure data. Thus, they argued that exposures could be characterized as high, intermediate, or low, depending upon factors such as intensity of cholinergic signs (e.g., rhinorrhea, salivation, neuromuscular effects, etc.), level of ChE inhibition, and type of medical treatment required. Clearly identified long-term effects have been noted at or above their defined Intermediate Level Exposure. Long-term health effects, according to Brown and Brix,<sup>14</sup> are not reported in individuals experiencing repeated low-level exposure alone.

In his brief review of chronic effects of low-level exposure to anticholinesterases, Roy<sup>15</sup> concluded that "Concerns about major adverse health effects of low-level exposure to anticholinesterases in general seem entirely unwarranted on the basis of currently available literature, but the data are at present insufficient to reflect the possibilities of subtle, agent-specific effects." In the section labeled "Chronic Health Effects of Repeated Low-Level Exposure," we will also review the scientific basis for these health concerns.

It is common practice for toxicologists to differentiate exposure to chemicals based on the dose and the duration of exposure. Four timeframes have been used to define duration of exposures: acute, subacute, subchronic, and chronic. It is useful in light of today's interest in "long-term, low-level" exposures to clarify these terms. Acute exposure is defined as exposure to a chemical for less than 24 h. Subacute exposure refers to an exposure of 1 month or less, subchronic for 1 to 3 months, and chronic for more than 3 months. These exposures can be by any route; for most chemicals it is the oral route with the chemical given in the diet.<sup>16</sup> However, the limited animal studies using nerve agents have usually employed parenteral administration

of the agent, and virtually all of them involve acute or subacute durations of exposure. All are intermittent, e.g., usually once a day. When referring to an inhalation exposure, the exposure duration most frequently used is 4h.

It is equally important to clearly define the term “low-level exposure.” This term has seen many different usages in the papers reviewed by these authors. These appear to range from any non-lethal exposure through “subtoxic” (defined by DeMenti<sup>17</sup> as no clinical signs) to “subclinical” (defined by DeMenti as no clinical signs and no significant depression of ChE). Exposure, then, is any contact with a chemical that may induce a biochemical effect. Each definition suffers from arbitrariness and we see no way around this. For the purposes of this review, we will attempt to characterize each paper in terms of presence/absence of either clinical signs or symptoms (in the case of human studies), and level and type of ChE inhibition.

## II. CHRONIC HEALTH EFFECTS OF ACUTE EXPOSURE

Much of the data regarding long-term neurological sequelae to exposures to cholinesterase inhibitors in man have been gathered following accidental exposures to organophosphate pesticides. While pertinent, extrapolation from these exposures to predictions of effects from nerve agents may be subject to risk. Several phenomena appear to differentiate nerve agent exposure from exposure to organophosphorus (OP) pesticides. These include:

1. The fact that the cholinergic crisis caused by acute, severe intoxication with the OP pesticides is generally much longer than that caused by OP nerve agents (days to weeks for pesticides vs. hours for nerve agents).
2. Many OP pesticides produce delayed peripheral neuropathy, a phenomenon known for more than 50 years, whereas nerve agents have caused polyneuropathy in animals only at doses manifold greater than the LD<sub>50</sub>—a phenomenon only seen in the presence of massive pretreatment and therapy with atropine and oxime.<sup>18</sup>
3. The “intermediate syndrome,” a delayed manifestation of OP poisoning seen in perhaps up to 100 accidentally poisoned patients,<sup>19</sup> has not been described after administration of nerve agents to animals, nor in the instances of nerve agent poisoning in man.<sup>20</sup>

Grob et al.<sup>21</sup> described the effects of acute to subacute short-term exposure of humans to DFP (1–2 mg, IM, daily for up to 7 days) on electroencephalographic (EEG) and psychological parameters. The changes produced by DFP included increases in EEG potential, frequency (especially noted was an increase in beta rhythm), more irregularities in rhythm, and by the intermittent appearance of abnormal waves similar to those seen in patients with grand mal epilepsy (high voltage waves of 3 to 6 Hz, usually most marked in frontal leads, and increased by hyperventilation). The CNS symptoms noted were excessive dreaming, insomnia, jitteriness and restlessness, increased tension, emotional lability, subjective tremulousness,

nightmares, headache, increased libido, giddiness, drowsiness, paresthesias, mental confusion, and tremor. The EEG changes usually followed the onset of CNS symptoms. CNS symptoms and EEG changes were correlated with the depression of red blood cell ChE to 70 and 60% of original activity, respectively. Central nervous system symptoms disappeared within 1 to 4 days after exposure was stopped, while the EEG changes persisted in a diminishing degree from 8 to 42 days (average of 29 days). Essentially similar CNS symptoms and EEG changes were described by Holmes and Gaon<sup>22</sup> as occurring acutely in OP-pesticide-exposed workers. They also noted that the more severely exposed individuals or those with multiple exposures, tended to display persistent symptoms that included forgetfulness, irritability, and confused thinking, although the duration of these persistent symptoms was never clearly defined.

These CNS symptoms and EEG changes are virtually identical to those that have been reported to occur following symptomatic exposure to different nerve agents. Grob and Harvey<sup>23,24</sup> described extensive studies of the effects of sarin in man, to include effects on ChE, EEG, and behavior. They noted behavioral and EEG effects virtually identical to those reported for DFP. These effects began coincident with the depression of plasma and red blood cell ChE activity to approximately 60 and 50% of original activity, respectively, following a single i.v. dose, or 34 and 22% of original activity, respectively, following oral administration. These differences between i.v. and oral administration of sarin suggest that the rate of ChE inhibition, and consequently the rate of increase in CNS ACh, are important factors in the development of symptoms of exposure. Bowers et al.<sup>25</sup> studied the effects of the nerve agent VX in man and described behavioral symptoms of anxiety, psychomotor depression, a general intellectual impairment consisting of difficulties in concentration and retention, and sleep impairments generally involving insomnia due to excessive dreaming.

Psychological/behavioral effects were typically evident before the occurrence of physical symptoms. These effects were associated with whole blood ChE inhibitions of > 60%. There have been descriptions of the acute toxic effects in humans that follow high-dose exposure ( $\geq$  LD<sub>50</sub>) to the nerve agents soman, sarin, and VX.<sup>8,26-29</sup> The same cluster of behavioral symptoms that are reported following lower doses (anxiety, psychomotor depression, intellectual impairment, sleep disturbances) dominate the clinical picture in the immediate period following resolution of the acute toxic signs of intoxication and then slowly fade with time, sometimes taking months to fully resolve.

There have been a number of investigations as to the possible long-term consequences of an acute symptomatic exposure to OP compounds. For the nerve agents, Burchfiel et al.<sup>30</sup> evaluated the long-term effects of an acute high dose (5  $\mu$ g/kg, i.v.) of sarin on the EEG of rhesus monkeys. The animals were paralyzed and artificially respiration during exposure since this dose of sarin produced generalized seizure activity on the EEG that lasted an average of 2.5 h. At both 24 h and 1 year following the exposure, there was a significant increase in the relative voltage in the beta frequency bands (13–22 Hz = beta-1; 22–50 Hz = beta-2) in the occipital-temporal EEG lead while the animals were awake in darkness. Similar EEG effects were seen in other animals in this study that were exposed to high doses of the chlorinated

hydrocarbon, dieldrin. Functional behavioral tests of other rhesus monkeys exposed to sarin under identical conditions revealed no deficits in performance of a previously learned delayed response test 24 h after the exposure.<sup>31</sup> Duffy et al.<sup>32</sup> performed a similar analysis of EEG of munitions workers accidentally exposed to the nerve agent sarin at doses that produced clinical signs and symptoms of exposure and produced a reduction of erythrocyte ChE at least 25% below the individual's pre-exposure baseline. Within the exposed group, there was a maximally exposed subgroup that had experienced three or more such exposures. The study was performed at least one year after the last exposure. Univariate and multivariate analysis of the data show that the exposed group, especially the maximally exposed subgroup, displayed:

1. Elevated amounts of spectral energy in high-frequency beta activity
2. Visual inspection of the EEG showed decreased amounts of alpha (9–12 Hz) activity along with increased amounts of slow activity (0–8 Hz, delta and theta) and an increased amount of “nonspecific” abnormalities in the EEG background.
3. Increased amounts of rapid eye movement (REM) sleep.

The functional consequences of these EEG changes were not established, but this group reportedly had a high incidence of self-reported memory disturbances, difficulty maintaining alertness and appropriate focusing of attention.<sup>33</sup>

Several studies of the long-term effects of the sarin-exposure victims from Japan have been published. Yokoyama et al.<sup>34,35</sup> evaluated 18 victims of the Tokyo subway incident 6 to 8 months after exposure. All but three of these victims had plasma ChE values below normal values on the day of exposure. Sarin-exposed individuals scored significantly lower than controls on a digit symbol substitution test; they scored significantly higher than controls on a general health questionnaire (GHQ; psychiatric symptoms) and a profile of mood states (POMS; fatigue). Additionally, they had elevated scores on a post-traumatic stress disorder (PTSD) checklist; they had significantly longer P300 latencies on event-related brain-evoked potentials and longer P100 latencies on brain visual-evoked potentials; and female exposed cases had significantly greater indexes of postural sway. The elevated scores on the GHQ and POMS were positively related to the increased PTSD scores and were considered to be due to PTSD. Nakajima et al.<sup>28,36</sup> performed a cohort study of victims of the Matsumoto City sarin exposure 1 and 3 years following the incident. At 1 year following the exposure, they report that 20 victims still felt some symptoms (fatigue, asthenopia, blurred vision, asthenia, shoulder stiffness, and husky voice), and they had lower erythrocyte ChE activity than those who did not have symptoms and had all lived close to the sarin release site. (Note: Not all the symptoms seen at 1 year have been related to nerve agent exposure historically.) At 3 years, some victims still complained of experiencing these symptoms, although with a reduced degree and frequency. There have been two brief reports of severely poisoned nerve agent victims (one sarin, one VX) in Japan who experienced retrograde amnesia, possibly due to prolonged periods of seizures and/or hypoxia.<sup>29,37</sup> Additionally, one of the Matsumoto victims who experienced prolonged seizure activity was followed for at least 1 year

and was found to have sporadic, sharp-wave complexes in the EEG during sleep and frequent premature ventricular contractions on Holter monitoring of the electrocardiogram.<sup>38</sup>

Finally, Yanno and Musiychuk published a short summary of 209 acute poisonings by sarin, soman, or VX in Russian nerve agent production facilities.<sup>39</sup> Twenty-eight percent of the victims required hospitalization that ranged from a few to 120 days. Long-term consequences of these exposures were described as memory loss, signs of asthenia, sleep disorders, diencephalic paroxysms, "vegetative changes in the cardiovascular system," and "microorganic disorders of the CNS" (not further defined in this paper). It was noted that CNS symptoms were most prominent and persistent following soman poisoning, confirming observations made by Sidell.<sup>8</sup>

In one of the major OP pesticide studies, Savage et al.<sup>40</sup> retrospectively (~9 years after the poisoning) examined 100 individuals with documented acute OP pesticide poisoning and compared them with matched-pair nonpoisoned controls. They reported no differences between the two groups in visually inspected EEG or a number of neurological tests. There were, however, significant differences between the two groups in their performance on a number of neuropsychological tests, as well as self- and family-assessment of functioning ratings. They stated that their results showed subtle, long-term neuropsychological sequelae to acute OP poisoning that are difficult to detect with standard neurological exams that stress sensory and motor function. Rosenstock et al.<sup>41</sup> performed a retrospective neuropsychological study of OP-poisoned agricultural workers and compared them with a matched control group. They found that when tested 2 years after exposure, poisoned workers self-reported significantly higher numbers of neuropsychological difficulties and had significantly lower test scores than controls on tests of verbal attention, visual memory, and visuomotor and motor functions, as well as tests of visuomotor sequencing and problem solving. Likewise, Steenland et al.<sup>42</sup> found deficits in vibrotactile sensitivity and sustained attention among previously intoxicated subjects vs. controls. These effects showed a rough dose-response relationship in that there were significant trends to worse performance on other neurobehavioral tests by those subjects who were more severely poisoned (longer hospitalization, took more time off from work). However, as with other studies, nerve conduction tests and neurological examinations were negative. There was no evidence of changes in postural sway in poisoned subjects as was reported for the sarin-exposed subjects from Tokyo indicating, perhaps, a difference between OP and nerve agents with regard to effects on motor activity.<sup>34,35</sup>

Studies in animals of long-term effects of acute, non-lethal exposures to nerve agents are numerous in the literature since 1980. Following high-dose exposure (~0.6 LD<sub>50</sub> and higher), seizures are a prominent sign of nerve agent intoxication, and these prolonged seizures can produce both neural and cardiovascular lesions if not promptly treated.<sup>43</sup> Neurological, behavioral and cardiac deficits are predictable long-term effects following exposure to such doses. Animals exposed to convulsant doses of nerve agent can develop spontaneous seizures, display hyperreactive and aggressive behavior (rats), and display profound deficits in learning and/or performance of a variety of behavioral tasks. In fact, animal studies have demonstrated deficits in acquisition of several types of operant tasks (differential reinforcement of



low rates, alternation), performance of serial probe recognition task, maze learning, and passive avoidance learning following acute intoxication with nerve agents.<sup>44–47</sup> Invariably, animals displaying such behavioral changes also are shown to have brain lesions in cortical and subcortical limbic structures. Exemplifying the relationships seen between such neuropathology and deficient behavior is the demonstration of EEG and performance changes following near-LD<sub>50</sub> challenge with soman (GD) in a comprehensive paper by Philippens et al.<sup>48</sup> In this study, rats were intoxicated with an LD<sub>50</sub> of soman and immediately treated with an antidotal combination of atropine and diazepam (described as a “low-dose combination”). These rats had previously trained to an over-learned criteria of 80% correct avoidance response (i.e., avoidance of a signaled foot shock). After a period of recovery of motor capacity, animals demonstrated impaired performance of the conditioned response for three test sessions before they approached the pre-challenge performance level. Similarly, electrographic correlates of lesions and ultimately, light microscopic observation of lesions, suggested the neuroanatomic basis for this deficit. By contrast, animals exposed to the same challenge dose of agent that received the “high-dose combination” of atropine and diazepam performed at or near the level of pre-challenge performance and were somewhat (but not completely) protected from electrographic and neuropathologic changes. This report demonstrates a general theme found in most of these “high-dose” exposure studies: animals exposed to nerve agents that develop seizures that are not promptly controlled, develop brain damage and consequent neurobehavioral problems; animals that do not develop seizures or those that seize and are rapidly and effectively treated with drugs that stop the seizures, suffer no brain lesions and display no long-term neurobehavioral deficits.

In the case of acute “low-level” exposures to nerve agents, exposures that produce minimal or no acute CNS signs of intoxication, an earlier study by Burchfiel et al.<sup>30</sup> suggested that a “clinically sign-free” dose of sarin (1 µg/kg, i.m.) given repeatedly (1/week for 10 weeks) to non-human primates (three rhesus monkeys) resulted in subtle, but persistent EEG changes (increases in the percentage of high frequency beta activity) that were virtually identical to those already described above that are seen after an acute high dose exposure (5 µg/kg, i.v.) that provoked seizures. More recently, Pearce et al.<sup>49</sup> followed behavioral and electrographic outcomes in nine marmoset monkeys for up to 15 months following exposure to a single, low dose of sarin (2.5–3 µg/kg, i.m.). Although the dose of sarin caused 36 to 67% inhibition of RBC AChE, there were no acute behavioral signs of intoxication (thus, the exposure was “subtoxic”), there was no significant change or decrement in performance on a series of touch screen mediated discrimination tasks either immediately or over a 12- to 15-month period following the exposure. There were also no significant long-term changes in EEG patterns in this study. Although there were changes in beta-2 amplitude that approached significance ( $p = 0.07$ ), this was entirely due to a long-term change in the EEG of a single subject of the nine animals that were exposed. The parameters chosen in this study were employed because they had been used to demonstrate deficits caused by cholinergic lesions or ChE inhibitors in previous studies.<sup>50,51</sup> Marrs et al.<sup>7</sup> have provided an extensive overview of human studies of nerve agent exposures conducted by the United States and United Kingdom military as well

as accidental exposures that occurred at production or test facilities. While this report does not come to any conclusions about long-term effects, there is no indication that asymptomatic exposures to nerve agents have produced long-term, adverse health effects. This is the same conclusion reached by the National Academy of Sciences committee that reviewed the then-available literature, to include the EEG studies of Burchfiel and Duffy.<sup>30,32</sup> They stated that while there may be subtle long-term EEG changes, the clinical significance and functional relevance of such changes had not been demonstrated.<sup>10</sup>

### **III. CHRONIC HEALTH EFFECTS OF REPEATED LOW-LEVEL EXPOSURE**

For chronic or repeated subclinical exposures to OP compounds, be they CW nerve agents or OP pesticides, the data in regards to long-term health effects are less consistent. In regards to the nerve agents, the report of Burchfiel et al.<sup>30</sup> about the effects of repeated low doses of sarin to rhesus monkeys producing a long-term increase in relative power in the EEG beta frequency bands is the most-cited study in support for a long-term health effect. There are no human studies known to the authors of this review other than the National Academy of Sciences report on the volunteer program mentioned earlier, that directly address the possible adverse, long-term health effects of repeated subclinical exposures to nerve agents.<sup>10</sup> Workers exposed to small amounts of nerve agents that produced mild, non-threatening medical signs of exposure, reported CNS effects such as headache, insomnia, excessive dreaming, restlessness, drowsiness, and weakness.<sup>52</sup> Medical officers describing these patients suggested that "Mental processes used in making judgments and decisions were also affected."<sup>52</sup> Of 53 patients with mild exposures not requiring antidotal therapy, CNS symptoms often were fully resolved within 3 days after exposure. However, Sidell and Hurst<sup>19</sup> caution that psychological symptoms are probably more common than usually recognized and may persist in more subtle forms for much longer (days, weeks) than physical symptoms.<sup>19</sup>

Reports in the literature of animal studies show that nerve agents can be administered repeatedly with minimal overt neurobehavioral effects if care is taken in choosing the dose and the time between doses.<sup>53,54</sup> Blood and brain AChE levels can be reduced to >20% of normal with no observable signs of toxicity with appropriate dosing schedules. Animal studies performed at the United States Air Force School of Aerospace Medicine have demonstrated a progressive and long-lasting inhibition of ChE in the CNS following repeated administration of low doses of the nerve agent soman,<sup>55</sup> a finding recently corroborated by Olson et al.<sup>56</sup> using the nerve agent sarin. There appear to be differential sensitivities among various brain regions, with frontal and piriform cortex being most sensitive to the ChE inhibiting effects of CW nerve agents, whereas the neostriatum and the hypothalamus are relatively less sensitive. These studies and others did not demonstrate a tolerance to the CNS ChE-inhibiting effects of repeated administration of low levels of CW nerve agents.

Recovery from the ChE inhibition produced by CW nerve agents or other OP compounds is not a simple matter, however. The recovery of CNS ChE does not

parallel the recovery of plasma ChE, with plasma ChE often recovering much more rapidly than RBC-AChE, which more closely parallels the recovery of brain AChE.<sup>57,58</sup> Thus, these and other tolerance studies suggest that behavior has often recovered to near baseline, while AChE is still significantly depressed.<sup>59,60</sup> This has been noted clinically and Sidell<sup>6</sup> cautions, "Analysis of blood for ChE is useful for occupational monitoring, but in an exposed patient, one treats the patient, not the ChE activity."

Recently, Olson and Benschop et al.<sup>56,61</sup> have provided reports of animal studies of effects of repeated low-level exposure to nerve CW agents. In rats, Olson determined the LOAEL and NOEL of subacute dosages of sarin, administered i.m. He found that the dose of sarin (GB) needed to produce a low but measurable blood ChE inhibition was 0.75  $\mu\text{g/kg}$  once a day for 4 days. Thus, the exposure in Olson's study would be described as "subclinical." GB was paired with a variety of other chemicals to include chlorpyrifos, DEET (N,N-diethyl-m-toluamide), carbaryl, and PB. No neurobehavioral or neuropathologic effects could be attributable to dosing with GB alone or in any combination with the other chemicals. Rats were also evaluated using a Functional Observational Battery and a Figure 8 Activity Monitor with no significant behavioral effects reported. Benschop et al.<sup>61</sup> reported on the toxicokinetics of low-level inhalation exposure to soman in atropinized guinea pigs. Animals were exposed to 200 ppb for 5 h of a toxic stereoisomer of soman, which resulted in a gradual inhibition of RBC-AChE to approximately 10% of baseline. This level of exposure resulted in an insignificant reduction of AChE activity in brain and diaphragm, although it was equivalent to a Ct value of 48  $\text{mg} \cdot \text{min}/\text{m}^3$ , a dose well above that sufficient to cause an incapacitating miosis. The observed lack of inhibition of AChE in brain and diaphragm at the end of the long-term, low-level exposure was interpreted to mean that systemic intoxication is unlikely despite extensive inhibition of blood AChE. Furthermore, Benschop et al. argued that the development of persistent neuropsychological disorders under these conditions would be unlikely. The authors cautioned that studies in animals without the benefit of carboxylesterase binding sites, such as primates, would most probably reflect a different outcome. This last study points out the influence of dose rate in determining whether a given exposure would be "nonlethal," "subtoxic," or "subclinical," a point made as long ago as 1975 by Sim.<sup>62</sup> The latter wrote that a patient appearing in a clinic without measurable ChE, yet not appearing to be intoxicated, "emphasizes that the poison is cumulative and if taken into the body slowly, can be accommodated without the appearance of critical illness."

The most notable effect of repeated low doses of nerve agents seen in animal experiments is the development of tolerance to the disruptive effects of each acute exposure on certain behaviors.<sup>58,60,63–65</sup> This is primarily thought to be brought on by downregulation (i.e., reduction in the number) of muscarinic receptors in the brain which will remain lowered (maximal reduction 30–40%) for the duration of the exposure and then recover in parallel with the recovery in erythrocyte ChE activity following the cessation of exposure.<sup>57,66</sup> During the period of reduction in muscarinic receptor numbers, the animals are subsensitive to anticholinesterases or direct acting muscarinic agonists and suprasensitive to the effects of antimuscarinic drugs

(e.g., atropine).<sup>67</sup> In this respect, nerve agents act much like other OP compounds and the possibility and mechanisms of tolerance development have been addressed in several studies (see the review by Russell and Overstreet<sup>68</sup> for an overview of these animal studies). The question of behavioral tolerance is seen by some authors as a masked toxicity in a vulnerable organism,<sup>17</sup> or as an adaptive response to a changed internal physiologic state.<sup>69</sup> Bignami et al.<sup>70</sup> have suggested that whereas some of the tolerance developed to OP may be attributable to cholinergic receptor changes, behavioral (test) variables may also play a role. These authors studied feeding and drinking responses and examined the role of practice factors in tolerance to paraoxon. Specifically, they measured the decrease in food consumption and the strength of a conditioned flavor aversion (CFA) produced by repeated doses of low levels of paraoxon. They qualified their finding of reductions in the depression of food intake or extinction of CFA by stating that treatment-behavior interactions may produce apparent attenuations of toxicity which are often not maintained when the situation is changed, leaving entirely open the nature of the purported response.

No review of subacute, subchronic, or chronic toxicity of chemical warfare nerve agents would be complete without discussion of the significant paper by Munro et al.<sup>71</sup> that reviewed both animal and human studies of the nerve agents tabun (GA), sarin (GB), and VX. These studies included subacute, subchronic, and chronic toxicity studies in animals. Special attention was paid to the phenomenon of Organophosphorus-Induced Delayed Neuropathy (OPIDN). Reproductive toxicity and carcinogenicity tests were reviewed as well as *in vitro* studies of mutagenicity. Munro et al.'s findings can be summarized as follows:

1. For the nerve agent GA, no evidence of subchronic toxicity was observed at any dose other than effects on ChE activity. No evidence of teratogenicity was found and GA was a weakly active mutagen.
2. For nerve agent GB, no evidence of acute or chronic toxicity was found at low intermittent exposure levels, sufficient to significantly depress AChE levels. No evidence for carcinogenicity, teratogenicity, or mutagenicity was found for GB, but a data gap in the area of reproductive toxicity was noted.
3. For nerve agent VX, no evidence for likely development of OPIDN was found. VX exposure sufficient to significantly depress RBC-AChE activity produced no subchronic toxicity, no evidence of carcinogenicity was found, and based on multiple studies, VX was not considered a potential mutagen or a teratogen.

Thus, the authors concluded that the overriding concern with regard to exposure to GA, GB, or VX was their extraordinarily high acute toxicity.<sup>71</sup>

For chronic or repeated subclinical exposures to OP pesticides, there are numerous studies of humans that have addressed this issue and there is no common consensus among the results. Korsak and Sato<sup>72</sup> reported that OP pesticide workers with relatively high occupational levels of exposure to OP pesticides, in comparison with workers with low levels of exposure, tended to have increased EEG power within the

beta frequencies, primarily in frontal areas of the brain. In addition, the high exposure level group had lower performance on a Trail-Making Test and the Bender Visual Motor Gestalt test of a neuropsychological test battery. The authors suggested that these results indicated subtle frontal lobe dysfunction in the exposed subjects. Stephens et al.<sup>73</sup> studied a population of 146 sheep dippers with an average of 15 years of potential exposure to several OP (diazinon, propetamphos, chlorfenvinphos) and found that these individuals compared to a control group (quarry workers) has slower simple reaction time latencies, slower latencies in a symbol-digit substitution test, and slower correct reaction times in a syntactic reasoning test. Only the syntactic reasoning effect showed a significant dose-effect relationship when analyzed with an analysis of covariance. Tests of memory and learning showed no effect and there were no self-reported drops in intellectual performance. On the other hand, Ames et al.<sup>74</sup> surveyed 45 pesticide applicators, each of whom had at least one documented episode of asymptomatic OPP exposures. He reported no CNS or PNS effects. Rodnitsky et al.<sup>75</sup> evaluated 23 workers chronically exposed to a mild degree to pesticides (farmers and commercial pesticide applicators) using a battery of neurobehavioral tests. They found no differences between the exposed vs. a control group on tests of memory, signal processing, vigilance, language, and proprioceptive feedback performance even though plasma cholinesterase levels of the exposed group were depressed below the control values. Similarly, Daniell et al.<sup>76</sup> studied 49 pesticide applicators over a 7-month period of pesticide spraying. Performance of the cohort was compared to a control group of 40 subjects (slaughterhouse workers). Both groups were given a computerized neuropsychological test battery. The test battery consisted of visuomotor coordination tests, memory and cognition tests, and tests of motor coordination. The pesticide applicators were known to have generally well-controlled, low, intermittent exposure, as part of a program of occupational health training and monitoring. The authors found no evidence for clinically significant decrements in neuropsychological performance following one 7-month season of such exposure among pesticide applicators, the main one being Guthion (azinphosmethyl). Maizlish et al.<sup>77</sup> examined 99 pest control workers (46 exposed workers vs. a group of non-applicators). The 46 workers applied diazinon to residential lawn properties. Both applicators and non-applicators were monitored for the appearance of diethylthiophosphate (DETP) in their urine. Subjects were given a comprehensive neurobehavioral test battery before and after their 8-h work shift, as well as having urine samples taken. The application season lasted 39 days. The following tests were included in the neurobehavioral battery: Continuous Performance test (measures attention/vigilance), Finger Tapping (motor speed), Digit Symbol Substitution (visual/motor speed), etc. Median diazinon exposure per workday for applicators vs. non-applicators was 2.1 mg vs. 0.03 mg, respectively. No adverse DETP-related changes were found in pre- or post-shift neurobehavioral function. The authors concluded that there were no demonstrable behavioral effects of short-term, low-level diazinon exposure in a pest control program characterized by adequate personal protective equipment and direct supervision.<sup>77</sup>

More recently Bazylewicz-Walczak et al.<sup>78</sup> published their study of greenhouse workers occupationally exposed to pesticides. They gave greenhouse workers and a

matched control group neuropsychological tests (simple reaction time, digit symbol, digit span, Benton visual retention test, Santa Ana test, aiming test, profile of mood states), and a subjective symptom questionnaire before and then 4 months later after the heaviest period of OP pesticide application. Overall, when compared to the controls (kitchen workers and administrative workers), the exposed subjects showed slower simple reaction times, lower hand movement efficiency on the aiming test, and reported a higher degree of anxiety, anger, depression, and fatigue-inertia. World Health Organization guidelines were used to characterize the level of exposure of these experimental groups and it was considered to be low. In addition they also reported more complaints relating to absent-mindedness and neurological symptoms. There were no differences in the exposed group over the one season. There was no change or improvement in scores on the neuropsychological tests across the season and there was an improvement in mood and general feeling scores between the pre-season test and the post-season test. The authors concluded that even low, long-term OP pesticide exposure may be associated with subtle adverse behavioral effects, characterized by increased tension and anxiety states, depression, fatigue, and a slow-down of perceptual-motor functions.

#### **IV. THE CONTRIBUTIONS OF *IN VITRO* STUDIES TO DETERMINE HEALTH EFFECTS OF LOW-LEVEL EXPOSURE**

By their nature, *in vitro* studies of the physiological effects of nerve agents tend to involve acute or short, subacute exposures. Isolated tissues and organ systems have a limited viability that limits the durations of nerve agent studies, as does the stability of an agent under physiological conditions. Even cultures of isolated cells have a limited useful lifetime *in vitro* although this can approach months under optimal conditions. However, the ability to dissect complicated phenomena into simpler processes *in vitro* often provides a unique opportunity to examine putative mechanisms of nerve agent toxicity. A critical examination of the range of nerve agent concentrations (rather than doses) that evoke these *in vitro* pathologies can then be used to assess the relative involvement of the mechanisms in nerve agent pathology.

Many of the earliest *in vitro* studies of nerve agent toxicity involved isolated smooth or striated muscle tissue and relatively high concentration of nerve CW agents. Under these conditions the actions of the nerve agents as inhibitors of AChE in the muscle tissues could be clearly discerned and related to the degree of AChE inhibition. Smooth muscle, with a predictable response to enhanced stimulation of muscarinic receptors, produced enhanced and sustained contractions in response to physiologic stimulation at relatively low concentrations and spontaneous contractions at high concentrations.<sup>79,80</sup> The actions of the nerve agents on skeletal muscle were more varied but clearly involved initial stimulation of neuromuscular transmission followed rapidly by desensitization of the postsynaptic nicotinic receptors during the course of physiologic tetanic stimulations, eventually leading to paralysis.<sup>81-85</sup> However, not all effects of organophosphorus nerve CW agents were completely

consistent with the degree of AChE inhibition.<sup>86,87</sup> These observations led to studies of direct interactions between nerve CW agents and various cholinergic receptors.

One direct receptor effect of various organophosphate cholinesterase inhibitors noted *in vitro* was an apparent increase of the desensitization rate for nicotinic receptors at the skeletal neuromuscular junction.<sup>88,89</sup> Since this is a major factor in skeletal muscle failure subsequent to AChE inhibition and subsequent accumulation of endogenous ACh, such a direct action of the nerve CW agent on the nicotinic receptor would be expected to be synergistic with its actions on AChE. Separation of these phenomena was initially accomplished by comparing rates of desensitization in *in vitro* preparations in which the presence or absence of receptor agonists was controlled and where the effects of AChE inhibition were masked by prior irreversible inhibition of the enzyme. Later studies have used a variety of techniques, including direct measurement of receptor activity without AChE present,<sup>90</sup> or with ligand competition binding studies as indicators of receptor state.<sup>91</sup> Although a variety of nerve CW agents are capable of enhancing desensitization in skeletal muscle receptors, they do so with different mechanisms. VX appears to bind to the open form of the nicotinic receptor ion channel, leading to a block of ion flow and subsequent receptor desensitization,<sup>90,92</sup> while GB and GD appear to bind to an allosteric site on the nicotinic receptor that stabilizes the desensitized form of the receptor.<sup>90,91</sup> A consistent observation of this phenomenon, regardless of agent or mechanism, was that the concentrations of nerve CW agent needed to produce a significant increase in the rate of nicotinic receptor desensitization were equal to or higher than those needed for complete inhibition of AChE activity, with concentrations in the micromolar range often required to show the effect. Clearly, these direct effects on skeletal muscle do not represent low-dose effects of nerve CW agent.

Similar phenomena have been noted for effects of nerve CW agents in ganglionic nicotinic receptor systems although the origins of these effects on transmission may be different and more complicated than in the skeletal muscle endplate. In mammalian superior cervical ganglia, the nerve CW agents produce an initial enhancement of transmission followed by depression of the postsynaptic response with repetitive stimulation.<sup>93,94</sup> However, unlike the endplate, these effects do not appear to follow from desensitization of the postsynaptic nicotinic receptors alone since, in both mammalian and amphibian ganglia, there was no evidence of desensitization to exogenous cholinergic agonists.<sup>94,95</sup> The frequency-dependent decrease in ganglionic transmission has been attributed variously to presynaptic inhibition of release mediated by muscarinic autoreceptors or by cumulative and tonic depolarization of postsynaptic neurons due to endogenous ACh build-up.<sup>94,95</sup> The relative contributions of these phenomena may be dependent upon the source and properties of the ganglia studied. However, as with the direct effects on the skeletal neuromuscular junction, the concentrations of nerve CW agents, or other organophosphorus AChE inhibitors, responsible for depression of ganglionic transmission are at least as high, 0.1 to 100 micromolar if not higher, than those required for nearly complete AChE inhibition.

As indicated in the ganglionic effects of nerve CW agents, a number of mammalian synapses have presynaptic muscarinic autoreceptors that can modulate release of acetylcholine.<sup>94</sup> The concept of presynaptic receptors that can modulate evoked

release of other classes of neurotransmitters suggests that organophosphorus nerve agents could have actions on cholinergic systems that would be expressed primarily in noncholinergic systems. Such interactions could occur either through inhibition of AChE and accumulation of extracellular ACh, or via direct actions of organophosphorus nerve agents on cholinergic membrane targets. Such phenomena are most readily observed in the central nervous system, where the organizational and pharmacological complexity of synaptic connections is substantially greater than in skeletal muscle. Since *in vitro* techniques to study function in such central systems have only recently become common, some of the details of such interactions are only now becoming apparent.

Studies in brain slices have indicated that presynaptic muscarinic receptors are involved in reductions of evoked transmitter release in dopaminergic, GABAergic, and glutaminergic synapses.<sup>96,97</sup> The effects on GABAergic neurotransmitter release were particularly potent with a small depression seen at 10 picomolar VX.<sup>97</sup> Although these presynaptic effects could be prevented with the general antagonist atropine, specific binding studies suggest that the effects are mediated via the M3 class of muscarinic receptors.<sup>96,98</sup> Studies in cloned cell lines have suggested that activation of these muscarinic receptors and the resulting activation of phospholipase C can occur with normal agonists at the orthosteric binding site and with certain organophosphorus anticholinesterases acting at an allosteric modulation site. The organophosphate anticholinesterases do not compete with the standard muscarinic ligands QNB or N-methyl scopolamine at the orthosteric site, normally occupied by agonists and competitive antagonists, except at micromolar concentrations in acute assays.<sup>99–102</sup> However, exposure of the muscarinic receptors for longer periods causes a non-competitive reduction in muscarinic receptor ligand binding that reaches a maximum at 24 h.<sup>100,103</sup> Unlike competitive reactions, the concentrations of organophosphorus anticholinesterases causing the allosteric modulation were in the picomolar to nanomolar range.<sup>99,104</sup> Further, use of an unconventional ligand, *cis*-methyldiololane, to examine a subset of muscarinic binding sites also indicated competition with many organophosphates, including the nerve CW agents, at picomolar to nanomolar concentrations.<sup>99,104</sup> The loss of orthosteric binding sites in response to either muscarinic agonists or anticholinesterases did not result in a corresponding loss of phospholipase C activity. Rather, IP<sub>3</sub> production remained activated while the receptor was in a “sequestered” state.<sup>103,105</sup> The concentrations of organophosphorus nerve agents involved in the allosteric modulation of muscarinic receptors, and, hence, possibly involved in modulation of synaptic transmitter release, are substantially less than those required for AChE inhibition and suggest that the effects may be observed at low-doses *in vivo*, particularly with prolonged acute or subacute exposures.

Other effects of nerve CW agents on presynaptic events have been identified *in vitro* that do not involve muscarinic receptor binding. Nanomolar concentrations of VX and micromolar concentrations of GD greatly enhance spontaneous release of both GABA and glutamate from rat hippocampal brain slice synapses.<sup>97</sup> A similar phenomenon was observed in glutaminergic synapses in the insect, where micromolar VX caused bursts of activity,<sup>92,106</sup> and in amphibian sympathetic ganglia, where repetitive firing of neurons was observed subsequent to a nerve CW agent-induced reduction in



a calcium-activated potassium after hypolarization.<sup>107</sup> These cellular effects are not mediated through muscarinic receptors. They appear to be direct actions upon ion channels in the cells. However, except for the effects of VX on spontaneous miniature postsynaptic potentials in the hippocampus, these effects are seen only at concentrations of nerve CW agent that would cause profound inhibition of AChE.

A potentially subtle role for AChE inhibition in affecting synaptic transmission in the central nervous system is suggested by the differential sensitivity of different neuronal nicotinic acetylcholine receptors. Neuronal nicotinic receptors include the  $\alpha 7$  and the  $\alpha 4\beta 2$  classes.<sup>108</sup> The  $\alpha 7$  class can be found presynaptically on both glutaminergic and GABAergic neurons where it enhances spontaneous and evoked transmitter release.<sup>109–111</sup> Similar functions can be ascribed to the  $\alpha 4\beta 2$  receptors, although their time course, particularly for desensitization, is slower. Significantly, the  $\alpha 7$  receptors are activated by both acetylcholine and choline with similar sensitivity while the  $\alpha 4\beta 2$  receptors are activated only by acetylcholine. This suggests that a decrease in the concentration of choline present due to AChE inhibition with nerve CW agents could alter the glutaminergic or GABAergic tone in regulated synapses. The exact result of the inhibition of AChE would depend upon a number of factors, including the number of each neuronal nicotinic receptor type present, the transmitter normally released by the presynaptic terminal, and the ambient level of ACh present in the tissue. These effects would be dependent upon concentrations of the nerve CW agents that would inhibit a substantial fraction of the available AChE in the tissue. Hence, the differential activity of the neuronal nicotinic receptors would be expected to be significant only at concentrations that would provoke serious symptoms of acute anticholinergic toxicity.

Of the various mechanisms of action ascribed to nerve CW agents in various *in vitro* model systems, most appear to be active at concentrations where significant inhibition of AChE would be expected to occur. These conditions are unlikely to be seen in the absence of recognizable cholinergic toxicity and hence would not be classified as a low-dose effect. The one effect that seems to occur at a sufficiently low concentration of organophosphorus anticholinesterase to be considered a low-dose phenomenon is the slow allosteric modulation of muscarinic receptors regulating presynaptic release of other neurotransmitters. The observation that this allosteric modulation requires several hours to develop suggests that this mechanism would most likely be applicable to a subacute or subchronic exposure rather than one seen after a brief, acute dose.

## V. SUMMARY AND CONCLUSIONS

We believe that studies to determine the potential long-term psychologic/neurologic sequelae following repeated low-level exposures to OP are confounded by factors such as low response rates, possible selection and follow-up biases (which is certainly the case for nerve CW agent), compensatory psychological response, possible co-exposures and the like. Although the recent national investment into additional research has emphasized animal research, we are hopeful that a more comprehensive assessment of

this problem is unfolding. However, the significance of and the biochemical basis for chronic health effects resulting from high-level, acute exposure should not be overlooked with all the current emphasis on repeated low-level exposure.

## ACKNOWLEDGMENTS

The authors wish to thank Mrs. Patricia Little for her skillful editorial assistance and persistence in coordinating the contributions of the four authors of this chapter.

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