

Withdrawal/Redaction Sheet

Clinton Library

DOCUMENT NO. AND TYPE	SUBJECT/TITLE	DATE	RESTRICTION
--------------------------	---------------	------	-------------

001. memo	Elisa D. Harris to Samuel R. Berger re: non-selection [partial] (2 pages)	03/07/1995	b(6)
-----------	---	------------	------

COLLECTION:

Clinton Presidential Records
First Lady's Office
Melanne Verveer
OA/Box Number: 20033

FOLDER TITLE:

Gulf War Illness - Gulf War Illnesses

2013-0534-S

rc1867

RESTRICTION CODES

Presidential Records Act - [44 U.S.C. 2204(a)]

- P1 National Security Classified Information [(a)(1) of the PRA]
- P2 Relating to the appointment to Federal office [(a)(2) of the PRA]
- P3 Release would violate a Federal statute [(a)(3) of the PRA]
- P4 Release would disclose trade secrets or confidential commercial or financial information [(a)(4) of the PRA]
- P5 Release would disclose confidential advice between the President and his advisors, or between such advisors [(a)(5) of the PRA]
- P6 Release would constitute a clearly unwarranted invasion of personal privacy [(a)(6) of the PRA]

C. Closed in accordance with restrictions contained in donor's deed of gift.

PRM. Personal record misfile defined in accordance with 44 U.S.C. 2201(3).

RR. Document will be reviewed upon request.

Freedom of Information Act - [5 U.S.C. 552(b)]

- b(1) National security classified information [(b)(1) of the FOIA]
- b(2) Release would disclose internal personnel rules and practices of an agency [(b)(2) of the FOIA]
- b(3) Release would violate a Federal statute [(b)(3) of the FOIA]
- b(4) Release would disclose trade secrets or confidential or financial information [(b)(4) of the FOIA]
- b(6) Release would constitute a clearly unwarranted invasion of personal privacy [(b)(6) of the FOIA]
- b(7) Release would disclose information compiled for law enforcement purposes [(b)(7) of the FOIA]
- b(8) Release would disclose information concerning the regulation of financial institutions [(b)(8) of the FOIA]
- b(9) Release would disclose geological or geophysical information concerning wells [(b)(9) of the FOIA]

PHOTOCOPY
PRESERVATION

GULF WAR ILLNESSES

Gulf War

NATIONAL SECURITY COUNCIL

GULF WAR ILLNESSES

DIRECTORATE

NATIONAL SECURITY COUNCIL

17th & Penn, N.W.
Washington, D.C.
20504

Did you get a complete,
clear transmission? If not,
please call:

(202) 456-9391

FAX 456-9390

From: Phil Heyl

**To: Thurgood Marshall Jr. & Ann McGulre; Jack
Gibbons & Cliff Gabriel; Melanne Verveer & Nicole
Rabner**

Agency: EOP

Fax Number: 62983/66704/66021/66027/66244/

Date/Time: 11 Jul 97/1655

No. of pages to follow: 5

Subject: Draft GWI Package to POTUS

**Please clear by 2:00 PM Monday, 14 July. Have a
nice weekend.**

Phil

DRAFT

July 11, 1997

ACTION

MEMORANDUM FOR SAMUEL R. BERGER
JACK GIBBONS
THURGOOD MARSHALL, JR.
MELANNE VERVEER

FROM: PHILLIP J. HEYL

SUBJECT: Recent Criticism of Efforts Relating to Gulf War
Illnesses (GWI) and Discussion of Next Steps

Attached as Tab I is the proposed response to the President's comment on the June 21, 1997 Shenon article in the New York Times. We also address the Chief of Staff's concern as to whether GWI issues are being handled appropriately.

The article's criticisms of the government's effort (that inadequate emphasis has been placed on chemical warfare agents and the interactive effects of multiple chemical exposure as possible causes for undiagnosed illnesses) are off-target. The Presidential Advisory Committee on Gulf War Veterans' Illnesses (PAC) recommended in December 1996 that new research should be directed in these two areas -- and projects are under way. Accordingly, the GAO report raising these criticisms received forceful responses from the PAC, DOD and VA. The PAC has also told us that they intend to respond to the critical letter drafted by Rep. Sanders referred to in the Shenon article. Congressional interest remains strong -- the House held hearings on the GAO report -- although there was little press interest.

You should also note that the proposed memorandum for the President takes this opportunity to brief him on the next public announcement -- the Khamisiyah demolition results. We are coordinating this announcement and troop notification carefully with the agencies in order to ensure that we have a clear message and that we reinforce public confidence in the Administration's process.

Concurrence by: Tony Blinken; Bill Danvers; David Johnson;
Randy Beers

RECOMMENDATION

That you sign and forward the information memorandum to the President at Tab I.

Attachment

Tab I Memorandum to the President

Tab A June 21, 1997 New York Times article (annotated)

DRAFT

DRAFT

INFORMATION

MEMORANDUM FOR THE PRESIDENT

FROM: SAMUEL R. BERGER
JACK GIBBONS
THURGOOD MARSHALL, JR.
MELANNE VERVEER

SUBJECT: Recent Criticism of Efforts Relating to Gulf War
Illnesses (GWI) and Discussion of Next Steps

Criticism

Philip Shenon's June 21st New York Times article, attached as Tab A, reports on recent claims that Administration GWI efforts continue to devote inadequate consideration to two possible sources of the illnesses reported by many Gulf War veterans. A June 20th letter, drafted by Congressman Bernard Sanders and signed by 86 other Members, criticizes your Presidential Advisory Committee on Gulf War Veterans' Illnesses (PAC) for downplaying risk factors other than stress and calls for the PAC to reconsider the interactive effects of deployed troops' routine exposure to multiple chemicals. In addition, a recent GAO report contains more general criticisms of the Administration's research and clinical programs and calls specifically for further investigation into possible exposure to chemical warfare (CW) agents released as the result of coalition air strikes and post-war demolition.

Response

We believe these criticisms of your Administration's efforts are off-target. Following its comprehensive survey of the peer-reviewed scientific literature, the PAC did find that stress is likely to be "an important contributing factor" to the broad range of GWI reported by Gulf veterans. However, the PAC also concluded that given the significant scientific unknowns remaining, new research should be directed at the principal uncertainties, specifically including the "long-term health effects of low-level exposure to chemical weapons" and "interactions between pyridostigmine bromide and other agents."

cc: Vice President
Chief of Staff

DRAFT

DRAFT

As a result, the PAC's point-by-point rebuttal to the GAO criticisms emphasizes the degree to which its previous findings remain valid. The PAC will respond to the Sanders letter separately. DOD and VA also prepared detailed responses to the GAO report. The bottom line is that despite earlier missteps -- which the PAC has been instrumental in identifying -- we are now devoting substantial resources to thoroughly pursue all important GWI risk factors, including CW exposure and multiple chemical interaction. DOD, HHS and VA are now funding more than \$9.5 million in research on these issues alone, and have requested proposals for another \$17 million in additional related work.

Next Steps

In communicating our efforts with the veteran community, we have focused on enhancing the government's credibility through commitment to a thorough process, rather than any pre-determined conclusions. During the upcoming months, we will release the results of a number of investigations and inspectors-general inquiries. Given the history of Congress' involvement in the GWI issue, we can also expect congressional interest in the future, although fewer headlines came out of recent House and Senate hearings on the GAO report than anticipated. We will continue to work with former Senator Rudman to ensure that the relevant committees receive the most complete and accurate information on GWI efforts. While not all of what is uncovered will be "good news," our demonstrated openness will help fulfill your pledge to "leave no stone unturned" and will underline your commitment to uncovering the truth -- no matter what it is.

The next significant public announcement will occur at the end of this month, when DOD and CIA present their joint determination of the specific military units likely to have been exposed to any quantity of CW agents. You should be aware that the total number of soldiers who received a limited duration exposure to low levels of CW agent is likely to be substantially greater than the 21,000 soldiers notified last summer following the first Khamisiyah announcement. This increase has been anticipated for some time, since it was publicly discussed at the PAC's March 18th public hearing in Salt Lake City. We are working intensively with DOD, VA and HHS to ensure that this announcement, which is the last in a four-part program of briefings reviewing the operational and meteorological conditions surrounding the demolition at Khamisiyah, is presented in a way that reinforces confidence in our current approach and, given the very low levels of chemicals involved, does not needlessly alarm veterans.

It was in large part to oversee the agencies' response to its recommendations regarding the government's GWI efforts that you

DRAFT

DRAFT
3

decided in January to extend the PAC's charter through October of this year. In that capacity, the PAC has continued to hold public hearings and effectively spur the agencies toward getting out all of the facts about GWI, including those concerning CW exposure and multiple chemical interaction. We will continue to ensure that the DOD, HHS and VA research and investigative efforts are thorough, that their results are quickly incorporated in improved health care programs, and that these initiatives are effectively communicated to veterans and the public at large.

Attachment

Tab A June 21, 1997 New York Times article (annotated)

DRAFT

FOR OFFICIAL USE ONLY

4571

THE WHITE HOUSE

WASHINGTON

INFORMATION

MEMORANDUM FOR THE PRESIDENT

FROM: SAMUEL R. BERGER
JACK GIBBONS
THURGOOD MARSHALL, JR.
MELANNE VERVEER

SUBJECT: Recent Criticism of Efforts Relating to Gulf War
Illnesses (GWI) and Discussion of Next Steps

Criticism

Philip Shenon's June 21st New York Times article, attached as Tab A, reports on recent claims that Administration GWI efforts continue to devote inadequate consideration to two possible causes of the illnesses reported by many Gulf War veterans. A June 20th letter, drafted by Congressman Bernard Sanders and signed by 86 other Members, criticizes your Presidential Advisory Committee on Gulf War Veterans' Illnesses (PAC) for downplaying risk factors other than stress and calls for the PAC to reconsider the interactive effect of deployed troops' routine exposure to multiple chemicals. A recent GAO report contains more general criticisms of the Administration's research and clinical programs and calls specifically for further investigation into possible exposure to chemical warfare (CW) agents released as the result of coalition air strikes and post-war demolition.

Response

We believe these criticisms of your Administration's efforts are off-target. Following its comprehensive survey of the peer-reviewed scientific literature, the PAC did find that stress is likely to be "an important contributing factor" to the broad range of GWI reported by Gulf veterans. However, the PAC also concluded that given the significant scientific uncertainties remaining, new research should be directed at the "long-term health effects of low-level exposure to chemical weapons" and "interactions between pyridostigmine bromide and other agents."

As a result, the PAC's point-by-point rebuttal to the GAO criticisms emphasizes the degree to which its previous findings

cc: Vice President
Chief of Staff

FOR OFFICIAL USE ONLY

2

remain valid. The PAC will respond to the Sanders letter separately. DOD and VA also provided detailed responses to the GAO report. These forceful responses helped to limit the media fallout from both the GAO report and related congressional hearings. The bottom line is that despite earlier missteps -- which the PAC was instrumental in identifying -- we are now devoting substantial resources to thoroughly pursue all important GWI risk factors, including CW exposure and multiple chemical interaction. DOD, HHS and VA are now funding more than \$9.5 million in research on these issues alone, and have requested proposals for another \$17 million in additional related work.

Next Steps

In communicating our efforts with the veteran community, we have focused on enhancing the government's credibility through commitment to a thorough process, rather than any predetermined conclusions. During the upcoming months, we will release the results of a number of investigations and inspectors-general inquiries. The next significant public announcement will occur later this month, when DOD and CIA present their joint determination of the specific military units likely to have been exposed to CW agents. *You should be aware that the total number of soldiers who received a limited duration exposure to low levels of CW agent is likely to be substantially greater than the 21,000 soldiers notified last year following the first Khamisiyah modeling announcement.* This increase has been anticipated for some time, since it was publicly discussed at the PAC's March public hearing in Salt Lake City. We are working intensively with DOD, VA and HHS to ensure that this announcement is presented in a way that reinforces confidence in our current approach and, given the very low levels of chemicals involved, does not needlessly alarm veterans.

It was in large part to oversee the agencies' response to its recommendations regarding the government's GWI efforts that you decided in January to extend the PAC's charter through October of this year. In that capacity, the PAC continues to hold public hearings and effectively spur the agencies toward getting out all of the facts about GWI, including those concerning CW exposure and multiple chemical interaction. We will continue to ensure that the DOD, VA and HHS research and investigative efforts are thorough, that their results are quickly incorporated in improved health care programs, and that these initiatives are effectively communicated to veterans and the public at large.

Given the history of Congress' involvement in the GWI problem, we can expect congressional interest in the future. DOD and CIA will work with former Senator Rudman to ensure that the relevant committees receive the most complete and accurate information on



FOR OFFICIAL USE ONLY

3

GWJ efforts. While not all of what is uncovered will be "good news," our demonstrated openness will help fulfill your pledge to "leave no stone unturned."

Attachment

Tab A June 21, 1997 New York Times article (annotated)

Internet Laws Overturned In New York and Georgia

Curbs on Smut and Anonymity Are Blocked

By ROBERT D. McFADDEN

In separate but related cases that could shape the future of the Internet, Federal judges in Manhattan and Atlanta ruled yesterday that the States of New York and Georgia could not enforce new laws intended to protect children from computer-network peepholes and to bar anonymous cyberspace communications.

In the New York case, Judge Loreta A. Preska held that a 1996 law making it a criminal offense to send pornographic materials to minors over the Internet violated a clause of the United States Constitution that prohibits states from unduly interfering in the commerce and activities of people in other states.

The judge ordered Gov. George E. Pataki and Attorney General Dennis C. Vacco, the defendants — and by extension all district attorneys in New York — not to prosecute anyone under the law, noting in a 63-page ruling that was a virtual primer on the Internet that users in California, Oregon, Oklahoma or anywhere might have run afoul of a law they did not even know about.

"The Internet is one of those areas of commerce that must be marked off as a national preserve to protect users from inconsistent legislation that, taken to its most extreme, could paralyze development of the Internet altogether," Judge Preska said, adding: "Only Congress can legislate in this area."

In the Georgia case, Judge Marvin Shoob blocked the enforcement of a recent state law making it a criminal offense to communicate anonymously or to use a pseudonym over the Internet, saying that the statute violated the free-speech protections of the First Amendment.

The decisions were the first by the Federal courts to limit growing state efforts to regulate the content of the Internet, a global network of computers that is used by more than 40 million people and is expected to grow to more than 100 million by 1998. The state laws were held to be well-intentioned but unconstitutional and injunctions were granted to bar any criminal prosecutions. Officials in both states said they were considering appeals.

The rulings were hailed by civil liberties groups, library and book-seller associations, publishers and others who were plaintiffs in the cases, who use the Internet to display and disseminate a wide range of communications, and who might have been exposed to prosecutions under the laws that were challenged.

"We believe this is a historic decision," said Norman Siegel, executive director of the New York Civil Liberties Union, which helped represent the plaintiffs in the New York case. "It is a huge win for the people of the State of New York. It vindicates what we've been telling Governor Pataki and the State Legislature — that they could not and should not censor the Internet."

Ann Beason, a national staff attorney for the American Civil Liberties Union, who was an attorney for the

plaintiffs in both cases, also praised the rulings as a victory for Internet users. "Together, these cases send the powerful message that states cannot constitutionally regulate speech on the Internet."

Mr. Beason said the New York law was "almost identical" to the Federal Communications Decency Act, which makes it a Federal crime to display "patently offensive," sexually explicit material over the Internet in a manner available to children. The United States Supreme Court is expected to rule soon on the constitutionality of that law.

Attorneys General Vacco of New York and Thurbert Baker of Georgia declined substantive comment on the rulings, saying they were under study and that appeals would be considered. A spokesman for Mr. Vacco, Marc Carey, called the New York law "a reasonable balance between First Amendment free-speech rights and protecting children against pornography on the Internet."

Signed by Governor Pataki last Nov. 1, the law made it a crime to

A Federal judge compares the Internet to interstate commerce.

send "indecent" materials to minors by E-mail or other direct means, although pornographic material posted on the World Wide Web or other locations for general viewing were not covered.

But it also outlawed generally posted Web sites or chat groups that made deliberate, specific efforts to lure children into viewing pornography. The law imposed prison terms of up to four years for anyone convicted of sending sexually explicit material to children on the Internet, and up to seven years for those convicted of making sexual advances to children on computer networks.

On-line services, electronic publishers, other Internet users and civil liberties groups filed suit soon after the law became official, and, pending the outcome, no one was prosecuted, officials said. The suit contended that the law violated the First Amendment free-speech rights of Internet users, and the so-called Commerce Clause of the Constitution, which was written by the framers to prevent overreaching by individual states that might jeopardize the growth of the nation and might interfere in its communications and trade.

The state, in response, argued that the law had been aimed solely at conduct within New York's borders, and had been narrowly drawn to criminalize only those who tried to harm children, and not to interfere with freedom of speech.

The New York Times

SATURDAY, JUNE 21, 1997

Lawmakers Ask White House To Revisit Gulf War Illnesses

By PHILIP SHENON

WASHINGTON, June 20 — Eighty-six members of the House today urged a special White House panel of experts to reverse itself and conclude that Iraqi chemical weapons were responsible for some of the health problems reported by veterans of the 1991 Persian Gulf war.

The lawmakers, responding to the draft of a new Government report that linked Iraqi nerve gas with the sores of illnesses seen among the veterans, said in a letter to the White House committee that "the evidence is clear that exposure to a wide variety of chemicals in the Persian Gulf may be a significant factor in Persian Gulf illness."

The panel, the Presidential Advisory Committee on Gulf War Veterans' Illnesses, concluded earlier this year that Iraqi chemical weapons were probably not responsible for the veterans' health problems, and that it was far more likely the ailments

were linked to wartime stress. Other G.A.O. efforts, and that "the errors of commission and omission are so serious that the publication of this draft in its current form would do a disservice to the Congress and President's efforts to address gulf war veterans' illnesses."

The Defense Department acknowledged last year that thousands of American troops may have been exposed to chemical weapons after the demolition of an Iraqi ammunition depot. But Pentagon officials have been reluctant to link their exposure to veterans' health problems.

"I hate to condemn these people," Mr. Sanders said of the White House committee, which includes prominent scientists and doctors. "But it's what happens in politics. They are backing themselves into a corner. And I think that's a very unfortunate."

The House letter was signed by lawmakers from both parties. "This is absolutely a bipartisan issue," Mr. Sanders said.

A Pentagon spokesman, Bryan Whitman, said lawmakers were wrong to suggest that the Defense Department was not aggressively seeking an answer to the health problems of veterans.

"We're embarked on a very extensive research program," Mr. Whitman said. "We take very seriously the reports of chemical detections."

The House letter was released as three senior Democratic Senators called on the accounting office to open a new investigation of American military doctrine on how to deal with low-level exposure to chemical weapons of the sort American troops may have faced in the gulf war.

Two Senators — Carl Levin of Michigan, ranking Democrat on the Armed Services Committee; Robert C. Byrd of West Virginia, ranking Democrat on the Appropriations Committee; and John Glenn of Ohio, ranking Democrat on the Governmental Affairs Committee — said in a letter to the accounting office that they were concerned "that U.S. military doctrine has not changed to reflect the lessons learned from the gulf war experience."

"We believe that the most effective course of action is to prevent the exposures or combination exposures from occurring," the Senators said.

James J. Tuite Jr., a former Senate investigator who is now the director of the Gulf War Research Foundation, said a new investigation was needed because "the Pentagon's steadfast denial of a cause and effect relationship between exposures and illness had resulted in policies and doctrine that continue to place our active duty forces in harm's way."

Capitol Hill skeptics of a Presidential panel's conclusions.

were linked to wartime stress. But the panel's findings have been challenged in recent weeks, most significantly by the General Accounting Office, the investigative arm of Congress, which is expected to release a report next week that will harshly criticize the committee.

A draft of the report said there was "substantial evidence" linking nerve gas to the sores of health problems seen among the veterans.

The author of the House letter, Representative Bernard Sanders, independent of Vermont, said today that the White House panel might have overlooked the possibility that chemical weapons had caused the veterans' illnesses because the committee depended on the Pentagon and the Department of Veterans Affairs "for a lot of its information."

The panel's chairman, Joyce C. Leach, the former dean of the public health school at the University of California at Berkeley, said the committee members stood by their findings and that their conclusions were reached "after an extensive review of the peer-reviewed literature — we looked at all of these issues."

In a response this month to the General Accounting Office, Dr. Leach said the draft report was "not

EB/Valus
We need to be
cautious in this
way. We need to
offer things
for
for

Copied
Bowles
Comanual.

JUN 30 6:22

6/26 Sandy -
I think anything we need to
make sure this is all
handled absolutely appropriate
The president wants to
make sure we don't
negotiate
K

file Grey war

Halley
PB - anti nerve gas
chemicals - pesticides

negligent
not to do
what
they did

single illness - w/ 3 variants

due to brain damage - minimal
low brain & "Can't tell you what
it is"
Special
card

[probably due to exposure
to combination of chemicals]

we think stress did affect longer
but went away.

{ no gd test to distinguish guys
who have this & not some
other malady

{ no gd treatment

Self reported risk factors are
legitimate because it's at
epidemic proportions

2

PHOTOCOPY
PRESERVATION

Clinton Presidential Records Digital Records Marker

This is not a presidential record. This is used as an administrative marker by the William J. Clinton Presidential Library Staff.

This marker identifies the place of a publication.

Publications have not been scanned in their entirety for the purpose of digitization. To see the full publication please search online or visit the Clinton Presidential Library's Research Room.

Original Contributions

Is There a Gulf War Syndrome?

Searching for Syndromes by Factor Analysis of Symptoms

Robert W. Haley, MD; Thomas L. Kurt, MD, MPH; Jim Hom, PhD

Objective.—To search for syndromes in Persian Gulf War veterans.**Participants.**—Two hundred forty-nine (41%) of the 606 Gulf War veterans of the Twenty-fourth Reserve Naval Mobile Construction Battalion living in 5 southeastern states participated; 145 (58%) had retired from service, and the rest were still serving in the battalion.**Design.**—Participants completed a standardized survey booklet measuring the anatomical distributions or characteristics of each symptom, a booklet measuring wartime exposures, and a standard psychological personality assessment inventory. Two-stage factor analysis was used to disentangle ambiguous symptoms and identify syndromes.**Main Outcome Measures.**—Factor analysis—derived syndromes.**Results.**—Of 249 participants, 175 (70%) reported having had serious health problems that most attributed to the war, and 74 (30%) reported no serious health problems. Principal factor analysis yielded 6 syndrome factors, explaining 71% of the variance. Dichotomized syndrome indicators identified the syndromes in 63 veterans (25%). Syndromes 1 ("impaired cognition," characterized by problems with attention, memory, and reasoning, as well as insomnia, depression, daytime sleepiness, and headaches), 2 ("confusion-ataxia," characterized by problems with thinking, disorientation, balance disturbances, vertigo, and impotence), and 3 ("arthro-myo-neuropathy," characterized by joint and muscle pains, muscle fatigue, difficulty lifting, and extremity paresthesias) represented strongly clustered symptoms; whereas, syndromes 4 ("phobia-apraxia"), 5 ("fever-adenopathy"), and 6 ("weakness-incontinence") involved weaker clustering and mostly overlapped syndromes 2 and 3. Veterans with syndrome 2 were 12.5 times (95% confidence interval, 3.5-44.8) more likely to be unemployed than those with no health problems. A psychological profile, found in 48.4% of those with the syndromes, differed from posttraumatic stress disorder, depression, somatoform disorder, and malingering.**Conclusion.**—These findings support the hypothesis that clusters of symptoms of many Gulf War veterans represent discrete factor analysis—derived syndromes that appear to reflect a spectrum of neurologic injury involving the central, peripheral, and autonomic nervous systems.

JAMA. 1997;277:215-222

FIVE YEARS after the Persian Gulf War, an estimated 5000 to 80 000 of the approximately 700 000 Gulf War veterans remain ill with vague symptoms that resemble chronic fatigue syndrome, but defy diagnosis.¹⁻³ Although some veterans

appear to be neurologically impaired, the array of symptoms in complaining veterans appears too broad to fit any single syndrome.^{2,6} The diversity of complaints has led many to believe that they are due either to intercurrent illnesses that would have occurred without participation in the war, or that they are psychologically based, possibly from posttraumatic stress disorder (PTSD), somatoform disorder, depression, malingering, or "compensation neurosis."^{7,9}

See also pp 223, 231, 238, and 259.

After reviewing registry reports of approximately 2000 ill veterans, the medical records of those who appeared most

ill, and reports of likely wartime environmental exposures, we hypothesized that small subsets of veterans with discrete syndromes lay hidden amid the larger number of veterans with intercurrent and psychologic illnesses, and that these might be due to neurotoxic exposures. To test this hypothesis, we designed a survey instrument for measuring symptoms in sufficient detail to apply factor analysis for detecting clusters that might represent hidden syndromes. We applied the survey instrument in both ill and well, active and retired veterans⁴ of a reserve construction battalion, because members of construction battalions, in contrast to combat units, traveled throughout the military theater of operations, and members of reserve units, in contrast to active-duty units, live in a defined geographical region accessible to survey even after retirement. To our knowledge, this report is the first to attempt to define syndromes by factor analysis of symptoms in both ill and well Gulf War veterans, including those remaining on active duty and those who have retired.

METHODS

We conducted an epidemiologic survey in both ill and well, active and retired members of the Twenty-fourth Reserve Naval Mobile Construction Battalion (RNMCB-24), called to active duty for the Persian Gulf War and known to have a high prevalence of postwar illness (S.W. Berg, et al, unpublished military report). In November 1994 we attempted to contact all 606 of the reserve battalion's Gulf War veterans living in Alabama, Georgia, Tennessee, South Carolina, or North Carolina (NMCB-24 Alpha Roster 29MAR91, official in-theater battalion roster). We sent 2 personal letters and made telephone calls to 429 for whom a presumptive address and telephone number were available; these measures directly reached approximately 350 of the target group. Each contact emphasized the importance of participation by those not ill as well as those with illness. Attempts to notify the approximately 250 Gulf War veterans whom we were unable to contact

From the Epidemiology Division, Department of Internal Medicine (Drs Haley and Kurt), and the Department of Neurology (Dr Hom), University of Texas Southwestern Medical Center at Dallas, and The Neuropsychology Center, Dallas, Tex (Dr Hom).

Presented at the Intergovernmental Coordinating Board for the Gulf War Illness and to the staff of the Presidential Advisory Committee on Gulf War Veterans' Illnesses, Washington, DC, September 16, 1995, and at a research conference on the Gulf War illness, Centers for Disease Control and Prevention, Atlanta, Ga, October 26, 1995.

Corresponding author: Robert W. Haley, MD, Epidemiology Division, Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75235-8874.

Increased Neurotoxicity Following Concurrent Exposure to Pyridostigmine Bromide, DEET, and Chlorpyrifos

MOHAMED B. ABOU-DONIA,*¹ KENNETH R. WILMARTH,*² ALI A. ABDEL-RAHMAN,* KARL F. JENSEN,†
FREDERICK W. OEHME,‡ AND THOMAS L. KURT§

*Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710; †Neurotoxicology Division, U.S. EPA, Research Triangle Park, North Carolina 27711; ‡Comparative Toxicology Laboratories, Department of Clinical Sciences, Kansas State University, Manhattan, Kansas 66506-5605; and §Department of Internal Medicine, University of Texas, Southwestern Medical School, Dallas, Texas 75235

Received January 19, 1996; accepted August 20, 1996

Increased Neurotoxicity Following Concurrent Exposure to Pyridostigmine Bromide, DEET, and Chlorpyrifos. ABOU-DONIA, M. B., WILMARTH, K. R., ABDEL-RAHMAN, A. A., JENSEN, K. F., OEHME, F. W., AND KURT, T. L. (1996). *Fundam. Appl. Toxicol.* 34, 201-222.

The operating environment of the service personnel during the Persian Gulf War involved psychological, biological, and chemical elements including exposure to pesticides such as the insect repellent DEET (*N,N*-diethyl-*m*-toluamide) and the insecticide chlorpyrifos (*O,O*-diethyl *O*-3,5,6-trichloropyridinyl phosphorothioate) and to pyridostigmine bromide (PB, 3-dimethylaminocarbonyloxy-*N*-methylpyridinium bromide) that was administered as a prophylactic agent against possible nerve gas attack. The present study was designed to determine the toxicity produced by individual or coexposure of hens 5 days/week for 2 months to 5 mg PB/kg/day in water, by gavage; 500 mg DEET/kg/day, neat, sc; and 10 mg chlorpyrifos kg/day in corn oil, sc. Coexposure to various binary treatments produced greater neurotoxicity than that caused by individual exposures and was characterized by severe neurologic deficit and neuropathological alterations. Also, neurotoxicity was further enhanced following concurrent administration of the three chemicals. Severe inhibition of plasma butyrylcholinesterase (BuChE) activity was produced in hens treated with PB (activity 17% of control) compared to those treated with chlorpyrifos (activity 51% of control) or DEET (activity 83% of control). BuChE inhibition was further increased in binary and tertiary treatment groups compared to individual treatment groups. In contrast, a significant inhibition of brain acetylcholinesterase (AChE) was produced in hens administered chlorpyrifos alone (activity 67% of control), while those given chlorpyrifos in combination with other compounds exhibited a significant inhibition of brain AChE activity ranging from 43 to 76%. Brain neurotoxicity target esterase (NTE) was not inhibited in any of the individual treatment groups or PB/DEET, but was significantly inhibited and had activity expressed as a percentage of control in groups administered combined chlorpyrifos with PB of 73% or DEET of 74% and in the tertiary treatment group of 71%. We hypothesize that test com-

pounds may compete for xenobiotic metabolizing enzymes in the liver and blood and may also compromise the integrity of the blood-brain barrier, leading to an increase in their "effective concentrations" in the nervous system to levels equivalent to the toxic doses of individual compounds. This is consistent with the present observation of increases in (1) the inhibition of brain AChE and NTE, (2) the extent of neurologic dysfunction, and (3) the severity and frequency of neuropathologic lesions in the combined treatment groups compared to those administered individual compounds. © 1996 Society of Toxicology.

Between the invasion of Kuwait by Iraq on August 2, 1990, and March 1991, the United States had 697,000 military personnel in the Persian Gulf region (Institute Of Medicine, 1995). Among the troops, women accounted for 7% of the force, while Reserve/National Guard personnel were 17%. Since their return, approximately 30,000 veterans have had unexplained complaints including chronic fatigue, muscle and joint pain, ataxia, rash, headache, difficulty concentrating, forgetfulness, and irritability. Approximately half of the veterans with complaints have been Reservists/National Guard. Among more than 100,000 non-U.S. coalition forces, including Canadian, Saudi, French, Egyptian, Syrian, Moroccan, and British Troops, only several hundred British and Canadian veterans complained of similar illnesses (Persian Gulf Veterans Coordinating Board, 1995).

The Persian Gulf War veterans were exposed in the Gulf area to a unique combination of biological, chemical, and psychological environments. In addition to extreme climatic changes, other exposures may have included sand and dirt, fumes and smoke from military operations, oil well spills and fires, petroleum products, diesel exhaust, depleted uranium, lead, multiple immunizations, and pesticides, e.g., the insect repellent DEET (*N,N*-diethyl-*m*-toluamide) and the insecticide chlorpyrifos (*O,O*-diethyl *O*-3,5,6-trichloropyridinyl phosphorothioate) (Fig. 1; Institute Of Medicine, 1995). Furthermore, all U.S., British, and Canadian military personnel were given, at the beginning of the war, 21 30-mg tablets

¹ To whom correspondence should be addressed.

² Present address: ENVIRON Corp., Arlington, VA 22203.

TABLE 1
Summary of the Number of Treated Hens That Died or Were Euthanized during the Study and at Termination and the Number of Animals Used for Neurotoxicity and Histopathological Studies^a

Group	No. of animals that died before the termination of the study	No. of animals euthanized before the termination of the study	No. of animals euthanized at the termination of the study	No. of animals used for neurochemistry	No. of animals used for histopathological examination
Control	0	0	5	5	5
Pyridostigmine bromide (PB)	0	0	5	5	4
DEET	0	0	5	5	4
Chlorpyrifos (CPF)	0	0	5	5	4
PB + DEET	2 (38, 41) ^b	0	4	4	4
DEET + CPF	3 (15, 17, 20) ^b	2 (20, 24) ^b	2	4	3
PB + CPF	1 (17) ^b	0	4	4	3
PB + DEET + CPF	3 (12, 19, 32) ^b	2 (34, 41) ^b	3	5	5

^a Hens were given a daily dose, 5 days/week, for 2 months, individual or combinations of 5 mg PB/kg in water by gavage, 500 mg DEET/kg sc, or 10 mg CPF/kg in corn oil sc.

^b Days after beginning of dosing when hens died or were euthanized.

of pyridostigmine bromide (PB) to protect against organophosphorus nerve gases (Persian Gulf Veterans Coordinating Board, 1995). The troops were to self-administer one PB tablet every 8 hr when the risk of chemical attack was imminent. In the light of the safety record of PB during drug development (Teichman *et al.*, 1987) and of the fact that its oral therapeutic dose for myasthenia gravis is in the range 200–1400 mg a day (Breyer-Pfaff *et al.*, 1985), it was surprising that up to 50% of those seen in health care facilities during the Gulf War complained of PB muscarinic side effects (Institute Of Medicine, 1995; Keeler *et al.*, 1991; Cook and Kolka, 1992).

The prophylactic action of PB against organophosphorus nerve gases is based on its reversible inhibition of 30–40% of acetylcholinesterase (AChE) activity in the peripheral nervous system, thus shielding it from long-lasting inactivation by the nerve gas (Blick *et al.*, 1991). PB, a dimethylcarbamate, has a half-life of elimination of 0.35 hr following a single oral dose of 60 mg in human volunteers (Eiermann *et al.*, 1993).

The aromatic amide DEET is used as a personal insect repellent (Pressley and Longbottom, 1982). Reported human

poisonings following dermal application of DEET include two deaths (Gryboski *et al.*, 1961; Zadikoff, 1979; Edwards and Johnson, 1987). Symptoms of DEET toxicity include tremor, restlessness, slurred speech, seizures, impaired cognitive functions, and coma (McConnell *et al.*, 1986). Neuro-pathologic lesions in rats treated with near-lethal doses of DEET are characterized by spongiform myelinopathy in the brainstem (Verschoyle *et al.*, 1990).

Chlorpyrifos, a phosphorothioate insecticide, undergoes first-pass metabolism to chlorpyrifos oxon (Abdel-Rahman *et al.*, 1993), which inhibits rat brain AChE *in vitro* with a bimolecular rate constant (k_i , $M^{-1} min^{-1}$) of 3.18×10^6 compared to 3.22×10^3 for the parent compound (Huff *et al.*, 1994). Chlorpyrifos toxicity results in muscarinic, nicotinic, and central nervous system symptoms (Abou-Donia, 1994). In addition to its acute neurotoxicity, near-lethal doses of chlorpyrifos produce organophosphorus compound-induced delayed neurotoxicity (OPIDN). Recently, Kaplan *et al.*, (1993) have reported sensory neuropathy associated with environmental exposure to chlorpyrifos. Following a suicide attempt with chlorpyrifos, OPIDN was reported in a 42-year-old man (Lotti *et al.*, 1986). Chlorpyrifos at

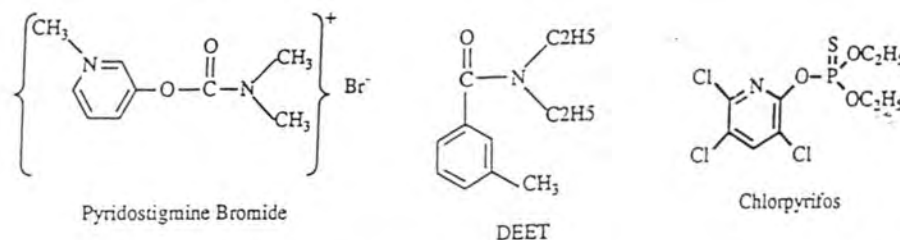


FIG. 1. Chemical structure of test compounds.

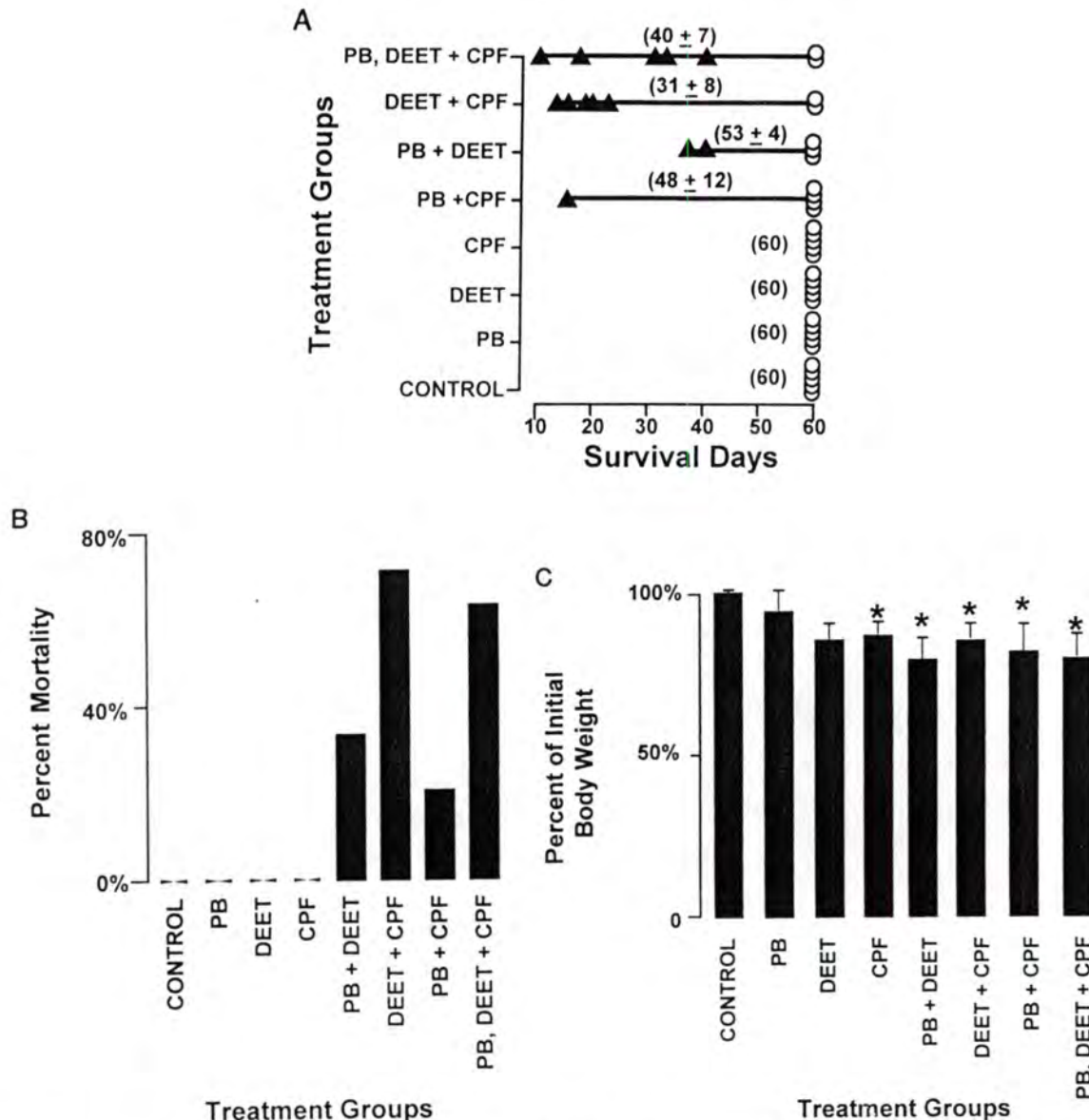


FIG. 2. Effect of daily administration of pyridostigmine bromide (PB), DEET, and chlorpyrifos (CPF) on survival and body weight of treated hens. Hens were dosed daily, 5 days/week, for 2 months with individual compounds or combinations of 5 mg/kg PB in water, po; 500 mg/kg DEET neat, sc; or 10 mg/kg CPF in oil, sc. (A) Number of days hens survived. Solid triangles represent hens euthanized before the termination of study because of severe morbidity. Open circles represent animals euthanized at the termination of the study. The mean and standard deviation of the number of survival days for hens in each treatment group are shown in parentheses. (B) Percentage mortality by treatment group. (C) Percentage of initial body weight at time of euthanization. Bars represent the mean and standard error from each treatment group ($n = 5$).

doses equivalent to 2–3 $\times$ LD₅₀ produced OPIDN in the hen (Capodicasa *et al.*, 1991; Abou-Donia and Wilmarth, 1995) and the cat (Fikes *et al.*, 1992). OPIDN is characterized by a delayed and protracted development of ataxia and paralysis accompanied by a Wallerian-type degeneration of the central and peripheral nervous systems (Smith *et al.*, 1930; Abou-Donia, 1981).

Our hypothesis that some of the Persian Gulf War veterans' illnesses may have resulted from concurrent exposure

to PB and other chemicals such as pesticides was prompted by two observations: (1) failure to identify bacterial, viral, or parasitic diseases as a source of the veterans' complaints; (2) concurrent exposure to multiple chemicals has been shown to cause increased toxicity of single compounds. For example, the nonneurotoxic chemical *iso*-butyl ketone increased the neurotoxicity of the weak neurotoxicant *n*-hexane (Lapadula *et al.*, 1991) and of EPN (*O*-ethyl 0–4-nitrophenyl phenylphosphonothioate)-induced delayed neuro-

TABLE 2
Clinical Condition and Neuropathological Changes in Hens Following Daily Administration of Individual and Coexposure of Pyridostigmine Bromide (PB), DEET, and Chlorpyrifos (CPF) in Hens^a

Treatment ^b	Clinical signs ^c		Locomotor dysfunction ^c		Histopathological changes ^d	
	Day of onset	Severity	Day of onset	Severity	Spinal cord	Sciatic nerve
Control	— ^e	— ^e	— ^e	— ^e	— ^e	— ^e
PB	12.8 ± 1.4	1.0 ± 0.0	— ^e	— ^e	— ^e	— ^e
DEET	9.4 ± 1.4	1.0 ± 0.0	— ^e	— ^e	— ^e	0.5 ± 0.3
CPF	16.4 ± 3.5	1.0 ± 0.0	— ^e	— ^e	1.0 ± 0.0	0.8 ± 0.2
PB + DEET	5.2 ± 1.4	1.8 ± 0.4	16.0 ± 4.6	1.0 ± 0.3	0.8 ± 0.2	1.0 ± 0.0
DEET + CPF	8.2 ± 1.2	1.6 ± 0.2	20.4 ± 3.8	1.4 ± 0.2	2.0 ± 0.0	1.0 ± 0.0
PB + CPF	3.4 ± 0.5	2.4 ± 0.3	20.4 ± 2.1	1.6 ± 0.2	2.0 ± 0.0	1.7 ± 0.3
PB + DEET + CPF	2.8 ± 0.2	2.6 ± 0.2	17.6 ± 2.9	2.6 ± 0.4	2.8 ± 0.2	1.2 ± 0.2

^a All values are means ± SEM from each treatment group.

^b Hens were given a daily dose, 5 days/week, individual or combinations of 5 mg/kg PB in water po, 500 mg/kg sc DEET, and 10 mg/kg sc CPF.

^c Animals were assessed daily for clinical signs, locomotor dysfunctions, and tremor and scored as indicated under Methods. Severity scores indicate the condition of the animal at the time of euthanization.

^d Severity of histopathological alterations was determined by examination of the cervical and thoracic spinal cord and sciatic nerve tissues.

^e Not different from control.

toxicity (Abou-Donia *et al.*, 1991). Also, propetamphos (1-methylethyl (E)-3-[[[(ethylamino) methoxyphosphinothioyl]-oxy]-2-butenate), an organophosphorus insecticide that does not produce OPIDN, decreased the threshold dose of chlorpyrifos that causes OPIDN (Abou-Donia and Wilmarth, 1995). Consistent with this view is the recommendation of the committee to review the Health Consequences of Service During the Persian Gulf War that "studies are needed to resolve uncertainties about whether PB, DEET, and permethrin have additive effects" (Institute of Medicine, 1995). In fact, we have demonstrated in a previous study that coexposure to these three chemicals resulted in increased neurotoxicity in the adult hen (Abou-Donia *et al.*, 1996).

The present study was carried out concurrently with the above-mentioned companion study to investigate if coexposure to PB, DEET, and chlorpyrifos enhances neurotoxicity compared to exposure to individual compounds.

METHODS

Chemicals

Technical grade (94%) chlorpyrifos (*O,O*-diethyl *O*-3,5,6-trichlorpyridinyl phosphorothioate) was obtained from DowElanco Co. (Indianapolis, IN). DEET (≥97%, *N,N*-diethyl-*m*-toluamide), pyridostigmine bromide (≥99%, 3-dimethylaminocarbonyloxy-*N*-methylpyridinium bromide), acetylthiocholine iodide, and butyrylthiocholine iodide were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade and obtained from commercially available sources.

Birds

The adult hen was used in this study to allow the investigation of both acute and OPIDN induced by exposure to chlorpyrifos alone and in combination with other chemicals. Although the clinical condition of the Gulf

War veterans may not be identical to that of OPIDN, it may have resulted from superimposition of several conditions including OPIDN because of the potential exposure to organophosphorus compounds.

Healthy adult laying hens, 18 months old, weighing approximately 1.5 kg, were obtained from Featherdown Farm (Raleigh, NC). The hens were vaccinated against common chicken diseases and were considered specific pathogen-free, without abnormalities of gait. Control and treated hens were randomly assigned into groups of five hens and housed in single-tier, 3 × 3 × 3-foot stainless-steel cages in a temperature (21–22°C)-controlled room with a 12-hr light, 12-hr dark cycle 1 week before dosing and throughout the study. The hens had a supply of feed (Layena Chicken Feed; Ralston, Purina Co., St. Louis, MO) and water *ad libitum*.

Dosing

An initial dose-response study was conducted to select the dose used in single and combined treatments. This dose was determined as the maximum dose that produced minimum changes in the clinical parameters following treatment for 5 days/week over a 2-month period that demonstrated the safety of test compounds at relatively large doses. The individual test compound study was carried out using the following dosages for 5 days/week for 2 months: PB, 5 mg/kg/day in water by gavage; DEET, 500 mg/kg/day, neat, sc; chlorpyrifos, 10 mg/kg/day, in corn oil, sc. A group of five hens was given a daily sc injection of the same volume of corn oil on treatment days. Four groups of five hens each were given the following combinations of the test compounds: PB/DEET, PB/chlorpyrifos, DEET/chlorpyrifos, and PB/DEET/chlorpyrifos. Some of the animals died prior to the termination of the study, and their tissues were unsuitable for analysis as the result of autolysis and were replaced. In addition, four animals were euthanized when they became moribund before the end of the study. The number of animals from each treatment group and those that died or were euthanized during the study and at termination are listed in Table 1.

Clinical Evaluation

Control and treated hens were observed daily both inside their cages and when they were allowed to freely move outside the cage. Clinical assessment included walking pattern, leg movement, flying, body tremor, and

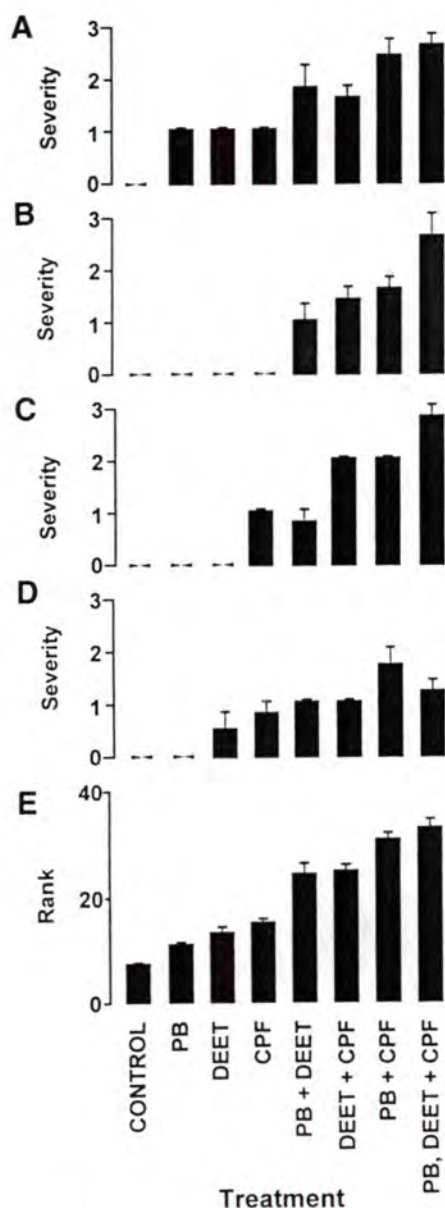


FIG. 3. Severity of clinical signs (A), locomotor dysfunction (B), histopathological changes, and mean rank of hens following daily administration of pyridostigmine bromide (PB), DEET, and chlorpyrifos (CPF), either individually or in combination. Experimental conditions are described in Fig. 2. Animals were assessed daily for clinical signs, locomotor dysfunctions, and scored as described under Methods. Severity of histopathological alterations was determined by examination of samples from the cervical and thoracic spinal cord (C) and the sciatic nerve (D). Mean rank (E) is the mean of the ranked scores of clinical signs, locomotor dysfunction, and histopathological alterations. The rank value of scores for individual parameters was obtained by ranking severity scores from the least to the greatest, and within a given severity value by the time of onset.

entering the home cage. Acute signs of toxicity that developed shortly after dosing consisted of (1) diarrhea, salivation; (2) reluctance to walk, decreased activity in cage; (3) shortness of breath; and/or (4) pupil constriction. Persis-

tent signs of neurotoxicity were characterized by locomotor dysfunctions and were classified into (1) body lowered, mild leg splay while standing and on landing after flying; (2) leg weakness demonstrated by preference to fly instead of walking, gait disturbance, e.g., linear or narrow gait, stumbling while entering the home cage, and moderately impaired flying activity; (3) leg sprawl out, body unsteadiness, stumbling gait, inability to enter the home cage, severely impaired flying activity; and (4) partial or complete paralysis. For quantification, hens with these signs were assigned numerical values of 1 to 4, corresponding to these conditions and assigned a value of 0 in the absence of any signs.

Histopathological Assessment

At 24 hr following administration of the last dose, treated and control hens were anesthetized using 0.25% halothane and then euthanized by decapitation. The spinal cord and sciatic nerve were excised immediately after euthanization and immersed in 4% phosphate-buffered formalin and postfixed for at least a week in the same fixative. Cross sections of cervical and thoracic spinal cord and longitudinal sections of proximal and distal sciatic nerve were stained for hematoxylin and eosin (H&E) and periodic acid Schiff's method. In addition, selected tissues were stained with Marsland and Glee's silver stain. Tissues were assessed with a step-down approach by first comparing sections from the control and triple treatment groups and then subsequently examining double and single treatments for any evidence of alterations observed in the triple treatment groups. Severity scores for the spinal cord were based on the frequency of enlarged axons in the ventral, lateral, and columns in cervical and thoracic regions (0, no detectable differences from control; 1, mild increase in the frequency of enlarged axons; 2, moderate; 3, severe). Severity scores for the sciatic nerve also ranged from 0 to 3 and were based on the combined frequency of prominent enlarged axons in the proximal and distal segments. Photomicrographs of illustrative examples of scores are presented under Results.

Enzymatic Studies

Brains were rapidly removed after euthanization 24 hr after the last dose and homogenized (10% w/v) in 50 mM Tris-HCl buffer, pH 8.0, with 0.1 mM EDTA. Blood samples were collected into heparinized tubes and spun in a Beckman Model J2-21 Centrifuge (Beckman Instruments Corporation, Palo Alto, CA) at 5000g for 30 min at 4°C using a JA-20 rotor to separate plasma and red blood cells. Cholinesterase activities in plasma and brain homogenates were determined using butyrylthiocholine iodide as substrate for plasma butyrylcholinesterase (BuChE) and acetylthiocholine iodide as substrate for brain AChE enzyme (Ellman *et al.*, 1961). Brain neurotoxicity target esterase (NTE) activity was determined immediately after brain homogenization using phenyl valerate as a substrate, according to the method of Johnson (1977). Protein concentrations for brain and plasma were determined using the bicinchoninic acid method (Smith *et al.*, 1985).

Evaluation of Neurotoxicity

Mean rank. Mean rank was calculated for each control and treatment group to compare the neurotoxicity produced following exposure to individual or combined treatments. Mean rank was calculated as described previously (Abou-Donia *et al.*, 1982) based on the method of Jonckheere (1954), by ranking the hens in the following six categories: (1) time of onset and (2) severity of acute clinical signs of toxicity, (3) time of onset and (4) severity of motor dysfunction, and (5) severity of neuropathologic lesions in the CNS and (6) PNS. Ranks were assigned starting with minimal changes in all parameters. In the case of ties, the same mean rank of birds involved was assigned to each of these birds. The mean rank for a treatment group was calculated as the mean of rank values for the six categories for the hens in that group.

Statistical analysis. For all unpaired data, groups of two or more were analyzed for overall treatment effect using analysis of variance. If an overall

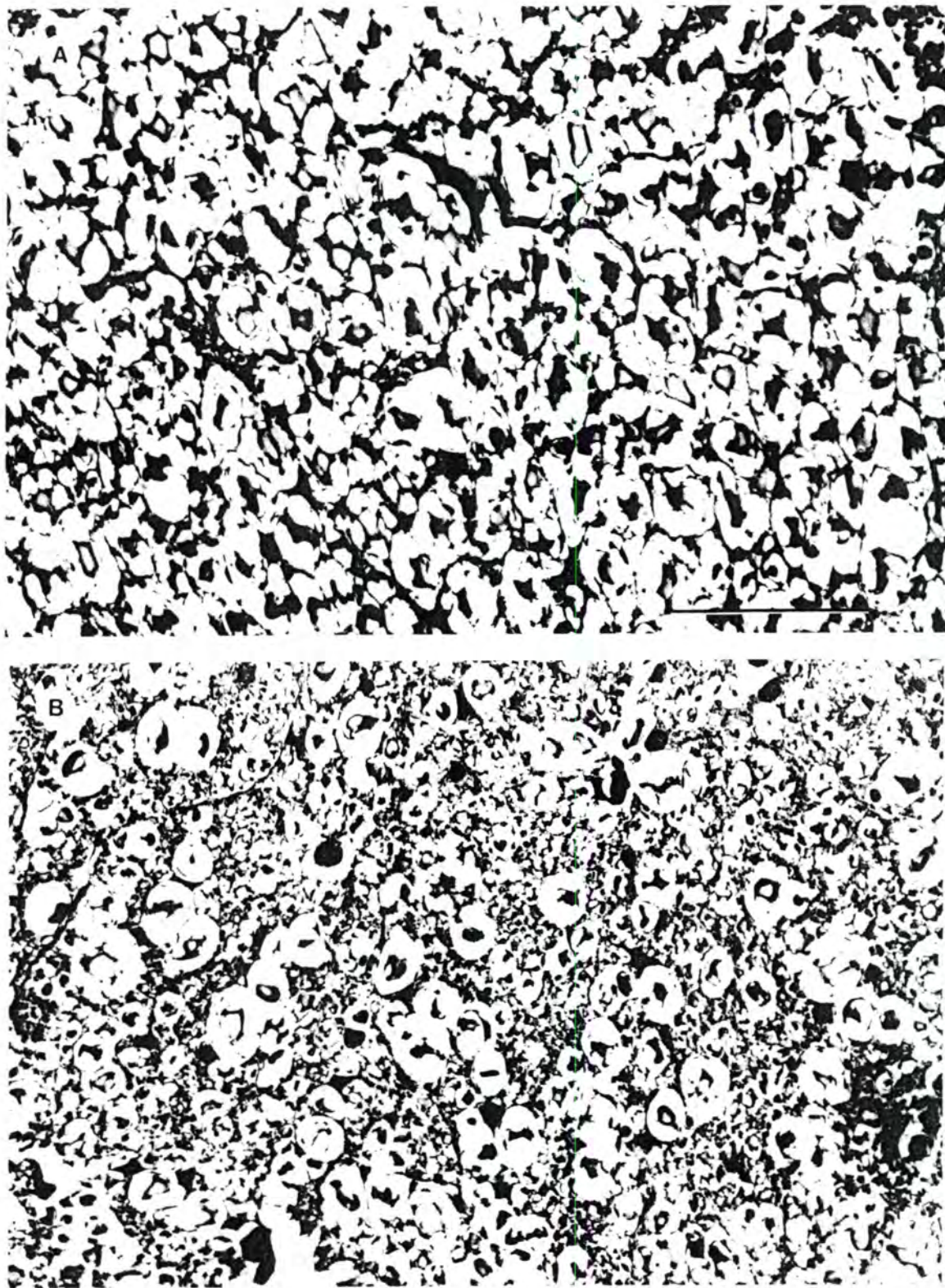


FIG. 4. Photomicrographs of cross sections of spinal cord from control hens and hens treated with chlorpyrifos (CPF). Ventral (A) and lateral (B) columns from a control animal (severity score, 0). Ventral (C) and lateral (D) columns from a hen treated with CPF exhibiting a mild increase in the frequency of enlarged axons (severity score, 1). Scale bar, 50 μ m. H&E.

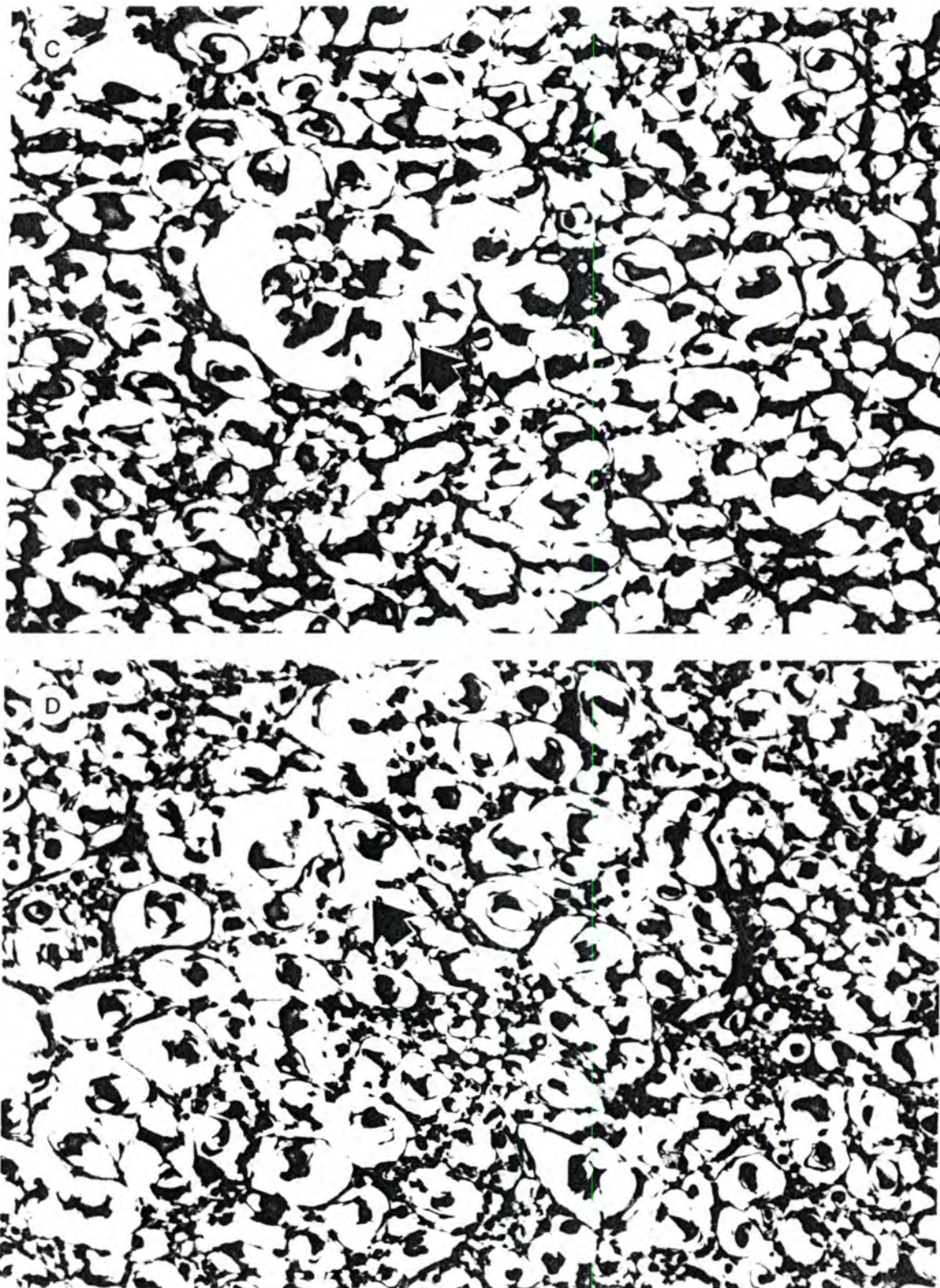


FIG. 4—Continued

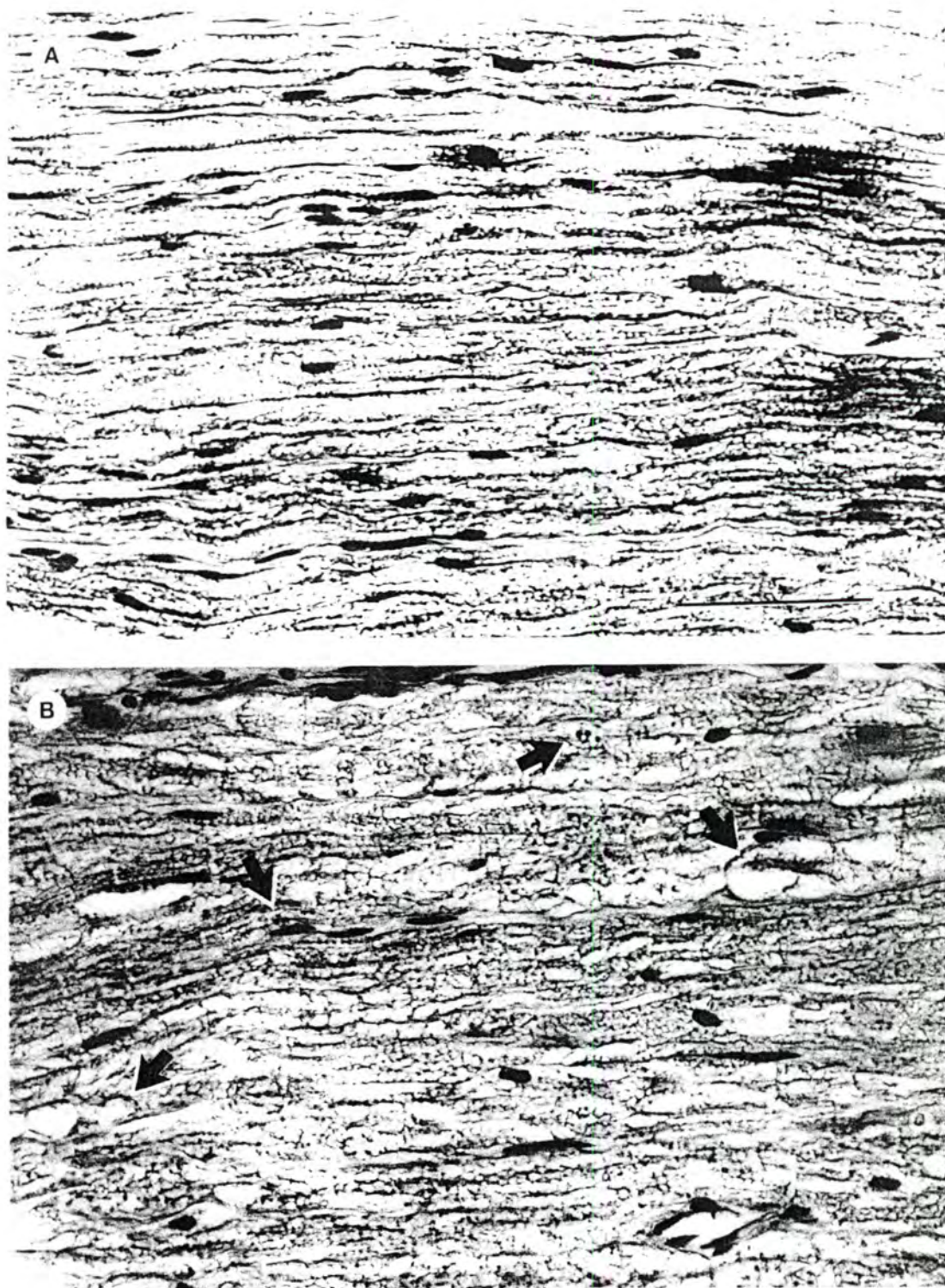


FIG. 5. Photomicrographs of transverse sections through sciatic nerves from (A) a control hen (severity score, 0) and (B) a hen treated with CPF exhibiting a mild increase in the frequency of enlarged axons (arrows) (severity score, 1). Scale bar, 50 μ m. H&E.

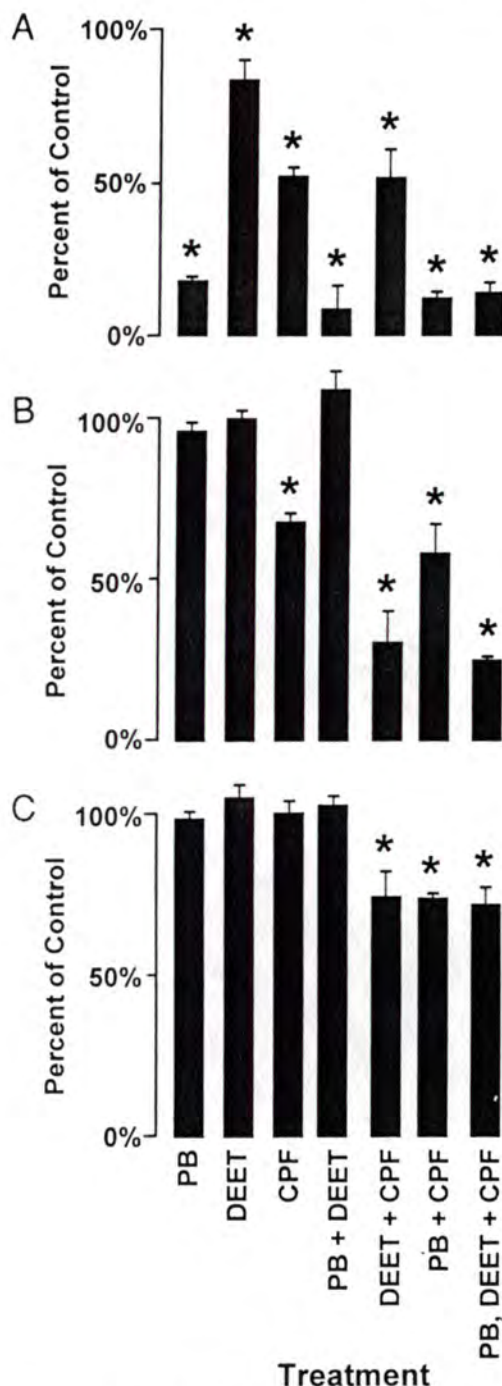


FIG. 6. Effect of daily administration of pyridostigmine bromide (PB), DEET, and chlorpyrifos (CPF) on hen (A) plasma butyrylcholinesterase, (B) brain acetylcholinesterase, and (C) brain neurotoxicity target esterase. Bars represent means and standard errors for treatment groups ($n = 5$). Significant differences between control and treatment groups were determined by Student's t test ($p < 0.01$) and indicated by an asterisk.

treatment effect was observed, then differences between groups were assessed using Fisher's least significant difference test. For paired data, Student's t test was used to assess differences between groups.

RESULTS

Treatment with Individual Compounds

Hens treated with PB exhibited transient mild cholinergic signs such as leg weakness and diarrhea with no significant loss of weight (Fig. 2). DEET caused an immediate rapid shallow breathing and inactivity which recovered within 24 hr (Table 2) of dosing daily. Hens treated with chlorpyrifos showed mild acute signs of cholinergic toxicity after 8 to 28 days of treatment such as transient diarrhea and leg weakness. These signs disappeared within 4 weeks despite continued treatment and the hens looked like controls at sacrifice. Hens treated with DEET or chlorpyrifos lost small but significant amounts of weight (Fig. 2). All birds treated with single compounds survived the experimental period (Fig. 2).

There was no detectable change in the spinal cords of hens treated with PB or DEET and those of control hens (Fig. 3 and Table 2). In all of the hens treated with chlorpyrifos, there was a small but detectable increase in the frequency of enlarged axons. Figure 4 shows ventral and lateral columns from a hen treated with chlorpyrifos showing a mild increase in the frequency of enlarged axons that had a score of 1. These enlarged axons tended to occur in clusters and were most obvious in the ventral and ventro-lateral tracts.

There were no detectable histopathologic differences in the sciatic nerves of hens treated with PB and those of control hens (Fig. 3 and Table 2). In two of the animals treated with DEET, there was a small but detectable increase in frequency of enlarged axons in the sciatic nerve (Fig. 3 and Table 2). In three of the animals treated with chlorpyrifos, there was a mild increase in frequency of enlarged axons in the sciatic nerves (severity score, 1; Fig. 5).

At the end of the treatment, PB strongly inhibited plasma BuChE activity to 17% of the control value (Fig. 6). DEET produced a slight inhibition of this enzymatic activity which was 83% of the control value. On the other hand, chlorpyrifos inhibited plasma BuChE to 51.3% of control activity. Of the three compounds, only chlorpyrifos resulted in significant inhibition of brain AChE to 67% of control activity. None of the three chemicals had any affect on brain NTE.

Treatment with Two Compounds

Concurrent treatment with both PB and DEET produced intermittent diarrhea, shallow breathing, and diminished leg movement (Fig. 3 and Table 2). Leg splay and altered stance were evident in four hens 9–32 days after the beginning of treatment. The clinical condition of three of these hens progressed to persistent unsteady gait. Chlorpyrifos and DEET developed diarrhea and acute signs similar to those produced by DEET such as body lowered, unsteadiness, reluctance to walk, and rapid shallow breathing. Clinical signs diminished during the course of the study and at euthaniza-

tion three had moderate effects and two exhibited mild signs. Locomotor dysfunction was mild in three hens and moderate in two. Combined treatment with chlorpyrifos and PB resulted in more severe clinical conditions than any other binary treatment. Onset of cholinergic signs was at 2 to 3 days after the beginning of treatment. These signs were severe diarrhea, salivation, reluctance to walk, and falling. With time, hens developed tolerance to the acute effects of the test compounds and by Day 28 had improved to moderate or severe acute toxicity. Locomotor dysfunction, such as change in gait, became evident after 18 to 26 days of dosing and was mild to moderate at euthanization. Hens treated with two compounds exhibited a small but significant loss of weight, despite their ability to reach their feed (Fig. 2). All binary treatments caused death in some of the hens while some were euthanized when they became moribund during the experiment as indicated in Table 1.

In the spinal cord of four of the hens treated with PB and DEET, there was a mild increase in the frequency of enlarged axons (severity score, 1; Figs. 7A and 7B). Three hens treated with PB and chlorpyrifos exhibited a moderate increase in the frequency of axonal swellings in the ventral and ventro-lateral tracts of the spinal cord (severity score, 2; Figs. 7C and 7D). There was also a moderate increase in the frequency of axonal swelling in three hens treated with DEET and chlorpyrifos (severity score, 2). As in other hens treated with chlorpyrifos alone or in combination with other agents, enlarged axons appeared to occur in clusters and were most obvious in the ventral, ventro-lateral, and lateral tracts.

The sciatic nerve of the hens treated with PB and DEET showed a mild increase in the frequency of enlarged axons (severity score, 1; Fig. 8A). In the sciatic nerve of three hens treated with PB and chlorpyrifos there was a moderate increase in the number of enlarged axons (severity score, 2; Fig. 8B) in two animals and in one of the animals, the extent alteration appeared mild (severity score, 1). In the sciatic nerve of the hens treated with chlorpyrifos and DEET, there was a mild but consistent increase in the number of detectable axonal swellings (severity score, 1).

Hens dosed with PB and DEET exhibited great inhibition of plasma BuChE activity to 7.7% of control. Plasma BuChE activity was markedly inhibited in hens dosed with DEET and chlorpyrifos to 50.8% of control while it was reduced to 11.3% of control following treatment with PB and chlorpyrifos (Fig. 6). While PB/DEET treatment did not affect brain AChE activity, the other two combined treatments produced significant inhibition of this enzyme and resulted in activity (percentage of control) of 57.3% for PB/chlorpyrifos and 29.5% for DEET/chlorpyrifos. Brain NTE activity was not significantly affected after coexposure to combined treatment of PB and DEET. Chlorpyrifos in combination with either PB or DEET produced similar inhibition of brain NTE, which had an activity of 73% of control (Fig. 6).

Treatment with Three Chemicals

Hens treated concurrently with PB, DEET, and chlorpyrifos exhibited severe acute cholinergic signs such as diarrhea, salivation, and leg weakness, as well as shallow breathing (Fig. 3 and Table 2). Severity of clinical signs regressed from severe to mild and moderate signs at euthanization. This treatment caused a small but significant loss of weight (Fig. 2). In this treatment group, one hen died and was replaced and two became moribund and were euthanized before the end of the experiment.

There was an increase in the frequency of enlarged axons in the spinal cords of hens treated with PB, DEET, and chlorpyrifos (Figs. 7E and 7F). These enlarged axons appear in clusters and were most obvious in the ventral, ventro-lateral, and lateral tracts. These clusters often appeared in close proximity to small blood vessels. In four of the hens treated with PB, DEET, and chlorpyrifos, the extent of increase in the frequency of these axonal enlargements was severe (severity score, 3; Figs. 7E and 7F) and in one hen the frequency of axonal enlargements was moderate (severity score, 2). The extent to which these alterations are indicative of axonal degeneration was most obvious in silver-stained longitudinal sections of the spinal cord (Figs. 9B and 9C), in which neurofibrillary alterations and axonal fragmentation were apparent.

There was an increase in the frequency of enlarged axons and prominent axonal swellings in the sciatic nerves of hens treated with PB, DEET, and chlorpyrifos (Fig. 3, Table 2). In four of these hens, the extent of this alteration was mild (severity score, 1) and in one it was moderate/severe (severity score, 3). Figure 8C shows a cross section from a hen treated with the three compounds exhibiting a severe increase in the frequency of enlarged axons (severity score, 3). A silver-stained longitudinal section of a sciatic nerve from a hen treated with the three compounds shows many focal enlargements, some of which contain cytoskeletal inclusions (Fig. 10B).

Coexposure to the three compounds PB, chlorpyrifos, and DEET greatly decreased plasma BuChE to 13.2% of control. Brain AChE activity was diminished to 24% while brain NTE was 71% of the control value (Fig. 6).

Relative Neurotoxicity of Individual and Multiple Exposures

The relative neurotoxic effect of individual test compounds and concurrent exposure to two or three chemical combinations were compared by calculating the mean rank. The mean rank encompasses the onset of severity of acute signs of toxicity and locomotor dysfunctions as well as the severity of peripheral nerve and spinal cord damage at the time of euthanization (Fig. 3, Table 3). Control hens were normal and had a mean rank value of 7.0. The mean rank

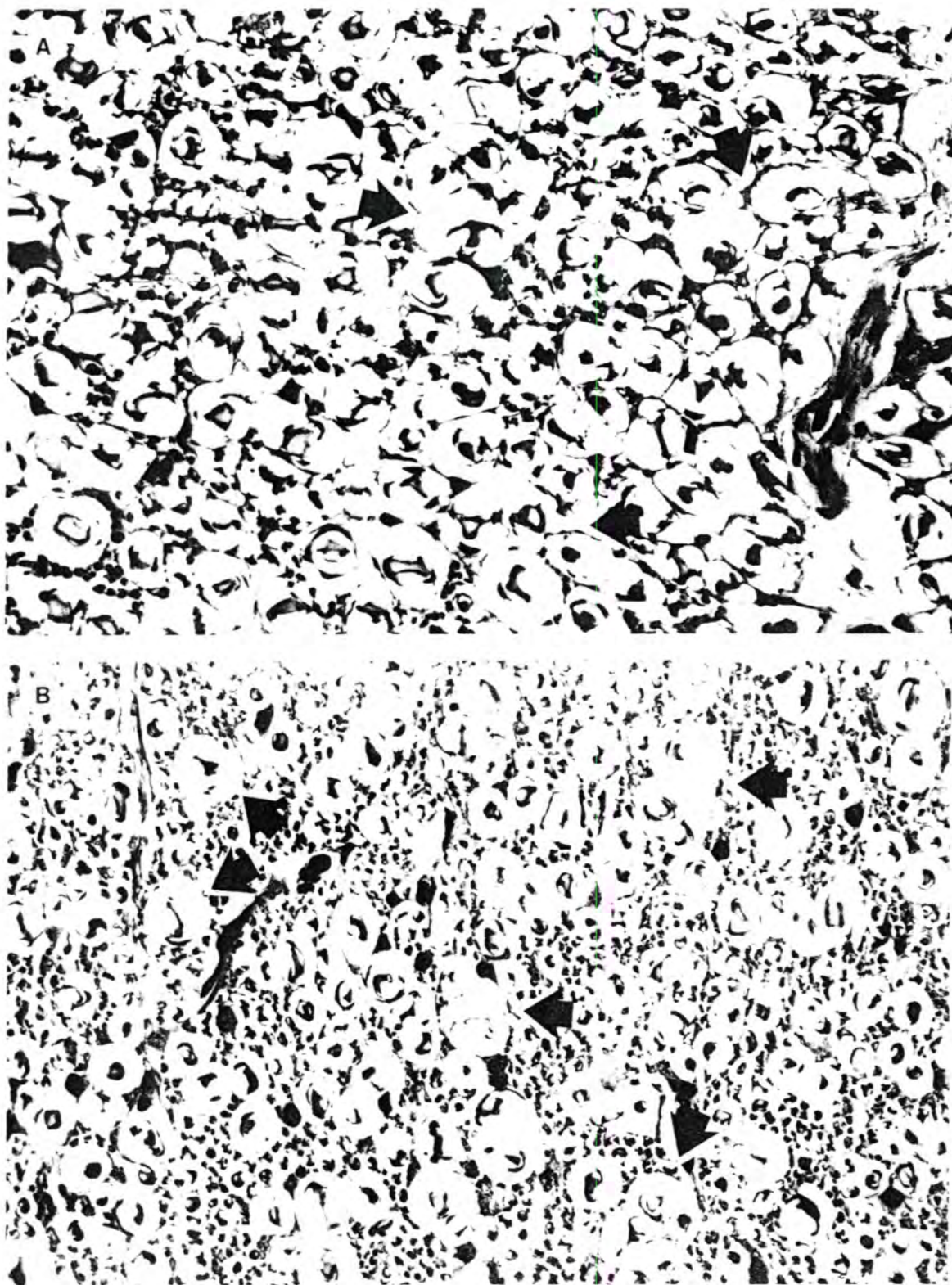


FIG. 7. Photomicrographs of cross sections of the ventral (A, C, E) and lateral (B, D, F) columns of the spinal cord from a hen treated with PB and DEET. (A, B) Section from a hen treated with PB/DEET exhibiting a mild increase in the frequency of enlarged axons (severity score, 1). (C, D) Section from a hen treated with PB/CPF exhibiting a moderate increase in the frequency of enlarged axons (severity score, 2). (E, F) Section from a hen treated with PB/DEET/CPF exhibiting a severe increase in the frequency of enlarged axons (severity score, 3). Details as in Fig. 4.

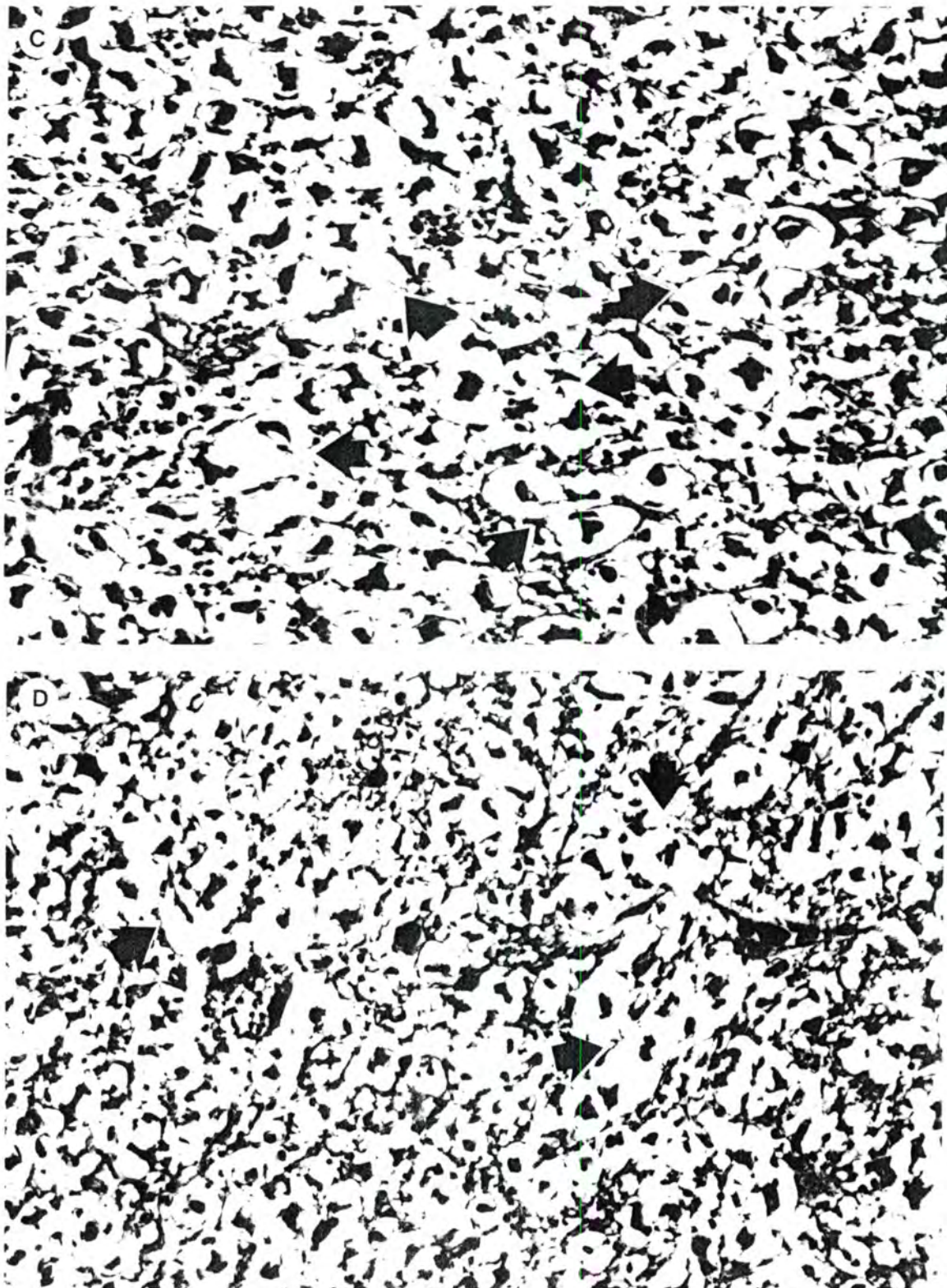


FIG. 7—Continued

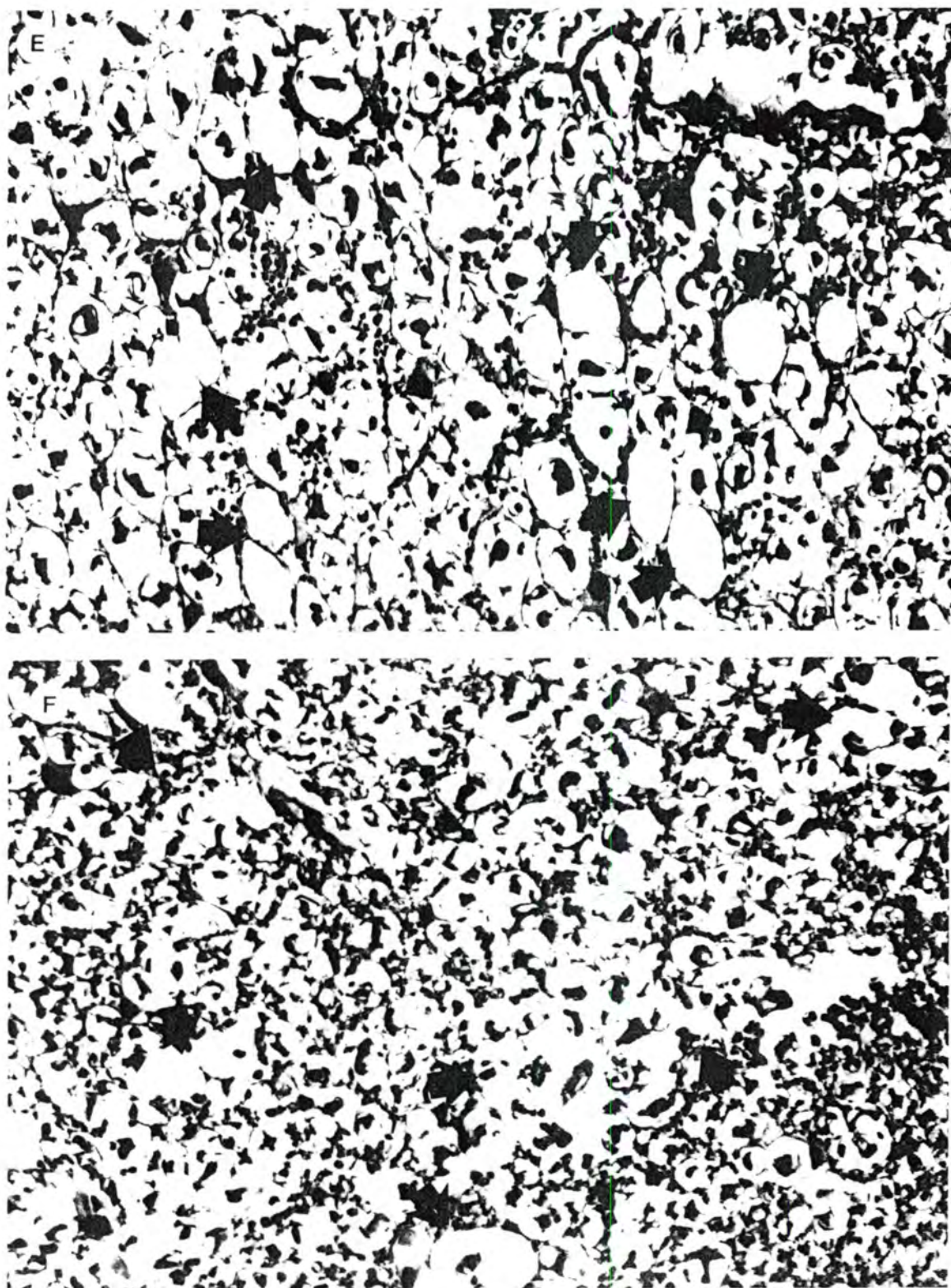


FIG. 7—Continued

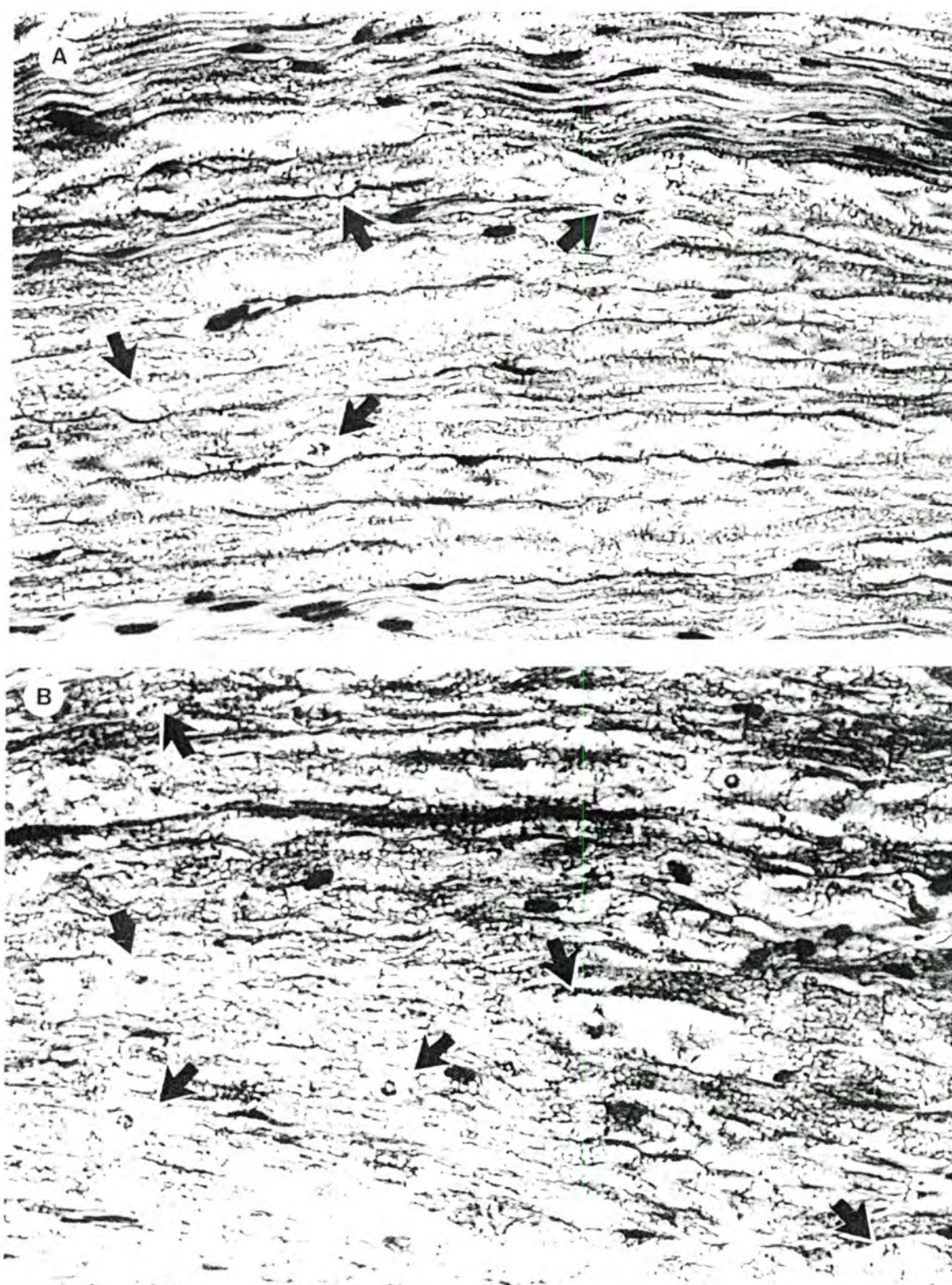


FIG. 8. Photomicrographs of transverse sections of sciatic nerve. (A) Section from a hen treated with PB and DEET exhibiting a mild increase in the frequency of enlarged axons (severity score, 1) and (B) section from a hen treated with PB/CPF exhibiting a moderate increase in the frequency of enlarged axons (severity score, 2). (C) Section from a hen treated with PB/DEET/CPF exhibiting a severe increase in the frequency of enlarged axons (severity score, 3). H&E. Details as in Fig. 5.

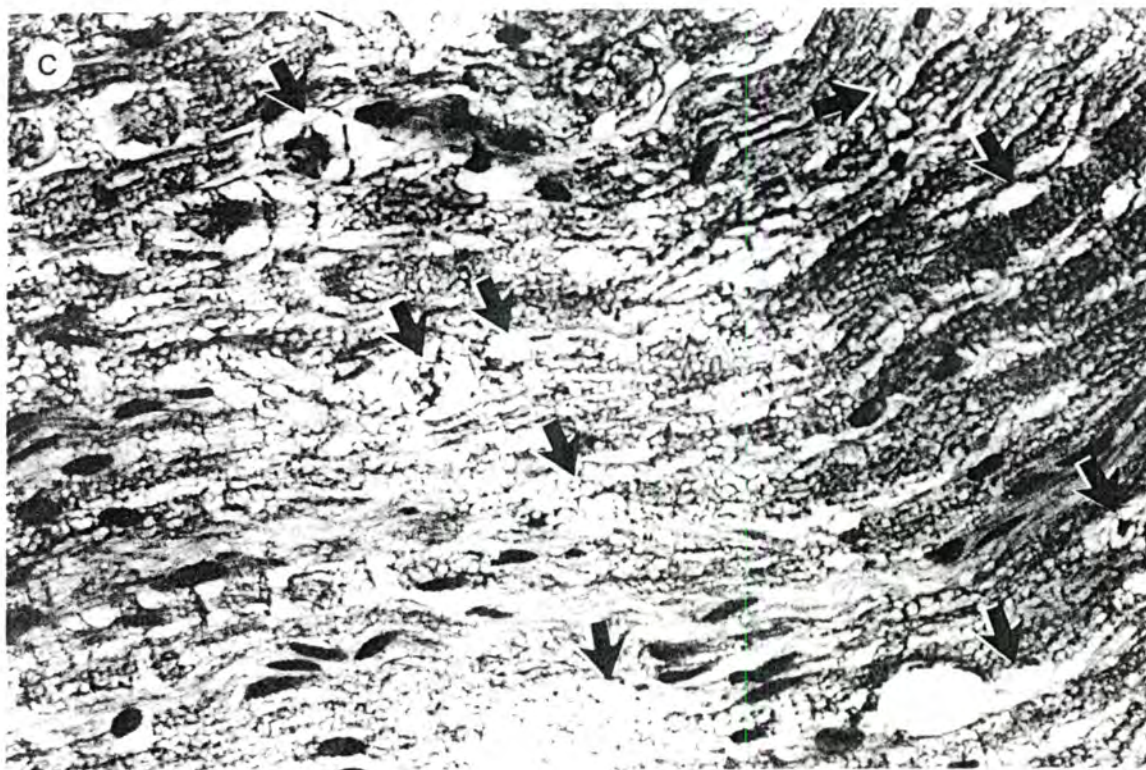


FIG. 8—Continued

values for individual treatments were significantly higher than the control value. The mean rank values indicated that PB (10.7), DEET (12.9), and chlorpyrifos (14.8) were not significantly different from each other (Table 4). Also, mean rank values indicated that combined exposures were more neurotoxic than individual compounds, with the PB/DEET and DEET/chlorpyrifos combinations having similar values, i.e., 23.8 and 24.3, respectively, and with PB/chlorpyrifos having a greater mean rank value of 30.2. Furthermore, the combined treatment of the three chemicals produced the largest mean rank value of 32.4. Among binary combinations, while the mean rank value for PB/DEET was not significantly different from that for DEET/chlorpyrifos, it was significantly lower than that for PB/chlorpyrifos. The mean rank value of PB/chlorpyrifos was significantly higher than that for DEET/chlorpyrifos (Table 4). On the other hand, the tertiary treatment was significantly higher than all other treatments except for that of PB/chlorpyrifos.

DISCUSSION

The present results demonstrate that individual chemicals used to protect Gulf War soldiers from nerve-gas poisoning and insect-borne diseases at doses that produced mild toxicity to hens were highly toxic when used in combination. The insect repellent DEET, the insecticide chlorpyrifos, and the

anti-nerve gas agent PB at large but mild doses when used alone caused neurologic deficits in the adult hen following concurrent subchronic exposure. Increased neurologic dysfunctions were accompanied by significant inhibition of plasma and brain cholinesterases and brain NTE activity as well as neuropathologic lesions. These findings suggest that an agent such as PB, which normally does not reach the brain, can augment the neurotoxicity produced by chlorpyrifos that does. Similarly, DEET that does not have cholinergic action can enhance those actions of an agent that does, such as chlorpyrifos. These findings have three important implications. First, concurrent exposure to test chemicals may decrease the body's normal mechanisms to remove these chemicals and/or increase their transport to the neurotoxicity target. Second, the use of PB concurrently with exposure to pesticides and other chemicals in the Gulf War may be related to some of the complaints of the service personnel. Third, genetic variations in plasma and liver esterases having reduced activity may be useful in identifying a subpopulation particularly susceptible to neurotoxic chemicals such as chlorpyrifos.

Although the three test compounds adversely affect the nervous system, they all have a distinct mode of action. The acute oral LD_{50} of PB in male rats is 61.6 mg/kg (McCain, 1995) and that of DEET is 3000 mg/kg (Ambrose *et al.*, 1959). The acute oral LD_{50} of chlorpyrifos in male rats is

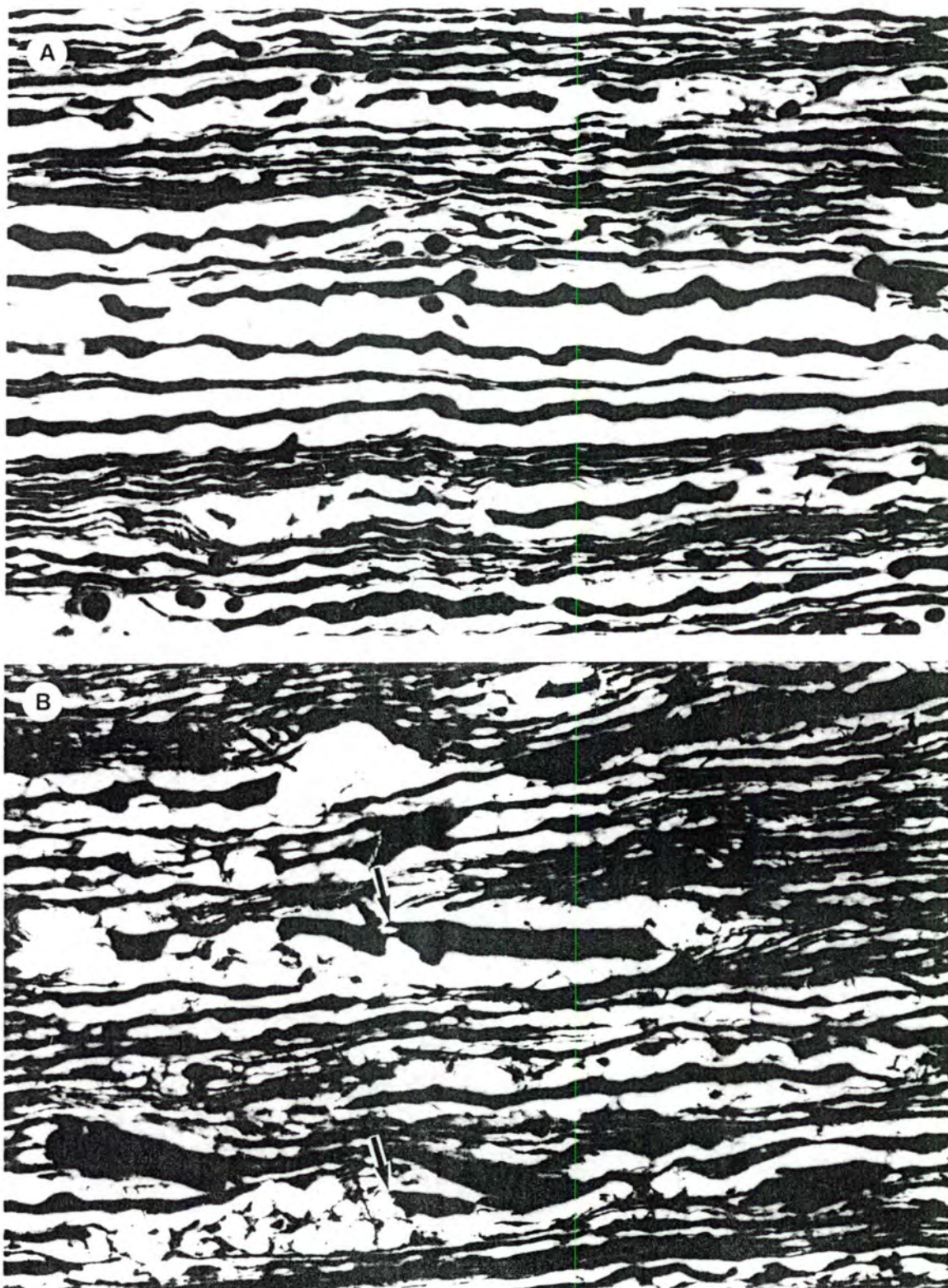


FIG. 9. Photomicrographs of transverse sections of spinal cord through the lateral columns stained with Marsland and Glee's silver stain. (A) Section from control hen. (B, C) Sections from hens treated with PB/DEET/CPF exhibiting fragmentation of axis cylinders (arrows) and focal enlargements. Scale bar, 50 μ m.

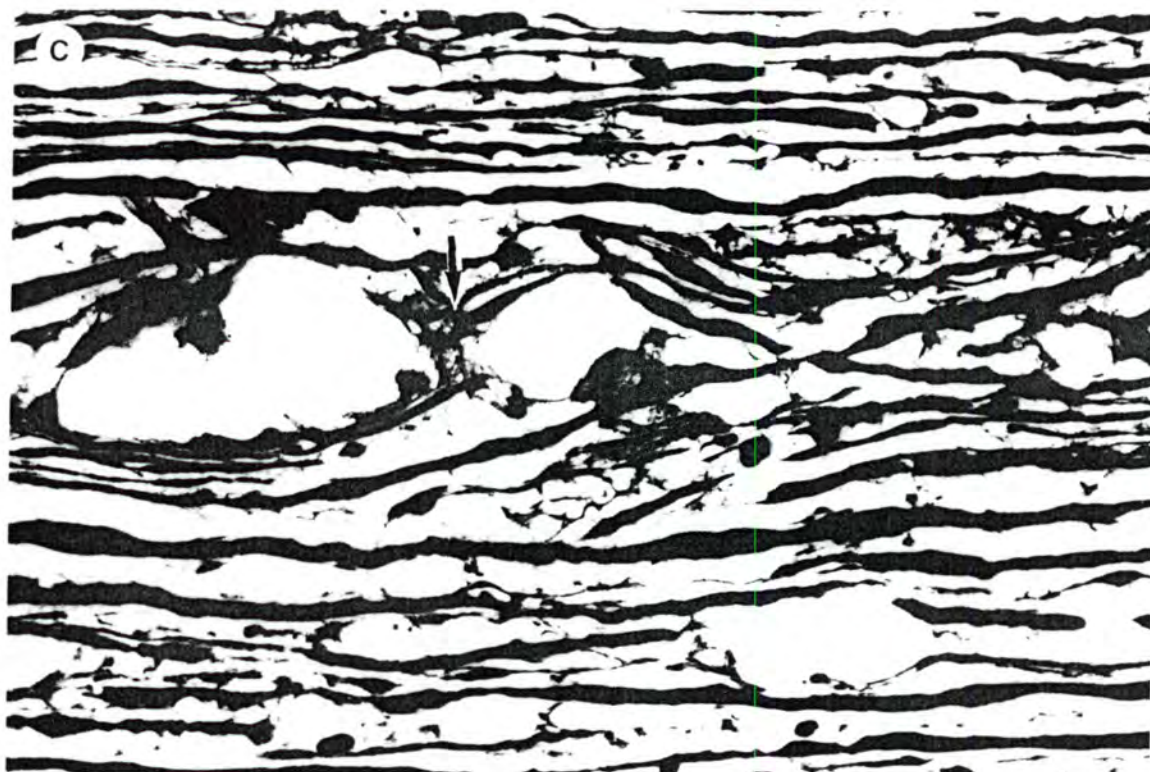


FIG. 9—Continued

150 mg/kg and in hens is 50 mg/kg (Lotti *et al.*, 1986; Abou-Donia and Wilmarth, 1995). Individual test compounds, under the present dosing regimen, produced inhibition of plasma BuChE in the following descending order: PB > chlorpyrifos > DEET. The large inhibition resulting from PB and chlorpyrifos is consistent with their anticholinesterase effect. On the other hand, DEET, an aryl amide, caused a slight but significant inhibition of plasma BuChE. Neither PB nor DEET alone or together had any effect on brain AChE, consistent with their properties that PB does not cross the blood-brain barrier (Birtley *et al.*, 1966) and that DEET does not have anticholinesterase activity. On the other hand, as expected, chlorpyrifos administration produced significant inhibition of brain AChE (Pope *et al.*, 1992; Richardson *et al.*, 1993; Huff and Abou-Donia, 1995). Furthermore, combined exposure to chlorpyrifos with PB, DEET, or both greatly increased brain AChE inhibition.

The effect of test compounds, singly and in combination, on NTE is also consistent with their mode of action and clinical manifestation. NTE is the putative target and a biomarker for OPIDN (Johnson, 1969). A single or multiple exposure of an organophosphorus ester that causes at least 70 (Johnson, 1969; Lotti, 1992) or 54% (Sprague *et al.*, 1981) inhibition of NTE, respectively, followed by "aging" results in OPIDN. The result that neither PB nor DEET inhibited hen brain NTE is in agreement with the fact that

these compounds do not produce OPIDN. Also, the chlorpyrifos dosage when used alone neither inhibited NTE nor produced OPIDN, indicating that it was below the threshold level to cause OPIDN. On the other hand, chlorpyrifos in combination with PB, DEET, or both resulted in a significant inhibition of NTE activity (27 to 29% inhibition). Since this inhibition was below the threshold level for NTE inhibition, neurologic deficits and neuropathologic lesions cannot be related to OPIDN. Neurologic dysfunctions resulting from coexposure to chlorpyrifos and other chemicals may have resulted from superimposition of neurotoxic effects of administered compounds. Inhibition of AChE in the PNS by the three compounds increases ACh in the synapse, leading to increased skeletal and smooth muscle contraction (Abou-Donia, 1994). This effect may be augmented by neuromuscular toxicity induced by PB (Hudson *et al.*, 1985). Acute and subacute exposure of rats to PB caused morphological alterations in the neuromuscular junction from the diaphragm, soleus, and extensor digitorum longus muscles. Combined treatment with the three chemicals caused less severe alterations in the cervical columns than those seen below the cervical level that included the descending tracts, i.e., corticospinal columns of the lateral and ventral regions.

The increased neurotoxicity of the test chemicals resulting in severe neurologic deficits in the experimental animal model reported in this study may explain some of the unex-

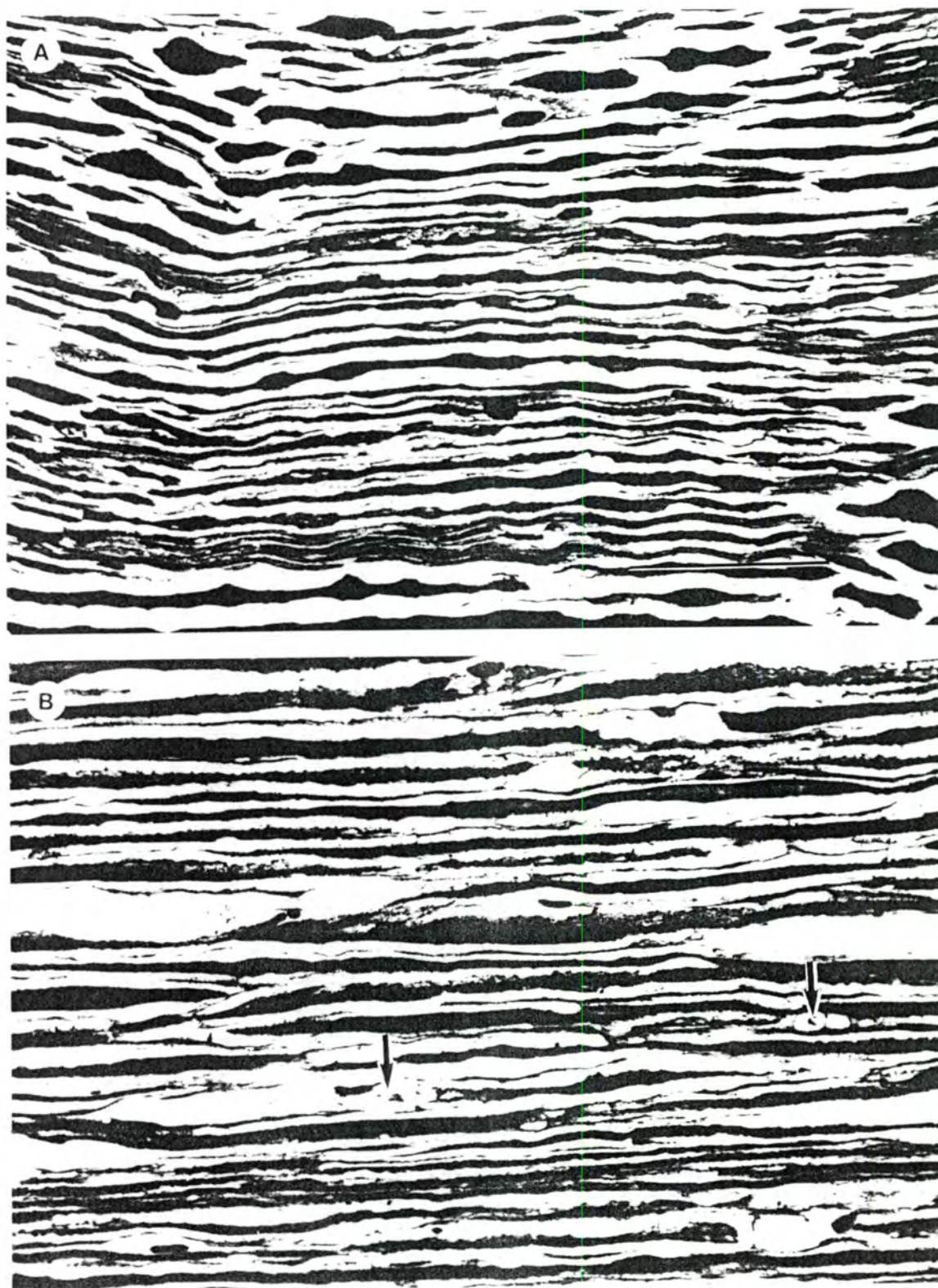


FIG. 10. Photomicrographs of transverse sections of sciatic nerve stained with Marsland and Glee's silver stain. (A) Section from a control hen. (B) Section from a hen treated with PB/DEET/CPF. Note the many focal enlargements, some of which exhibit cytoskeletal inclusions (arrows). Scale bar, 50 μ m.

TABLE 3
Ranked Neurotoxicity Scores Following Daily Administration of Individual and Coexposure of Pyridostigmine Bromide (PB), DEET, and Chlorpyrifos (CPF)^a

Treatment ^b	Clinical signs ^c		Locomotor dysfunction ^c		Histopathological changes ^d		Mean rank ^e (NTI)
	Day of onset	Severity	Day of onset	Severity	Spinal cord	Sciatic nerve	
Control	3	3	11	11	8	7	7
PB	13	15	11	11	8	7	11
DEET	19	15	11	11	8	14	13
CPF	12	15	11	11	19	22	15
PB + DEET	28	25	27	24	16	22	24
DEET + CPF	21	24	25	29	26	22	25
PB + CPF	33	33	31	31	26	29	31
PB + DEET + CPF	35	34	36	36	30	25	33

^a All values are means from each treatment group.

^b Hens were given a daily dose, 5 days/week, individual or combinations of 5 mg/kg PB in water po, 500 mg/kg sc DEET, and 10 mg/kg sc CPF.

^c Animals were assessed daily for clinical signs, locomotor dysfunctions, and tremor and scored as indicated under Methods. Severity scores indicate the condition of the animal at the time of euthanization.

^d Severity of histopathological alterations was determined by examination of the cervical and thoracic spinal cord and sciatic nerve tissues.

^e Mean rank or neurotoxicity index (NTI) is the mean score of clinical signs, locomotor dysfunction, tremor, and histopathological alteration shown in Table 2. Every parameter was ranked according to severity scores from least to greatest, and within the severity according to time of onset to obtain ranked scores.

pected neurologic complaints of some of the Persian Gulf War veterans (Keeler *et al.*, 1991). Although this experimental study was not designed to determine the cause of the Persian Gulf War veterans' illnesses, its findings suggest that coexposure to test compounds may have contributed to their complaints. In an attempt to approximate actual exposure conditions, PB which was taken orally by service personnel as a 30-mg tablet three times daily (equivalent to 1.3 mg/kg/day) was administered (5 mg/kg) to hens by gavage (Institute of Medicine, 1995). Exposure to DEET and chlorpyrifos by the service personnel was through the skin and by inhalation. In this study both chemicals were applied subcutaneously for accurate quantification of the dose. DEET is efficiently absorbed through the skin; thus, over

90% of DEET was diffused across the hairless mouse skin in 24 hr (Windheuser *et al.*, 1982) and its dermal absorption was nearly complete in 48 hr in rats (Valdez *et al.*, 1996), while 50% of the applied dose to human skin was absorbed (Spencer *et al.*, 1979). Thus, absorbed DEET following dermal application may approximate that of subcutaneous injection. The levels of exposure to the two pesticides during the Gulf War are not known. A 1986 NIOSH study, however, estimated the upper 1% of the weekly application dose of DEET by the employees in the Everglades National Park to be 1122 mg/kg/day (392.6 g/week).

Although the mechanisms of increased neurotoxicity following concurrent exposure to test chemicals are not known, we hypothesize that they are related to the increased effective

TABLE 4
Differences in the Mean Rank of Clinical Signs, Locomotor Dysfunction, and Neuropathological Alterations of Hens Administered Pyridostigmine Bromide (PB), DEET (D), or Chlorpyrifos (CPF) Either Individually or in Combination

	Single treatments			Binary treatments			Tertiary treatment
	PB	DEET	CPF	PB + DEET	DEET + CPF	PB + CPF	PB, DEET, + CPF
Single treatments	3.7	5.9	7.8	16.8	17.3	23.2	25.4
PB				13.1	13.9	19.5	21.7
DEET				10.9	11.4	17.3	19.5
CPF				9.0	9.5	15.4	17.6
Binary treatments							
PB + DEET							8.6
DEET + CPF							8.1
PB + CPF							n.s.

concentration of test compounds at the neurotoxicity target by altering one or more of the following processes: (a) Absorption. Concurrent exposure to chemicals may increase their absorption. Because it is efficiently absorbed through the skin (Windheuser *et al.*, 1982; Spencer *et al.*, 1979), DEET has been used as a transdermal delivery enhancer of many drugs (Windheuser *et al.*, 1982; Hussain and Ritschel, 1988). By inference, DEET may accelerate skin penetration of chlorpyrifos. Furthermore, the epidermis may serve as a depot for dermally applied DEET, resulting in its slow release into the circulation (Snodgrass *et al.*, 1982; Blomquist and Thorsell, 1977; Spencer *et al.*, 1979). (b) Metabolism. The three test compounds are metabolized via liver cytochrome P-450 and plasma hydrolases, esterases or amidases (Selim *et al.*, 1995; Abdel-Rahman *et al.*, 1993; Eiermann *et al.*, 1993; George and Balasubramanian, 1981). Such enzymes function to protect the body from the toxicity of xenobiotics by enhancing their removal from the circulation via either scavenging or breakdown into water-soluble metabolites (Williams, 1987; Abou-Donia, 1995). Thus, concurrently administered chemicals may compete for metabolizing enzymes, leading to their decreased breakdown and increased transport to nervous tissues. This mechanism is consistent with the results that inhibition of brain AChE and NTE was greatly increased after combined exposure with PB, DEET, or both with chlorpyrifos that may have resulted from increased chlorpyrifos oxon in the brain tissue. (c) Permeability of cerebrovasculature. Concurrently administered chemicals may cause trauma to the cerebrovasculature, resulting in altered blood flow to the brain and/or increased vascular permeability. This leads to their enhanced entry from the circulation, thereby increasing their neurotoxic action (Simonati *et al.*, 1983; Abou-Donia *et al.*, 1985). (d) Blood-brain barrier (BBB). Combined treatment with test compounds may compromise the integrity of the BBB and renders it more permeable to xenobiotics in circulation. This may be attributed to the inhibition of BuChE and AChE, located in the capillary wall and astrocytes (Koelle, 1952; Joó and Várkonyi, 1969). This suggestion is consistent with the increased permeability of the BBB by PB analog, physostigmine, and ACh (Greig and Holland, 1949).

The present results along with an earlier report (Abou-Donia *et al.*, 1996) may also explain the variable occurrence of the Gulf War Syndrome. This may be related both to concurrent exposure to specific chemicals and to individual susceptibility to adverse effects of these chemicals. Both studies have demonstrated that coexposure to chemicals causes increased neurologic deficits. Clinical signs, biochemical alterations, and pathological lesions varied and depended on both the nature and the dosage of each chemical. These studies also suggest that the idiosyncrasy of individuals may play a major role in their reaction to concurrent exposure to chemicals. Thus, individuals with diminished

ability to metabolize drugs and pesticides, such as those with the "atypical" genetic variant of plasma BuChE (Viby-Mogensen, 1981), are potentially vulnerable and at high risk to anticholinesterase and related chemicals and this may account for the more severe signs seen in some of the Gulf War veterans. This suggestion is supported by the recent finding that a soldier, homozygous for atypical BuChE, who had exhibited succinylcholine-induced apnea during surgery, also suffered severe cholinergic symptoms following prophylactic treatment with PB during the Persian Gulf War (Loewenstein-Lichtenstein *et al.*, 1995).

This study has expanded and confirmed our previous finding (Abou-Donia *et al.*, 1996) that coexposure to subneurotoxic doses of PB and pesticide chemicals resulted in increased neurotoxicity characterized by neurologic dysfunction, inhibition of plasma BuChE and brain AChE, and NTE and neuropathologic lesions. Further studies are needed to investigate the potential neurologic deficits resulting from coexposure to these chemicals at dosages to which the Gulf War veterans may have been exposed. Currently, we are investigating the specific mechanisms by which concurrent exposure to chemicals results in increased neurotoxicity.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the Perot Foundation. The technical assistance of Jeanene Olin, the statistical expertise of Judy Schmid, and the secretarial assistance of Diane Brooks are appreciated.

REFERENCES

- Abdel-Rahman, A. A., Wilmarth, K. R., Abou-Donia, S. A., and Abou-Donia, M. B. (1993). Distribution and pharmacokinetics of chlorpyrifos and selected metabolites in tissues of neonatal rats and milk following a single oral dose of [14 C]chlorpyrifos to the mother. *Pharmacologist* **35**, 176.
- Abou-Donia, M. B. (1981). Organophosphorus ester-induced delayed neurotoxicity. *Annu. Rev. Pharmacol. Toxicol.* **21**, 511-548.
- Abou-Donia, M. B. (1994). Organophosphorus Pesticides. In *Handbook of Neurotoxicology* (L. W. Chang and R. S. Dyer, Eds.), pp. 419-473. Dekker, New York.
- Abou-Donia, M. B. (1995). Metabolism and Toxicokinetics of Xenobiotic. In *CRC Practical Handbook of Toxicology* (Derelanko and Hollinger, Eds.), pp. 539-589. CRC Press, Boca Raton, FL.
- Abou-Donia, M. B., Hu, Z., Lapadula, D. M., and Gupta, R. P. (1991). Mechanisms of joint neurotoxicity of *n*-hexane, methyl isobutyl ketone and *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate in hens. *J. Pharmacol. Exp. Ther.* **257**, 282-289.
- Abou-Donia, M. B., Lapadula, D. M., Campbell, G., and Abdo, K. M. (1985). The joint neurotoxic action of inhaled methyl butyl ketone vapor and dermally applied *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate. *Toxicol. Appl. Pharmacol.* **75**, 69-81.
- Abou-Donia, M. B., Makkawy, H. M., and Graham, D. G. (1982). The relative neurotoxicities of 2-hexane, methyl *n*-butyl ketone, 2,5-hexanediol, and 2,5-hexanedione, following oral or intraperitoneal administration to hens. *Toxicol. Appl. Pharmacol.* **62**, 369-389.
- Abou-Donia, M. B., and Wilmarth, K. R. (1995). The joint action of chlor-

- pyrifos and saftrotin on the development of delayed neurotoxicity in the hen. *Toxicologist* **15**, 205.
- Abou-Donia, M. B., Wilmarth, K. R., Jensen, K. F., Oehme, F. M., and Kurt, T. L. (1996). Neurotoxicity resulting from coexposure to pyridostigmine bromide, DEET, and permethrin. *J. Toxicol. Environ. Health* **48**, 35–56.
- Ambrose, A. M., Huffman, D. K., and Salamone, R. T. (1959). Pharmacological and toxicologic studies on *N,N*-diethyltoluamide *N,N*-diethyl-m-toluamide. *Toxicol. Appl. Pharmacol.* **1**, 99–115.
- Birtley, R. D., Roberts, J. B., Thomas, B. H., and Wilson, A. (1966). Excretion and metabolism of [¹⁴C]-pyridostigmine in the rat. *Br. J. Pharmacol.* **26**, 393–402.
- Blick, D. W., Kerenyi, S. Z., Miller, S., Murphy, M. R., Brown, G. C., and Hartgraves, S. L. (1991). Behavioral toxicity of anticholinesterases in primates: Chronic pyridostigmine and soman interactions. *Pharmacol. Biochem. Behav.* **38**, 527–532.
- Blomquist, L., and Thorsell, W. (1977). Distribution and fate of the insect repellent ¹⁴C-*N,N*-diethyl-*m*-toluamide in the animal body. II. Distribution and excretion after cutaneous application. *Acta Pharmacol. Toxicol.* **41**, 235–243.
- Breyer-Pfaff, U., Maier, U., Brinkmann, A. M., and Schumm, F. (1985). Pyridostigmine kinetics in healthy subjects and patients with myasthenia gravis. *Clin. Pharmacol. Ther.* **37**, 495–501.
- Capodicasa, E., Scapellato, M. L., Moretto, A., Caroli, S., and Lotti, M. (1991). Chlorpyrifos-induced delayed polyneuropathy. *Arch. Toxicol.* **65**, 150–155.
- Cook, J. E., and Kolka, M. A. (1992). Chronic pyridostigmine bromide administration: Side effects among soldiers working in a desert environment. *Milit. Med.* **157**, 250–254.
- Edwards, D. L., and Johnson, C. E. (1987). Insect-repellent-induced toxic encephalopathy in a child. *Clin. Pharmacol.* **6**, 496–498.
- Eiermann, B., Sommer, N., Winne, D., Schumm, F., Maier, U., and Breyer-Pfaff, U. (1993). Renal clearance of pyridostigmine in myasthenic patients and volunteers under the influence of ranitidine and pirenzepine. *Xenobiotica* **23**, 1263–1275.
- Ellman, G. L., Courtney, K. D., Andres, V., and Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88–95.
- Fikes, J. D., Zachary, J. F., Parker, A. J., and Beasley, V. R. (1992). Clinical, biochemical, electrophysiologic, and histologic assessment of chlorpyrifos induced delayed neuropathy in the cat. *Neurotoxicol.* **13**, 663–678.
- George, S. T., and Balasubramanian, A. S. (1981). The aryl acylamidases and their relationship to cholinesterases in human serum, erythrocyte and liver. *Eur. J. Biochem.* **2**, 177–186.
- Greig, M. E., and Holland, W. C. (1949). Increased permeability of hemioencephalic barrier produced by physostigmine and acetylcholine. *Science* **110**, 237.
- Gryboski, J., Weinstein, D., and Ordway, N. K. (1961). Toxic encephalopathy apparently related to the use of an insect repellent. *N. Engl. J. Med.* **264**, 289–291.
- Hudson, C. S., Foster, R. E., and Kahng, M. W. (1985). Neuromuscular toxicity of pyridostigmine bromide in the diaphragm, extensor digitorum longus, and soleus muscles of the rat. *Fundam. Appl. Toxicol.* **5**, S260–S269.
- Huff, R. A., and Abou-Donia, M. B. (1995). *In Vitro* effect of chlorpyrifos oxon on muscarinic receptors and adenylate cyclase. *Neurotoxicology* **16**, 281–290.
- Huff, R. A., Corcoran, J. J., Anderson, J. K., and Abou-Donia, M. B. (1994). Chlorpyrifos oxon binds directly to muscarinic receptors and inhibits cAMP accumulation in rat striatum. *J. Pharmacol. Exp. Ther.* **269**, 329–335.
- Hussain, A. S., and Ritschel, W. A. (1988). Influence of dimethylacetamide, *N,N*-diethyl-*m*-toluamide and 1-dodecylazacycloheptan-2-one on *ex vivo* permeation of phosphonoformic and through rat skin. *Methods Find. Exp. Clin. Pharmacol.* **10**, 691–694.
- Institute of Medicine (1995). *Health Consequences of Service during the Persian Gulf War: Initial Findings and Recommendations for Immediate Action*. National Academy Press, Washington, DC.
- Johnson, M. K. (1969). A phosphorylation site in brain and the delayed neurotoxic effect of some organophosphorus compounds. *Biochem. J.* **111**, 487–495.
- Johnson, M. K. (1977). Improved assay of neurotoxic esterase for screening organophosphates for delayed neurotoxicity potential. *Arch. Toxicol.* **37**, 113–115.
- Jonckheere, A. R. (1954). A distribution-free *k*-sample test against ordered alternations. *Biometrika* **41**, 133–145.
- Joó, F., and Várkonyi, T. (1969). Correlation between the cholinesterase activity of capillaries and the blood-brain barrier in the rat. *Acta Biol. Acad. Sci. Hung.* **20**, 359–372.
- Kaplan, J. G., Kessler, J., Rosenberg, N., and Schaumburg, H. H. (1993). Sensory neuropathy associated with Dursban (chlorpyrifos) exposure. *Neurology* **43**, 2193–2196.
- Keeler, J. R., Hurst, C. G., and Dunn, M. A. (1991). Pyridostigmine used as a nerve agent pretreatment under wartime conditions. *JAMA* **266**, 693–695.
- Koelle, G. B. (1952). Histochemical localization of cholinesterases in the central nervous system of the rat. *J. Pharmacol. Exp. Ther.* **106**, 401.
- Lapadula, D. M., Habig, C., Gupta, R. P., and Abou-Donia, M. B. (1991). Induction of cytochrome P450 isozymes by simultaneous inhalation exposure of hens to *n*-hexane and methyl *iso*-butyl ketone (MiBK). *Biochem. Pharmacol.* **41**, 877–883.
- Loewenstein-Lichtenstein, Y., Schwarz, M., Glick, D., Nørgaard-Pedersen, B., Zakut, H., and Soreq, H. (1995). Genetic predisposition to adverse consequences of anti-cholinesterases in "atypical" BChE carriers. *Nat. Med.* **10**, 1082–1085.
- Lotti, M. (1992). The pathogenesis of organophosphate polyneuropathy. *CRC Crit. Rev. Toxicol.* **21**, 465–467.
- Lotti, M., Moretto, A., Zoppellari, R., Dainese, R., Rizzuto, N., and Barusco, G. (1986). Inhibition of lymphocytic neuropathy target esterase predicts the development of organophosphate-induced polyneuropathy. *Arch. Toxicol.* **59**, 176–179.
- McCain, W. C. (1995). Acute oral toxicity study of pyridostigmine bromide, permethrin, and DEET in the laboratory rat. *Toxicological Study 75-48-2665*. U.S. Army Medical Research and Material Command, Fort Detrick, Fredrick, MD.
- McConnell, R., Fidler, A. T., and Chrislip, D. (1986). Health Hazard Evaluation Determination Report No. 83-085. NIOSH, U.S. Department of Health and Human Services.
- Persian Gulf Veterans Coordinating Board (1995). Unexplained illnesses among desert storm veterans. *Arch. Intern. Med.* **155**, 262–268.
- Pope, C. N., Chakraborti, T. K., Chapman, M. L., and Farrar, J. D. (1992). Long-term neurochemical and behavioral effects induced by acute chlorpyrifos treatment. *Pharmacol. Biochem. Behav.* **42**, 251–256.
- Pressley, T. A., and Longbottom, J. E. (1982). The determination of organonitrogen pesticides in industrial and municipal wastewater. Method 633, U.S. Environmental Protection Agency, Cincinnati, OH.
- Richardson, R. J., Moore, T. B., Kayyali, V. S., Fowke, J. F., and Randall, J. C. (1993). Inhibition of hen acetylcholinesterase and neurotoxic esterase by chlorpyrifos *in vivo*, and kinetics of inhibition by chlorpyrifos

- axon *in vitro*: Application to assessment of neuropathic risk. *Fundam. Appl. Toxicol.* **20**, 273–279.
- Selim, S., Hartnagel, R. E., Jr., Osimitz, T. G., Gabriel, K. L., and Schoenig, G. P. (1995). Absorption, metabolism, and excretion of *N,N*-diethyl-*m*-tobiamide following dermal application to human volunteers. *Fundam. Appl. Toxicol.* **25**, 95–100.
- Simonati, A., Rizzuto, N., and Cavanagh, J. B. (1983). The effects of 2,5-hexanedione on axonal regeneration after nerve crush in the rat. *Acta Neuropathol.* **59**, 216–224.
- Smith, M. I., Elvove, E., Valer, P. J., Frazier, W. H., and Mallory, G. E. (1930). Pharmacology and clinic studies of the cause of the so-called ginger paralysis. *U.S. Public Health Rep.* **45**, 1703–1716.
- Smith, P. K., Krahn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, L. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Dlenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76–85.
- Snodgrass, H. L., Nelson, D. C., and Weeks, M. H. (1982). Dermal penetration and potential for placental transfer of the insect repellent, *N,N*-diethyl-*m*-toluamide. *Am. Ind. Hyg. Assoc. J.* **43**, 747–753.
- Spencer, T. S., Hill, J. A., Feldmann, R. J., and Maibach, H. I. (1979). Evaporation of diethyltoluamide from human skin *in vivo* and *in vitro*. *J. Invest. Dermatol.* **72**, 317–319.
- Sprague, G. L., Sandvik, L. L., and Bickford, A. A. (1981). Time course for neurotoxic esterase inhibition in hens given multiple diisopropyl fluorophosphate injections. *Neurotoxicology* **2**, 523–532.
- Teichman, S. L., Ferrick, A., Kim, S. G., Matos, J. A., Waspe, L. E., and Fisher, J. D. (1987). Disopyramide–pyridostigmine interaction: Selection reversal of anticholinergic symptoms with preservation of antiarrhythmic effect. *JACC* **10**, 633–641.
- Valdez, J., Kakkar, T., Martin, D. K., and Mayersohn, M. (1996). Disposition kinetics and dermal absorption of *N,N*-diethyl-*m*-toluamide (DEET). *Toxicologist* **30**, 39.
- Verschoye, R. D., Brown, A. W., Nolan, C., Ray, D. E., and Lister, T. (1990). A comparison of the acute toxicity, neuropathology, and electrophysiology of *N,N*-diethyl-*m*-toluamide and *N,N*-dimethyl-2,2-diphenylacetamide in rats. *Fundam. Appl. Toxicol.* **18**, 79–88.
- Viby-Mogensen, J. (1981). Succinylcholine neuromuscular blockade in subjects heterozygous for abnormal plasma cholinesterase. *Anesthesiology* **55**, 235.
- Williams, F. M. (1987). Clinical significance of esterase in man. *Clin. Pharmacokinet.* **10**, 392–403.
- Windheuser, J. J., Haslan, J. L., Caldwell, L., and Shaffer, R. D. (1982). The use of *N,N*-diethyl-*m*-toluamide to enhance dermal and transdermal delivery of drugs. *J. Pharmacol. Sci.* **71**, 1211–1213.
- Zadikoff, C. M. (1979). Toxic encephalopathy associated with use of insect repellent. *J. Pediatr.* **95**, 140–142.

NEUROTOXICITY RESULTING FROM COEXPOSURE TO PYRIDOSTIGMINE BROMIDE, DEET, AND PERMETHRIN: IMPLICATIONS OF GULF WAR CHEMICAL EXPOSURES

Mohamed B. Abou-Donia, Kenneth R. Wilmarth

Department of Pharmacology, Duke University Medical Center, Durham, North Carolina, USA

Karl F. Jensen

Neurotoxicology Division, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina, USA

Frederick W. Oehme

Department of Clinical Sciences, Comparative Toxicology Laboratories, Kansas State University, Manhattan, Kansas, USA

Thomas L. Kurt

Department of Internal Medicine, University of Texas, Southwestern Medical School, Dallas, Texas, USA

Of the three-quarters of a million service personnel involved in the Persian Gulf War, approximately 30,000 have complained of neurological symptoms of unknown etiology. One contributing factor to the emergence of such symptoms may be the simultaneous exposure to multiple agents used to protect the health of service personnel, in particular, the anti-nerve agent pyridostigmine bromide (PB; 3-dimethylaminocarbonyloxy-N-methylpyridinium bromide), the insect repellent DEET (N,N-diethyl-m-toluamide), and the insecticide permethrin (3-(2,2-dichloro-ethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester). This study investigated neurotoxicity produced in hens by individual or simultaneous exposure to these agents (5 d/wk for 2 months to 5 mg/kg/d PB in water, po; 500 mg/kg/d DEET, neat, sc; and 500 mg/kg/d permethrin in corn oil, sc). At these dosages, exposure to single compounds resulted in minimal toxicity. Combinations of two agents produced greater neurotoxicity than that caused by individual agents. Neurotoxicity was further enhanced following concurrent administration of all three agents. We hypothesize that competition for liver and plasma esterases by these compounds leads to their decreased breakdown and increased transport of the parent compound to nervous tissues. Thus, carbamylation of peripheral esterases by PB reduces the hydrolysis of DEET and permethrin and increases their availability to the nervous system. In

Received 6 October 1995; accepted 20 December 1995.

This study was supported in part by a grant from the Perot Foundation. The technical assistance of Jeanene Olin and John P. Locklear, the statistical expertise of Judy Schmid, and the secretarial assistance of Diane Brooks are appreciated. This document has been reviewed by the National Health and Environmental Effects Research Laboratory of the U.S. Environmental Protection Agency and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

effect, PB "pumps" more DEET and permethrin into the central nervous system. Consistent with this hypothesis, hens exposed to the combination of the three agents exhibited neuropathological lesions with several characteristics similar to those previously reported in studies of near-lethal doses of DEET and permethrin. If this hypothesis is correct, then blood and liver esterases play an important "buffering" role in protecting against neurotoxicity in the population at large. It also suggests that individuals with low plasma esterase activity may be predisposed to neurologic deficits produced by exposure to certain chemical mixtures.

Approximately three quarters of a million U.S. service personnel participated in the air, sea, and ground war in the Persian Gulf region between August 1990 and April 1991. During the war, service personnel were concurrently exposed to biological, chemical, and psychological environments. Some veterans have reported chronic symptoms including headache, loss of memory, fatigue, muscle and joint pain, ataxia, skin rash, respiratory difficulties, and gastrointestinal disturbances. Potential exposure was to fumes and smoke from military operations, oil well fires, diesel exhaust, toxic paints, pesticides, fire sand, depleted uranium, chemoprophylactic agents, and multiple immunization (Institute of Medicine, 1995). The reported chemical exposure included DEET and permethrin to protect against insect-borne disease and pyridostigmine bromide (PB) (Figure 1) to protect against

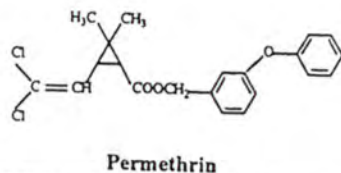
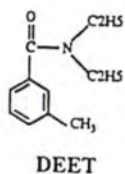
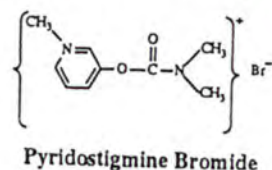


FIGURE 1. Chemical structure of test compounds.

possible nerve gas attack. Although the major site of toxicity for these chemicals is the nervous system, they differ in many aspects such as chemical structure, use, mechanisms of action, and metabolic pathways.

PB, a quaternary dimethyl carbamate, is used primarily for treatment of myasthenia gravis at a therapeutic oral dose range of 200–1,400 mg/d (Breyer-Pfaff et al., 1985, 1990) and for prophylactic protection against organophosphorus nerve gases at 30 mg three doses a day. It acts by shielding AChE from organophosphorus compound poisoning by reversibly inhibiting 30–40% of the enzyme in the peripheral nervous system, protecting it from permanent inactivation by the nerve gas. The enzyme activity is restored following spontaneous decarbamylation resulting in near-normal neuromuscular and autonomic functions (Blick et al., 1991). Toxicity of an overdose of PB results from the accumulation of acetylcholine at nicotine and muscarinic acetylcholine receptors in the peripheral nervous system (PNS) resulting in exaggerated cholinergic effects such as muscle fasciculations, cramps, weakness, muscle twitching, respiratory difficulty, tremor, gastrointestinal tract disturbances, and paralysis (Abou-Donia, 1994). Death results from asphyxia. PB does not cross the blood-brain barrier presumably because it has a positive charge on the pyridinium nitrogen (Birtley et al., 1966). The major metabolic product of PB is 3-hydroxy-N-methylpyridium resulting from the carbamate hydrolysis that abolishes its cholinergic action (Kornfeld et al., 1970, 1971). The half-life value of pyridostigmine administered iv in humans is 1–2 h (Eiermann et al., 1993).

Permethrin is a Type I synthetic pyrethroid insecticide that has four stereoisomers—(±), *cis/trans*—and often provides insecticidal activity for several weeks following a single application (Casida et al., 1983). Permethrin modifies sodium channel to open longer during a depolarizing pulse, evoking repetitive after-discharges by a single stimulus (Narahashi, 1985). This repetitive nerve action is associated with hyperactivity, tremor, ataxia, convulsion, and eventually paralysis. Aldridge (1990) reported peripheral nerve damage produced by a near-lethal dose of permethrin in rats (Rose & Dewar, 1983). Permethrin, like other pyrethroids, is metabolized by hydrolases and oxidases (Casida et al., 1983). Consequently, both esterase and oxidase inhibitors may create unanticipated hazards by enhancing pyrethroid toxicity to mammals (Pelligrini & Santi, 1972).

DEET is an aromatic amide used as a personal insect repellent against mosquitoes, biting flies, and ticks, among other insects. It has been used since 1946 by the U.S. Army and since 1957 by the general population (Pressley & Longbottom, 1982). Approximately 30% of the U.S. population uses DEET as a lotion, stick, or spray at concentrations between 10 and 100% active ingredient. Dermal exposure has been documented in humans (Robbins & Cherniack, 1986; McConnell et al., 1986). Extensive and repeated topical applications of DEET resulted in human poisoning including two deaths (Edwards & Johnson, 1987; Gryboski et al., 1961; Zadikoff, 1979; Heick et al., 1980; de Garbino & Laborde, 1983; Roland et al., 1985). Symptoms of poisoning are characterized by tremor, restless-

ness, slurred speech, seizures, impaired cognitive functions, and con (McConnell et al., 1986). Spongiform myelinopathy has been reported the brainstem of rats exposed to near-lethal doses of DEET (Verschoyle al., 1990). Metabolic biotransformation of DEET results in its detoxification by cytochrome P-450-mediated oxidation of the aromatic methyl group at N-deethylation of the amide group (McMahon & Sullivan, 1965; Yeung Taylor, 1988). Plasma amidases may also play an important role in the hydrolysis of the amide bond. Absorption, storage, and metabolism of DEET are influenced by the size of the applied dose. For example, high doses (10–15 g) significant amounts of the parent compound can be recovered in the urine, while at lower doses (15 mg) DEET is completely metabolized prior to being excreted in the urine (Selim et al., 1995; V et al., 1979; Moody et al., 1987). While DEET is efficiently absorbed through the skin (Windheuser et al., 1982; Spencer et al., 1979), the epidermis may serve as a depot for DEET such that it is slowly released in the circulation (Snodgrass et al., 1982; Blomquist & Thorsell, 1979; Spencer et al., 1979). The high efficiency by which DEET permeates the skin has led to its use as a transdermal delivery enhancer of many drugs (Windheuser et al., 1982; Hussain & Ritschel, 1988). By inference, DEET may enhance drug penetration across the blood-brain barrier to the brain.

In light of the established safety of PB (Teichman et al., 1987), it was surprising that approximately half of all military personnel seen in health care facilities during the Gulf War complained of PB side effects consistent with muscarinic stimulation (Institute of Medicine, 1995). One of the conclusions reached by the Committee to Review the Health Consequences of Service During the Persian Gulf War was that "studies are needed to resolve uncertainties about whether PB, DEET, and permethrin have additive effects" (Institute of Medicine, 1995, p. 15). The committee also recommended immediate action that "appropriate laboratory animal studies of interaction between DEET, PB, and permethrin should be conducted" (p. 15).

The present study anticipated this recommendation and investigated neurotoxicity produced by exposure to these chemicals when used individually or in combination in hens. The adult hen was chosen for the test animal because of its known susceptibility to anticholinesterase compounds and to allow direct comparison with ongoing studies of organophosphorus compounds in this laboratory.

MATERIALS AND METHODS

Chemicals

Technical-grade (93.6%) permethrin ($\pm$ -*cis/trans*-3-(2,2-dichloroethoxy)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester) was obtained from Roussel Uclaf Corp., Pasadena, TX. DEET ($\geq$ 97%, *N*, diethyl-*m*-toluamide), pyridostigmine bromide ($\geq$ 99%, 3-dimethylamino carbonyloxy-*N*-methylpyridinium bromide), acetylthiocholine iodide, a

butyrylthiocholine iodide were purchased from Sigma Chemical Co., St. Louis, MO. All other reagents were of analytical grade and obtained from commercially available sources.

Birds

Healthy adult leghorn laying hens (*Gallus gallus domesticus*), 18 mo old, weighing 1.6 ± 0.2 kg (mean $\pm$ SEM), were obtained from Featherdown Farm, Raleigh, NC. The hens were vaccinated against common chicken diseases, and were considered specific-pathogen-free and medication-free, without abnormalities of gait. Groups of five hens, randomly assigned, were housed in single-tier, $3 \times 3 \times 3$ ft stainless-steel cages in a temperature ($21\text{--}22^\circ\text{C}$) controlled room with a 12-h light, 12-h dark cycle before and during the study. The chickens were allowed to adjust to the environment for a week before treatment. They were supplied with feed (Layena Chicken Feed, Ralston Purina Co., St. Louis, MO) and water ad libitum. Animal care was in accordance with institutional guidelines.

Treatment

Initially, a dose finding study was carried out to choose the treatment dose. The selected dose was one that caused minimum changes in the clinical parameters after treatment for 5 d/wk over a 2-mo period. Individual test compound studies were performed using the following dosages over the test period: PB, 5 mg/kg/d in water, po; DEET, 500 mg/kg/d, neat, sc; and permethrin, 500 mg/kg/d in corn oil, sc. A group of five hens was used as an untreated control group. Four groups of hens were given the following combinations: PB/DEET, PB/permethrin, DEET/permethrin, and PB/DEET/permethrin.

Clinical Evaluation

Control and treated hens were observed daily inside and when they were allowed to freely move outside the cage. Observations included walking pattern, leg movement, flying, body tremor, and ability to enter the home cage. Other observations such as diarrhea, salivation, or pupil constriction were recorded. Clinical signs that developed shortly after dosing with test compounds were (1) reluctance to walk and decreased activity in cage, (2) diarrhea, and/or (3) shortness of breath. Persistent signs of neurotoxicity were categorized into two classes: locomotor dysfunctions and whole-body tremor. Locomotor dysfunctions were classified into (1) body lowered, mild leg splay while standing and on landing after flying, (2) leg weakness demonstrated by preference to fly instead of walking, gait disturbance such as linear or narrow gait, stumbling while entering home cage, and moderately impaired flying activity, (3) leg sprawled out, body unsteadiness, stumbling gait, inability to enter home cage, severely impaired flying activity, and (4) partial or complete paralysis. Signs of tremors were ranked on the following scale: (1) fine whole-body tremor, (2) fine body

tremor with intermittent head jerking, (3) coarse whole-body tremor, and (4) severe coarse body tremor. For quantification, hens were assigned numerical values corresponding to these conditions and in the absence of any signs received the value of zero.

Histopathological Assessment

Spinal cord and sciatic nerve were excised immediately after sacrifice and immersed in 4% phosphate-buffered formalin and postfixed for at least a week in the same fixative. Cross and longitudinal sections of cervical and thoracic spinal cord and longitudinal sections of proximal and distal sciatic nerve were stained with hematoxylin and eosin (H&E), Glee's and Marsland, and Periodic Acid Schiff's (PAS) method stains. Tissues were assessed with a step-down approach by first comparing sections from control and triple treatment groups, then subsequently examining double and single treatments for any evidence of alterations observed in the triple treatment groups. Severity scores for the spinal cord were based on the combined frequency of enlarged axons in white matter of the cervical and thoracic regions (0 = no detectable difference from control, 1 = mild, 2 = moderate, 3 = severe). Severity scores for the sciatic nerve also ranged from 0 to 3 and were based on the combined frequency of prominent axonal varicosities in proximal and distal segments.

Cholinesterase Determination

At 24 h following administration of the last dose, treated and control hens were anesthetized using 0.25% halothane, then sacrificed by decapitation. Brains were rapidly removed and homogenized (10% w/v) in 50 mM Tris-HCl buffer, pH 8.0 with 0.1 mM EDTA. Blood samples were collected into heparinized tubes and spun in a Beckman Model J2-21 Centrifuge (Beckman Instruments Corporation, Palo Alto, CA) at 5,000 g for 30 min at 4°C using a JA-20 rotor to separate plasma and red blood cells. All samples were stored at -70°C until use. Cholinesterase activities in plasma and brain homogenates were determined by the method of Ellman et al. (1961) using butyrylthiocholine iodide as substrate for plasma BuChE and acetylthiocholine iodide as substrate for brain AChE enzyme. Protein concentrations for brain and plasma were determined using the bicinchoninic acid method (Smith et al., 1985).

Determination of Neurotoxicity

Mean Rank Neurotoxicity was quantified by ranking control and treated hens according to severity scores from least to greatest in the following eight categories: (a) day of onset and (b) severity of acute clinical signs of neurotoxicity, (c) day of onset and (d) severity of motor dysfunction, (e) day of onset and (f) severity of tremor, and severity of neuropathologic lesions in the (g) CNS and (h) PNS (Abou-Donia et al., 1982). In case of ties, the mean rank of birds involved was assigned to each of these birds. The mean rank score for each bird is the mean of the eight ranked scores

(Jonckheere, 1954). The mean rank was used as a neurotoxicity index (NTI) to quantitate and compare neurotoxicity among treated hens.

Statistics Statistical analysis was carried out by a permutation test, which is suitable in cases where the sample size is small and the distribution of variables may not be normal. The test considers the population to consist of the scores that are present, and by calculating the difference in mean scores for all possible ways of assigning the animals to treatment groups (using the same sample sizes), calculates an exact *p*-value for the observed mean difference (Randles & Wolfe, 1979).

RESULTS

Treatment with Single Compounds

Control hens remained normal and gained slight weight by the end of the 2-mo experimental period (Figure 2). Animals treated with PB developed

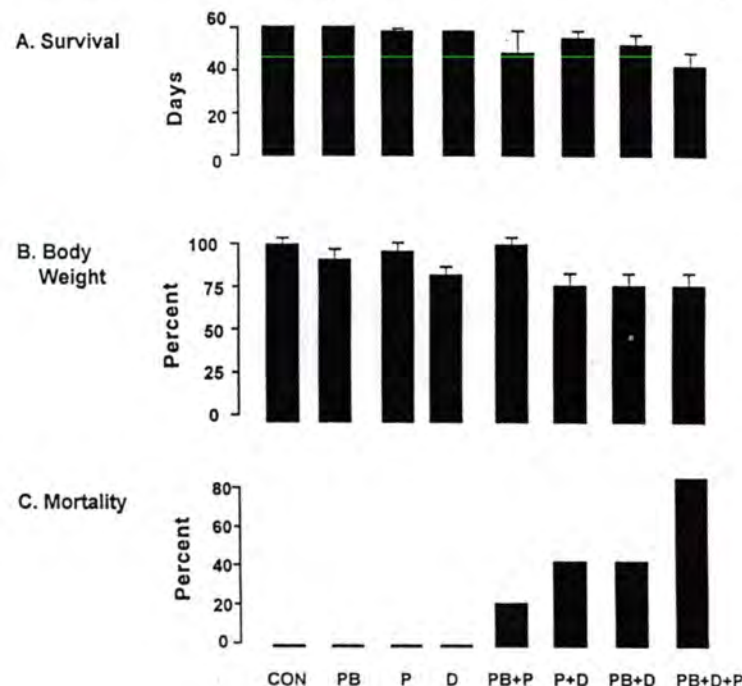


FIGURE 2. Effect of daily administration of pyridostigmine bromide (PB), DEET, and permethrin to hens on survival, body weight, and mortality. All values are means $\pm$ SEM from each treatment group (*n* = 5). Hens were given a daily dose, 5 d/wk individual or combinations of 5 mg/kg PB in water, po; 500 mg/kg sc DEET or permethrin.

TABLE 1. Clinical condition and histopathological alterations in hens following daily administration of pyridostigmine bromide (PB), DEET, and permethrin

Treatment ^a	Clinical signs ^b			Locomotor dysfunction			Tremor			Severity of histopathological changes ^c		
	Day of onset	Severity		Day of onset	Severity		Day of onset	Severity		Spinal cord		Sciatic nerve
Control	d	d		d	d		d	d		d		d
PB	15.8 ± 2.1	1.0 ± 0.0		d	d		d	d		d		d
DEET	17.4 ± 4.3	1.0 ± 0.0		d	d		d	d		d		d
Permethrin	d	d		d	d		d	d		d		d
PB/DEET	28.9 ± 2.1	1.8 ± 0.4		14.6 ± 4.0	1.0 ± 0.3		14.2 ± 8.9	0.2 ± 0.2		0.2 ± 0.2	0.4 ± 0.2	0.2 ± 0.2
PB/permethrin	19.9 ± 2.2	1.0 ± 0.0		9.6 ± 9.6	0.2 ± 0.2		10.8 ± 10.8	0.2 ± 0.2		0.8 ± 0.2	0.2 ± 0.2	1.0 ± 0.0
DEET/permethrin	23.8 ± 1.5	1.4 ± 0.2		18.6 ± 7.7	0.8 ± 0.6		20.6 ± 9.1	0.6 ± 0.2		0.2 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
PB/DEET/permethrin	2.6 ± 0.2	2.2 ± 0.4		18.8 ± 2.0	2.6 ± 0.4		26.0 ± 2.4	1.6 ± 0.5		1.8 ± 0.4	2.0 ± 0.3	2.0 ± 0.3

Note. All values are means ± SEM from each treatment group (*n* = 5).

^aHens were given a daily dose, 5 d/week individual or combinations of: 5 mg/kg PB in water, po; 500 mg/kg sc DEET or permethrin.

^bAnimals were assessed daily for clinical signs, locomotor dysfunctions, and tremor and scored as indicated under Methods. Severity scores indicate the condition of the animal at the time of sacrifice.

^cSeverity of histopathological alterations was determined by examination of the cervical and thoracic spinal cord and sciatic nerve tissues.

^dNot different from control.

transient mild signs of cholinergic toxicity characterized by decreased activity and slight diarrhea (Table 1, Figures 3 and 4). Animals treated with DEET developed rapid shallow breathing and tendency toward inactivity shortly after dosing but recovered within 24 h after dosing. Animals treated with permethrin did not exhibit any clinical signs. Only DEET-treated hens had significantly less weight at termination (Figure 2). All birds treated with single compounds survived the experiment.

Neuropathological examinations of tissues revealed no difference between controls and PB-treated animals (Table 1, Figures 3 and 4). Some animals treated with permethrin or DEET exhibited minor neuropathological changes that consisted of a small increase in the frequency of slightly enlarged axons.

At termination, animals treated with PB exhibited a marked inhibition of plasma BuChE activity to 17% of control levels, while animals exposed

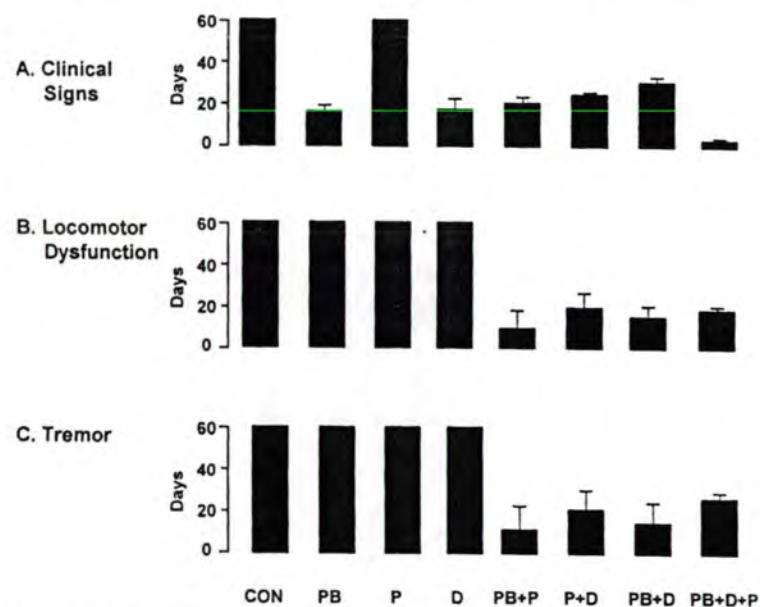


FIGURE 3. Days before onset of clinical signs, locomotor dysfunctions, and tremor in hens following daily administration of pyridostigmine bromide (PB), DEET, and permethrin. Sixty days indicate that no signs were developed by the end of the 60-d experimental period. All values are means ± SEM from each treatment group (*n* = 5). Hens were given a daily dose, 5 d/wk individual or combinations of 5 mg/kg PB in water, po; 500 mg/kg sc DEET or permethrin. Animals were assessed daily for clinical signs, locomotor dysfunctions, and tremor and scored as indicated under Methods. Severity scores indicate the condition of the animal at the time of sacrifice.

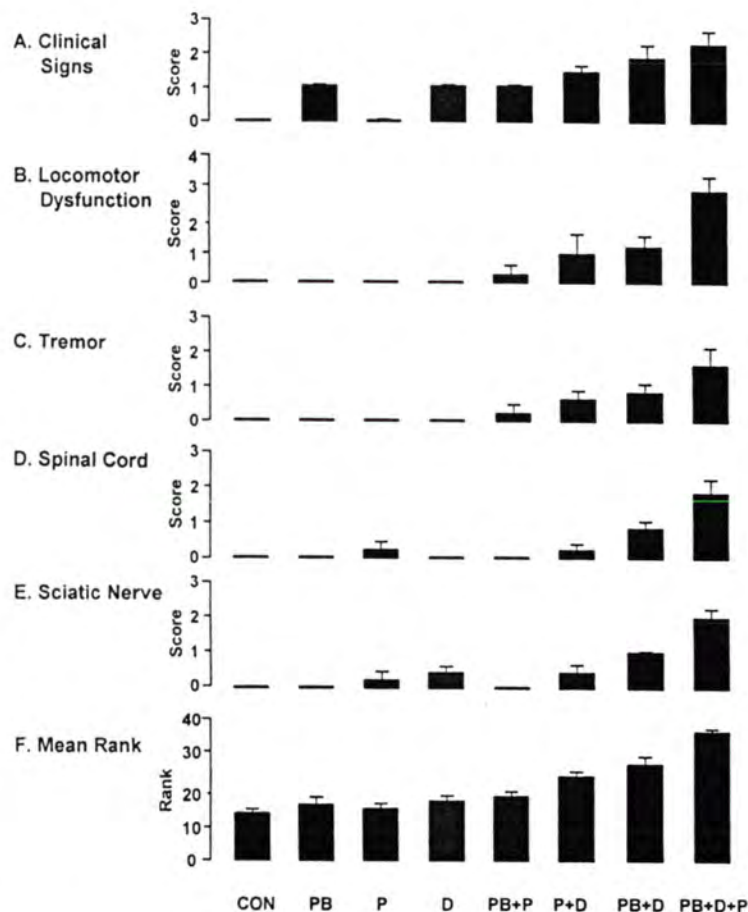


FIGURE 4. Severity of clinical signs, locomotor dysfunction, tremor, and histopathological changes and mean ranks following daily administration of pyridostigmine bromide (PB), DEET, and permethrin. All values are means $\pm$ SEM from each treatment group ($n = 5$). Hens were given a daily dose, 5 d/wk individual or combinations of: 5 mg/kg PB in water, po; 500 mg/kg sc DEET or permethrin. Animals were assessed daily for clinical signs, locomotor dysfunctions, and tremor and scored as indicated under Methods. Severity scores indicate the condition of the animal at the time of sacrifice. Severity of histopathological alterations was determined by examination of the cervical and thoracic spinal cord and sciatic nerve tissues. Mean rank or neurotoxicity index (NTI) is the mean score of clinical signs, locomotor dysfunction, tremor, and histopathological alteration. Every parameter was ranked according to severity scores from least to greatest, and within the severity according to time of onset to obtain ranked scores.

to DEET exhibited a lesser inhibition of plasma BuChE to 83% of control values (Figure 5). Animals treated with permethrin showed no alteration in plasma BuChE activity. Brain AChE was not inhibited by individual treatment with any of the compounds.

Concurrent Treatment with Two Compounds

Of the five animals treated with PB/permethrin, one developed a reluctance to walk and mild gait disturbance and another developed a fine body tremor (Table 1, Figures 3 and 4). Animals treated with DEET/permethrin or DEET/PB exhibited transient hyperexcitability between 1 and 4 wk of dosing. In addition to clinical signs observed in animals treated with the single compounds, animals treated with DEET/permethrin developed a transient leg weakness 9–14 d after the beginning of dosing. In one animal, a mild gait disturbance was detectable at 27 d that progressed to a stumbling and unsteady gait accompanied by moderate fine body tremors (Table 1, Figures 3 and 4). Altogether, three animals developed body tremors from 27 to 47 d after beginning dosing.

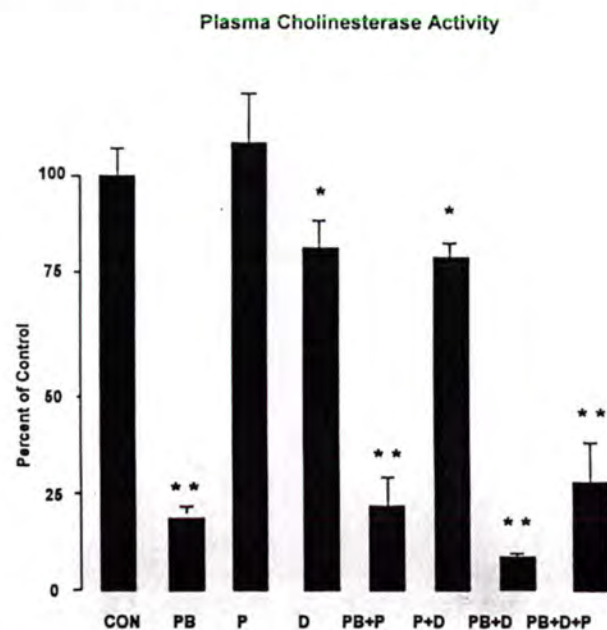


FIGURE 5. Effect of daily administration of pyridostigmine bromide (PB), DEET (D), and permethrin (P) to hens on plasma butyrylcholinesterase. Significant differences between control and treatment groups were determined by Student's *t* tests; * $p < .05$, ** $p < .01$.

Animals treated with PB/DEET also exhibited intermittent diarrhea, marked shallow breathing, and decreased locomotor activity. In four of these, altered stance and leg splay was detected from 9 to 32 d after the initiation of dosing. In three, this impairment progressed to a persistent unsteady gait. Animals treated with PB/DEET and those treated with DEET/permethrin significantly lost weight at termination (Figure 2). Two hens (40%) from each of these groups did not survive the experimental period compared to one (20%) from the group treated with PB/permethrin (Figure 2).

Microscopic examination of spinal cord and sciatic nerve did not reveal any differences between control animals and those treated with PB/permethrin (Table 1, Figures 3 and 4). Mild neuropathological alterations were observed in two of the animals treated with permethrin/DEET and mild to moderate alterations were observed in all animals treated with PB/DEET. Mild alterations, comparable to those observed after single treatments, consisted of a small but perceptible increase in the frequency of slightly enlarged axons. Moderate alterations consisted of a more significant increase in both the frequency and degree of enlargement of the axons.

Plasma BuChE activity was markedly decreased to 8% in animals treated with PB/DEET and to 20% of control activity in those treated with PB/permethrin (Figure 5). DEET/permethrin decreased plasma BuChE activity to that of treatment with DEET alone (83% of control). Brain AChE was not affected by these treatments.

Concurrent Treatment with Three Compounds

Concurrent treatment with PB, DEET, and permethrin caused severe diarrhea, shallow rapid breathing, and moderate inactivity within 15 min of dosing starting on the first day. All animals developed a gait disturbance, and in one this progressed to paralysis (Table 1, Figures 2–4). All animals exhibited body tremors 22–34 d after dosing. Two animals exhibited convulsions after 22 and 34 d into the study and subsequently died at 35 and 36 d, respectively. A third animal died 42 d into the study and another became moribund and was killed 35 d after initiation of dosing. At termination, hens from this group significantly lost weight. A total of four (80%) of the five treated hens did not survive the experimental period (Figure 2).

Animals treated with the three compounds exhibited neuropathological changes that ranged from mild to severe. There was an increase from baseline in the number of enlarged axons in the dorsal, lateral, and ventral columns of the spinal cord (Figure 6). In longitudinal sections of the thoracic spinal cord, the large number of axonal varicosities and prominent intraaxonal cytoskeletal aggregations was indicative of overt axonal degeneration. In the sciatic nerve, the most prominent effect was an increase in the number of axonal varicosities characteristic of peripheral nerve damage (Figure 7).

Concurrent treatment with the three compounds resulted in inhibition of plasma BuChE to 26.2% of control levels, similar to that of PB alone (Figure 5). No effect was detected on brain AChE.

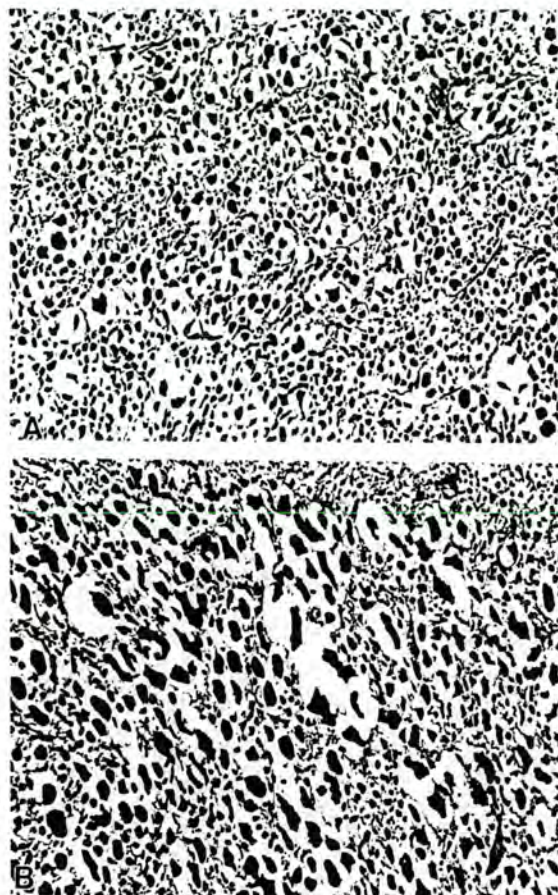


FIGURE 6. Photomicrographs of cross sections of the dorsal columns of the spinal cord (A,B). Compared to those of a control hen (A), many of the axons of the dorsal columns of a hen from the tertiary treatment group (B) appear swollen. Hematoxylin and eosin stain. $\times 100$.

Comparison of the Effects: Individual and Multiple Compounds

In this study, the mean rank value was used to quantify and compare the neurotoxic effects of various treatments (Table 1, Figure 4). The mean rank value for control was significantly less than the values for all treated groups except for hens treated with permethrin alone (Table 1, Figure 4). Hens treated with two compounds—PB/DEET, PB/permethrin, and DEET/permethrin—had mean rank values significantly higher than for single treatments

EXPOSURE TO PYRIDO
permethrin/DEET/PB
in the mean ranks
group (Table 2). For
was significantly
with an increase
cord. Mean rank
in the lo
for PB/per

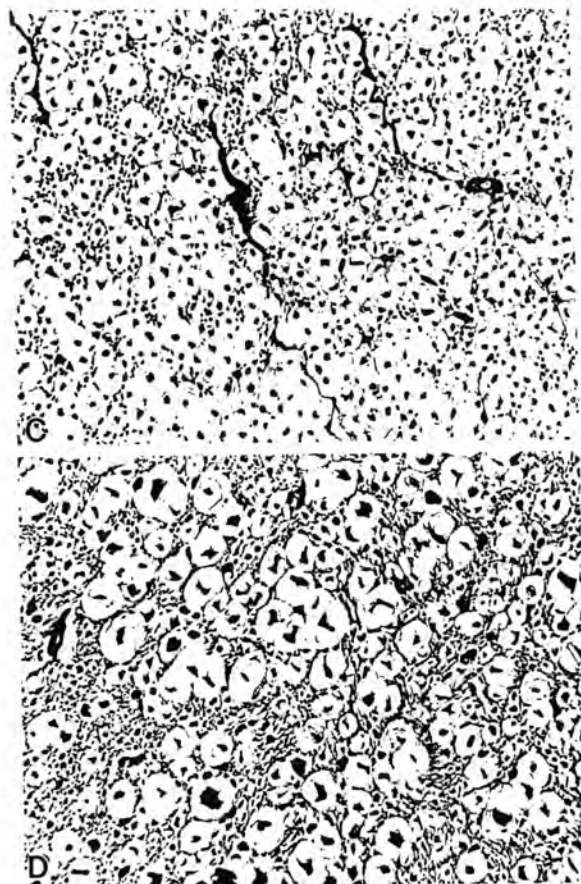


FIGURE 6. (Continued) Photomicrographs of cross sections of the lateral columns of the spinal cord (C,D). Compared to those of a control hen (C), the lateral columns of a hen from the tertiary treatment group (D) exhibit numerous enlarged axons. Hematoxylin and eosin stain. $\times 100$.

except for PB/permethrin, which was not significantly different from DEET alone (Table 2). Treatments with the three compounds had a mean rank value that was significantly higher than single- and two-compound treatments.

The results demonstrate that PB significantly increased the neurotoxic effect when combined with individual chemicals. For comparison of the



permethrin/DEET/PB group with the permethrin/DEET group, the difference in the mean ranks was significantly higher in the permethrin/DEET/PB group (Table 2). For the PB/DEET versus DEET comparison, the mean rank was significantly higher with PB/DEET, and this appeared to be associated with an increase in the locomotor dysfunction and changes in the spinal cord. Mean rank for PB/permethrin was also significantly greater than that

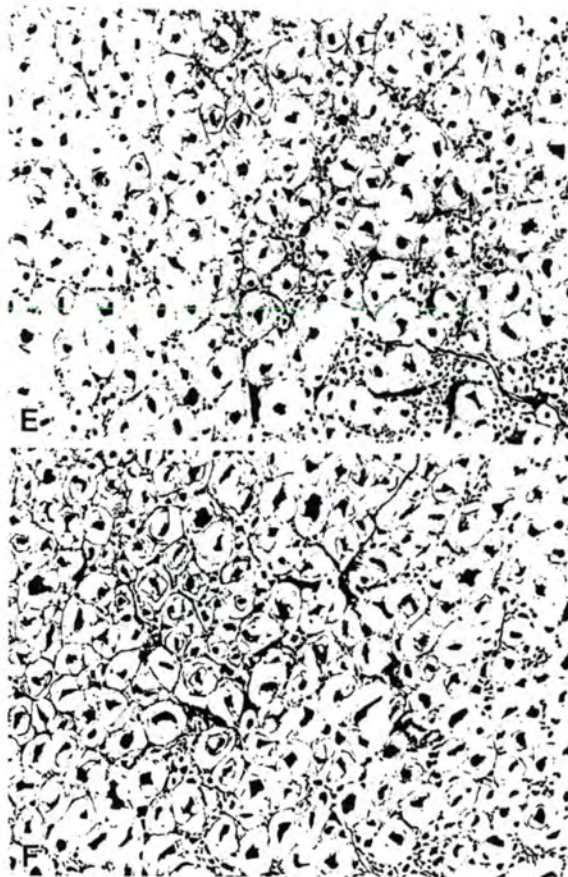


FIGURE 6. (Continued) Photomicrographs of cross sections of the ventral columns of the spinal cord (E,F). Compared to those of a control hen (E), the ventral columns of a hen from the tertiary treatment group (F) exhibit an increased frequency of swollen axons. Hematoxylin and eosin stain, $\times 100$.

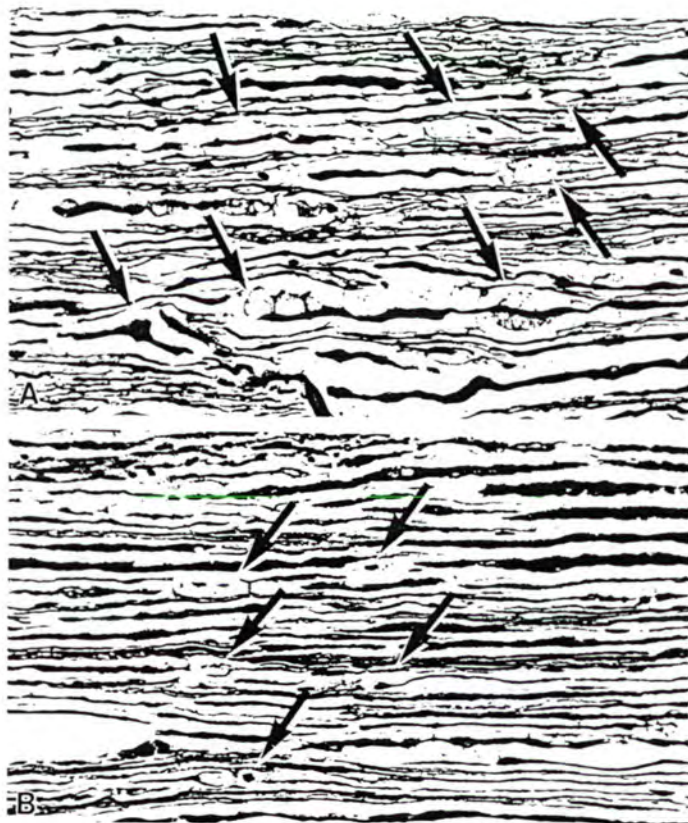


FIGURE 7. Photomicrographs of longitudinal sections through the lateral columns of the spinal cord (A, $\times 50$) and sciatic nerve (B, $\times 100$) from hens in the tertiary treatment group. There are a substantial number of axonal varicosities (arrows), similar to those observed following near-lethal doses of permethrin. Neurofibrillary alterations were apparent in many of these varicosities. Glees and Marsland silver stain.

for permethrin. The combined results also show the significant increase in neurotoxicity of the triple-compound treatment over the individual and binary treatments.

DISCUSSION

This study demonstrates that concurrent administration of any two compounds of PB, DEET, and permethrin results in neurotoxicity that is

markedly greater than that resulting from treatment with any individual compound. Additionally, neurotoxicity is further enhanced by the concurrent administration of all three compounds. These findings have been confirmed in part by a recently completed LD50 rodent study (McCain, 1995).

Although this study was not intended to simulate actual exposure conditions that may have existed during the Persian Gulf War, nor was it designed as a dose-response study, from it one can hypothesize why coexposure to test compounds may have contributed to Gulf War veterans' illnesses. PB was administered by gavage, since the service personnel took it as a 30-mg tablet, three times daily (equivalent to 1.3 mg/kg/d). Service personnel were dermally exposed to DEET and permethrin (Institute of Medicine, 1995). In this study, these pesticides were applied subcutaneously to account accurately for the absorbed dose. There are no reliable estimates of potential level of DEET exposure among service personnel during the Gulf War. The only available data to estimate potential DEET exposure in humans come from a 1986 NIOSH study of employees in the Everglades National Park (McConnell et al., 1986). This study indicated that the mean weekly estimated dermal application of DEET was 14.6 g (42 mg/kg/d for a 70-kg person). Also, 5% of the workers applied 66.3 g (189 mg/kg/d) or more to the skin weekly and 1% applied 392.6 g/wk (1,122 mg/kg/d). By analogy, heavy use of DEET by 1–5% of the Persian Gulf War veterans (7,000–35,000 persons) may be related to the unexpected complaints of some of the veterans, which included fatigue, joint pain, ataxia, and rash (Keeler et al., 1991; Institute of Medicine, 1995). Other complaints reported by "heavy users" of DEET were stumbling, weakness, and muscle cramps (McConnell et al., 1986).

TABLE 2. Differences in the combined rank^a for clinical signs, locomotor dysfunction, tremor, and neuropathological alterations in hens given a daily dose of pyridostigmine bromide (PB), DEET, and permethrin.

Treatment group ^a	Tertiary treatment, PB/DEET/ permethrin	Binary treatments			Individual treatments		
		DEET/ permethrin	PB/ DEET	PB/ permethrin	PB	DEET	Permethrin
Binary treatments							
DEET/permethrin	10.7 ^c						
PB/DEET	8.7 ^c	2.0 ^d					
PB/permethrin	15.8 ^c	5.1 ^c	7.1 ^c				
Individual treatments							
PB	19.9 ^c	9.2 ^c	11.2 ^c	4.1 ^c			
DEET	18.2 ^c	7.5 ^c	9.5 ^c	2.4 ^d			
Permethrin	22.7 ^c	12.0 ^c	14.0 ^c	6.9 ^c			
Control	23.2 ^c	12.5 ^c	14.5 ^c	7.4 ^c	3.3 ^c	5.0 ^c	0.5 ^d

^aMean rank is the mean score of clinical signs, locomotor dysfunction, tremor, and neuropathological lesions.

^bTreatments are indicated in Table 1.

^cStatistically significant, $p < .05$.

^dNot statistically significant.

Because combined treatments increased neurological deficits characterized by both PNS and CNS injury, one might assume that this neurotoxicity resulted primarily from the direct action of DEET and permethrin, with PB playing an indirect role because it does not cross the blood-brain barrier. Both DEET and permethrin have been shown to produce tremors and hyperexcitability in experimental animals (Ambrose et al., 1959; Schoening et al., 1993). However, the severity of neurologic deficits following exposure to combined chemicals in this study would not be readily predicted from their acute toxicity. The acute oral LD50 of PB in male rats is 61.6 mg/kg (McCain, 1995) and of DEET is 3,000 mg/kg (Ambrose et al., 1959). The acute oral LD50 of permethrin in the chicken is 9,000 mg/kg (Aldridge, 1990). The increased frequency of enlarged axons and axonal varicosities in the PNS tissues in the tertiary treatment is similar to those produced by lethal doses of permethrin (Rose & Dewar, 1983; Narahashi, 1985; Aldridge, 1990). Also, the observed axonal degeneration in the spinal cord may correspond to the CNS lesions produced by lethal doses of DEET (Verschoyle et al., 1990). Thus, the concomitant increase in PNS and CNS morphologic alterations may be related to levels of these two compounds acting independently but with increased potency.

Inhibition of plasma BuChE enzymatic activity is consistent with the mode of action of the test compounds. PB alone or in combination strongly inhibited plasma BuChE. As expected, PB did not affect brain AChE activity, because it does not cross the blood-brain barrier due to the positive charge on its pyridinyl nitrogen (Birtley et al., 1966). DEET caused slight but significant inhibition of plasma BuChE, perhaps because of its structural similarity to phenyl carbamates, known inhibitors of cholinesterases (Ahmad & Forgash, 1976). Permethrin, a noncholinergic insecticide, had no effect on either plasma or brain cholinesterase. Combination treatments including PB did not inhibit brain AChE activity, indicating that the increased neurotoxicity did not result from increased permeability of the blood-brain barrier.

We hypothesize that combined treatment-enhanced neurotoxicity is the consequence of increased "effective concentration" of DEET and permethrin at the neurotoxicity target. The three test compounds are metabolized via liver cytochrome P-450 and plasma hydrolyzing enzymes, esterases or amidases (Gaughau et al., 1978; Casida et al., 1983; Eiermann et al., 1993; Selim et al., 1995). Esterases function to protect the nervous system from xenobiotics by enhancing their removal from the body by acting as scavengers or through hydrolysis of xenobiotics into water-soluble breakdown products (Williams, 1987). The prophylactic action of PB depends on the reversible binding and temporary shielding of PNS AChE from irreversible attack by organophosphorus nerve agents. This agrees with our findings that PB, alone or in combination, significantly inhibited plasma BuChE. Thus, in combined treatments, lowered esterase activity in the blood results in diminished metabolic biotransformation of DEET and permethrin and increased bioavailability of the parent compounds in the nervous system.

so, DEET, alone or in combination with permethrin, may produce some inhibition of plasma BuChE, resulting in competition for hydrolases and a diminished breakdown of both chemicals. This would increase their effective concentration at the neurotoxicity target in the nervous system and may explain the increased toxicity observed following concurrent administration of DEET and permethrin. Also, DEET is used to enhance transdermal delivery of drugs (Windheuser et al., 1982; Hussain & Ritschel, 1988), suggesting that it may accelerate the skin penetration of permethrin following dermal exposure to both pesticides in humans.

Increased severity of clinical signs and tremor following coexposure to test compounds may be related to their effects on neurotoxicity target proteins. The severe and mild inhibitions of AChE in the PNS by PB and DEET, respectively, increase ACh in the synapse, thereby increasing skeletal and smooth muscle contraction (Abou-Donia, 1994). This effect may be augmented by permethrin, which keeps axonal and muscle sodium channels open longer, resulting in hyperexcitability of the nervous system and muscles (Narahashi, 1985).

The PB-induced increase in toxicity of DEET and/or permethrin found in this study agrees with reports of its enhanced neurotoxicity with nerve agents. Oral doses of PB in rats that produced 73% inhibition of plasma BuChE caused a significant increase in the neurotoxicity of soman (Shiloff & Clement, 1986; Koplovitz et al., 1992). In contrast, the PB dose that produced less than 30% inhibition was prophylactic against the nerve gas.

The variety of symptoms reported by veterans make it unlikely that a single etiologic cause is responsible for producing the Gulf War illnesses. The postulated interaction between plasma BuChE activity and effective CNS concentrations of concurrently administered toxicants, however, does provide a basis for characterizing a subpopulation potentially susceptible to adverse effects of permethrin, PB, and DEET. This may be explained in part by the relatively low incidence of genetic variants of plasma BuChE known as "atypical" BuChE in the general population that have a diminished ability to inactivate drugs or pesticides (Whittaker, 1964; Lehmann & Liddell, 1964). Usual BuChE, but not its genetic variants, acts as a scavenger for antiesterases, for example, anticholinesterases. The atypical enzyme in which an aspartate at position 70 is substituted by glycine (McGuire et al., 1989; Neville et al., 1992), is incapable of hydrolyzing succinylcholine used during surgery (Kalow & Davis, 1958; Lockridge, 1990). This variant is also much less sensitive than the usual enzyme to inhibition by some inhibitors (Kalow & Davis, 1958; McGuire et al., 1989; Lockridge, 1990; Neville et al., 1992). The homozygous usual enzyme (E^u, E^u) is 80% inhibited by dibucaine and is present in most of the Caucasian population (Viby-Mogensen, 1981). The heterozygous atypical enzyme (E^u, E^a) with 60% inhibition by dibucaine is present in about 4% of the population. The homozygous atypical enzyme (E^a, E^a) that is 20% inhibited by dibucaine occurs with an incidence under 0.04% in Europe, but up to

0.6% in specific populations (Ehrlich et al., 1994). Individuals with genetic types of decreased plasma BuChE activity should be considered potentially predisposed and at high risk to anticholinesterase and related compounds and may account for some of the more severe symptoms seen in up to 4% of the Gulf War veterans (Institute of Medicine, 1995). Consistent with our hypothesis, it has been reported that a soldier, homozygous for atypical BuChE who had exhibited succinylcholine-induced apnea during surgery, experienced cholinergic symptoms following treatment with PB during the Persian Gulf War (Loewenstein-Lichtenstein et al., 1995).

This study shows that relatively high doses of PB, DEET, and permethrin appear to cause minimal health risk when used individually. It demonstrates, however, the increased neurotoxicity associated with coexposure to the same doses of test compounds. In addition, these findings suggest the need for additional studies into potential health risks associated with coexposure of humans to these agents at dosages likely to have been used by the Gulf War veterans.

REFERENCES

- Abou-Donia, M. B., Makkawy, H. M., and Graham, D. G. 1982. The relative neurotoxicities of 2-hexane, methyl *n*-butyl ketone, 2,5-hexanediol, and 2,5-hexanedione, following oral or intraperitoneal administration to hens. *Toxicol. Appl. Pharmacol.* 62:369-389.
- Abou-Donia, M. B. 1994. Organophosphorus pesticides. In *Handbook of Neurotoxicology*, eds. L. W. Chang and R. S. Dyer, pp. 419-473. New York: Marcel Dekker.
- Ahmad, S., and Forgash, K. A. J. 1976. Nonoxidative enzymes in the metabolism of insecticides. *Drug Metab. Rev.* 5:141-164.
- Aldridge, W. N. 1990. An assessment of the toxicological properties of pyrethroids and their neurotoxicity. *CRC Crit. Rev. Toxicol.* 21:89-104.
- Ambrose, A. M., Huffman, D. K., and Salamone, R. T. 1959. Pharmacological and toxicologic studies on *N,N*-diethyltoluamide *N,N*-diethyl-*m*-toluamide. *Toxicol. Appl. Pharmacol.* 1:99-115.
- Birtley, R. D., Roberts, J. B., Thomas, B. H., and Wilson, A. 1966. Excretion and metabolism of [¹⁴C]-pyridostigmine in the rat. *Br. J. Pharmacol.* 26:393-402.
- Blick, D. W., Kerenyi, S. Z., Miller, S., Murphy, M. R., Brown, G. C., and Hartgraves, S. L. 1991. Behavioral toxicity of anticholinesterases in primates: Chronic pyridostigmine and soman interactions. *Pharmacol. Biochem. Behav.* 38:527-532.
- Blomquist, L., and Thorsell, W. 1977. Distribution and fate of the insect repellent ¹⁴C-*N,N*-diethyl-*m*-toluamide in the animal body. II. Distribution and excretion after cutaneous application. *Acta Pharmacol. Toxicol.* 41:235-243.
- Breyer-Pfaff, U., Maier, U., Brinkmann, A. M., and Schumm, F. 1985. Pyridostigmine kinetics in healthy subjects and patients with myasthenia gravis. *Clin. Pharmacol. Therap.* 37:495-501.
- Breyer-Pfaff, U., Schmezer, A., Maier, U., Brinkmann, A., and Schumm, F. 1990. Neuromuscular function and plasma drug levels in pyridostigmine treatment of myasthenia gravis. *J. Neurol. Neurosurg. Psychiatr.* 53:502-506.
- Casida, J. E., Gamman, D. W., Glickman, A. H., and Lawrence, L. J. 1983. Mechanisms of selective action of pyrethroid insecticides. *Ann. Rev. Pharmacol. Toxicol.* 23:413-438.
- de Garbino, J. P., and Laborde, A. 1983. Toxicity of an insect repellent: *N,N*-diethyltoluamide. *Vet. Hum. Toxicol.* 25:422-423.
- Edwards, D. L., and Johnson, C. E. 1987. Insect-repellent-induced toxic encephalopathy in a child. *Clin. Pharmacol.* 6:496-498.
- Ehrlich, G., Ginzberg, D., Loewenstein, Y., Glick, D., Kerem, B., Ben-Ari, S., Zakut, H., and Soreq, H. 1994. Population diversity and distinct haplotype frequencies associated with AChE and BChE genes of Israeli Jews from trans-caucasian Georgia and from Europe. *Genomics* 22:288-295.
- Eiermann, B., Sommer, N., Winne, D., Schumm, F., Maier, U., and Breyer-Pfaff, U. 1993. Renal clearance of pyridostigmine in myasthenic patients and volunteers under the influence of ranitidine and pirenzepine. *Xenobiotica* 23:1263-1275.
- Ellman, G. L., Courtney, K. D., Andres, V., and Featherstone, R. M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7:88-95.
- Gaughau, L. C., Robinson, R. A., and Casida, J. E. 1978. Distribution and metabolic fate of trans- and cis-permethrin in lactating jersey cows. *J. Agric. Food Chem.* 26:613-618.
- Gryboski, J., Weinstein, D., and Ordway, N. K. 1961. Toxic encephalopathy apparently related to the use of an insect repellent. *N. Engl. J. Med.* 264:289-291.
- Heick, H. M. C., Shipman, R. T., Norman, M. E., and James, W. 1980. Reye-like syndrome associated with the use of insect repellent in a presumed heterozygote for ornithine carbamoyl transferase deficiency. *J. Pediatr.* 97:471-473.
- Hussain, A. S., and Ritschel, W. A. 1988. Influence of dimethylacetamide, *N,N*-diethyl-*m*-toluamide and 1-dodecylazacycloheptan-2-one on ex vivo permeation of phosphonoformic and through rat skin. *Meth. Find. Expert. Clin. Pharmacol.* 10:691-694.
- Institute of Medicine. 1995. *Health consequences of service during the Persian Gulf War: Initial findings and recommendations for immediate action*. Washington, DC: National Academy Press.
- Jonckheere, A. R. 1954. A distribution-free *k*-sample test against ordered alternations. *Biometrik* 41:133-145.
- Kalow, W., and Davis, R. O. 1958. The activity of various esterase inhibitors towards atypical human serum cholinesterase. *Biochem. Pharmacol.* 1:183-192.
- Keeler, J. R., Hurst, C. G., and Dunn, M. A. 1991. Pyridostigmine used as a nerve agent pretreatment under wartime conditions. *JAMA* 266:693-695.
- Koplovitz, I., Harris, L. W., Anderson, D. R., Lennox, W. J., and Stewart, J. R. 1992. Reduction by pyridostigmine pretreatment of the efficacy of atropine and 2-PAM treatment of sarin and VX poisoning in rodents. *Fund. Appl. Toxicol.* 18:102-106.
- Kornfeld, P., Samuels, A. J., Wolf, R. I., and Osserman, K. E. 1970. Metabolism of ¹⁴C-labeled pyridostigmine in myasthenia gravis: Evidence for multiple metabolites. *Neurology* 20:634-641.
- Kornfeld, P., Wolf, R. I., Samuels, A. J., and Osserman, K. E. 1971. The effect of chronic pyridostigmine administration on pyridostigmine-¹⁴C metabolism in myasthenia gravis. *Neurology* 21:550-552.
- Lehmann, H., and Liddell, J. 1964. Genetical variants of human serum pseudocholinesterase. *Prog. Med. Gen.* 3:75-105.
- Lockridge, O. 1990. Genetic variants of serum cholinesterase influence metabolism of the muscle relaxant succinylcholine. *Pharmacol. Ther.* 47:35-60.
- Loewenstein-Lichtenstein, Y., Schwarz, M., Glick, D., Norgaard-Pedersen, B., Zakut, H., and Soreq, H. 1995. Genetic predisposition to adverse consequences of anti-cholinesterases n "atypical" BChE carriers. *Nat. Med.* 10:1082-1085.
- McCain, W. C. 1995. Acute oral toxicity study of pyridostigmine bromide, permethrin, and DEET in the laboratory rat. Toxicological study 75-48-2665, Fredrick, MD: U.S. Army Medical Research and Material Command, Fort Detrick.
- McConnell, R., Fidler, A. T., and Chrislip, D. 1986. Health hazard evaluation determination report no. 83-085. Washington, DC: NIOSH, U.S. Department of Health and Human Services.
- McGuire, M. C., Nogueira, C. P., Bartels, C. F., Lightstone, H., Hajra, A., Van der Spek, A. F., Lockridge, O., and La Du, B. N. 1989. Identification of the structural mutation responsible for the dibucaine-resistant (atypical) variant form of human serum cholinesterase. *Proc. Natl. Acad. Sci. U.S.A.* 86:953-957.
- McMahon, R. E., and Sullivan, H. R. 1965. The metabolism of the herbicide diphenamid in rats. *Biochem. Pharmacol.* 14:1085-1092.
- Moody, R. P., Riedel, D., Ritter, L., and Franklin, C. A. 1987. The effect of DEET (*N,N*-diethyl-*m*-toluamide) on dermal persistence and absorption of the insecticide fenitrothion in rats and monkeys. *J. Toxicol. Environ. Health* 22:471-479.
- Narahashi, T. 1985. Nerve membrane ionic channels as the primary target of pyrethroid. *Neurotoxicology* 6:3-22.
- Neville, L. F., Gnat, A., Loewenstein, Y., Seidman, S., Ehrlich, G., and Soreq, H. 1992. Intramolecular

- relationships in cholinesterases revealed by oocyte expression of site-directed and natural variants of human BChE. *EMBO J.* 11:1641-1649.
- Pelligrini, G., and Santi, R. 1972. Potentiation of toxicity of organophosphorus compounds containing carboxylic acid functions towards warm-blooded animals by some organophosphorus impurities. *J. Agric. Food Chem.* 20:944-950.
- Pressley, T. A., and Longbottom, J. E. 1982. The determination of organonitrogen pesticides in industrial and municipal wastewater. Method 633, U.S. Environmental Protection Agency, Cincinnati, OH.
- Randles, R. H., and Wolfe, D. A. 1979. *Introduction to the theory of nonparametric statistics*. New York: Wiley.
- Robbins, P. J., and Cherniack, M. G. 1986. Review of the biodistribution and toxicity of the insect repellent *N,N*-diethyl-*m*-toluamide (DEET). *J. Toxicol. Environ. Health* 18:503-525.
- Roland, E. H., Jan, J. E., and Rigg, J. M. 1985. Toxic encephalopathy in a child after brief exposure to insect repellents. *Can. Med. Assoc. J.* 132:155-156.
- Rose, G. P., and Dewar, A. J. 1983. Intoxication with four synthetic pyrethroids fails to show any correlation between neuromuscular dysfunction and neurochemical abnormalities in rats. *Arch. Toxicol.* 53:297-316.
- Schoening, G. P., Hartnagel, Jr., R. E., Schardein, J. L., and Vorhees, C. V. 1993. Neurotoxicity evaluation of *N,N*-diethyl-*m*-toluamide (DEET) in rats. *Fund. Appl. Toxicol.* 21:355-365.
- Selim, S., Hartnagel, Jr., R. E., Osimitz, T. G., Gabriel, K. L., and Schoenig, G. P. 1995. Absorption, metabolism, and excretion of *N,N*-diethyl-*m*-toluamide following dermal application to human volunteers. *Fund. Appl. Toxicol.* 25:95-100.
- Shiloff, J. D., and Clement, J. D. 1986. Effects of subchronic pyridostigmine pretreatment on the toxicity of soman. *Can. J. Physiol. Pharmacol.* 64:1047-1049.
- Smith, P. K., Krahn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, L. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Dlenk, D. D. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76-85.
- Snodgrass, H. L., Nelson, D. C., and Weeks, M. H. 1982. Dermal penetration and potential for placental transfer of the insect repellent, *N,N*-diethyl-*m*-toluamide. *Am. Ind. Hyg. Assoc. J.* 43:747-753.
- Spencer, T. S., Hill, J. A., Feldmann, R. J., and Maibach, H. I. 1979. Evaporation of diethyltoluamide from human skin in vivo and in vitro. *J. Invest. Dermatol.* 72:317-319.
- Teichman, S. L., Ferrick, A., Kim, S. G., Matos, J. A., Waspe, L. E., and Fisher, J. D. 1987. Disopyramide-pyridostigmine interaction: Selective reversal of anticholinergic symptoms with preservation of antiarrhythmic effect. *JACC* 10:633-641.
- Verschöyle, R. D., Brown, A. W., Nolan, C., Ray, D. E., and Lister, T. 1990. A comparison of the acute toxicity, neuropathology, and electrophysiology of *N,N*-diethyl-*m*-toluamide and *N,N*-dimethyl-2,2-diphenylacetamide in rats. *Fund. Appl. Toxicol.* 18:79-88.
- Viby-Mogensen, J. 1981. Succinylcholine neuromuscular blockade in subjects heterozygous for abnormal plasma cholinesterase. *Anesthesiology* 55:429-434.
- Whittaker, M. 1964. The pseudocholinesterase variants: Esterase levels and increased resistance to fluoride. *Acta Genet. Basel.* 14:281-285.
- Williams, F. M. 1987. Clinical significance of esterase in man. *Clin. Pharmacokinetic.* 10:392-403.
- Windheuser, J. J., Haslan, J. L., Caldwell, L., and Shaffer, R. D. 1982. The use of *N,N*-diethyl-*m*-toluamide to enhance dermal and transdermal delivery of drugs. *J. Pharm. Sci.* 71:1211-1213.
- Wu, A., Pearson, M. L., Shekoski, D. L., Soto, R. J., and Stewart, R. D. 1979. High resolution gas chromatography/mass spectrometric characterization of urinary metabolites of *N,N*-diethyl-*m*-toluamide (DEET) in man. *J. High Resolut. Chromatogr. Commun.* 2:558-562.
- Yeung, J. M., and Taylor, W. G. 1988. Metabolism of *N,N*-diethyl-*m*-toluamide (DEET) by liver microsomes from male and female rats. *J. Metab. Dispos.* 16:600-604.
- Zadikoff, C. M. 1979. Toxic encephalopathy associated with use of insect repellent. *J. Pediatr.* 95:140-142.

SHORT REPORT

The "Gulf War syndrome". Is there evidence of dysfunction in the nervous system?

G A Jamal, S Hansen, F Apartopoulos, A Peden

Abstract

In a pilot study, 14 Gulf War veterans were randomly selected from a large list of those with unexplained illness, to compare the functional integrity of the peripheral and central nervous system with a group of 13 healthy civilian control subjects using predetermined outcome measures. The controls were matched closely for age, sex, handedness, and physical activity. Outcome measures included scoring of symptoms and clinical neurological signs, quantitative sensory testing of heat, cold and vibration sensibilities, motor and sensory nerve conduction studies on upper and lower limbs, needle EMG of distal and proximal muscles and multimodality evoked potential (visual, brainstem, and somatosensory) studies.

Three measurements, all related to peripheral nerve function (cold threshold ($P = 0.0002$), sural nerve latency ($P = 0.034$), and median nerve sensory action potential ($P = 0.030$) were abnormal in the veterans compared with the controls. There may be a dysfunction in the veterans but more studies are required to investigate the findings further and to characterise the dysfunction if confirmed.

(*J Neurol Neurosurg Psychiatry* 1996;60:449-451)

Keywords: gulf war syndrome; neurological dysfunction; quantitative sensory testing; neurophysiological tests

anthrax, yellow fever, and cholera. They were also prescribed tablets known as NAPTS and BATS with the aim of protecting them against the potential use of chemical and biological weapons during the war. These tablets contain pyridostigmine bromide and pentavalent botulinum toxoid respectively. A recent study showed that giving pyridostigmine at the equivalent prescribed dose to the soldiers caused a breakdown of skeletal muscles after physical exercise in rats.¹ There is also evidence that troops were exposed to pesticides² during the war which have potential neurotoxic effects.³ Furthermore, although the toxic and biological effects of each substance are well identified, their combined effects are not.

It is desirable to establish whether or not there is any scientific evidence of organic neurological dysfunction underlying this syndrome given the nature of the symptoms reported. This pilot study was designed to look for any evidence of peripheral and central nervous system dysfunction in veterans with GWSUI. The neurological symptoms and clinical signs were scored by standard methods⁴ and a battery of neurophysiological techniques was used to assess the function of both the peripheral and the central nervous system in a group of 14 soldiers, randomly selected, and in a group of age and sex matched control subjects. These neurophysiological techniques are sufficiently sensitive to detect early or sub-clinical signs of neurotoxicity in humans.^{5,6}

Subjects and methods

Fourteen veterans were investigated, 12 men and two women, all of whom had taken part in the Gulf War and who complained of unexplained illness afterwards. These soldiers were randomly selected by a standard computer program (Lotus 1-2-3 using a congruential multiplicative generator) from a list of 40 veterans from all over the United Kingdom with GWSUI, made available to us by a voluntary organisation. All the subjects selected by computer were studied and none were rejected for other reasons. The mean age was 34.2 (SD 8.0); range 24-50 years. They had no known causes of peripheral nerve dysfunction, no family history of neuromuscular illnesses, and none were on any neurotoxic medication or excessive alcohol intake. Thirteen healthy

There has been increasing public concern about the so-called Gulf War syndrome in veterans who participated in Operation Desert Storm in 1991. Many veterans were found to have diagnosable diseases but in many the symptoms were not readily explained and this bizarre collection of symptoms has been referred to as the Gulf War syndrome unexplained illness (GWSUI). The symptoms include excessive fatigue, sensory symptoms such as paraesthesiae and numbness, headaches, skin rashes, myalgia, arthralgia, dyspnoea, chest pain, memory loss, sleep disturbances, and diarrhoea. Soldiers in the Gulf were given vaccines against polio, hepatitis B,

Departments of
Neurology and Clinical
Physics, Institute of
Neurological Sciences,
Southern General
Hospital NHS Trust,
Glasgow, UK
G A Jamal
S Hansen
F Apartopoulos
A Peden

Correspondence to:
Dr Goran A Jamal,
Department of Neurology,
Institute of Neurological
Sciences, Southern General
Hospital NHS Trust,
Glasgow G51 4TF, UK.

Received 16 March 1995
and in final revised form
25 September 1995
Accepted 17 November 1995

civilian control subjects (mean age 35.1 (SD 7.8); range 23–49 years) matched for age, sex, handedness, and as closely as possible for physical activity (similar quality and average hours of daily physical exercise) were also studied. The controls were not matched for height or weight. The study had been approved by the local hospital ethics committee and all those participating gave their informed, written consent.

The symptoms and signs were scored according to international standard techniques.⁴ Clinical symptoms were scored as 0: absent or 1: present without further quantification. Symptoms scored included fatigability, weakness, paraesthesiae, numbness, spontaneous sensation of heat and cold, and pain with an overall symptoms score ranging from 0 to 6. Five reflexes (biceps, triceps, supinator, knee, and ankle) were scored on the right side. Each reflex was graded 0: normal, 1: present only on reinforcement and 2: absent, thereby providing an overall reflex score ranging from 0 to 10. Power in the distal, intermediate, and proximal group of muscles in the right upper and lower limbs was assessed and scored ranging from 0: normal to 4: complete paralysis using the same criteria as the MRC scale and was combined into an overall score ranging from 0 to 24. Sensations of pin prick, vibration, fine touch, and position on the right side were scored separately as follows; 0: normal, 1: reduced below ankle, 2: reduced below knee, 3: reduced in hand and below knee, and 4: reduced below elbow and

below knee. These were combined into a single score ranging from 0 to 16 for each person.

Motor nerve conduction studies in the median and the common peroneal nerves and sensory conduction studies in the right median and sural nerves were carried out with standard techniques.⁷ The shortest distal motor latencies, fastest motor conduction velocities, compound muscle action potential amplitudes, shortest F wave latencies of 10 supramaximal stimulations, sensory latencies, and amplitudes were all measured. Needle EMG was performed in the right tibialis anterior and extensor digitorum brevis muscles.

Quantitative sensory testing was undertaken by measuring thermal thresholds (heat and cold) on the dorsum of the right foot⁸ and vibration threshold over the middle of the right first metatarsal bone.⁹ The first two assess the small fibre afferent pathways and the last tests the large fibre afferent pathways.¹⁰ Pattern reversal visual, brainstem auditory, and somatosensory evoked potentials (to stimulation of the right median nerve at the wrist) were recorded with standard techniques.¹¹

The clinical assessments and the battery of neurophysiological investigations were performed by three independent teams with no prior knowledge of each others' outcome for both the patients and the control subjects.

The median and the 25 and 75 percentiles are given for the quantitative sensory testing data which were not normally distributed and the statistical significance was evaluated by the Mann-Whitney *U* test. The remaining neurophysiological variables, which were normally distributed, are described in terms of their mean values, SE, and 95% confidence interval of the control mean. The statistical significance of the difference in mean values between the control and veteran groups were calculated by two tailed Student's *t* test.

Neurophysiological variables

	Controls (<i>n</i> = 13)		Veterans (<i>n</i> = 14)	
	Mean (SEM)	95% CI	Mean (SEM)	<i>P</i> value (2 tailed) [†]
Lower limb (motor):				
SDML (ms)	4.11 (0.21)	3.63–4.59	4.50 (0.12)	0.106
FMNCV (m/s)	50.1 (1.27)	46.6–52.7	48.8 (1.15)	0.442
Amplitude (mV)	5.02 (0.58)	3.35–6.68	5.14 (0.45)	0.883
F wave latency (ms)	48.53 (1.16)	45.59–51.47	51.36 (0.85)	0.059
Lower limb (sensory):				
Sural nerve latency (ms)	3.13 (0.09)	2.86–3.40	3.42 (0.09)	0.034*
Sural nerve amplitude (μV)	9.6 (1.51)	5.0–14.3	11.0 (1.66)	0.538
Upper limb (motor):				
SDML (ms)	3.33 (0.12)	3.03–3.64	3.23 (0.09)	0.481
FMNCV (m/s)	61.1 (1.59)	57.2–65.0	61.5 (1.07)	0.846
Amplitude (mV)	8.6 (0.80)	6.5–10.6	8.32 (0.60)	0.804
F wave latency (ms)	27.5 (0.47)	26.2–28.8	26.9 (0.43)	0.378
Upper limb (sensory):				
Median nerve latency (ms)	2.66 (0.05)	2.50–2.82	2.70 (0.06)	0.647
Median nerve amplitude (μV)	24.2 (2.26)	18.7–29.6	18.0 (1.48)	0.030*
Evoked potential latencies:				
Visual—P100 (ms)	101.2 (2.02)	96.1–106.3	102.6 (1.45)	0.574
Brainstem auditory:				
I (ms)	1.56 (0.02)	1.49–1.63	1.55 (0.02)	0.876
I–III (ms)	2.21 (0.05)	2.07–2.34	2.21 (0.04)	0.988
III–V (ms)	1.95 (0.07)	1.76–2.13	1.88 (0.06)	0.482
Somatosensory:				
Erb (ms)	9.86 (0.19)	9.35–10.37	9.92 (0.16)	0.788
Spine (ms)	13.16 (0.24)	12.58–13.74	13.47 (0.16)	0.282
Cortex (ms)	19.2 (0.29)	18.2–20.2	20.1 (0.39)	0.085

	Controls (<i>n</i> = 13)			Veterans (<i>n</i> = 14)	
	Median	Quartile 1st	3rd	Median	<i>P</i> value [‡]
Quantitative sensory testing:					
Vibration (μm)	0.19	0.12	0.47	0.55	0.072
Hot threshold (°C)	0.80	0.60	1.65	1.00	0.422
Cold threshold (°C)	0.25	0.18	0.28	0.55	0.0002*

**P* < 0.05; [†]Parametric test (Student's *t*); [‡]Non-parametric test (Mann-Whitney).
SDML = Shortest distal motor latency; FMNCV = fastest motor nerve conduction velocity.

Results

The table shows a summary of the results. In the patient group, the symptom score was increased (*P* = 0.00002) as was the mean score for clinical signs although at a lower level of significance (*P* = 0.0198). The cold sensation threshold was raised (*P* = 0.00018). The mean sural sensory potential latency was increased in the patient population (*P* = 0.034) whereas the mean amplitude of the sensory potential recorded from the median nerve was reduced (*P* = 0.030). Neither the needle EMG investigation nor any of the evoked potential variables showed convincing evidence of abnormality.

Discussion

The difference between the two populations in terms of symptom scoring, although large (*P* = 0.00002), is non-specific and can only be relevant if accompanied by abnormalities in the objective variables. It is interesting to note that the mean scores of clinical neurological signs showed a significant difference with a *P* value of 0.0198. In addition, two variables

from sensory nerve conduction studies, both testing large fibre peripheral nerve function, were significantly different ($P = 0.030$ and 0.034). One of the quantitative sensory testing variables (cold threshold, testing thinly myelinated small fibre function) was abnormal ($P = 0.00018$).

In performing multiple statistical tests on a set of variables, there is a risk that some of the comparisons will be statistically significant purely by chance. After applying the Bonferroni correction factor,¹² two of the tests remained significantly different at the 95% confidence level. It is therefore unlikely that the differences found between the two study populations occurred at random, and raises the question of possible organic elements underlying some of the complaints in the patient group. The exact clinical relevance of these findings is unknown and further studies of larger groups are required for verification and to characterise the nature and cause of any dysfunction found.

This study was supported by the Joseph Rowntree Charitable Trust and the Polden Puckham Charitable Foundation. We thank Professor Gordon Murray, University of Glasgow, for statistical advice and Miss Elizabeth Jackson for secretarial support.

- 1 Hubert M, Lison D. Study of muscular effects of short-term pyridostigmine treatment in resting and exercising rats. *Hum Exp Toxicol* 1995;14:49-54.
- 2 Reigle DW, D'Amato AM. US chemical and biological warfare-related dual use exports to Iraq and their possible impact on the health consequences of the Persian Gulf war. A report of the Committee on Banking, Housing and Urban Affairs with respect to Export Administration. Washington: United States Senate, 25 May 1994.
- 3 Schaumburg HH, Berger AR. Human toxic neuropathy due to industrial agents. In: Dyck PJ, Thomas PK, eds. *Peripheral neuropathy*. London: Saunders, 1993:1533-48.
- 4 Dyck PJ, Sherman WR, Hallcher LM, Service FJ, O'Brien PC, Grina L, et al. Human diabetic endoneurial sorbitol, fructose, and myo-inositol related to sural nerve morphometry. *Ann Neurol* 1980;8:590-6.
- 5 LeQuesne PM. Neurophysiological investigation of subclinical and minimal toxic neuropathies. *Muscle Nerve* 1978;1:392-5.
- 6 Seppäläinen AM. Neurophysiological approaches to the detection of early neurotoxicity in humans. *Crit Rev Toxicol* 1988;18:245-98.
- 7 Jamal GA, Weir AI, Hansen S, Ballantyne JP. Myotonic dystrophy. A reassessment by conventional and more recently introduced neurophysiological techniques. *Brain* 1986;109:1279-96.
- 8 Jamal GA, Hansen S, Weir AI, Ballantyne JP. An improved automated method for the measurement of thermal thresholds. 1. normal subjects and 2. patients with peripheral neuropathy. *J Neurol Neurosurg Psychiatry* 1985;48:354-60; 361-6.
- 9 Jamal GA, Hansen S, Ballantyne JP. An automated system for quantification of the sense of vibration in man. *J Physiol (Lond)* 1991;438:340P.
- 10 Jamal GA, Weir AI, Hansen S, Ballantyne JP. The neurophysiologic investigation of small fibre neuropathy. *Muscle Nerve* 1987;10:537-45.
- 11 Chiappa KH. *Evoked potentials in clinical medicine*. New York: Raven Press, 1990.
- 12 Snedecor GW, Cochran WG. *Statistical methods*. 7th ed. Ames: Iowa State University Press, 1982.

Sensory neuropathy associated with Dursban (chlorpyrifos) exposure

J.G. Kaplan, MD; J. Kessler, MD; N. Rosenberg, MD; D. Pack, MD*; and H.H. Schaumburg, MD

Article abstract—Chlorpyrifos (Dursban) is an organophosphate insecticide with extensive domestic and agricultural applications. It is regarded as safe for these purposes; one report of neurotoxicity is attributed to massive ingestion in a suicide attempt. We report eight people who developed peripheral neuropathy after exposure to exterminator-applied commercial Dursban; five also experienced memory loss and cognitive slowing. Evaluation failed to reveal other causes of neurologic dysfunction; symptoms recurred in one patient following accidental reexposure. We conclude that environmental contact with chlorpyrifos can cause sensory neuropathy and CNS dysfunction and that this agent should be used with caution.

NEUROLOGY 1993;43:2193-2196

Chlorpyrifos is a potent and widely used organophosphate (OP) insecticide¹ that is considered safe for use in home and industry when applied properly.² We report eight previously healthy people who developed peripheral sensory neuropathy after environmental exposure to commercial chlorpyrifos (Dursban); five also experienced memory loss and mental slowing.

Case presentations. Group I: Peripheral neuropathy alone. *Case 1.* A 33-year-old physician developed paresthesias in his feet, accompanied by urinary frequency and pain in the suprapubic, groin, and thigh regions. Approximately 4 weeks earlier, an exterminator had inadvertently sprayed Dursban into the ventilating system at his place of employment. Immediately following the application, the physician experienced nausea, vomiting, and lightheadedness. A strong residual odor remained for several weeks; during this interval, paresthesias in the feet developed, accompanied by chronic groin aching and urinary frequency. Neurologic examination performed 2 weeks after exposure was normal, including sensory, motor, and reflex testing. A diagnosis of demyelinating disease was entertained. A therapeutic trial of prednisone, started at 120 mg daily and tapering rapidly to 40 mg daily, did not relieve the symptoms. He was then admitted to New York Hospital, where a lumbar puncture revealed CSF protein to be 68 mg/dl with a glucose of 64 mg/dl; a myelogram was normal. CBC, electrolytes, BUN, glucose, B₁₂, erythrocyte sedimentation rate (ESR), serum protein electrophoresis (SPEP), and immunoprotein electrophoresis (IPEP) were normal. The fluorescent antinuclear antibody (FANA), cryoglobulins, and urinary heavy metal screen were negative. EMG and nerve conduction studies (NCSs) were consistent with

distal axonopathy (table 1). He then experienced mild paresthesias in his hands and feet and lability of both pulse and blood pressure. All symptoms slowly resolved during the next 8 to 10 weeks. Several months later, Dursban was again sprayed at his place of employment. During the next several weeks, the patient again developed paresthesias in both feet as well as urinary frequency, but symptoms were milder than with the initial episode and resolved within 5 to 8 weeks. He has been asymptomatic in the 10 years since exposure. NCSs and late-response studies performed 5 years after the hospital admission were normal (table 1).

Case 2. A 33-year-old housewife noted burning, numbness, cramps, and a sensation of vibration in her hands and feet 4 weeks after an exterminator sprayed Dursban around her radiators. She was first evaluated by us 6 months later. Examination at that point revealed normal sensation to all modalities tested, normal strength, and reflexes, which were mildly attenuated at the ankles. Widespread abnormalities were present on NCSs (table 1). She slowly improved after the pesticide was eliminated from her house. Repeat NCSs 3 months later were within normal limits, save for borderline sural nerve action potential amplitudes. A third study performed 3 months later disclosed normal sural amplitudes (table 1). Quantitative sensory testing of vibration, obtained sequentially during the recovery phase, also showed improvement that paralleled the clinical and electrophysiologic recovery.

Case 3. A 40-year-old exterminator developed diarrhea, muscle twitching, excessive tearing, paresthesias, and numbness after repeated exposure to Dursban in a closed environment over a 6-month interval. Erythrocyte cholinesterase levels, done at the onset of symptoms, were initially low; these returned to normal within 2 months after removal of Dursban. Neurologic evaluation

*Deceased.

From the Department of Neurology, Albert Einstein College of Medicine, Bronx, NY.

Received September 29, 1992. Accepted for publication in final form April 20, 1993.

Address correspondence and reprint requests to Dr. Jerry G. Kaplan, Montefiore Medical Park, Department of Neurology, 1575 Blondell Avenue, Suite 225, Bronx, NY 10461.

Table 1. Nerve conduction studies after Dursban exposure and recovery

Pt no.:	Postexposure							Recovery						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Date	11/80	1/85	12/86	6/91	6/91	6/91	6/91	10/85	10/85	5/87	4/92	4/92	4/92	4/92
SNAP in mV														
Sural (>4)	Abs	Abs	3.2	2	2	3.5	2.5	6.2	3.0	4.1	3.0	4.2	4.5	4.6
Ulnar (>4)	NR	3.4	3.4	4	14.2	NR	NR	NR	4.0	5	NR	NR	NR	NR
Median (>4)	NR	3.8	NR	10.8	20	23	7.5	NR	4.2	NR	NR	NR	NR	NR
CMAP in mV														
Peroneal (>2.5)	2.2	2.5	2.2	2.6	7.2	5.7	5.5	2.6	2.7	2.9	2.7	NR	NR	NR
Tibial (>2.5)	2.0	2.6	2.4	NR	NR	NR	NR	2.5	2.6	3.1	NR	NR	NR	NR
Median (>4.5)	5.0	6	6.4	6.1	8	14	6.6	5.6	5.8	6.2	NR	NR	NR	NR
Late responses														
Tibial H	34*		34*	NR	NR	NR	NR	32	30	32	NR	NR	NR	NR
Peroneal F	56*		48	49	38	42	40	52	45	48	49	NR	NR	NR
Tibial F	56*		49	NR	NR	NR	NR	53	46	48	NR	NR	NR	NR

SNAP Sensory nerve action potential performed orthodromically.

CMAP Compound muscle action potential.

H H reflex.

F F response.

Abs Absent.

NR Not recorded by original observer.

* Prolonged latency relative to patient's height; unless otherwise noted, all latency and velocity measurements were normal.

Normal values in parentheses.

6 weeks later revealed sensory loss of all modalities in a stocking-glove distribution, as well as mild distal weakness and areflexia in the lower extremities. No fasciculations or tremor were present. NCSs and quantitative sensory threshold studies (QSTs) revealed changes consistent with peripheral neuropathy of the distal axonopathy type. SMA 6 and 12, 2-hour postprandial glucose, CBC, ESR, SPEP, IPEP, urine protein electrophoresis, cryoglobulins, FANA, rheumatoid factor, and Lyme titer were normal. Follow-up after 1 year revealed normalization of the neurologic examination, NCS, and QSTs and remission of all symptoms.

Group II: Peripheral neuropathy with cognitive dysfunction. Cases 4 to 7. A family of four became ill after their house was sprayed by an exterminator. Immediate symptoms were headaches, nausea, and painful muscle cramps. One month later, all developed numbness and paresthesias, most prominent in the legs, and reported memory impairment. Both children (ages 14 and 15 years) noted decline in school performance that lasted for 6 months.

One month later, elevated levels of Dursban were found in their home, especially in the kitchen; other OPs were not detected (table 2).

Neurologic examination, performed 6 months after exposure, revealed mild short-term memory loss on all routine mental status testing of recall of multiple objects. Neuropsychological testing was declined. The neurologic examination was otherwise normal.

NCSs revealed low-amplitude sural nerve action potentials in all. Motor and upper-extremity sensory nerve action potential studies were normal. Extensive evaluation for other causes of neuropathy and CNS dysfunction, including MRI, were normal. NCSs 6 months later disclosed normal sural nerve amplitudes in all but one (table 1). At that time, there were no longer complaints or evidence of cognitive dysfunction.

Case 8. Three weeks after Dursban was applied in her

Table 2. Levels at exposure site

Compound	Concentration (µg/kg)	PQL (µg/kg)
Azinphos	U	7,000
Bolstar	U	700
Chlorpyrifos	66,000	1,400
Coumaphos	U	7,000
Dementon-O	U	1,176
Dementon-S	U	1,176
Diazinon	U	2,800
Dichlorvos	U	462
Disulfoton	U	938
Ethprop	U	1,176
Fensulfothion	U	7,000
Fenthion	U	462
Merphos	U	1,176
Mevinphos	U	1,400
Naled	U	462
Parathion methyl	U	140
Phorate	U	700
Ronnel	U	1,400
Stirophos (tetrachlorvinphos)	U	23,380
Tokuthion (Prothiofos)	U	2,338
Trichloronate	U	700

Surrogate recovery (diphenylamine): 13%.

PQL Practical quantitation limit.

U None detected.

basement exercise room, a 42-year-old physician noted impaired memory and slowed thinking. This was accompanied by electrical sensations in the hands and feet and pins-and-needles sensations in both legs. There were eight applications over 3 weeks before onset of symptoms. No other member of her family had occasion to go

to the basement and none had symptoms. Examination revealed sensory loss in the feet; vibration sensitivity was more affected than were light touch, pin, or thermal sensitivity. Ankle reflexes were depressed. Brain MRI, T3 and T4 studies, heavy metal screen, Lyme serology, and connective tissue disease evaluation were normal. Spraying ceased after onset of symptoms, and during the ensuing 3 months the abnormal acral sensations abated. Neurologic examination performed 6 months later was normal.

Eighteen months later, the patient was reevaluated because of complaints of persistent cognitive slowing, memory loss, and word-finding difficulties. Neurologic examination and NCSs were normal.

Neuropsychological evaluation revealed intellectual functioning well below her superior premorbid level; performance on recent memory tests was especially impaired. The patient had considerable difficulty on the Embedded Figures Test, which involves identifying objects from overlapping figures. She failed to perceive objects even when the outline of part of an object was pointed out to her. A similar problem was displayed on the Picture Completion Subtest of the Wechsler Adult Intelligence Scale-Revised, which involves identifying the important missing detail from a visual scene. She performed poorly on tasks that involved deciding whether pairs of computer-generated checkerboard patterns were the same or different and had difficulty copying a complex geometric form that involved the distortion of a major internal detail. Memory for short paragraphs recalled immediately after hearing them was in the low average range, as was serial order recall of seven-digit telephone numbers after 15 seconds of interference.

Discussion. Chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) is a pyridyl phosphorothioate derivative, OP insecticide effective against a broad spectrum of agricultural and household pest species.^{2,3} It is the only peripheral neurotoxic component of the nine commercial preparations of Dursban; other ingredients are xylene, aromatic solvents, emulsifiers, and chlorinated solvents,⁴ none of which has been associated with peripheral neuropathy. When used properly, Dursban is considered safe.^{3,5} One instance of severe sensory motor neuropathy has occurred following massive oral ingestion of chlorpyrifos in a suicide attempt.³ Domestic application of OP insecticides has not been associated with peripheral neuropathy.⁶

Our patients experienced symptoms of peripheral neuropathy after repeated respiratory and dermal exposure to chlorpyrifos in enclosed, commercially fumigated sites. Extensive medical evaluation failed to reveal other sources of neuropathy. Distal upper- and lower-extremity paresthesias and leg pain heralded the onset in every case; some had mild sensory loss on examination. All who were tested (seven of eight) had clear abnormalities of nerve conduction and late-response abnormalities that improved following removal from exposure (table 1). In one, elevated vibration and thermal thresholds were present on QST testing. Recovery was gradual, complete, and accompanied by recovery of nerve conduction (table 1) and sensory perception. Transient recurrence occurred in one after

repeat exposure. Mild, largely reversible memory impairment occurred in five.

The classic syndrome of OP intoxication is a two-stage illness.^{3,6-10} An initial cholinergic reaction stage consists of gastrointestinal distress, lacrimation, and diarrhea; this is followed (if not fatal) in several weeks by a sensorimotor peripheral neuropathy stage, with cramping, lower-extremity pain,^{3,6-10} hyporeflexia, distal numbness, paresthesias, ataxia, and weakness. The second condition is denoted as OP-induced delayed polyneuropathy (OPIDP).^{2,8,9} Varying degrees of CNS axonal degeneration can occur concomitantly with OPIDP, but may be masked by the peripheral neuropathy; severely affected individuals may eventually develop spasticity when the neuropathy recovers.^{3,6,8-10} Most cases with lasting disability have been ascribed to intoxication with triorthocresyl phosphate and related OP compounds.⁸ Prolonged lower-level exposure may also cause neuropathy that is usually mild and frequently not heralded by dramatic cholinergic symptoms as in our cases.

OPIDP is a large-fiber distal axonopathy whose pathophysiology has been partially elucidated.⁷⁻⁹ Originally, it was thought that only OPs that can covalently transfer a phosphate moiety to the active site of the enzyme neuropathy target esterase (NTE), in a process referred to as aging, cause OPIDP; it was thought that at least 70% of the enzyme must be so deactivated (aged) for this to occur.⁷⁻⁹ This reaction is independent of anticholinesterase activity; OPs that fail to cause aging (nonagers) will protect from OPIDP if given immediately prior to exposure to agers.⁷⁻⁹ Recent studies indicate further complexity in the relationship between aging of NTE and OPIDP.^{7,9} Nonagers that protect from OPIDP, when given *prior* to exposure to agers, may actually enhance the neuropathic potential of agers when they are given soon *after* exposure (promotion).^{7,9} Apparently, depending on the sequence of dosing, two opposite results, either promotion or protection, may be obtained by combined treatment with a neuropathic OP and a non-neuropathic NTE inhibitor.^{7,9} Further, OP-induced peripheral neuropathy has been demonstrated after exposure to two nonaging NTE inhibitors, and the nonaging NTE inhibitor methamidophos has been reported to cause neuropathy in hens in a form of self-promoted OPIDP.¹⁰ These studies suggest that the aging phenomenon is not as quantitatively important (>70% requirement) as previously thought. Richardson⁹ showed that lymphocyte NTE was markedly depressed in patients with alcoholic neuropathy and suggested that it may be advisable to examine compounds not currently thought to act as NTE inhibitors (eg, commonly employed solvents, drugs, and neurotoxicants thought to act by non-NTE mechanisms) to discover any effects on NTE and examine possible interactions with OP compounds.

No prior reports have implicated fumigant chlorpyrifos as a cause of peripheral neuropathy.^{3,6,7,11}

6	7
4/92	4/92
4.5	4.6
NR	NR
NR	NR
NR	NR
NR	NR
NR	NR
NR	NR
NR	NR

PQL (µg/kg)
7,000
700
1,400
7,000
1,176
1,176
2,800
462
938
1,176
7,000
462
1,176
1,400
462
140
700
1,400
23,380
2,338
700

ician noted
was accom-
and feet and
There were
of symp-
-asion to go

Because it is a potent inhibitor of acetylcholine esterase (AChE), its therapeutic index (the relationship between AChE and NTE inhibition) suggests that it does not have strong neurotoxic potency.^{7-9,11,12} The therapeutic index is based on acute exposure data^{7,9} and may not be relevant in instances of repeated low-level exposure as experienced by our individuals. NTE inhibition and levels of chlorpyrifos in body fluids were unavailable in our patients because they were referred after the period of exposure; none was recognized as chlorpyrifos intoxication at initial evaluation. Pesticide analysis showed high levels of chlorpyrifos without evidence of other toxins in the one instance (cases 4 to 7) where site levels were measured. Because NTE assays were not performed at the time of exposure, it is not clear whether the mild sensory neuropathy encountered in our patients was due to self-promotion with long-standing, low-level exposure to chlorpyrifos, was due to interaction of NTE inhibition with other unrecognized causes of neuropathy, or was due to other idiosyncratic reactions to chlorpyrifos unrelated to NTE inhibition.

Transient weakness, areflexia, and vocal cord paralysis have been described in a 3-year-old child found playing next to an open spilled bottle of chlorpyrifos.¹³ This was attributed to OPIDP,¹³ although this diagnosis appears to be virtually excluded by normal NCSs and clinical improvement within weeks of the onset of this illness. The presence of widespread late-response abnormalities and the clinical course suggest acute inflammatory demyelinating polyradiculoneuropathy (AIDP), which frequently gives rise to normal NCSs early in the course of illness.¹⁴ AIDP has previously been associated with OP exposure¹⁵; whether this rare association is related as cause and effect or as coincidence is not clear. Another possibility for the transient weakness in the child is the "intermediate syndrome" of OP intoxication, which produces cranial neuropathy and proximal weakness.¹⁶ This was discounted by the authors on the basis of normal NCSs although the results of repetitive stimulation studies were not reported.¹³

Cognitive dysfunction with deficits of concentration, word-finding, and memory occurred in five; this was reversible in four. Neuropsychological testing performed in one patient revealed persistent dysfunction in memory, word-finding, and visual perception. Previous studies of cognitive effects of OP compounds usually attribute these effects to a CNS cholinergic reaction.¹⁷ With chronic exposure, OP compounds may cause persistent cerebral effects; confusion and poor recent memory remain even after biochemical indices of exposure, such as erythrocyte cholinesterase determination,

return to normal.^{17,18} We conclude that repeated environmental exposure to commercial chlorpyrifos (Dursban) can cause both reversible distal symmetric sensory neuropathy and mild cognitive dysfunction. This substance should be deployed with caution and its safety reassessed.

References

1. Lotti M, Becker CE, Aminoff MJ. Organophosphate polyneuropathy: pathogenesis and prevention. *Neurology* 1984;34:658-662.
2. Racke K. Degradation of organophosphorus insecticides in environmental matrices. In: Chambers J, Levi P, eds. *Organophosphates*. San Diego: Academic Press, 1992:47-78.
3. Lotti M, Moretto A, Zoppellari R, Dainese R, Rizzuto N, Barusco G. Inhibition of lymphocytic neuropathy target esterase predicts the development of organophosphate-induced delayed polyneuropathy. *Arch Toxicol* 1986;59:176-179.
4. Braddock J, Greeno P, Lee W, Lum G, Fleckenstein L. Trade name index. In: Gosselin RE, Smith RP, Hodge HC, eds. *Clinical toxicology of commercial products*, 5th ed. London: Williams & Wilkins, 1984:V217-V218.
5. Hayes W. *Pesticide studies in man*. London: Williams & Wilkins, 1982:398.
6. LeQuessne P. Toxic neuropathies. In: Asbury A, Gilliat R, eds. *Peripheral nerve disorders*. London: Butterworths, 1984:184-204.
7. Lotti M. The pathogenesis of organophosphate polyneuropathy in humans: perspectives for biomonitoring. *Trends Pharmacol Sci* 1987;8:175-179. *
8. Davis C, Richardson R. Organophosphorus compounds. In: Spencer P, Schaumburg H, eds. *Experimental and clinical neurotoxicology*. Baltimore: Williams & Wilkins, 1980:527-544. 148
9. Richardson R. Interactions of organophosphorus compounds with neurotoxic esterase. In: Chambers J, Levi P, eds. *Organophosphates*. San Diego: Academic Press, 1992:299-323. 149
10. Moretto A, Bertolazzi M, Capodicasa E, et al. Phenylmethanesulfonyl fluoride elicits and intensifies the clinical expression of neuropathic insults. *Arch Toxicol* 1992;66:67-72.
11. Lotti M, Johnson M. Neurotoxicity of organophosphorus pesticides: predictions can be based on in vitro studies with hen and human enzymes. *Arch Toxicol* 1978;41:215-221.
12. Annau Z. Neurobehavioral effects of organophosphorus compounds. In: Chambers J, Levi P, eds. *Organophosphates*. San Diego: Academic Press, 1992:419-429.
13. Aiuto LA, Pavlakis SG, Boxer RA. Life-threatening organophosphate-induced delayed polyneuropathy in a child after accidental chlorpyrifos ingestion. *J Pediatr* 1993;122:658-660.
14. Arnason BG, Soliven B. Acute inflammatory demyelinating polyradiculoneuropathy. In: Dyck PJ, Thomas PK, Griffin JW, et al, eds. *Peripheral neuropathy*, 3rd ed. Philadelphia: WB Saunders, 1993:1437-1497.
15. Fisher JR. Guillain-Barré syndrome following organophosphate poisoning. *JAMA* 1977;238:1950-1951.
16. Senanayake N, Karralliede L. Neurotoxic effects of organophosphorus insecticides: an intermediate syndrome. *N Engl J Med* 1987;316:761-763.
17. Chambers J. Summary and conclusions. In: Chambers J, Levi P, eds. *Organophosphates*. San Diego: Academic Press, 1992:433-438.
18. Savage E, Keefe T, Mounce L, et al. Chronic neurologic sequelae of acute organophosphate pesticide poisoning. *Arch Environ Health* 1988;43:38-45.

* Johnson MK, Receptor or enzyme: the puzzle of NTE and organophosphate-induced delayed polyneuropathy. *Trends in Pharm. Sci.* 1987; 8:174-8.

Delayed Neurotoxic Effect of Sarin in Mice after Repeated Inhalation Exposure

46740823



① K. Husain,^{1,†} R. Vijayaraghavan,¹ S. C. Pant,¹ S. K. Raza² and K. S. Pandey²

¹Division of Pharmacology and Toxicology, Defence Research and Development Establishment, Gwalior-474002, India.

²Division of Synthetic Chemistry, Defence Research and Development Establishment, Gwalior-474002, India

Key words: delayed neurotoxicity; sarin; inhalation exposure; mice; NTE: brain; spinal cord; platelets

Delayed neurotoxicity of sarin in mice after repeated inhalation exposure has been studied. Female mice exposed to atmospheric sarin (5 mg m^{-3} for 20 min) daily for 10 days developed muscular weakness of the limbs and slight ataxia on the 14th day after the start of the exposure. These changes were accompanied by significant inhibition of neurotoxic esterase (NTE) activity in the brain, spinal cord and platelets. Histopathology of the spinal cord of exposed animals showed focal axonal degeneration. These changes were comparatively less than in animals treated with the neurotoxic organophosphate, mipafox. Results from this study indicate that sarin may induce delayed neurotoxic effects in mice following repeated inhalation exposure.

INTRODUCTION

Sarin (isopropyl methyl phosphonofluoridate) is an organophosphorus nerve agent.¹ It is a potent inhibitor of cholinesterase² and causes delayed neurotoxicity in hens.³⁻⁶ Organophosphate-induced delayed neurotoxicity (OPIDN) is characterized by a delay period of 4-21 days after intoxication before the clinical signs and symptoms (ataxia followed by paralysis) are manifested, especially in humans, birds and certain species of mammals.^{7,8} Histopathological changes¹ consist of degeneration of axon and myelin of the nervous system.^{7,9} There is a significant correlation between an organophosphate to inhibit neurotoxic esterase (NTE) after dosing and the production of neurotoxic symptoms.^{8,10} Studies demonstrated that delayed neurotoxic organophosphates produce neurotoxic effects in rats and mice following repeated exposure by subcutaneous and oral routes.^{11,12} Inhalation is one of the major routes of entry of neurotoxic organophosphate compounds into the body, and information on whether organophosphates cause delayed neurotoxicity by this route is sparse. Therefore, the present study has been carried out to investigate the susceptibility of mouse to OPIDN following repeated inhalation exposure of sarin.

EXPERIMENTAL

Sarin, mipafox and phenyl valerate were synthesized in our establishment and their purities were checked by TLC, GLC, IR, UV and NMR. Fourteen female Swiss albino mice (25-30 g) bred in the animal house of the establishment were used in the present study. In this strain, the LC_{50} of sarin is $600 \text{ mg m}^{-3} \text{ min}^{-1}$. They were divided randomly into two groups of eight

and six animals, respectively, provided with food (Lipton Feed, India) and water *ad libitum* and maintained at 25°C .

A 10- μl aliquot of sarin was diluted in 10 ml of distilled water in a graduated tube and pumped through a syringe infusion pump at a rate of 7.2 ml h^{-1} (Braun Perfusor, Germany) into the liquid pick-up capillary of a glass air-blast nebulizer. The air pressure applied (1.0 kg cm^{-2}) assured the evaporation of all sarin delivered to the tip of the capillary, as shown by its absence in the waste. The total filtered air passed into the chamber was 26 l min^{-1} and the nominal concentration was 5 mg m^{-3} . The temperature inside the chamber was $26 \pm 1^\circ\text{C}$.

The animals in group 1 were exposed to filtered fresh air for 20 min daily for 10 days in a dynamically operated whole-body exposure chamber. The animals in group 2 were exposed to sarin vapour (5 mg m^{-3}) for the same periods. In a separate group, mice ($n = 6$) were treated with mipafox (2.5 mg kg^{-1} , s.c.) daily for 10 days and served as positive controls. All animals were observed carefully for the occurrence of any changes in gross behaviour and were sacrificed on the 14th day after the start of the exposure. Blood was collected in vials containing 0.1 M citrate buffer ($\text{pH } 7.4$) and platelets were isolated as described elsewhere.¹³ Platelets were lysed with saponin and diluted with 50 mM Tris-HCl/ 0.20 mM EDTA buffer ($\text{pH } 8.0$). The brain and spinal cord were removed, weighed quickly and homogenized in Tris-HCl EDTA buffer ($\text{pH } 8.0$) to give 5% homogenate (w/v). Specimens of the spinal cord were fixed in neutral phosphate-buffered formalin and embedded in paraffin. Paraffin sections were stained with haematoxylin and eosin, luxol fast blue and Holm's silver stain and gold chloride¹⁴ for examination under the light microscope. Neurotoxic esterase (NTE) activities in the brain, spinal cord and platelets were assayed according to the method of Johnson¹⁵ and platelet proteins were estimated as described by Lowry *et al.*¹⁶ Data were analysed statistically using Student's *t*-test.

† Author to whom correspondence should be addressed.

Table 1. Effect of repeated exposure of sarin (5 mg m^{-3} for 20 min) and mipafox (2.5 mg kg^{-1} , s.c.) daily for 10 days on neurotoxic esterase (NTE) activity in the brain, spinal cord and platelets of mice (Animals were sacrificed on the 14th day after the start of the exposure; each value represents mean $\pm$ SE)

Group	Neurotoxic esterase (NTE)*		
	Brain	Spinal cord	Platelets
1. Control ($n = 8$)	680.5 ± 12.8	258.8 ± 10.3	4.04 ± 0.18
2. Sarin ($n = 6$)	277.0 ^{b,c} ± 11.4	135.8 ^d ± 9.5	1.80 ^b ± 0.22
% Inhibition	59.2	47.4	55.4
3. Mipafox ($n = 6$)	235.0 ^b ± 8.8	112.5 ^b ± 3.8	1.57 ^b ± 0.20
% Inhibition	65.4	56.5	61.1

* Units: nmol phenyl valerate hydrolysed $\text{min}^{-1} \text{g}^{-1}$ tissue (or mg^{-1} protein).

^b Significantly different from control ($P < 0.001$).

^c Significantly different from group 3 ($P < 0.02$).

^d Significantly different from group 3 ($P < 0.05$).

RESULTS

The animals exposed to sarin exhibited muscular weakness of the limbs, muscle twitching and slight ataxis on the 14th day after the start of the exposure (i.e. the 4th day after the last exposure) whereas mipafox-treated animals showed severe ataxia. They did not show any anti-acetylcholinesterase (AChE) symptoms at any time.

The effects of sarin and mipafox on neurotoxic esterase (NTE) activity in different tissues are given in Table 1. Neurotoxic esterase activity was significantly inhibited in the brain, spinal cord and platelets. Sarin inhibition of NTE in all tissues was less than that with mipafox. The incidence of axonal degeneration in the spinal cord of mice exposed to sarin and mipafox is shown in Table 2. The degeneration densities were light and moderate in mice exposed to sarin and moderate and heavy in mipafox-treated animals.

Comparison of histological sections of the spinal



Figure 1. Spinal cord (left side) of control mouse showing normal neurons with axon ($\times 500$).



Figure 2. Spinal cord (left side) of sarin-exposed mouse showing degenerating axons ($\times 500$).

cord of control and sarin-exposed animals showed that sarin caused focal axonal degeneration in the lateral columns of the cord (Figs 1 and 2).

DISCUSSION

The present study demonstrates the susceptibility of mice to sarin, as evidenced by NTE inhibition and lesions in the spinal cord. A relationship between NTE inhibition and lesions has been observed in the hen, a species more sensitive to delayed neurotoxicity.^{3,17} However, the mouse is a better model than the rat^{11,18} for OPIDN studies. The results of the present study are in agreement with a recent finding, which indicated that platelet NTE is a good peripheral marker for OPIDN.¹² However, it must be pointed out that the behavioural, biochemical and histopathological changes induced by sarin were less than in animals given mipafox. It is concluded that repeated inhalation exposure of sarin may cause delayed neurotoxicity, which may be related to NTE inhibition, spinal cord damage and subsequent appearance of clinical symptoms.

The role of acetylcholinesterase (AChE) inhibition was also considered but it was rejected for the following reason. The same exposure inhibited AChE in blood by 27.3% and in brain by 19.2%. Such an inhibition is not able to cause anti-AChE symptoms.

Table 2. The incidence of axonal degeneration in the spinal cord of mice exposed to sarin and mipafox*

Animal no.	Control	Sarin	Mipafox (positive control)
1	-	+	+++
2	-	++	+++
3	-	+	++
4	-	++	++
5	-	+	+++
6	-	+	+++

* Degeneration densities in the lateral columns of spinal cord: -, none; +, light; ++, moderate; +++, heavy.

Ku922

Acknowledgements

The authors are thankful to Dr R. V. Swamy, Director, Defence R & D Establishment, Gwalior, India for his keen interest in this

study and to Dr S. Das Gupta, Head, Division of Pharmacology and Toxicology for his encouragement.

REFERENCES

1. J. R. Wood, Chemical defence. *J. Am. Med. Assoc.* **145**, 1264-1268 (1951).
2. D. Grob and J. C. Harvey, Effect in man of the anticholinesterase compound sarin. *J. Clin. Invest.* **37**, 350-357 (1958).
3. M. K. Johnson, organophosphorus and other inhibitors of brain neurotoxic esterase and the development of delayed neurotoxicity in hens. *Biochem. J.* **120**, 523-537 (1970).
4. D. R. Davies, P. Holland and M. J. Rumens, The relationship between the chemical structure and neurotoxicity of alkyl organophosphorus compounds. *Br. J. Pharmacol.* **15**, 271-278 (1960).
5. M. A. Vranken, H. C. DeBisschop and J. L. Willems, *In vitro* inhibition of neurotoxic esterase by organophosphorus nerve agents. *Arch. Int. Pharmacodyn.* **260**, 316-318 (1982).
6. J. J. Gordon, R. H. Inns, M. K. Johnson, L. Leadbeater, M. P. Maidment, D. G. Upshall, G. H. Cooper and R. L. Rickard, The delayed neuropathic effects of nerve agents and some other organophosphorus compounds. *Arch. Toxicol.* **52**, 71-82 (1983).
7. M. B. Abou-Donia, Organophosphorous ester-induced delayed neurotoxicity. *Ann. Rev. Pharmacol. Toxicol.* **21**, 511-548 (1981).
8. M. K. Johnson, The target for initiation of delayed neurotoxicity by organophosphorus esters: biochemical studies and neurotoxicological applications. *Rev. Biochem. Toxicol.* **4**, 141-212 (1982).
9. M. B. Abou-Donia and D. M. Lapadula, Mechanisms of organophosphorus ester-induced delayed neurotoxicity: type I and type II. *Ann. Rev. Pharmacol. Toxicol.* **30**, 405-440 (1990).
10. M. Lotti and M. K. Johnson, Repeated small doses of a neurotoxic organophosphate: monitoring of NTE in brain and spinal cord. *Arch. Toxicol.* **45**, 263-271 (1980).
11. M. A. Marin and K. Husain, Striatal neurochemical changes and motor dysfunction in mipafox treated animals. *Methods and Findings in Experimental and Clinical Pharmacology* **7**, 79-81 (1985).
12. D. M. Lapadula, S. E. Patton, G. M. Campbell and M. B. Abou-Donia, Characterization of delayed neurotoxicity in the mouse following chronic oral administration of tri-*o*-cresyl phosphate. *Toxicol. Appl. Pharmacol.* **79**, 83-90 (1985).
13. K. Husain, Peripheral biochemical marker for organophosphate induced delayed neurotoxicity. *Biochem. Int.* **24**, 1051-1056 (1991).
14. J. F. A. McManus and R. W. Mowry, *Staining Methods: Histologic and Histochemical*, pp. 324-353. Paul B. Hoeber Inc., Medical Division of Harper and Brother, New York (1985).
15. M. K. Johnson, Improved assay of neurotoxic esterase for screening organophosphates for delayed neurotoxicity potential. *Arch. Toxicol.* **37**, 113-115 (1977).
16. O. H. Lowry, J. N. Rosenbrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275 (1951).
17. S. Caroli and M. Lotti, Neurotoxic esterase in peripheral nerve, assay, inhibition and rate of resynthesis. *Toxicol. Appl. Pharmacol.* **62**, 498-501 (1982).

THIS ARTICLE IS FOR INDIVIDUAL USE ONLY
AND MAY NOT BE FURTHER REPRODUCED OR
STORED ELECTRONICALLY WITHOUT WRITTEN
PERMISSION FROM THE COPYRIGHT HOLDER.
PERMISSION FROM THE COPYRIGHT HOLDER
REPRODUCTION PENALTIES.

OPIDN developed in hens on 14th day after start of 10 daily doses of mipafox, sarin but not parathion. Demonstrates relative severity.

Indian J Physiol Pharmacol 1995; 39(1) : 47-50

A COMPARATIVE STUDY OF DELAYED NEUROTOXICITY IN HENS FOLLOWING REPEATED ADMINISTRATION OF ORGANOPHOSPHORUS COMPOUNDS

K. HUSAIN, S. C. PANT*, S. K. RAZA**, R. SINGH AND S. DAS GUPTA

Division of Pharmacology and Toxicology,

***Division of Synthetic Chemistry,*

Defence Research and Development Establishment,

Gwalior - 474 002

(Received on December 8, 1993)

Abstract: Hens treated with Mipafox (10 mg/kg, sc), sarin (50 µg/kg, sc) or parathion (1 mg/kg, sc) daily for 10 days exhibited severe, moderate and no ataxia respectively on 14th day after the start of exposure. The neurotoxic esterase (NTE) activity was significantly inhibited in the brain, spinal cord and platelets of hens treated with mipafox or sarin whereas no change was noticed with parathion treatment. All three compounds significantly inhibited acetylcholinesterase (AChE) activity in the platelets. Spinal cord of hens treated with mipafox, sarin or parathion showed axonal degeneration heavy, moderate and none respectively. It is concluded that repeated administration of equitoxic doses of mipafox, sarin and parathion to hens are marked, moderate and non-delayed neurotoxic respectively.

Key words: delayed neurotoxicity

organophosphorus compounds

NTE

axonal degeneration

ataxia

INTRODUCTION

Organophosphorus (OP) compounds are widely used in agriculture, industry and also as potent chemical warfare agents (1). Some of these compounds, beside producing acute cholinergic toxicity, have been reported to induce delayed neurotoxicity in human, hen and certain species of mammals (2, 3). OP compound-induced delayed neurotoxicity (OPIDN) is a syndrome which is characterised by a delay period of 4-21 days after intoxication before the clinical symptoms (ataxia and paralysis) are manifested (3). The initial biochemical step is inhibition of the enzyme neurotoxic esterase (NTE) of the nervous system (4). Minimum 70% NTE inhibition after single exposure and 45% after multiple exposure to OP compounds and subsequent aging of NTE is biochemical prerequisite for the development of OPIDN (5, 7). Morphologically there is axonal degeneration followed by demyelination

(2). Recently a peripheral biochemical marker for OPIDN has been demonstrated (5). Adult hens are used to provide a model of OPIDN for laboratory studies (2). Since most of the previous studies have been performed in hens after a single exposure of OP compounds, however, population in general, military troops and farm animals may also be exposed to these compounds repeatedly. The aim of the present study was to assess the delayed neurotoxic effects in hens using behavioural, biochemical and histopathological analysis following repeated administration of equitoxic doses of three OP compounds - mipafox, sarin and parathion.

METHODS

Twentyfour adult white leghorn hens (*Gallus domesticus*) weighing 1.01-1.35 kg were used. They were divided into 4 groups of 6 hens each and housed

*Corresponding Author

in aluminium cages (2 per cage) and were provided food and tap water *ad libitum*. Hens in group 1 were administered normal saline subcutaneously daily for 10 days and served as control. Those in group 2, 3 and 4 were injected with mipafox (10 mg/kg, sc), sarin (50 µg/kg, sc) and parathion (1.0 mg/kg, sc) respectively daily for 10 days. The doses of three compounds administered were 1/10th of their respective LD₅₀ values. The birds were carefully observed for occurrence of delayed neurotoxic symptoms (ataxia and paralysis) and assessed on 4-point scale as described by Sprague et al (6). Birds were sacrificed on 14th day after the start of the exposure.

Platelets were isolated from the blood as described previously (5). Neurotoxic esterase (NTE) was assayed in the brain, spinal cord and platelets

light microscope. The data were analysed statistically by Student's 't' test.

RESULTS

Mipafox treated hens exhibited severe ataxia whereas hens treated with sarin developed moderate ataxia on 14th day after the start of the exposure. Those treated with parathion did not show any delayed neurotoxic symptoms (Table I).

A significant inhibition of NTE activity in the brain, spinal cord and platelets was noticed in hens treated with mipafox and sarin. However, no significant change in enzyme activity was observed in hens treated with parathion (Table I). Acetylcholinesterase (AChE) activity in the platelets was significantly inhibited in hens treated with mipafox, sarin or parathion (Table

TABLE I: Effect of repeated administration of mipafox, sarin and parathion on behavioural pattern (ataxia) and NTE in various tissues and AChE in the platelets of hens after 14 day exposure. Each value represents mean ± S.E.

Groups	Ataxia	Neurotoxic esterase (NTE) ^a			Acetylcholine- ^b sterase (AChE) platelets
		Brain	Spinal cord	Platelets	
Control (n = 6)	-	2177.0 ±26.5	791.0 ±12.6	10.80 ±0.64	1091.5 ±20.4
Mipafox (n = 6)	++	689.5 ^a ±26.4	401.6 ^a ±10.3	4.74 ^a ±0.57	409.9 ^a ±8.5
Sarin (n = 5)	+	1013.6 ^a ±26.1	488.2 ^a ±13.1	4.93 ^a ±0.30	310.6 ^a ±9.2
Parathion (n = 6)	—	2087.9 ±42.2	750.0 ±16.9	10.85 ±0.78	501.7 ^a ±15.9

Ataxia: + Moderate ++ Severe

a = n moles of phenyl valerate hydrolysed /min/gm tissue or mg of platelet protein.

b = n moles of acetylthiocholine hydrolysed/min/mg protein.

^a = v/s control (P < 0.001).

according to the method of Johnson (7). Acetylcholinesterase (AChE) was assayed in the platelets by the method of Ellman et al (8).

Specimens of the spinal cord were fixed in neutral phosphate buffered formalin and embedded in paraffin. Paraffin sections (10 µm) were stained with hematoxylin and eosin with luxol fast blue and Holm's silver stain and gold chloride (9) for examination under

I). Spinal cord section of mipafox treated birds showed heavy degeneration of axon and myelin of the lateral columns of cord (Fig. 1). Reticular fibers of small blood vessels were also frequently impregnated. Degeneration caused by sarin was moderate (Fig. 2) and axonal swellings were accompanied by macrophages accumulation.



Fig. 1 : Mipafos exposed illustrating axonal degeneration. Arrows indicate axonal degeneration while arrow head indicates capillary reticular fibers.



Fig. 2 : Sarin exposed showing light degeneration and swollen axons, which are surrounded by macrophages (arrow).
Luxol Fast blue & Holm's silver nitrate method.
Scale bar = 10 μm.

DISCUSSION

In the present study mipafos induced delayed neurotoxic symptoms (severe ataxia) in hens (Table I).

However, when treated with a single dose also caused production of clinical symptoms and inhibition of NTE in hens (2, 3). Sarin which is a nerve gas has been reported to inhibit NTE *in vitro* but NTE inhibition was below the threshold level *in vivo* (10). Moreover, sarin can induce OPIDN in hens after a single massive dose (11). In the present study sarin after repeated exposure to hens produced moderate ataxia and significantly inhibited NTE in all the tissues (Table I) suggested that it can induce OPIDN in hens after repeated exposure. Massive parathion exposure to human can induce neurotoxic symptoms (12). However, other reports indicated that single exposure to parathion did not induce OPIDN in hens (4). In the present study even after repeated exposure, parathion is devoid of producing OPIDN in hens. The results of the present study show that the NTE inhibition by mipafos and sarin is within the threshold level (Table I) and the inhibition of NTE in the brain and platelets is similar thus support the platelet NTE as a peripheral marker for OPIDN (5). There is a significant correlation between NTE inhibition and the lesions in the spinal cord of hens treated with mipafos or sarin (Table I). This relationship has been reported to occur in animals after single exposure to neurotoxic OP compounds (2, 11). The noticeable feature of the present study is the correlation of neurotoxic symptoms, NTE inhibition and spinal cord lesions in hens after repeated administration of OP compounds. It is concluded that mipafos and sarin are most and moderate delayed neurotoxic compounds to hens after repeated exposure. However, parathion is not a delayed neurotoxic.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. R. V. Swamy, Director, Defence Research and Development Establishment, Gwalior and Dr. R. Vijayaraghavan, Head, Division of Pharmacology and Toxicology for their valuable suggestions in the present study.

REFERENCES

1. Davis CS, Richardson RJ. In "Experimental and Clinical Neurotoxicology" (P.S. Spencer & H.H. Schaumburg eds.), pp. 527-544. Williams and Wilkins, Baltimore, USA: 1980.
2. Abou-Domia MB. Organophosphorus ester-induced delayed neurotoxicity. *Ann Rev Pharmacol Toxicol* 1981; 21 : 511-548.
3. Johnson MK. The delayed neuropathy caused by some organophosphorus esters : mechanism and challenge. *Crit Rev Toxicol* 1975; 3 : 289-316.

4. Johnson MK. The target for the initiation of delayed neurotoxicity by organophosphorus esters : Biochemical studies and toxicological applications. *Rev Biochem Toxicol* 1982; 4 : 141-212.
5. Husain K. Peripheral biochemical marker for organophosphate induced delayed neurotoxicity. *Biochem Int* 1991; 24 : 1051-1056.
6. Sprague GL, Sandvik LL, Bickford AA, Castles TR. Evaluation of sensitive grading system for assessing acute and subchronic delayed neurotoxicity in hens. *Life Sci* 1980; 27 : 2523-2528.
7. Johnson MK. Improved assay of neurotoxic esterase for screening organophosphate for delayed neurotoxicity potential. *Arch Toxicol* 1977; 37 : 113-116.
8. Ellman GL, Courtney KD, Andre VJ, Feathenstone RM. A new and colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 1961; 7 : 88-95.
9. McManus JFA, Mowry RW. "Staining methods : Histologic and histochemical", pp. 324-353. Paul B. Hoeber Inc. Medical Div. of Harper and Brothers, NY, 1965.
10. Crowell JA, Parker RM, Bocci TJ, Dacre JC. Neuropathy target esterase in hen after soman and sarin. *J Biochem Toxicol* 1989; 4 : 15-20.
11. Gordon JJ, Inns RH, Johnson MK, Leadbeater L, Maidment MP, Upshall DG, Cooper GH, Rickard RL. The delayed neuropathic effects of nerve agents and some other organophosphorus compounds. *Arch Toxicol* 1983; 52 : 71-82.
12. Dejager AEJ, VanWeerden TW, Houthof HJ, deMonchy JGR. Polynuropathy after massive exposure to parathion. *Neurology* 1981; 31 : 603-605.

1990 Vol. 31, No. 4 of 4
December 1990

Promotion 1990

Presented at 7th Int. Neurotoxicology Conference,
Little Rock, Ark, Abstract #21. (see Richardson FO# 149 Biblio)

1989

New published in 1990

OPIDN

164

GWS

223

1. First report of Promotion
Verone & Padilla 1985 p. 270
2. Great Summary of the
small mechanism of
OPIDN. p. 268

POTENTIATION OF ORGANOPHOSPHORUS- INDUCED DELAYED NEUROTOXICITY BY PHENYLMETHYLSULFONYL FLUORIDE

C. N. Pope

School of Pharmacy, Northeast Louisiana University,
Monroe, Louisiana

S. Padilla

Neurotoxicology Division, United States Environmental
Protection Agency, Research Triangle Park,
North Carolina

It is well known that pretreatment with the serine esterase inhibitor phenylmethylsulfonyl fluoride (PMSF) can protect experimental animals from organophosphorus-induced delayed neurotoxicity (OPIDN), presumably by blocking the active site of neurotoxic esterase (NTE) such that binding and "aging" of the neuropathic OP is thwarted. We report here that while PMSF (60 mg/kg, sc) given 4 h before the neuropathic organophosphate (OP) mipafox (50 mg/kg, im) completely prevented the clinical expression of OPIDN in hens, the identical PMSF treatment markedly amplified the delayed neurotoxicity (relative to hens treated with OP only) if administered 4 h after mipafox (5 or 50 mg/kg, im). Moreover, in a separate experiment using diisopropylphosphorofluoridate (DFP) as the neurotoxicant in place of mipafox, posttreatment with PMSF 4 h after DFP (0.5 mg/kg) also accentuated the severity of ataxia. These data indicate that PMSF only protects against OPIDN if given prior to exposure to the neurotoxicant; treatment with PMSF after OP exposure critically exacerbates the delayed neurotoxicity from exposure to organophosphorus compounds.

INTRODUCTION

Organophosphorus compounds (OPs) are a diverse group of chemicals used primarily as pesticides, plasticizers, flame retardants, and lubri-

Presented in part at the 7th International Neurotoxicology Conference, September 18-21, 1989, Little Rock, Ark. C. N. Pope was supported by a National Research Council postdoctoral associateship.

The authors wish to thank Valerie Wilson, Donald Lyrly, and Mark Chapman for excellent technical assistance and Dr. A. M. Crider for distillation of the phenyl valerate. The research described in this article has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Requests for reprints should be sent to Dr. Carey Pope, School of Pharmacy, Northeast Louisiana University, Monroe, LA 71209.

cants (Davis and Richardson, 1980). Some of these compounds are known to induce a central-peripheral distal axonopathy in humans and other species (Smith and Lillie, 1931; Cavanagh, 1954). This condition, termed organophosphorus-induced delayed neurotoxicity (OPIDN), is characterized by a delayed (1 wk to several weeks following exposure), progressive neuropathy with associated sensory and motor functional deficits (Abou-Donia, 1981). The adult chicken has proven to be a highly sensitive animal model for OPIDN, and tests in chickens for delayed neurotoxicity are required for all OP pesticides produced for use in the United States (*Federal Register*, 1985).

Extensive (>70%) inhibition of a membrane-bound carboxylesterase (neurotoxic esterase, NTE) in the nervous system is proposed to be the initial molecular event in the induction of OPIDN (for a review, see Johnson, 1990). It is well known that some compounds, such as the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF), can prevent the development of OPIDN if administered *prior* to exposure to the delayed neurotoxicant (Johnson, 1970; Baker et al., 1980; Caroldi et al., 1984; Veronesi and Padilla, 1985). In contrast, we report here that PMSF can markedly exacerbate the clinical signs of OPIDN if administered *after* exposure to the OP. While it is generally thought that PMSF pretreatment prevents OPIDN by blocking the active site of NTE so that the neuropathic OP cannot bind and undergo subsequent "aging," the mechanism for the potentiation of OPIDN by PMSF is presently unknown.

METHODS

Materials and General Methods

Mipafox (purity $\geq 98\%$ by NMR, melting point, and elemental analysis) was purchased from Chemsyn, Lenexa, Kans. Paraoxon (O,O-diethyl-p-nitrophenyl phosphate) was purchased from Aldrich Chemical Co., Milwaukee, Wis. Phenyl valerate was synthesized essentially as described by Johnson (1977) and distilled (boiling point 70–70.5°C, 0.5 mm Hg). Phenylmethanesulfonyl fluoride (PMSF), atropine sulfate, eserine hemisulfate, glycerol formal, and O,O-diisopropylphosphorofluoridate (DFP) were purchased from Sigma Chemical, St. Louis, Mo. All other chemicals were reagent grade.

For biochemical determinations, hens were decapitated and tissues were removed and frozen at -55 to -70°C until assay (less than 14 d). All samples from each tissue type (e.g., all spinal cord samples) were thawed, homogenized, and assayed on the same day. The tissues were homogenized in an ice bath with a Polytron homogenizer (Brinkmann Instruments) in 50 mM tris(hydroxymethyl)aminomethane buffer containing 0.2 mM disodium ethylenediamine tetraacetic acid (EDTA), adjusted to pH 8.0 at 25°C with hydrochloric acid (Tris-EDTA). Brain samples were

homogenized in 7 volumes (w/v) Tris-EDTA (20 s, setting 6), cervical spinal cord samples were homogenized in 2.5 ml Tris-EDTA (20 s, setting 7), and both sciatic nerves were homogenized 3 times in 4 ml Tris-EDTA (20 s, setting 7). After homogenization, samples were centrifuged at $9000 \times g$ for 15 min at 4°C. Supernatants were collected and assayed for NTE activity by the method of Johnson (1977) as modified by Soliman et al. (1982). Protein content of the supernatants was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard, and data are reported as nmol phenyl valerate hydrolyzed/min · mg protein.

Range-Finding Experiments

Preliminary dose-finding studies were performed to determine a dose of PMSF that would extensively inhibit (>90%) hen brain NTE activity 4 h after treatment. Additionally, other groups of hens were given incremental doses of mipafox or DFP, and exposure conditions were identified that resulted in graded levels of NTE inhibition 4–24 h after treatment.

Organophosphorus Compound/PMSF Interactions

Mipafox Adult, female, white Leghorn chickens (body weight 1.5–2.1 kg, at least 12 mo old) were obtained from Featherdown Farms, Apex, N.C. The animals were housed at the Duke Vivarium, Duke University, Durham, N.C., and supplied with feed (Purina Layena) and water ad libitum. A detailed analysis of NTE inhibition in the brain, cervical spinal cord, and sciatic nerve was performed 24 h after mipafox treatment at the selected doses (see below). These levels of mipafox were then used to investigate the effects of the relative order of administration (when both mipafox and PMSF were sequentially administered) on the development of clinical signs of OPIDN.

Twenty minutes before challenge with either PMSF (60 mg/kg), mipafox (1.5, 5.0, or 50 mg/kg), or their appropriate vehicles, hens were pretreated with atropine sulfate (10 mg/kg) and eserine hemisulfate (0.1 mg/kg) for prophylaxis against acute cholinergic toxicity and to prevent long-term cholinesterase inhibition, respectively (Johnson, 1969). Four hours after such treatment the hens were again pretreated with atropine and eserine and then treated with the alternate inhibitor or its respective vehicle (see Table 1 for details of the different dosing regimens). Hens were observed for signs of motor dysfunction at 6, 8, 10, 12, 14, and 16 d after treatment and graded on an 8-point scale (0 = normal ambulation; 1–2 = slight, infrequent hindlimb incoordination; 3–4 = moderate but definite hindlimb incoordination; 5–6 = severe, frequent difficulties in walking and standing erect; 7–8 = virtual to complete hindlimb paralysis; from Roberts et al., 1983). All hens were coded such that the observer did not know their respective treatments.

DFP Adult, female white Leghorn chickens (body weight 1.0–1.8 kg,

TABLE 1. Treatment Protocols

Group	Time			
	0 min	20 min	240 min	260 min
Control	Atropine ^a , eserine	Saline ^b	Atropine, eserine	Glycerol formal ^c
Mipafox only	Atropine, eserine	Mipafox ^b in saline	Atropine, eserine	Glycerol formal
Mipafox and PMSF	Atropine, eserine	Mipafox in saline	Atropine, eserine	PMSF in glycerol formal
DFP only	Atropine, eserine	DFP ^b in peanut oil	Atropine, eserine	Glycerol formal
DFP and PMSF	Atropine, eserine	DFP in peanut oil	Atropine, eserine	PMSF in glycerol formal

^aAtropine (10 mg/kg, sc) and eserine (0.1 mg/kg, sc) were administered at a volume of 0.1 ml saline/kg.

^bSaline, mipafox (im) in saline, or DFP (sc) in peanut oil was administered at a volume of 1.0 ml/kg.

^cGlycerol formal or phenylmethylsulfonyl fluoride (PMSF, 60 mg/kg, sc) in glycerol formal was administered at a volume of 0.67 ml/kg.

at least 12 mo old) were obtained from Gunter Egg Farms, Calcasieu, La. The animals were housed at the School of Pharmacy, Northeast Louisiana University, Monroe, La., and maintained as above. A detailed analysis of NTE inhibition in brain, cervical spinal cord, and sciatic nerve was performed following DFP treatment at selected doses. The identified doses of DFP producing low (0.125 mg/kg), moderate (0.25 mg/kg), and high (0.5 mg/kg) nervous system NTE inhibition were used to investigate the effects of PMSF posttreatment on the development of clinical signs of OPIDN. Hens were treated as in the above schedule except that DFP was used in place of mipafox (see Table 1). PMSF was not administered prior to DFP treatment to reconfirm protection, as this effect is well documented (Johnson, 1970; Baker et al., 1980; Caroldi et al., 1984).

Statistical Analyses

Differences in clinical signs between groups were analyzed by ranking the scores on the last day of observation (i.e., 16 d after treatment) and testing for significance by the Mann-Whitney *U*-test. A probability level of .01 was used for significance.

RESULTS

Effects of PMSF, Mipafox, and DFP on Neurotoxic Esterase Activity

Four hours after PMSF treatment (60 mg/kg, sc), hen brain NTE activity was extensively (97%) inhibited (control = 15.9 ± 0.1 , PMSF = $0.5 \pm$

0.1 nmol phenyl valerate hydrolyzed/min·mg protein). Preliminary studies (data not shown) determined doses of mipafox and DFP that produced graded levels of NTE inhibition (50, 5, and 1.5 mg/kg mipafox or 0.5, 0.25, and 0.125 mg/kg DFP). Two separate experiments were then performed with these doses of mipafox and DFP to investigate possible potentiation of OPIDN by PMSF.

Mipafox/PMSF Interactions In Vivo

Figure 1a shows the inhibition of brain, cervical spinal cord, and sciatic nerve NTE activity 24 h following mipafox treatment. All tissues from each treatment group showed the same relative extent of NTE inhibition. NTE inhibition in all three tissues indicated that hens treated with 50 mg/kg mipafox would be expected to develop clinical signs of OPIDN, since the degree of inhibition of NTE was above the 70% "threshold" (Johnson, 1990). Indeed, animals treated with 50 mg/kg mipafox exhibited a progressive ataxia beginning 8 d after treatment, with several hens showing complete hindlimb paralysis by 14 d after treatment (Fig. 2a). PMSF completely prevented the clinical signs of OPIDN induced by mipafox (50 mg/kg) when administered 4 h prior to OP treatment. Alternatively, when the identical dose of PMSF was administered 4 h after mipafox treatment, hens in this group showed an increased severity of ataxia (Fig. 2a). In addition, hens treated with mipafox followed by PMSF exhibited an earlier onset of ambulatory deficit than hens given mipafox only: hens receiving only mipafox did not show grade 4 ataxia until 14 d after dosing, while hens given PMSF 4 h after mipafox showed grade 4 ataxia by 10 d after treatment.

Hens treated with 5 mg/kg mipafox showed >70% NTE inhibition 24 h after treatment in all three areas of the nervous system (Fig. 1a) and therefore were expected to exhibit clinical signs of OPIDN. Little evidence of a progressive motor deficit was noted in this group of hens, however (Fig. 2b). Alternatively, in hens given this dose of mipafox followed 4 h later by PMSF, a significant increase ($p < .01$ on d 16) in motor dysfunction developed (Fig. 2b).

The lowest dose of mipafox tested (1.5 mg/kg) caused $\leq 53\%$ inhibition of NTE in all three areas of the nervous system (Fig. 1a). This level of inhibition was not associated with significant ataxia, as was expected, and no significant increase in motor dysfunction was apparent in hens posttreated with PMSF at this dose level (Fig. 2c).

DFP/PMSF Interactions In Vivo

Figure 1b shows the inhibition of brain, cervical spinal cord, and sciatic nerve NTE activity 24 h after DFP treatment (0.125, 0.25, and 0.5 mg/kg). As with mipafox treatment, the three tissues showed equivalent degrees of NTE inhibition within the treatment groups. Hens treated with the highest DFP dose (0.5 mg/kg) exhibited >70% inhibition of NTE activ-

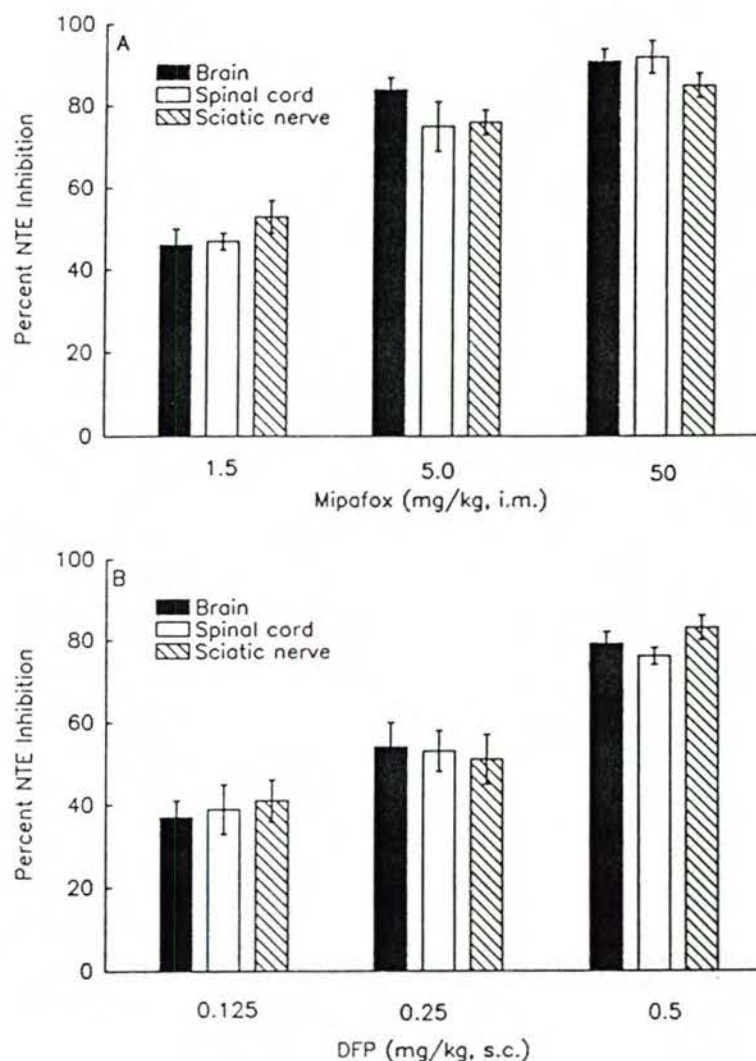


FIGURE 1. Inhibition of neurotoxic esterase (NTE) in brain, cervical spinal cord, and sciatic nerve following organophosphate treatment, represented as mean $\pm$ SE percent inhibition relative to control values ($n = 5/\text{group}$). (a) Inhibition of NTE activity 24 h after mipafox treatment (control values: brain = 17.16 ± 1.7 ; spinal cord = 6.03 ± 0.46 ; sciatic nerve = 3.15 ± 0.41 nmol phenyl valerate hydrolyzed/min·mg protein). (b) Inhibition of NTE activity 24 h after diisopropylphosphorofluoridate (DFP) treatment (control values: brain = 20.00 ± 0.54 ; spinal cord = 9.06 ± 0.30 ; sciatic nerve = 3.17 ± 0.08 nmol phenyl valerate hydrolyzed/min·mg protein).

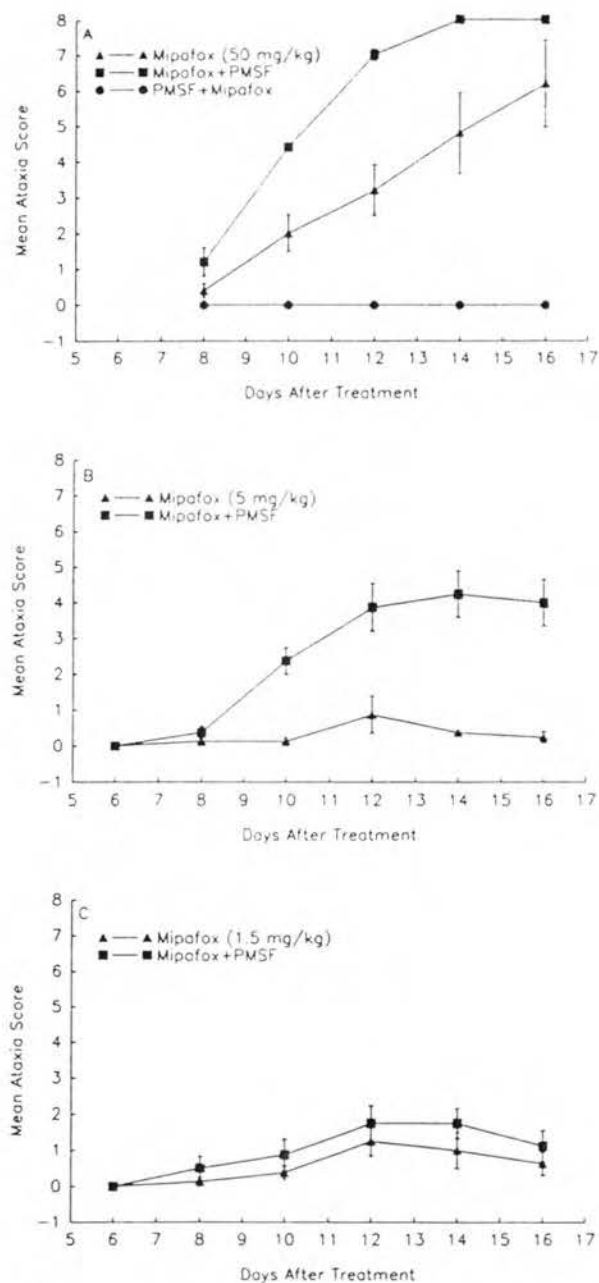


FIGURE 2. Effects of PMSF on development of clinical signs of OPIDN after mipafox treatment. Hens were treated (see Table 1) and observed (blind) on alternate days from 6 to 16 d using an 8-point graded scale (ataxia score of 0 = no signs to ataxia score of 8 = complete hindlimb paralysis; see Methods section for details). (a) Hens ($n = 5/\text{group}$) were treated with 50 mg/kg mipafox alone, pretreated with PMSF (60 mg/kg) 4 h before mipafox (PMSF + mipafox), or treated with mipafox 4 h before PMSF (mipafox + PMSF). (b) Hens ($n = 5/\text{group}$) were treated with 5.0 mg/kg mipafox alone or treated with mipafox followed by PMSF 4 hours later. (c) Hens ($n = 5/\text{group}$) were treated with 1.5 mg/kg mipafox alone or treated with mipafox followed by PMSF 4 h later.

ity in all three tissues and were expected to develop a progressive motor deficit. No signs or slight to moderate gait disturbances (grade 0 to grade 4 ataxia) were shown by this group during the experimental observation period, however. In contrast, hens treated with the same DFP dose and subsequently administered PMSF showed more extensive clinical deficits (grade 6 to grade 8 ataxia, $p < .01$ on d 16) during the observation period (Fig. 3a).

NTE activity was inhibited in hens treated with 0.25 mg/kg DFP by 50–54% in all three tissues (Fig. 1b). Hens treated with this level of DFP alone showed no signs of motor deficits during the entire observation period. In contrast, in the group of hens given 0.25 mg/kg DFP followed by PMSF 4 h later, 1 hen ($n = 5$) exhibited grade 8 ataxia (i.e., complete hindlimb paralysis) and another hen showed moderate ataxia (grade 3) during the observation period. The remaining three hens from this group (0.25 mg/kg DFP + PMSF) showed no signs of ataxia during the observation period, however.

NTE activity in the low-dose DFP group (0.125 mg/kg) was reduced by 36–41% in all three tissues (Fig. 1b). No signs of motor dysfunction were evident in any hens treated with this dose of DFP alone or those treated with DFP followed by PMSF (data not shown).

DISCUSSION AND CONCLUSIONS

The extensive inhibition of NTE by neuropathic OPs has been correlated with the subsequent development of clinical and neuropathological signs of OPIDN (for a review, see Johnson, 1990). The recognition of NTE inhibitors that did not cause OPIDN (e.g., PMSF, phenyl benzyl carbamate, di-*n*-pentyl phosphinate), however, suggested that NTE inhibition alone could not be the causal biochemical event precipitating OPIDN. Demonstration that pretreatment with these nonneuropathic NTE inhibitors actually protected experimental animals from the delayed neurotoxicity led Johnson to hypothesize that the inhibition of the esteratic activity itself was not the critical event: it was suggested that a secondary reaction, analogous to that occurring with OP inhibition of acetylcholinesterase (i.e., the loss of an alkyl group from the phosphorylated residue or "aging"), must be requisite for neuropathogenicity. Accordingly, NTE inhibitors could be divided into two subgroups: those that have labile alkyl groups are capable of aging and initiating OPIDN, while those that do not have labile alkyl groups cannot undergo aging or induce OPIDN but can protect the active site of NTE such that subsequent neuropathic challenges are thwarted.

The present study extends previous findings regarding the *in vivo* interaction between neuropathic OPs and nonneuropathic NTE inhibitors. Previous reports (Johnson, 1970; Baker et al., 1980; Carrington and Abou-Donia, 1983) have shown that PMSF is a potent inhibitor of NTE

Great summary
of OPIDN mechanism

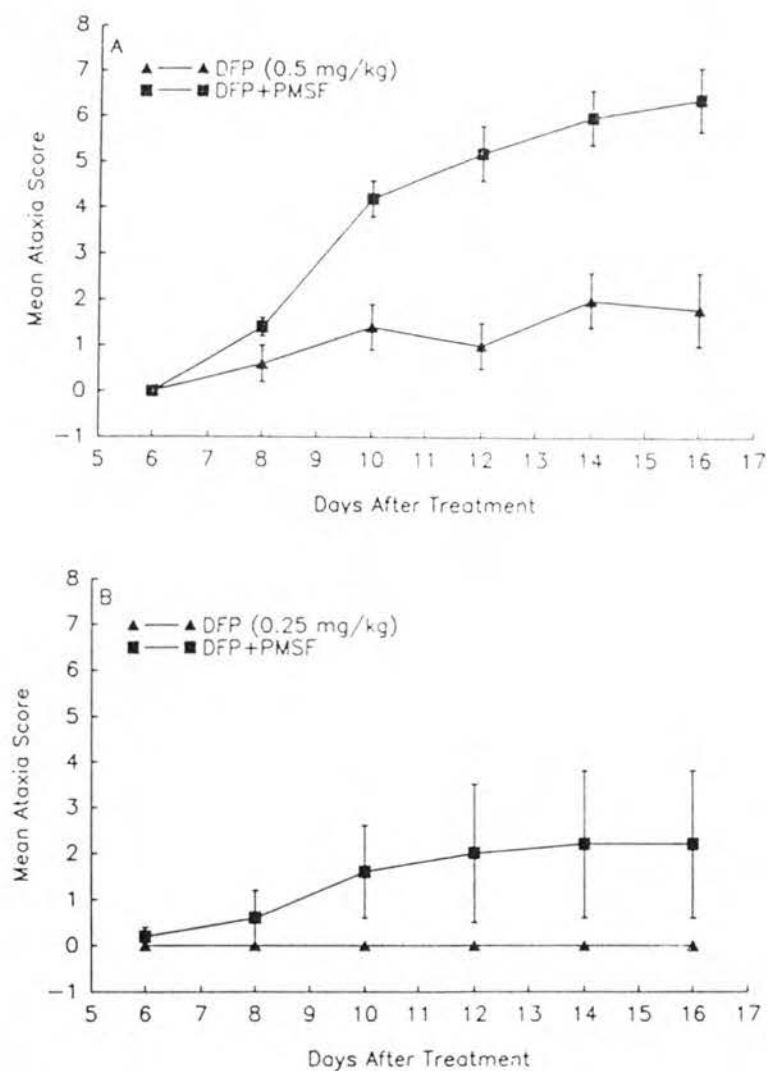


FIGURE 3. Effects of PMSF on development of clinical signs of OPIDN after diisopropylphosphorofluoridate (DFP) treatment. Hens were treated (see Table 1) and observed as described in Methods section and Figure 2. (a) Hens ($n = 5/\text{group}$) were treated with 0.5 mg/kg DFP alone or treated with DFP followed 4 h later by PMSF (60 mg/kg). (b) Hens ($n = 5/\text{group}$) were treated with 0.25 mg/kg DFP alone or treated with DFP followed by PMSF 4 h later.

activity, capable of preventing the clinical expression of OPIDN in hens when administered before exposure to DFP. In addition, Veronesi and Padilla (1985) reported that PMSF pretreatment prevented the neuropathology associated with mipafox exposure in rats. The results described herein show that PMSF pretreatment can prevent the clinical expression of mipafox-induced delayed neurotoxicity in hens. More importantly,

however, when PMSF was administered 4 h after mipafox or DFP treatment, a marked increase in the severity of clinical signs was observed. In the only previous study that examined neuropathic effects of OP and PMSF treatment as a function of their relative sequence of administration (Veronesi and Padilla, 1985), a higher incidence of severe neuropathology was noted in rats exposed to mipafox followed by PMSF relative to rats exposed only to mipafox. While no histopathological assessment was performed in the present study, the results reported by Veronesi and Padilla (1985) suggest that potentiation of the clinical deficits in hens by PMSF posttreatment may coincide with a higher incidence of severe neuropathology.

In some cases PMSF posttreatment amplified the degree of ataxia present in the OP-only groups (Figs. 2a and 3a), while in other cases PMSF posttreatment induced definitive clinical signs at OP doses that caused no signs in the respective OP-only groups (Figs. 2b and 3b). For example, hens exposed to mipafox (5 mg/kg) alone showed no progressive clinical evidence of OPIDN whereas hens treated with 5 mg/kg mipafox followed by PMSF exhibited moderate to severe gait disturbances. The lack of clinical response in the mipafox-only group was unexpected in light of the extensive NTE inhibition in all three nervous system tissues (Fig. 1).

A somewhat analogous situation was evident following DFP administration where hens treated with 0.5 mg/kg DFP showed >70% NTE inhibition in all three tissues yet showed little evidence of OPIDN (Fig. 3a). In contrast, hens treated with 0.5 mg/kg DFP and then given PMSF 4 h later exhibited severe signs of delayed neurotoxicity (Fig. 3a). In one DFP-treated group where nervous system NTE inhibition was only 51–54% (0.25 mg/kg DFP), complete hindlimb paralysis occurred in 1 hen (of 5) from the group administered PMSF afterward. The lack of potentiation of clinical signs with the lower doses of both mipafox and DFP (i.e., those doses that resulted in approximately 50% NTE inhibition or less) suggests that some critical "threshold" of prior binding by a neuropathic compound is necessary for potentiation to occur from later administration of PMSF.

These data therefore indicate that PMSF can either prevent or exacerbate OPIDN, the critical difference between either protection or potentiation residing in the sequence of the PMSF and OP exposures. The relative timing of the treatments is crucial to the ultimate outcome: PMSF given 4 h before OP administration can prevent delayed neurotoxicity, while PMSF given 4 h after OP administration can potentiate delayed neurotoxicity. It is difficult to construct a unifying hypothesis regarding the mechanism of action for both the protection afforded by PMSF pretreatment and the potentiation exerted by PMSF posttreatment. While blocking the neuropathy target site by previously administered PMSF seems a plausible mechanism for protection from OPIDN (Johnson, 1990),

posttreatment binding to additional unoccupied target sites would not (on the basis of known target site-PMSF interactions) be expected to enhance delayed neurotoxicity. Thus, we propose that another PMSF binding site(s) modifies the response to the neurotoxicant.

Numerous studies (Frawley et al., 1957; Murphy et al., 1959; Cohen et al., 1972; Boskovic, 1979; Clement, 1984; Gupta and Dettbarn, 1987) have reported potentiation of the acute toxicity of OPs by compounds that alter OP detoxification mechanisms, although consequences due to the relative order of administration were largely unexplored. Binding of PMSF to proteins involved in the enzymatic or nonenzymatic detoxification of OPs could also enhance the delayed neurotoxicity from OP exposure. Both mipafox and DFP are direct-acting neurotoxicants requiring no metabolic activation; thus if potentiation of OPIDN occurs through alteration of the pharmacodynamics of the OP, PMSF must be altering OP detoxification and/or distribution. This seems improbable based on (1) an extremely rapid distribution of DFP into the nervous system (Martin, 1985) and (2) preliminary findings from our laboratory indicating that PMSF can potentiate DFP-induced delayed neurotoxicity when given as long as 72 h after OP administration (data not shown).

Mipafox, DFP, and PMSF are all esterase inhibitors. DFP and PMSF are prototype serine protease inhibitors (for a review, see Bond and Butler, 1987), and all are documented inhibitors of cholinesterase and NTE (Johnson, 1990; Moss et al., 1985). The binding site specificities for these inhibitors therefore overlap, but the relative affinities of these inhibitors for various binding sites are unknown. A simple explanation for the post-treatment effects of PMSF on the development of delayed neurotoxicity would be that PMSF has a higher affinity than the OP for some population of the nontarget esterase/protease "pool." In that case, posttreatment with PMSF would displace the OP from these nonneuropathic sites, freeing the OP for subsequent binding to neuropathic sites. This explanation would require reversible binding (e.g., Michaelis complex formation) between the OP and the nontarget binding sites. In essence, when given sufficiently before OP treatment such that protection occurs, PMSF may irreversibly block the neuropathy target site (Johnson, 1990) and prevent its phosphorylation by a neuropathic compound. Under potentiating conditions, PMSF may displace OP molecules from reversible binding sites, thereby effectively increasing the burden of neurotoxicant for interaction with target sites within the nervous system.

Another possibility is that PMSF disrupts the cellular repair processes that are activated after exposure to neurotoxic levels of OPs. Generally, however, protease inhibitors have been reported to alleviate rather than exacerbate postlesion neuronal damage. For example, the calcium-activated neutral protease inhibitor leupeptin has been reported to reduce axonal damage following acute spinal cord injury in rats (Iwasaki et al., 1985) and to facilitate repair in nerve branches following median

Mechanism?

*Even when
given 72h
after OP*

*First
Hypothesized
mechanism
of 1st list ..*

*Second
Hypothesized
mechanism*

nerve section in the monkey (Badalamente et al., 1989). Interestingly, a recent study (Takauchi and Miyoshi, 1989) reported that infusion of leupeptin into the lateral ventricle in rats produced severe degeneration of neuronal processes.

Regardless of the mechanism for PMSF-induced potentiation of delayed neurotoxicity, the probability for multiple, temporally related chemical exposures in the "real" world makes the study of this phenomenon pertinent (Cherniak, 1988). While PMSF is not a common environmental toxicant, other chemicals may also uncover latent neurotoxicity due to prior exposure to neuropathic compounds. Knowledge concerning the potentiation of delayed neurotoxicity may indicate whether this phenomenon is of environmental relevance as well as provide insight into the pathogenesis of OPIDN.

REFERENCES

- Abou-Donia, M. B. 1981. Organophosphorus ester-induced delayed neurotoxicity. *Annu. Rev. Pharmacol. Toxicol.* 21:311-348.
- Badalamente, M. A., Hurst, L. C., and Stracher, A. 1989. Neuromuscular recovery using calcium protease inhibitor after median nerve repair in primates. *Proc. Natl. Acad. Sci. USA* 86:5983-5987.
- Baker, T., Lowndes, H. E., Johnson, M. K., and Sandberg, I. C. 1980. The effects of phenylmethane-sulfonyl fluoride on delayed organophosphorus neuropathy. *Arch. Toxicol.* 46:305-311.
- Bond, J. S., and Butler, P. E. 1987. Intracellular proteases. *Annu. Rev. Biochem.* 56:333-364.
- Boskovic, B. 1979. The influence of 2-(*o*-cresyl)4*H*-1:3:2-benzodioxophosphorin-2-oxide (CBDP) on organophosphate poisoning and its therapy. *Arch. Toxicol.* 42:207-216.
- Caroldi, S., Lotti, M., and Masutti, A. 1984. Intraarterial injection of diisopropylfluorophosphate or phenylmethane sulfonyl fluoride produces unilateral neuropathy or protection, respectively, in hens. *Biochem. Pharmacol.* 33:3213-3217.
- Carrington, C. D., and Abou-Donia, M. B. 1983. The time course of protection from delayed neurotoxicity induced by tri-*o*-cresyl phosphate and *o,o'*-diisopropyl phosphorofluoridate by phenyl methyl sulfonyl fluoride in chickens. *Toxicol. Appl. Pharmacol.* 18:251-256.
- Cavanagh, J. B. 1954. The toxic effects of triorthocresyl phosphate on the nervous system: An experimental study in hens. *J. Neurol. Neurosurg. Psychiatr.* 17:163-172.
- Cherniak, M. G. 1988. Toxicological screening for organophosphorus-induced delayed neurotoxicity: Complications in toxicity testing. *Neurotoxicology* 9:249-272.
- Clement, J. G. 1984. Role of aliesterase in organophosphate poisoning. *Fundam. Appl. Toxicol.* 4:S96-S105.
- Cohen, S. D., Callaghan, J. E., and Murphy, S. D. 1972. Investigation of multiple mechanisms for potentiation of malaoxon (MX) by triorthotolyl phosphate. *Toxicol. Appl. Pharmacol.* 22:300.
- Davis, C. S., and Richardson, R. J. 1980. Organophosphorus compounds. In *Experimental and Clinical Neurotoxicology*, eds. P. S. Spencer and H. H. Schaumburg, pp. 527-544. Baltimore: Williams & Wilkins.
- Federal Register. 1985. Rules and Regulations, vol. 50, no. 188, Sept. 1985, p. 39464.
- Frawley, J. P., Fuyat, H. N., Hagan, E. C., Blake, J. R., and Fitzhugh, O. G. 1957. Marked potentiation in mammalian toxicity from simultaneous administration of two anticholinesterase compounds. *J. Pharmacol. Exp. Ther.* 121:96-106.
- Gupta, R. C., and Dettbarn, W.-D. 1987. *iso*-OMPA-induced potentiation of soman toxicity in rat. *Arch. Toxicol.* 61:58-62.
- Iwasaki, Y., Iizuka, H., Yamamoto, T., Konno, H., and Kadoya, S. 1985. Alleviation of axonal damage

- in acute spinal cord injury by a protease inhibitor: Automated morphometric analysis of drug-effects. *Brain Res.* 347:124-126.
- Johnson, M. K. 1969. A phosphorylation site in brain and the delayed neurotoxic effect of some organophosphorus compounds. *Biochem. J.* 111:487-495.
- Johnson, M. K. 1970. Organophosphorus and other inhibitors of brain "neurotoxic esterase" and the development of delayed neurotoxicity in hens. *Biochem. J.* 120:523-531.
- Johnson, M. K. 1977. Improved assay of neurotoxic esterase for screening organophosphates for delayed neurotoxicity potential. *Arch. Toxicol.* 37:113-115.
- Johnson, M. K. 1990. Organophosphates and delayed neuropathy—Is NTE alive and well? *Toxicol. Appl. Pharmacol.* 102:385-399.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Martin, B. R. 1985. Biodisposition of [³H]diisopropylfluorophosphate in mice. *Toxicol. Appl. Pharmacol.* 77:275-284.
- Moss, D. E., Rodriguez, L. A., and McMaster, S. B. 1985. Comparative behavioral effects of CNS cholinesterase inhibitors. *Pharmacol. Biochem. Behav.* 22:479-482.
- Murphy, S. D., Anderson, R. L., and DuBois, K. P. 1959. Potentiation of toxicity of malathion by triorthotolyl phosphate. *Proc. Soc. Exp. Biol. Med.* 100:482-487.
- Roberts, N. L., Fairley, C., and Phillips, C. 1983. Screening acute delayed and subchronic neurotoxicity studies in the hen: Measurements and evaluations of clinical signs following administration of TOCP. *Neurotoxicology* 4:263-270.
- Smith, M. L., and Lillie, R. D. 1931. The histopathology of tri-ortho-cresyl phosphate poisoning. *Arch. Neurol. Psychiatry* 26:976-999.
- Soliman, S. A., Linder, R., Farmer, J., and Curley, A. 1982. Species susceptibility to delayed toxic neuropathy in relation to in vivo inhibition of neurotoxic esterase by organophosphorus esters. *J. Toxicol. Environ. Health* 9:187-197.
- Takauchi, S., and Miyoshi, K. 1989. Degeneration of neuronal processes in rats induced by a protease inhibitor, leupeptin. *Acta Neuropathol.* 78:380-387.
- Veronesi, B., and Padilla, S. 1985. Phenylmethylsulfonyl fluoride protects rats from mipatox-induced delayed neuropathy. *Toxicol. Appl. Pharmacol.* 81:258-264.

Received February 6, 1990

Accepted June 4, 1990

SYMPOSIUM PROCEEDINGS

LONG TERM EFFECTS OF THE ORGANOPHOSPHATE SARIN ON EEGs IN MONKEYS AND HUMANS

Frank H. Duffy and James L. Burchfiel

Developmental Neurophysiology Laboratory, Children's Hospital Medical Center, Harvard Medical School, 300 Longwood Avenue, Boston, Massachusetts 02115

INTRODUCTION

The insecticidal activity of organophosphorus esters (OP) was first recognized in 1937 by Gerhard Schrader. They are now among our major chemical weapons against insects and acarids; currently over 100 OP biocides are in use. Casida and Baron (1976) conservatively estimate an annual worldwide production in excess of 10^6 kilograms with wide applicability and importance in agricultural, veterinary, and medical areas.

The discovery of the biocidal activity of OP compounds led initially to their development by Germany during World War II as potential chemical warfare agents, e.g., the "nerve gases" soman, sarin, and tabun (Koelle, 1968). Within the last two decades, many OP compounds less toxic to man have been developed as pest control agents, e.g., malathion, parathion, mipafox. Nonetheless, all OP compounds share similar chemical structures and pharmacological actions. Toxic properties appear related to an ability to inhibit esterases throughout the central and peripheral nervous systems. OP compounds differ mainly as regards potency and use.

Detailed studies of the structure activity relationships of thousands of OP compounds have led to 1) a reduction in mammalian toxicity, and 2) a diversification of uses, e.g., nematocides, rodenticides, avicides, insect chemosterilants, insecticide synergists, fungicides, herbicides, and plant growth regulators (Casida and Baron, 1976). With the reduction in acute human toxicity, however, increasing attention has been focused upon secondary or late-developing effects of these compounds. This is of particular concern due to the widespread and growing use of OP agents. In this article we will present new evidence that low level exposure or exposures to an OP compound can produce unexpectedly long term changes in brain function as measured by the electroencephalogram (EEG).

RECOVERY FROM A SINGLE EXPOSURE

Low level human exposure is known to produce a variety of signs and symptoms including miosis, muscular fasciculations, and apprehension

¹Supported in part by:

- A grant from Agrippa Data Systems of Boston.
- National Institute of Health Mental Retardation Core Grant HD06276, and
- Department of the Army Contract DADA03-77-W-0400

Please send requests for reprints to Dr. Frank Hopkins Duffy.

Neurotoxicology 1:667-689

Copyright © 1980 by Pathotox Publishers, Inc.

(Koelle, 1968; Wadia *et al.*, 1974). These acute manifestations, particularly those involving behavioral alterations, are usually accompanied by marked desynchronization of the electroencephalogram (EEG), a triad of changes consisting of increased high frequency activity, decreased low frequency activity, and lowered background voltage (Burchfiel *et al.*, 1976). At higher dose levels, OP compounds can produce convulsions, muscular paralysis, and death (Koelle, 1968). High OP levels cause slowing of the EEG followed by the development of spike wave discharges which accompany clinical convulsions (Burchfiel *et al.*, 1976). Most of these drug actions are attributable to phosphorylation of a serine residue within the active site of acetylcholinesterase. The result is inactivation of this critical nervous system enzyme and an overabundance of the excitatory neurotransmitter, acetylcholine. Symptomatic recovery from OP exposure is normally complete within 2 to 9 weeks at which time cholinesterase usually has returned to normal (Grob and Harvey, 1958; Bowers *et al.*, 1964).

MULTIPLE OR CONTINUAL EXPOSURES

Sustained low level administration of OP compounds can produce symptoms and signs not seen following single exposures of the same dose level. For example, subjects given daily injections of diisopropyl fluorophosphate (DFP) showed the additional symptomatology of insomnia, excessive dreaming, emotional lability, increased libido, paresthesias, visual hallucination, and tremor (Grob *et al.*, 1947). However, such symptomatology usually disappears after cessation of exposure. In most instances, EEG abnormalities are reported to disappear within two weeks of an acute exposure or termination of chronic exposure (Grob *et al.*, 1947; Holmes and Gaon, 1956; Santolucito and Morrison, 1971).

LONG TERM EFFECTS — DELAYED NEUROTOXICITY

In the late 1920s and early 1930s, cases of severe peripheral neuropathy occurred in the United States and elsewhere (Smith *et al.*, 1930). Adulteration of the beverage, "Jamaica Ginger" with the OP compound tri-o-cresyl phosphate (TOCP) was determined to be the cause. The syndrome was initially referred to as "ginger jake paralysis". A second outbreak occurred in Morocco in 1959, when humans consumed olive oil contaminated with a lubricating oil containing TOCP (Smith and Spaulding, 1959).

Since then, a number of OP compounds - but by no means all - have been found to cause delayed neurotoxicity, e.g. DFP and the closely related systemic insecticide, Mipafox.

The symptoms are those of peripheral weakness, sensory dysesthesias and severe ataxia often followed by respiratory failure and death. Lesions of the myelin sheath (demyelination) and axonal degeneration are found in the peripheral nerve, spinal cords, brain stem, and cerebellum of affected humans or animals. Few, if any, lesions are found in the cerebral hemispheres (Bradley, 1976).

Many OP compounds simply fail to produce delayed toxicity. Others produce delayed toxicity in some animal species but not in others. The mechanisms underlying delayed neurotoxicity are not well understood. However, Johnson (1975) has shown that inhibition of a specific nerve esterase is associated with the delayed neurotoxicity in hens.

LONG TERM EFFECTS — PSYCHIATRIC AND EEG

More recently, studies have appeared suggesting that long term effects other than delayed neurotoxicity may be present. Many of these studies, however, have been challenged.

A number of studies suggest that some subjects may experience long term sequelae from a single OP exposure or a limited period of low level exposure. Gershon and Shaw (1961) reported histories of memory difficulty, depression, schizophrenic reactions, and emotional instability lasting up to one year following cessation of prolonged low level OP exposure in 8 of 16 subjects. Similar but less severe abnormalities (lasting more than six months) were reported by Tabershaw and Cooper (1966) in 38 percent of 114 subjects after acute OP poisoning. Rowntree, *et al.* (1940), suggested that OP exposure might exacerbate pre-existing psychiatric problems. Metcalfe and Holmes (1969) suggested that OP exposure may lead to chronic EEG changes. They reported that workers with past histories of both OP and chlorinated hydrocarbon exposure, but with no recent exposures, had EEGs which showed a high incidence of excessive slowing. All night sleep EEGs showed a high incidence of "narcoleptic sleep records." Psychological dysfunctions in this group included disturbed memory and difficulty maintaining alertness and appropriate focusing of attention.

None of these studies included a detailed statistical analysis or a comparison with control (unexposed) subjects. Thus, as of the early 1970s, the issue of delayed or persistent toxicity affecting the cerebral hemispheres (as manifested by psychiatric or EEG changes) was not conclusively settled.

PROJECT LEACHE (LATE EFFECTS OF ANTICHOLINESTERASE EXPOSURE)

In the late 1960s and early 1970s, the U.S. Government became aware that a number of its civilian employees working with or in the vicinity of the potent OP, sarin, were complaining of a variety of symptoms, including excessive dreaming, loss of libido, memory loss, irritability, trouble concentrating, etc. On clinical grounds it was impossible to ascribe these symptoms to any single cause - including sarin exposure. But as some of these employees had been involved in the study by Metcalfe and Holmes (1969), it was decided to investigate the question in a more rigorous manner. First, it was decided to determine whether long term EEG changes occurred in monkeys under well controlled laboratory conditions. Second, if the results of this study of a non-human primate were positive, it was decided to carefully evaluate the EEGs of the government employees for any deviation from normal.

THE MONKEY STUDY

Rhesus monkeys (*Malacca Mulatta*) weighing 2.5 - 4.0 kg were utilized in a study to compare the EEGs of monkeys one year after exposure to the OP, sarin, with their pre-exposure baseline EEGs. To control for the effects of changes attributable to aging or other unknown factors, a comparable group of control monkeys was similarly studied before and one year after sham exposure. Changes attributable to sarin exposure should be seen only in the exposed group, whereas, nonspecific effects would be seen in both groups.

Clinical EEGs are routinely evaluated by visual inspection. To avoid the possibility of introducing subjective bias and to permit a more quantitative analysis, EEG data were preprocessed by the technique of spectral analysis, using the Fast Fourier Transform (FFT). This analysis quantitatively determines the EEG energy at different frequencies.

Sarin was administered according to one of two schedules. In one, animals received a single "large dose" of the drug: 5 ug/kg of sarin, i.v. In the other, animals received multiple "small doses": a series of 10 injections of 1 ug/kg of sarin i.m., given at one-week intervals. Hereafter, these will be referred to respectively as the "large-dose" and "small-dose" schedules. These dose levels were arrived at through pilot studies in which an attempt was made to find dose levels which would approximate, respectively, (1) a serious but sublethal exposure showing all the signs of poisoning, and (2) a series of sub-clinical but near-threshold exposures showing few, if any, signs of overt poisoning. At the large dose level, all animals had generalized convulsions. To prevent the possibility of secondary anoxic brain damage, animals were paralyzed by intravenous infusion of 4-12 mg/hr Gallamine Triethiodide (Flaxedil) and artificially respired. Controls were similarly treated but sarin was not given. Small dose animals did not convulse and did not require paralysis and respiration.

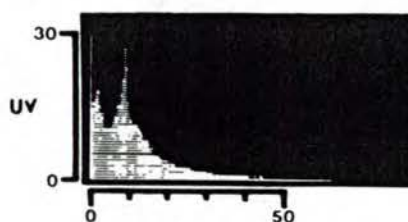
EEG was recorded before drug administration and one year later for all animals. Via spectral analysis on a digital computer, a calculation was made of the percentage of total energy present in each of the classical EEG frequency bands: delta = 0.5 - 3.75 Hz, theta = 4.0 - 7.75 Hz, alpha = 8.0 - 12.75 Hz, beta 1 = 13 - 21.75 Hz, beta 2 = 22.0 - 50.0 Hz. Data were recorded and analyzed from dural electrodes implanted in the skull; bipolar recordings were done from the fronto-central and occipital-temporal regions. Animals were studied in the awake (light), awake (darkness), and drowsy (darkness) states.

Statistical analysis was performed as a two-step procedure. First, a longitudinal comparison was made for each animal between his pre-exposure and one year post-exposure records on the basis of a two-tailed Student's *t*-test. The second step was to determine which differences, if any, revealed by *t*-test were consistently and exclusively seen among the drug-treated animals. Contingency tables (2 x 2) were set up and analyzed by Fisher's exact probability test (Fisher, 1934).

Figures 1 and 2 illustrate typical EEG spectra derived from an animal exposed to 5 ug/kg sarin IV and a control animal, respectively. Note in Fig. 1

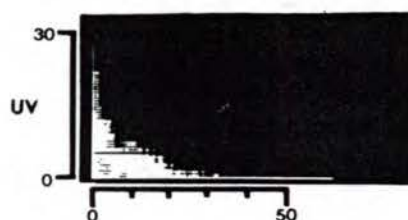
the significant EEG changes seen one day after exposure and how the increase in beta activity was still present one year later; comparable changes are not seen in the control animal (Fig. 2). This increase in beta activity was consistently observed in monkeys administered sarin. This is shown in Table 1, which summarizes the results of this study. For example, 2 of the 3 monkeys on the

CONTROL



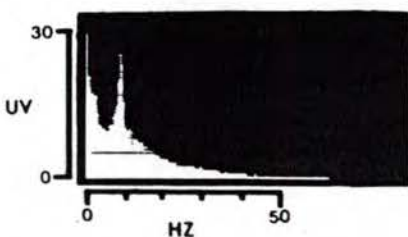
BAND	PERCENT ENERGY	S. E.
D	19.89	0.92
T	19.32	1.05
A	32.50	0.94
B1	18.72	0.78
B2	13.25	0.41

24 HOURS



BAND	PERCENT ENERGY	S. E.	t VALUE vs CONTROL
D	26.80	2.12	3.53**
T	19.05	0.65	0.14
A	19.39	0.33	7.64**
B1	19.65	1.44	0.59
B2	18.26	0.22	6.68**

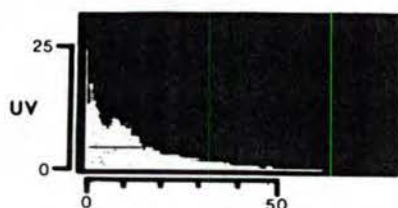
1 YEAR



BAND	PERCENT ENERGY	S. E.	t VALUE vs CONTROL
D	21.05	0.29	0.99
T	17.16	0.73	1.51
A	30.36	0.93	1.55
B1	18.64	0.58	0.07
B2	16.29	1.23	2.77*

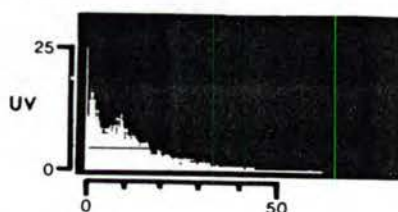
FIGURE 1. An example of one monkey's EEG frequency spectrum before, immediately after, and 1 year after administration of a single large dose of the organophosphate, sarin. The ordinate for each curve is in microvolts. The abscissa is frequency in Hertz or cycles per second. The "control" spectrum represents the EEG spectrum in the pre-exposure state. The "24 hour" curve refers to the spectrum 1 day after administration of a large (5 ug/kg) dose of sarin. The "1 year" curve refers to the spectrum one year after the large dose of sarin. To the right of each curve is the quantitative summary of each curves values. D = delta (0.5 - 3.75 Hz); T = theta (4 - 7.75 Hz); A = alpha (8 - 12.75 Hz); B1 = beta1 (13 - 21.75 Hz); B2 = beta 2 (22-50 Hz). These are the standard EEG frequency ranges of clinical interest. The "t value" is the result of the Student's t test between the current curve and the control state. Significant differences are indicated by asterisks; $p \leq 0.05^*$ and $p \leq 0.01^{**}$. Note there are significant differences in delta, alpha and beta 2 at 24 hours. Note that there is still a significant difference between the control and 1 year curves in the beta 2 EEG range. The delta and alpha differences reverted to pre-exposure baseline values.

CONTROL



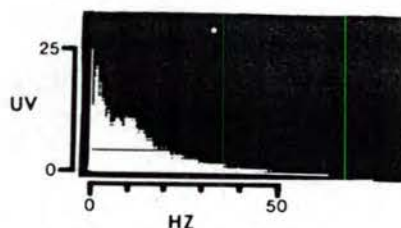
BAND	PERCENT ENERGY	S. E.
D	23.54	0.62
T	18.35	0.62
A	22.22	1.00
B1	18.47	0.77
B2	20.86	1.00

24 HOURS



BAND	PERCENT ENERGY	S. E.	t VALUE vs CONTROL
D	22.74	1.87	0.56
T	17.02	0.42	1.16
A	22.26	1.56	0.02
B1	20.68	0.53	1.55
B2	20.86	0.42	0.01

1 YEAR



BAND	PERCENT ENERGY	S. E.	t VALUE vs CONTROL
D	23.46	1.57	0.05
T	18.00	0.75	0.36
A	21.95	0.95	0.20
B1	20.14	0.97	1.34
B2	19.94	0.72	0.75

FIGURE 2. An example of one monkey's EEG frequency spectrum handled in the same way as the large dose monkeys but not administered any sarin. Note the absence of significant difference between the control values and the 24 hour and 1 year values.

small dose schedule of sarin showed a significant increase of temporal lobe beta one year after administration. By contrast, none of the 10 control monkeys did. Fisher's exact probability test indicates that this is a significant difference at the 0.039 level. When the results from all six monkeys receiving sarin (3 large dose and 3 small dose) are pooled and compared to the control animals for the temporal lead, the distributions are significantly different in the states of eyes closed in darkness and drowsy. These results indicate that a single symptomatic exposure to sarin or a series of subclinical exposures can alter the frequency spectrum of spontaneous EEG for at least one year. Significant alterations occurred in the temporal region in the beta frequency ranges.

THE HUMAN STUDY

In view of the positive results of the monkey study, it was decided to proceed with a search for persistent EEG changes in human subjects with

TABLE 1. Persistent Effects of Sarin Exposure on Monkey EEG One Year After Exposure

TABLE 1. Persistent Effects of Sarin Exposure on Monkey EEG One Year After Exposure																		
increased temporal lobe beta: (exact probability test)	exposed animals:		control animals:		signif increase	no increase												
					signif increase	no increase												
	SMALL DOSE		LARGE DOSE		COMBINED													
EYES CLOSED	<table><tr><td>2</td><td>1</td></tr><tr><td>0</td><td>10</td></tr></table>	2	1	0	10	.039	<table><tr><td>2</td><td>1</td></tr><tr><td>0</td><td>10</td></tr></table>	2	1	0	10	.039	<table><tr><td>4</td><td>2</td></tr><tr><td>0</td><td>10</td></tr></table>	4	2	0	10	.008
2	1																	
0	10																	
2	1																	
0	10																	
4	2																	
0	10																	
DARKNESS																		
DROWSY	<table><tr><td>3</td><td>0</td></tr><tr><td>2</td><td>8</td></tr></table>	3	0	2	8	.035	<table><tr><td>3</td><td>0</td></tr><tr><td>2</td><td>8</td></tr></table>	3	0	2	8	.035	<table><tr><td>6</td><td>0</td></tr><tr><td>2</td><td>8</td></tr></table>	6	0	2	8	.003
3	0																	
2	8																	
3	0																	
2	8																	
6	0																	
2	8																	

Each 2 x 2 table represents the number of animals who showed a significant increase in beta (left column), versus the number of animals who did not (right column). The E group is the top row in each table and the C group the bottom row. Next to each 2 x 2 table is the result of the Fishers exact probability test. This test is ordinarily employed in lieu of the Chi-Square test when the number in any place is smaller than 5. Values less than 0.05 are considered significant. Note that all values shown above are less than this value. These data were recorded from the occipital-temporal electrode pair and represent beta activity only. Other electrode pairs and frequency ranges did not show significant differences.

histories of exposure to sarin. Here, however, the experimental design would have to differ from the monkey study as all humans had accidental exposures. No pre-exposure baseline records were available. Thus, the study was designed to statistically compare the EEGs of exposed subjects with those of a comparable group of unexposed control subjects. The exposure group was carefully chosen to exclude any individual with a history of sarin exposure within the preceding year.

The exposure (E) Group consisted of 77 industrial workers. Each had a documented history of at least one exposure to sarin. In addition, 41 workers within the E group had had 3 or more exposures within the six years preceding the study. This subgroup will be referred to as the maximum exposure (ME) group. The exposure criteria were defined as (1) history of exposure, (2) resultant clinical signs and symptoms consistent with exposure, and (3) reduction in erythrocyte cholinesterase to a level at least 25 percent below the individual's pre-exposure baseline. No E group member had a history of exposure to chlorinated hydrocarbon or carbamate compounds.

Thirty-eight industrial workers from the same plant comprised the control (C) group. No member of this group had access to OP compounds at work, had a history of OP, chlorinated hydrocarbon, or carbamate exposure, had ever complained of symptoms associated with OP exposure, or had ever shown significant fluctuations in semiannual or spot check cholinesterase levels.

Each subject in the study had two EEG recording sessions. In the first, EEG data were obtained under the usual clinical laboratory conditions. These data were recorded on paper for visual interpretation and on magnetic tape for computer analysis. In the second session, an all-night sleep EEG record was obtained. For analysis of the sleep data, we excluded those few subjects below age 30 so as to render the C, E, and ME populations more comparable

(Williams *et al.*, 1974; Brezinova, 1975). Consequently, for the sleep analysis, there were 26 members in the C group, 67 in the E group, and 38 in the ME group.

In addition to the conventional group comparisons of individual parameters as outlined above, we wished to consider the combined effect of the mutual interdependence of all variables upon group differences. Accordingly, we employed the standard multivariate technique of discriminant analysis (Cooley and Lohnes, 1971) to the problem of establishing a single statistically quantifiable measure of group difference. Here, we calculated Wilks' Lambda to assess the significance of group separation. To reduce the dimensionality of the data representation prior to discriminant analysis, we submitted all variables, or "features" to a merit rating process. The "merit function" is based on (1) the computation of an ambiguity function (Genchi and Mori, 1965), and (2) the computation of a measure of detectability from the Receiver Operating Characteristic Curve (Selin, 1965). Furthermore, the merit function takes into account the mutual correlation of all features selected by the first two criteria.

For all analyses, control subjects were compared to all the exposed subjects (CxE analysis) and again to the subgroup of subjects with multiple (3 or more) exposures (CxME analysis). We considered doing a regression analysis but sufficient medical information to permit quantification of an individual's exposure history was not available.

Results of the CxE comparison of the spectral analysis data are shown in Table 2. The corresponding CxME comparison is shown in Table 3. Note that we analyzed three electrode locations: temporal (T3-T5), fronto-central (F3-C3), and occipital-central (Oz-Cz). Note that there were five clinical states - eyes open (EO), eyes closed (EC), drowsy (D), hyperventilation (HV), and recovery from hyperventilation (pHV). For each lead and state, the frequency range from 0.5 to 30 Hz was broken down into 10 bands, each 3 Hz wide. The CxE analysis (Fig. 4) showed only 8 significant comparisons. This raises the important question of how many individual comparisons must be statistically significant to have a "physiological significance". For example, there are 50 comparisons - 5 states and 10 bands - for the CxE analysis of F3-C3. At the 0.05 level, 0.05 x 50 or 2.5 comparisons might be expected to show significance by chance. As only eight comparisons in the entire CxE comparisons showed significance, this could have arisen by chance alone, and have no definite physiological significance. However, since five comparisons within the Oz-Cz linkage were statistically significant, and since two of the entire CxE comparisons reached the 0.02 level, it is unlikely that chance alone determined this outcome. Furthermore, six of the eight significant comparisons represented increases in beta activity (12-30 Hz) making the likelihood of a physiologically significant difference between the C and E groups more secure.

On the other hand, the C:ME analysis (Table 3) showed a more definitive picture. There were 14 significant comparisons for T3-T5, 5 for F3-C3, and 14 for Oz-Cz. Clearly, we have exceeded the "chance level" for all linkages (especially T3-T5 and Oz-Cz). Note that 13 of the 24 CxME comparisons were significant at the 0.02 level, six at the 0.01 level, and one at the 0.001 level. The

TABLE 2. Power Spectra Analysis of EEG Records (Human C x E Significant Differences)

		Spectral EEG band (Hz)									
		0.5-3	3-6	6-9	9-12	12-15	15-18	18-21	21-24	24-27	27-30
T3-T5	EO										
	EC										
	D										
	HV										
	pHV										
F3-C3	EO	↓ T*									
	EC										
	D										
	HV								* U	↑ T	
	pHV										
OZ-CZ	EO										
	EC										
	D				↑ U		↑ U	↑ T			
	HV									↑ T*U*	
	pHV								↑ TU		

This figure summarizes the results of the comparison between the spectral data of the control group (C) and the entire exposed population (E); the C x E comparison. The ten columns represent the ten EEG frequency bands from approximately 0 to 30 Hz in 3 Hz steps. The rows represent the different recording locations and different clinical states.

Electrodes: T3T5 = temporal; F3C3 = fronto-central; OzCz = occipital-central.

States: EO = eyes open, alert; EC = eyes closed, alert; D = drowsy; HV = hyperventilation; pHV = recovery from hyperventilation.

Each entry in the table represents the significance level of the C x E comparison for that particular frequency band, electrode pair, and state. The absence of an entry indicates the failure to reach the 0.05 probability level of the 2-tailed Student's t test or Mann-Whitney U-test. The presence of an arrow indicates the direction of the change in the E group with respect to the C group mean value. The presence of a T implies a significant t test at the 0.05 level. U implies the same level for the U-test. Asterisks indicate higher levels of significant difference, i.e., *0.02; **0.01; ***0.001. For example, the 24-27 Hz band in the E group was of greater value than the 24-27 Hz value for the C group in the Oz-Cz electrode pair in the HV state. This difference was significant at the $p \leq 0.02$ level.

physiological significance is increased by the clear tendency for the results to cluster in the beta range and to represent an *increase* of beta in every case.

Thus, the results of the univariate spectral analysis show (a) probable differences between the C and E populations, (b) highly significant differences between the C and ME populations, and (c) a tendency of the differences to

TABLE 3. Power Spectra Analysis of EEG Records (Human C x ME Significant Differences)

		Spectral EEG band (Hz)									
		0.5-3	3-6	6-9	9-12	12-15	15-18	18-21	21-24	24-27	27-30
T3-T5	EO						† T**U	† T			
	EC					† TU	† T**U*	† T*U	† TU	† T*U	† T*U
	D		† TU				† T*U	† T*U	† T*U	† T*	† T
	HV										
	pHV										
F3-C3	EO										
	EC										
	D								† T	† U	
	HV								† T**U**	† T**U**	† U
	pHV										
OZ-CZ	EO										
	EC							† TU		† TU	
	D				† U		† TU*	† T*U	† T*U*	† T*U*	† T*U
	HV								† T**U*	† T***U**	† TU
	pHV							† TU	† T*U*	† T**U*	

Results of the spectral analysis between the control subjects (C) and the maximally exposed subjects (ME) with histories of 3 or more exposures. Convention is the same as in Table 2. Note the clustering of significant results in the beta range. Note also that all changes represent an increase of beta for the maximum exposure group. * $p \leq 0.02$ ** $p \leq 0.01$ *** $p \leq 0.001$

cluster in the beta range and represent an increase in beta within the exposed group.

MULTIVARIATE ANALYSIS

The merit function values for the selection of best features for discrimination were computed for all 150 (10 spectral bands x 5 states x 3 electrode pairs) features available per subject. The percent time spent in REM sleep was added as the 151st feature. The ten best features were chosen for inclusion in the discriminant analysis. All had highly significant differences for the comparison between controls and subjects who suffered maximum exposure. The data show that the spectral values in the beta frequency range contribute most to the

discriminating function. All of the selected features showed approximately the same high correlation with the discriminant function.

The overall group separation is measured by the value of Wilks' Lambda, which was found to be 0.644, at 10 and 93 degrees of freedom respectively (CxME) for the data without the REM feature, and at 0.620, as 9 and 94 degrees of freedom for the data set including the REM sleep feature. This statistic is also known as "determinant ratio" and since the determinant of a multivariate distribution expresses the scatter, Wilks' Lambda may be seen as a multivariate analogue to the F-ratio in univariate statistics, which is a variance ratio. The asymptotic distribution of Wilks' Lambda can be approximated by the F-distribution. The value for Wilks' lambda for the CxME comparison, with and without the sleep feature, rendered a highly significant F-ratio, $p = 0.00001$. Very similar data, with only slightly decreased level, were obtained for the CxE discrimination, $p = 0.0001$.

Thus, the results of the multivariate spectral analysis show (a) significant differences between the C and E populations, (b) significant differences between the C and ME sub-populations, and (c) that beta frequencies contribute most heavily to the differentiation.

In addition to spectral analysis of the tape recorded EEG data, the paper EEG records were visually interpreted by a qualified clinical electroencephalographer (clinician). For each record, a set of EEG parameters was evaluated. Based upon his clinical experience, the electroencephalographer designated each parameter as normal or abnormal. These data were then tabulated and statistically analyzed using Fisher's test. The results of CxE and CxME analyses are shown in Table 4.

TABLE 4. Human EEG Readings — Exact Probability Test

	Waking	Drowsy	Sleep	Hyper-ventilation	Post-hyper-ventilation	Strobe	Back-ground voltage	Back-ground rhythms	Background rhythms	
									High	Low
C	33/5 ^b	33/5	36/2	28/10	34/4	38/0	31/7	32/6	32/4	32/2
E	61/16	57/20	67/10	58/19	70/7	73/4	66/11	54/23	54/13	54/10
C × E	0.13	0.06	0.13	0.18	0.25	0.20	0.18	0.05*	0.13	0.11
M	30/11	30/11	37/4	32/9	38/3	39/2	33/8	26/15	26/9	26/6
C × M	0.07	0.07	0.26	0.19	0.27	0.27	0.22	0.02*	0.07	0.09
	Parox. Activity	Focal Activity	Asym-metries	Delta	Theta	Alpha	Beta	Doctor's guess	Entire record	
C	23/15	37/1	36/2	35/3	27/11	29/9	32/6	18/20	29/9	
E	44/33	75/2	76/1	58/19	39/38	44/33	58/19	39/39	55/22	
C × E	0.15	0.45	0.22	0.02*	0.02*	0.02*	0.11	0.15	0.16	
M	25/16	39/2	40/1	32/9	21/20	22/19	28/13	18/23	29/12	
C × M	0.18	0.39	0.36	0.06	0.04*	0.02*	0.06	0.17	0.17	

NORMAL/ABNORMAL

Results of analysis, by visual inspection of standard clinical EEG, on all subjects. C, E, ME, CxE, and CxME have the same meaning as in previous tables. Results of the exact probability test are shown. Statistically significant levels ($p < 0.05$) are flagged with an asterisk.

Note the significant results for the CxE and CxME comparisons in the "Bkgnd Rhythms" column. This indicates that EEG background activity was more likely to deviate from normal in the E group and especially the ME sub-group than in the C group. As the "Hi" and "Lo" columns failed to show significant levels, the background EEG abnormalities in the E and ME groups were not consistently toward either more slow or more fast activity. However, the significant values in the "Delta" and "Theta" columns indicate that more E and ME group EEGs showed excessive amounts of slow activity at some time during the record than for the C group EEGs. Furthermore, the "Alpha" column revealed more EEGs with diminished or absent alpha activity in the E and ME populations. No other columns showed significant values. Note that there were no more significant numbers of abnormal EEGs in the E and ME groups than in the C group ("Entire Record"). Also, the clinician was not able to place EEGs into their correct group ("Doctor's Guess"). He was able to correctly categorize 47% of the C, 51% of the E, and 56% of the ME group EEGs. The overall classification success using inspection techniques was 49.6%, clearly at the chance level.

The large number of EEG records read as "abnormal" deserve comment. Note that 29 percent of the E records (22 of 77) and ME records (12 of 41) were read as abnormal. However, as 24 percent of the C records (9 of 38) were also "abnormal", the CxE and CxME comparisons were not statistically significant. It is not clear why so many records in all groups were "abnormal" in comparison to the clinician's hypothetically normal model. It is possible that some unknown environmental factor was present, e.g., effect of living at 1,500 to 2,000 m elevation. Whatever the explanation, the effect was equally distributed across all groups. The essence of the experimental method used in this study involved the comparison of groups chosen to be as similar as possible with the exception of a single factor, in this case, OP exposure. Thus, any statistically significant differences between the EEGs of the groups could be argued to be attributable to this single factor. The effect of other factors (reader bias, etc.) should be randomly distributed across all groups.

Thus, the visual inspection of the clinical EEG revealed more EEGs in the E and ME groups containing (1) diminished alpha, (2) increased epochs of slow activity, and (3) deviation of background activity to either faster or slower rhythms. However, these were not clearly diagnostic features since the encephalographer's classification success was at a chance level.

Table 5 shows the results of the CxE and CxME analyses of the all night sleep EEG records. Note the highly significant increase in absolute and percent Rapid Eye Movement (REM) sleep for both CxE and CxME comparisons. There were no such changes for any single slow wave sleep stage, for combined stages 3 and 4, or for total sleep time. The decreased stage 2 sleep for the CxME percent comparison suggests that the additional time spent in REM came at the expense of stage 2 sleep.

Percent REM for all three of our groups, including the controls, fell below published normals for middle aged men (Agnew *et al.*, 1967; Williams *et al.*, 1974). This may be due to either or both of the following reasons. First, REM is usually decreased during the first night of sleep in a laboratory situation, the

TABLE 5. Results of analysis of all night sleep EEG on human subjects.

	HUMAN SLEEP EEG												
	REM	ABSOLUTE TIME					Total Sleep	REM	Stage 1	PERCENT TIME			
		Stage 1	Stage 2	Stage 3	Stage 4	Stages 3 + 4				Stage 2	Stage 3	Stage 4	Stages 3 + 4
C	34.865	39.078	196.790	16.639	6.332	22.970	289.857	11.414	15.732	67.718	5.575	1.967	7.548
E	46.318 ^{1,1}	43.305	193.913	16.483	4.141	20.595	304.194	15.117 ^{2,1}	13.967	64.319	5.419	1.168	6.583
C x E	T ¹ U ¹							T ² U ¹					
ME	47.736 ^{2,4}	46.996	183.161	20.701	6.663	27.364	305.261	15.645 ^{2,4}	14.974	60.710 ^{1,4}	6.818	1.841	8.669
C x ME	T ² U ²							T ² U ²		T ¹ U ²			

C, E and ME have the same meaning as in previous tables. Time in each phase of sleep is shown in minutes and again as percent of total sleep time. Level of significance is indicated by appropriate reference for comparison by t-test (¹P 0.05, ²P 0.01); for U-test (¹P < 0.05, ⁴P < 0.01).

first night effect (Mendels and Hawkins, 1967). Second, most REM tends to occur in the final third of a night's sleep (Williams 1974). All our subjects were awakened at 0530, approximately 30 to 45 minutes before their usual waking time. Thus, a significant portion of this lost sleep might have been REM.

The results of the all night sleep study show that the entire exposed group showed an increase in REM sleep and that this was of slightly greater magnitude for the maximum exposure group (Duffy *et al.*, 1976, 1979).

DEVELOPMENT OF AUTOMATIC CLASSIFICATION RULES

Results of the initial human study demonstrated significant differences between sarin-exposed workers and controls. The existence of group differences for certain parameters, however, does not necessarily mean that an individual can be correctly assigned to one group or the other based upon his individual scores for these parameters. The diagnosis of an individual worker as sarin exposed is a different problem from the demonstration of group differences between sarin exposed and control populations. Therefore, a new study was undertaken to re-examine the EEG data gathered from the control and exposed industrial workers to see if rules could be developed to permit classification of each individual as being a member either of the control or the exposed population.

The overall approach involves three stages. These are:

First, a search for features which show potential as components in a discriminant function. In other words, one must determine which features, derived from the EEG analysis, would be most useful in diagnosing an individual subject.

Second, classification rules have to be developed; this stage is also referred to as "system training". It is carried out by submitting sets of data known to represent controls, or exposed workers to self-learning algorithms. In other words, once one has determined which EEG features are useful in individual diagnosis, one must make up the diagnostic rules using groups of these features.

Third, these rules have to be tested for consistency on test data sets, i.e., on data not used to derive the decision rules. In other words, once one has determined the useful features and the diagnostic rules via a "training" data set, one must test their validity on new data or "test" data sets.

All stages of the above process were run under a version of the TICAS software system, modified to analyze EEG data. The TICAS system, originally designed to differentiate between normal and malignant tissue cells, includes automated feature selection, system training, and classification algorithms (Wied *et al.*, 1968).

Selection of features for use in setting up discrimination rules was done as follows: Initially, features showing high statistical differences in the group comparison study were evaluated. Various univariate techniques, such as Student's t-test, the Mann Whitney U-test, and the Kruskal Wallis test statistic were employed. Next, the best features were selected by an algorithm computing a merit function. For each feature the value of the ambiguity function after

Genchi and Mori (1965), and the measure of detectability from the receiver operating characteristic curve (Selin, 1965) of the discrimination problem at hand were computed. Features showing the lowest values of an intermediate merit function according to these two criteria were then chosen in a sequential procedure so that the average correlation between all selected features was minimized.

Three different algorithms were employed to derive optimized decision rules. These were the supervised learning program DSELECT, the program EXTRACT, and the program CLASSIFY.

DSELECT is a non-parametric procedure which has in the past proven to be robust, and particularly valuable for data with very close group centroids, high correlations between feature vector components, and markedly different eigenstructure. DSELECT is, however, prone to lead to system overtraining when sample sizes are small as compared to the number of available features.

EXTRACT forms a linear combination based on the mean differences and respective variances of two groups. The weights are optimized to produce a minimum number of misclassifications.

CLASSIFY is a parametric procedure. The probability densities of two or more populations are estimated from the data. Test set data are then classified on the basis of the maximum likelihood criterion. Using a slightly enlarged data set, an initial set of features and classification rules were developed. There were 39 controls, and 82 recordings from exposed individuals. Of the exposed group, 36 individuals had received three or more exposures; this group was designated as maximum exposed. Out of the total available features, a set of 24 were flagged as exhibiting statistically significant differences. Rules were developed to individually classify the ME group members and the C group members with 100 percent success. It is not unusual, however, to achieve complete classification success on the same set of subjects used to generate the classification rules. The ultimate test of one's power of automatic diagnosis is to apply rules developed on one set of subjects to the problem of classifying a second set of subjects not used in the ongoing rule generation.

So, we applied the rules developed above to a set of 16 subjects (8 controls and 8 max-exposures) not previously analyzed. The results of this are shown in Table 6, along with the electroencephalographer's (clinician's) designations.

Therefore, the computer correctly classified 13 of 16 subjects for an 81.3 percent success rate. The clinician correctly classified 7 of 16 subjects for a 43.8 percent success rate, slightly below the chance level. These results were encouraging.

TABLE 6

actual	COMPUTER CLASSIFICATION			CLINICIAN'S CLASSIFICATION	
	control	max. exp.	no decision	control	max. exp.
controls	6	0	2	5	3
max. exp.	1	7	0	6	2

TABLE 7. Computer Classification (Percent correct)

	C	ME
C	93.3	6.7
ME	44.8	55.2

Subsequently, the data set was significantly enlarged and the test of the rules applied to this larger population. We were surprised to find that the classification success for subsequent test sets, although superior to the inspection procedure in every case, did not match the high values achieved in the initial test. The success rate of the computer analysis is shown in Table 7. It soon became apparent that we were consistently classifying correctly all or most of the C group, but only about half of the ME group. Accordingly, we explored the data structure within the C and ME groups as regards discriminant function score (Fig. 3 and 4) for two separate sets of C and ME subjects. Note the unimodal distribution of the C group but the clearly bimodal distribution of the ME group. Furthermore, one ME group mode appears to coincide with the distribution of the control population. The most obvious implication is that not every subject with a history of exposure to OP compounds suffers changes in

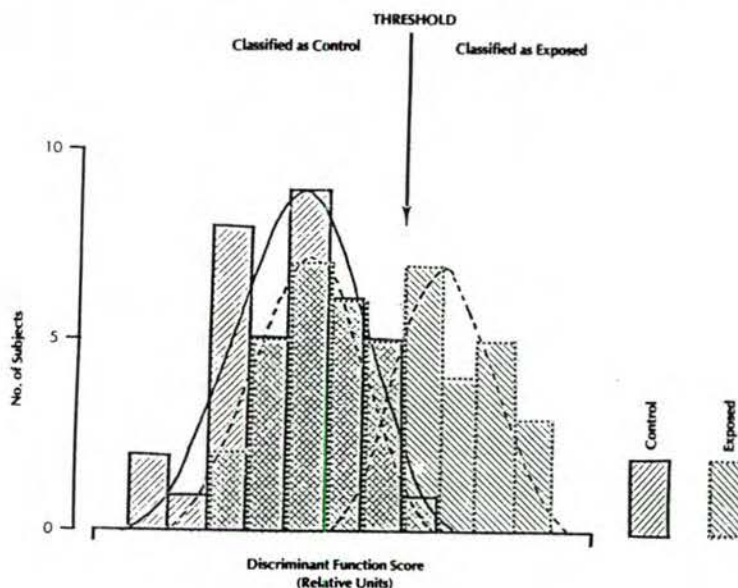


FIGURE 3. This figure shows the relative distribution of the control and maximally exposed populations within discriminant function space. Note that the control population tends to have a unimodal distribution and the exposed population a bimodal distribution. Note also that one mode of the exposed population seems to coincide with the control population's distribution. This is suggestive but not rigorous evidence to suggest that not all brains will show the same degree of EEG change following sarin exposure. Does the lesser exposed population mode represent recovery or do some brains fail to show the persistent effects at all?

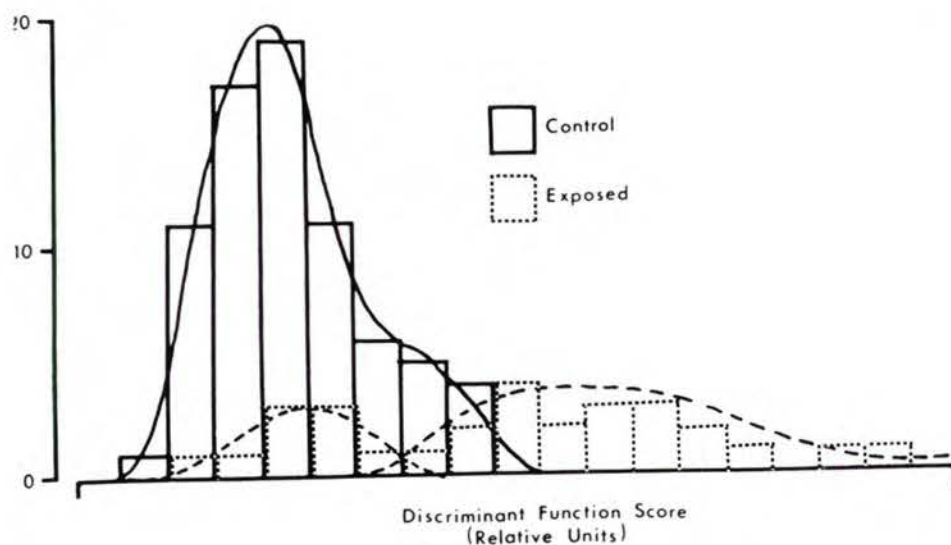


FIGURE 4. This figure represents the same analysis on another set of control and exposed subjects. Note the similar bi-modal distribution for the exposed subjects.

brain function. Alternatively, the bimodal distribution may reflect spontaneous recovery.

In any event, when the automatic classification procedure "correctly" classified only 50 percent of the ME group, it may have been accurately defining those ME group members with and without persistent OP effects. The success of the initial classification rules upon the training data set used to derive them may be due to the choice of exposed subjects for this set. The exposed subjects in the training set tended to be those with the best documented and most recent sarin exposures (but not more recent than a year before the study). Subjects exposed to sarin a long time before the study or with borderline histories of exposure were more likely to be members of the later, test M groups.

CONCLUSIONS

The results obtained from these studies support four main conclusions:

First, there is sufficient evidence to state that EEG data of individuals with exposure to the organophosphate sarin exhibit highly significant group differences from EEG data of controls. These changes, seen in subjects free of OP exposure for one year or more, suggest that OP compounds have persistent effects upon brain wave (EEG) activity. The multivariate discriminant analysis shows that the control (C) group, when represented as a multidimensional vector, can be statistically separated from the entire exposure (E) group at the $P = 0.001$ level and the maximum exposure (ME) group at $P = 0.00001$ level. Similarly, the univariate CxE and CxME comparisons indicated statistically

significant group differences, the nature of which lend themselves, directly, to physiological and pharmacological discussion.

Results of the univariate analysis showed the E and ME groups to differ from the C group in the following manner:

1. Spectral analysis - increased high frequency activity (12-30 Hz, beta).
2. Visual reading of standard EEG - decreased amounts of alpha (9-12 Hz), increased amounts of slow activity (0-8 Hz, delta and theta), and non-specific abnormalities in EEG background.
3. Sleep EEG - increased amounts of REM sleep.

Second, the multivariate analysis shows clearly evident differences in the eigenstructure of the frequency data between controls and exposed workers, an observation which may agree with reports in the literature that organophosphates lead to "desynchronization" in the EEG. Furthermore, the majority of features selected as being optimum for classification were in the beta frequency range. This supports the finding by univariate statistics that the major spectral differences fall in the beta range as for the monkey study.

Third, the difference between the control and maximally (> 3) exposed populations is such that automated classification rules can be developed to diagnose subjects with classification accuracies exceeding those of an experienced electroencephalographer.

Fourth, the data structure of the maximum exposure group shows a clear, bimodal distribution (Fig. 3 and Fig. 4). Furthermore, one mode appears to coincide with the distribution of the control population. The most obvious meaning is that not all workers exposed to sarin undergo persisting changes in brain function. On the other hand, the bimodal distribution may also reflect recovery from change. At the moment there exists no behavioral or psychological data on these subjects which might allow one to test this hypothesis.

DISCUSSION

The increased beta activity noted in this study is of particular interest because similar increases in temporal lobe beta were also found in monkeys one year after sarin exposure (Burchfiel *et al.*, 1976). The significance of this finding is not yet established. Beta activity may be due to any number of factors. It is commonly increased during drowsiness and sleep (Kiloh *et al.*, 1972), by many drugs (Fink, 1969), and by aging (Mundy-Castle, 1951). Rarer causes are mental retardation (Kooi, 1971), organic brain lesions (Joffe and Jacobs, 1972; Nealis and Duffy, 1978), epilepsy (Nealis and Duffy, 1978), and emotional tension (Kamp *et al.*, 1972). Many pharmacological agents are especially prone to produce beta as part of generalized desynchronization. These include some of the most potent psychoactive agents, e.g., amphetamine, DMT, mescaline, and LSD (Fink, 1969). OP compounds are also among those known to cause desynchronization and increased beta at subconvulsive levels (Grob *et al.*, 1947; Grob and Harvey, 1953; Burchfiel *et al.*, 1976).

The significance of increased amounts of REM sleep is unclear. Of 20 or more pathological states, other than narcolepsy, associated with clinical de-

rangements of sleep (Williams *et al.*, 1974), almost all involve no change or a decrease in REM. In a review of REM sleep pharmacology, Hartman (1968) listed over 20 drugs causing a depression in REM (including barbiturates, common tranquilizers, mood elevators, alcohol, and amphetamine), but only four causing an increase in REM (Reserpine, L-tryptophane, 5-OH-tryptophane, and LSD). Thus, it is unusual to find classes of drugs that increase REM.

OP compounds, however, form one such class. Hernandez-Peon (1963, 1965) was among the first to suggest that the sleep-wake cycle was partially under cholinergic control. More recently, several reports have demonstrated a cholinergic link in REM sleep mechanisms (Magherini *et al.*, 1972; Thoden *et al.*, 1972; Matsuzaki *et al.*, 1968; Henrichsen *et al.*, 1972; Sitaram *et al.*, 1976.) It has been postulated that cholinergic mechanisms are involved in REM initiation, based upon the demonstration that cholinesterase inhibitors, such as OP compounds, can elicit REM sleep in both intact animals and brain stem preparations.

It is possible, therefore, that the long term EEG changes in this study represent the unexpected persistence of the well known effects of acute OP administration.

What might the pharmacological mechanisms underlying this persistent effect be? Symptoms and signs of acute OP exposure are believed to occur secondarily to reductions in cholinesterase levels. Accordingly, recovery is believed to parallel return of CNS cholinesterase to normal. Since tissue cholinesterase returns to normal levels at an approximate rate of 1 percent per day (Grob and Harvey, 1958; Milby, 1971), recovery should be complete 3 to 4 months following exposure. On the other hand, monkeys exposed to sarin showed EEG abnormalities one year after exposure (Burchfiel *et al.*, 1976). Furthermore, the abnormalities reported in the current study were found in subjects with normal RBC cholinesterase values at the time they were studied and who were exposure free for more than one year. Thus, evidence of CNS abnormality attributable to OP compounds can be found in monkey and man at a time when tissue cholinesterase levels are probably within normal limits.

One explanation may be that the low tissue cholinesterase levels, and resultant acetylcholine excess, at time of exposure, induce long-term changes in synaptic morphology or biochemical organization which do not completely reverse when tissue cholinesterase levels return to normal. For example, Fernichel *et al.* (1972, 1974) found that excessive acetylcholine levels could produce skeletal muscle necrosis in rats even where cholinesterase systems were intact. A central analogue of this effect could be postulated. We suggest that high acetylcholine levels at the time of exposure might produce long term changes in the post-synaptic receptor which render it more sensitive to endogenous acetylcholine. We have recently demonstrated the presence of such a mechanism in the hippocampus of the rat (Burchfiel *et al.*, 1979). After an electrically induced seizure discharge we found that hippocampal pyramidal cells showed prolonged hypersensitivity to iontophoretically applied acetylcholine.

Another explanation may be that sarin has actions beside that of a potent anticholinesterase. Similar compounds, triethocresylphosphate (TOCP) and

difluorophosphate (DFP), are known to produce peripheral neuropathies of delayed onset. The primary lesion is axonal degeneration with secondary demyelination. When the peripheral nerves are involved, lesions are always found in the spinal cord, often in the brain stem, and occasionally in the hemispheres (Johnson, 1970, 1974, 1975). However, sarin is not reported to produce delayed neurotoxic responses. There is no evidence to suggest that the persistent CNS effects of sarin in primates are related to a direct action on central axons or myelin.

In any event, the long term findings are also seen as part of the acute exposure syndrome. Thus, the pathogenesis of the long term effects may well represent an unexpected extension of the primary drug effect, the exact details of which do not appear to be completely established.

Regardless of the pathogenic mechanisms, results of the current study confirm the ability of OP compounds to induce persistent abnormalities in the electrical activity of the human brain. Whether these long-term neurophysiological changes can be directly related to reported behavioral abnormalities is not known. On the other hand, the long-term behavioral changes and long-term electrophysiological abnormalities provide parallel evidence that OP exposure can produce long-term change in the brain function of monkey and man.

IMPLICATIONS

(1) Although the current study has dealt exclusively with the OP, sarin, it seems quite reasonable to extend the implication of long term effects to many other OP compounds. As previously discussed, sarin is very similar, structurally, to many more common OP compounds.

(2) It seems reasonable to wonder about possible long term effects in humans of exposure to other pesticides (carbamates, sulfonates, chlorinated hydrocarbons, etc.). Although the appropriate human studies have not yet been done, there is evidence to support such concerns. In parallel with our study of the effects of sarin upon the monkey EEG, we also studied the effects of dieldrin-a chlorinated hydrocarbon. Long term effects were found (Burchfiel et al., 1976)!

(3) The most obvious question raised by our findings is: "What is the significance of the brain wave differences seen in the exposed population?"

There is no simple answer. On one hand, the EEG changes may have no psychological or physiological significance. The persisting EEG changes following OP exposure may have no more significance than the persisting changes in skin pigment that follow first pregnancy. On the other hand, other drugs which desynchronize the EEG, increase beta, and increase REM sleep are among the most potent psychoactive drugs known. Thus, the persisting EEG changes following OP exposure may signal subtle disturbances in intellectual or emotional function.

There is only one way to resolve this issue and that is to concurrently perform both EEG and detailed psychological tests on a population of control and exposed subjects. This has not been done. In the absence of such data, the

most conservative and most ethical position must be to presume that the EEG changes are associated with functional abnormalities until proven otherwise.

(4) The next most obvious question is: "How persistent are these EEG effects?" We do not know. The bimodal distributions in Figs. 3 and 4 suggest a potential for recovery, but the only way this could be resolved is to study the same exposed population at differing points in time.

(5) And, finally, comes the question: "What is the minimal dose necessary to precipitate long term changes?" Unfortunately, the answer to this question is not obtainable from the human data. The problems are 1) how would one quantitate the magnitude or degree of an accidental exposure? and 2) how would one compare the effects of such exposures (even if a good measure could be found) occurring at differing points in the past. The monkey study does provide a partial answer and is cause for some concern. The degree of EEG change seen one year after a series of subclinical administrations was as large or larger than that produced by a single large exposure. Thus, it is possible that there is some long term effect for very small levels of exposure - just how low requires further study.

REFERENCES

- Agnew, H.W., Webb, W.W., Williams, R.L.: Sleep patterns in late middle age male: An EEG study. *Electroenceph. Clin. Neurophysiol.* 23:168-171, 1967.
- Bowers, M.B., Goodman, E. and Sim, V.M.: Some behavioral changes in man following anticholinesterase administration. *J. Nerv. Ment. Dis.* 138:383-389, 1964.
- Bradley, W.A.: The Pathology of Delayed Neurotoxicity Due to Organophosphates in Pesticide Induced Delayed Neurotoxicity. L.E. Baron, Ed., U.S. Environmental Protection Agency; EPA - 600/1 - 76 - 025, July, p. 84, 1976.
- Brezinova, V.: The number and duration of the episodes of the various EEG stages of sleep in young and older people. *Electroenceph. clin. Neurophysiol.* 39:273-278, 1975.
- Burchfiel, J.L., Duffy, F.H., and Sim, V.M.: Persistent effects of sarin and dieldrin upon the primate electroencephalogram. *Toxicol. Appl. Pharmacol.* 35:365-379, 1976.
- Burchfiel, J.L., Duchowny, M., and Duffy, F.H. Acetylcholine hypersensitivity in rat hippocampus - induction by kindling stimulus. *Science* 204:1096-1098, 1979.
- Casida, J.E. and Baron, R.L.: Recognition and overview of the organophosphate induced delayed neurotoxicity problem. In R.L. Baron, (Editor): *Pesticide Induced Delayed Neurotoxicity*, U.S. Environmental Protection Agency EPA-600/1-76-025, Research Triangle Park, N.C., 1976.
- Cooley, W.W. and Lohnes, P.R.: *Multivariate Data Analysis*. John Wiley, New York, 1971.
- Davies, D.R., Holland, P. and Rurnens, H.J.: The relationship between the chemical structure and neurotoxicity of alkyl organophosphate compounds. *Brit. J. Pharmacol.* 15:271-278, 1960.
- Duffy, F.H., Burchfiel, J.L. and Sim, V.M.: Persistent effects of organophosphate exposure as evidenced by electroencephalographic measurements. In Baron, R.L. (Editor): *Pesticide Induced Delayed Neurotoxicity*. U.S. Environmental Protection Agency, EPA-600/1-76-025, July, p. 102, 1976.
- Duffy, F.H., Burchfiel, J.L., Bartels, P.H., Gaon, M., and Sim, V.M.: Long term effects of an organophosphate upon the human electroencephalogram. *Toxicol. and Appl. Pharmacol.* 47:161-176, 1979.
- Fenichel, G.M., Dettbarn, W.D. and Newman, T.M.: An experimental myopathy secondary to excessive acetylcholine release. *Neurology* 24:41-45, 1974.
- Fenichel, G.M., Kilbler, W.B., Olson, W.H. and Dettbarn, W.D.: Chronic inhibition of cholinesterase as a cause of myopathy. *Neurology* 22:1026-1033, 1972.
- Fink, M.: EEG and human psychopharmacology. *Annu. Rev. Pharmacol.* 9:241-258, 1969.
- Fisher, R.A.: *Statistical Methods for Research Workers*, Oliver and Boyd, London, 1934.
- Genchi, H. and Mori, K.: Evaluation and feature extraction on automated pattern recognition system. *Denki-Tsushin Gakki, Part 1 (Jap.)*, 1965.

- Gershon, S. and Shaw, F.H.: Psychiatric sequelae of chronic exposure to organophosphorous insecticides. *Lancet* 1:1371-1374, 1961.
- Grob, D. and Harvey, A.M.: The effects and treatment of nerve gas poisoning. *Am. J. Med.* 14:52-63, 1953.
- Grob, D. and Harvey, J.C.: Effects in man of the anticholinesterase compound warin (isopropyl methyl phosphonofluoridate). *J. Clin. Invest.* 37:350-368, 1958.
- Grob, D., Harvey, A.M., Langworthy, O.R. and Lilienthal, J.L., Jr.: The administration of diisopropyl fluorophosphate (DFP) to man. III. Effect of the central nervous system with special reference to the electrical activity of the brain. *Bull. Johns Hopkins Hosp.* 81:257-266, 1947.
- Hartmann, E.: On the pharmacology of dreaming sleep (the D. state). *J. nerv. Ment. Dis.* 146:165-173, 1968.
- Henricksen, S.J., Jacobs, B.L., and Dement, W.C.: Dependence of REM sleep PGO waves on cholinergic mechanisms. *Brain Res.* 48:412-416, 1972.
- Hernandez-Peon, R.: Central neuro-humoral transmission in sleep and wakefulness. In Bally, C. and Schade, J.P., (Editors): *Sleep Mechanism, Progress in Brain Research.* 18, Elsevier, Amsterdam; 1965.
- Hernandez-Peon, R., and Chavez-Ibarra, G.: Sleep induced by electrical or chemical stimulation of the forebrain. *Electroenceph. clin. Neurophysiol. Suppl.* 24:188-198, 1963.
- Holmes, J.H. and Gaon, M.D.: Observations on acute and multiple exposure to anticholinesterase agents. *Trans. Am. Clin. Climat. Assn.* 68:86-101, 1956.
- Joffe, R. and Jacobs, L.: The Beta Focus: its nature and significance. *Acta Neurol. Scand.* 48:191-203, 1972.
- Johnson, M.K.: Organophosphorus and other inhibitors of "neurotoxic esterase" and the development of delayed neurotoxicity in hens. *Biochem. J.* 120:523-531, 1970.
- Johnson, M.K.: The primary biochemical lesion leading to the delayed neurotoxic effects of some organophosphorus compounds. *J. Neurochem.* 23:785-789, 1974.
- Johnson, M.K.: Organophosphorous esters causing delayed neurotoxic effects: Mechanism of action and structure/activity studies. *Arch. Toxicol.* 34:259-288, 1975.
- Kamp, A., Schrijer, C.F.M., and Storm Van Leeuwen, W.: Occurrence of "beta bursts" in human frontal cortex related to psychological parameters. *Electroenceph. clin. Neurophysiol.* 33:257-268, 1972.
- Kiloh, L.G., McCornas, A.J. and Osselson, J.W.: *Clinical Electroencephalography.* Appleton-Century Crofts, N.Y., 1972.
- Koelle, G.B.: Anticholinesterase agents. In Goodman, L.S. and Gelman, A. (Editors): *The Pharmacological Bases of Therapeutics.* The Macmillan Co., N.Y., 1968.
- Kooi, K.A.: *Fundamentals of Electroencephalography.* Harper & Row, N.Y., 1971.
- Magherini, P.C., Pompeiano, O., and Thoden, U.: Cholinergic mechanisms related to REM sleep. I: Rhythmic activity of the vestibulo-oculomotor system induced by an anticholinesterase in the male decerebrate cat. *Arch. Ital. Biol.* 110:234-259, 1972.
- Matsuzaki, M., Obada, Y. and Shoto, S.: Cholinergic agents related to parasleep state in acute brain stem preparations. *Brain Res.* 9:253-267, 1968.
- Mendels, J. and Hawkins, D.R.: Sleep Laboratory adaptation in normal subjects and depressed patients ("first night effects"). *Electroenceph. clin. Neurophysiol.* 22:556-558, 1967.
- Metcalfe, D.R. and Holmes, J.H.: EEG, psychological, and neurological alterations in humans with organophosphate exposure. *Ann. N.Y. Acad. Sci.* 160:357-365, 1969.
- Milby, T.: Prevention and management of organophosphate poisoning. *J.A. Med. Assoc.* 216:2131-2133, 1971.
- Mundy Castle, A.C.: Theta and beta rhythm in the electroencephalograms of normal adults. *Electroenceph. clin. Neurophysiol.* 4:165-179, 1952.
- Nealis, J.G.T. and Duffy, F.H.: Paroxysmal beta activity in the pediatric electroencephalogram. *Ann. Neurol.* 4:112-116, 1978.
- Rowntree, D.W., Nevin, S. and Wilson, A.: The effects of diisopropyl fluorophosphate in schizophrenic and manic depressive psychosis. *J. Neurol. Neurosurg. Psychiat.* 13:47-62, 1950.
- Santolucito, J.A. and Morrison, G.: EEG of rhesus monkeys following prolonged low-level feeding of pesticides. *Toxicol. Appl. Pharmacol.* 19:147-154, 1971.
- Sitaram, N., Wyatt, R.J., Dawson, S. and Gillin, J.C.: REM sleep induction by physostigmine infusion during sleep. *Science* 191:1281-1282, 1976.
- Selin, I.: *Detection Theory.* Princeton University Press, Princeton, N.J., 1965.
- Smith, M.I., Elvove, E. and Frazier, W.H.: The Pharmacological Action of Certain Phenol Esters, with Special Reference to the Etiology of So-called Ginger Paralysis. *U.S. Publ. Hlth. Rep.* 45:2509-2524, 1930.

- Smith, H.V. and Spaulding, N.M.K.: Outbreak of paralysis in Morocco due to ortho-cresyl phosphate poisoning. *Lancet* 2: 1019-1021, 1959.
- Tabershaw, I.R. and Cooper, W.C.: Sequelae of acute organic phosphate poisoning. *J. Occup. Med.* 8:5-10, 1966.
- Thoden, U., Magherini, P.C. and Pompeiano, O.: Cholinergic mechanisms related to REM sleep. II. Effects of an anticholinesterase on the discharge of central vestibular neurons in the decerebrate cat. *Arch. Stat. Biol.* 110:260-283, 1972.
- Wadia, R.S., Sadagopau, C., Amin, R.B. and Sardesai, H.V.: Neurological manifestations of organophosphorous insecticide poisoning. *J. Neurol., Neurosurg. and Psychiat.* 37:841-847, 1974.
- Wied, G.L., Bartels, P.H., Bahr, G.F., and Oldfield, D.G.: Taxonomic intracellular analytic system (TICAS) for cell identification. *Acta Cytologica* 12:127-210, 1968.
- Williams, R.L., Karacan, I.K. and Hirsch, C.J.: *EEG of Human Sleep*. John Wiley & Sons, Inc., New York, N.Y., 1974.

NEUROTOXICOLOGY MS N-54

Received November 9, 1979

Accepted November 29, 1979

NATIONAL SECURITY COUNCIL
WASHINGTON, D.C. 20504

November 14, 1996

7513

file Gulf War

INFORMATION

MEMORANDUM FOR ANTHONY LAKE

FROM: PAUL E. BUSICK *PEB*

SUBJECT: Gulf War Illnesses -- Strategic Overview

Last Friday you asked the Senior Directors to submit a paper looking ahead at the next four years in terms of their respective portfolios. This memo responds to your request.

The attached Strategic Overview represents my initial thoughts on the nature and scope of the Gulf War Illnesses problem as well as how I propose to address the relevant issues. I will work with NSC Strategic Planning and the interested White House staffs to refine my approach.

Current: 14 November 1996

GULF WAR ILLNESSES: STRATEGIC OVERVIEW

PROBLEM STATEMENT

While Gulf War veterans have generally been well cared for, the government has been slow to determine all of the relevant facts and address the various issues relating to possible health problems related to chemical or biological exposures (e.g., delayed-onset effects of low-level exposures). Moreover, the slow government response, coupled with the inherent complexity and sensitivity of the subject, have led to a public perception that the overall process of caring for Gulf War veterans has been flawed -- thus calling into question DOD's commitment to the well-being of its troops both when deployed and afterwards.

CHALLENGE

Ensure that: (1) all Gulf War veterans with diagnosed or undiagnosed illnesses are appropriately cared for; (2) thorough investigations are conducted to determine whether certain Gulf War veterans are at long-term risk owing to theater exposures to chemical and/or biological agents; (3) lessons learned from both the deployment process and the post-conflict follow-up are fully implemented by DOD and other involved agencies; and (4) public confidence in the government's commitment to and process for taking care of Gulf War and other veterans is restored.

CRITERIA FOR SUCCESS

Agency reports, consensus feedback from Congressional, VSO and media sources, and any other relevant information indicate that: (1) Gulf War veterans -- including those with current diagnosed or undiagnosed illnesses, as well as those who may be at long-term risk -- are receiving appropriate care; (2) all that should and reasonably can be known about what happened in theater either is known or the investigative process firmly in place gives every indication that that will happen; (3) the lessons learned either have been or are in the process of being implemented by DOD and other involved agencies; and (4) public confidence in the government's commitment to caring for troops and veterans has been restored in substantial measure.

Encl: Synopsis -- Intended Approach and Implementing Objectives

Enclosure to Gulf War Illnesses: Strategic Overview

SYNOPSIS: INTENDED APPROACH AND IMPLEMENTING OBJECTIVES

INTENDED APPROACH

Focus our efforts prospectively, continually asking the question -- how can we most effectively promote two outcomes: (1) all concerned doing their best for Gulf War veterans from here on; and (2) an enhanced readiness posture for future deployments? This emphasis would entail four fundamental steps, with the Final Report of the Presidential Advisory Committee (due out by year's end) as the principal basis for assessing progress.

First, closely monitor the ongoing and intensifying Gulf War Illness-related efforts of DOD, VA and other involved agencies. Secondly, where appropriate, orchestrate quality improvements in the overall process of Gulf War follow-up, both at the agency-specific and interagency levels. Thirdly, review the ongoing implementation of relevant Gulf War lessons learned for future deployments. Finally, take appropriate measures to communicate these efforts to the public so as to restore confidence in the government's commitment to and effectiveness in discharging its obligations to Gulf War and future veterans.

IMPLEMENTING OBJECTIVES

-- Acquire a thorough understanding of the basic issues, emphasizing inputs from interested White House staffs as well as NSC/Non-Pro, NSC/SP and OSTP...

-- Establish NSC/GWI as focal point within EOP for monitoring this issue and facilitating any quality improvements in the overall federal process for addressing this issue...

-- Determine who at an appropriately high level (likely Assistant Secretary) is going to speak on this issue for each of the involved agencies...

-- Constructively engage all of the stakeholders (e.g., DOD, VA, CIA, HHS, Congress, and VSOs) in the process...

-- Cultivate and maintain good relations with DOD, and seek to preclude any possibility of a White House/DOD schism...

-- Coordinate with OMB, DOD, VA and others as necessary to ensure the overall effort is adequately resourced...

Page two...

-- Identify which of the related issues are interagency as opposed to agency-specific...

-- Determine the required Presidential action(s) for the PAC Final Report and plan the overall Administration "roll out" at the end of the year (including any significant announcements the President should make); this effort may include the establishment of a follow-on independent entity to audit the continuing DOD investigative efforts...

-- Promote development of a plan that effectively utilizes all available resources to tackle what the PAC has identified needs to be done, and then engage the Strategic Planning Staff to resource the plan and ensure its implementation; plan needs not only to be workable in the practical sense of accomplishing a vital task, but also needs to come across effectively to the public so that the effort is perceived as appropriate and successful...

-- Review pending and planned research priorities in terms of unmet needs...

-- Monitor the ongoing implementation of lessons learned from both the Gulf War deployment and post-conflict follow-up...

-- Organize appropriate interagency working groups to this end, as well as a periodic schedule for briefing appropriate senior officials...

**National Unity Conference Press Statement
Gulf War Veterans, Families, and Civilian Participants
Irving, Texas, March 10-12, 1995**

**Press Release:
12 March 1995**

Contact: Paul Sullivan

These Are The Facts:

- Tens of thousands of us are ill and many more have died from Gulf War Syndrome. We know why we are ill, yet we do not receive adequate treatment for our illnesses.

- We have Department of Defense documents obtained under the Freedom of Information Act proving that the DoD (and Deputy Secretary of Defense Deuch) is actively engaged in a cover-up of the presence and exposure to chemical and biological warfare agents. The credibility of Deuch is in question, and the issue of a DoD cover-up must be resolved before he is confirmed as Director of Central Intelligence.

- Last week, President Bill Clinton announced the formation of a Presidential Advisory Committee to investigate the cover-up and create a plan of action to ensure prompt, comprehensive health care for war veterans, their families, and civilian participants of the war. **We support the concept, but we have reservations about the make-up and mission of the Committee.**

- We thank Congress and the Clinton Administration for the high level of attention they have given this issue. If Congress and the White House had not taken such a pro-active stand in support of Gulf War veterans, then the Departments of Defense, Veterans Affairs, and Health and Human Services may have buried this issue along with many more Gulf War veterans and their family members.

THE WHITE HOUSE

Melanne V. —

As we discussed!

Judy

|

Gulf War Veterans of Georgia

April 21, 1995

Honorable Bill Clinton
President
United States of America
1600 Pennsylvania Avenue, NW
Washington, DC 20500

Attn: Mr. Anthony Lake
Re: Presidential Gulf War Advisory Committee

Dear Mr. President:

Dr. Diana Zuckerman, a special assistant to First Lady Hillary Rodham Clinton, called me yesterday about your Gulf War Advisory Committee. We appreciate her contacting us on a regular basis to ask us for our input. As a result of her questions and comments yesterday, we wish to present the following suggestions to you for review and response directly by your White House staff:

1. Will the Presidential Gulf War Committee formulate policy? If so, will it formulate DoD, VA, and HHS policy? If so, by what means?
2. Will the Committee have a budget? If so, how much and for how long?
3. Will the Committee prepare a finalized, written, published report? If so, will it be unclassified and promptly released to the public?
4. Will the Committee have a professional investigative staff and other administrative staff support?
5. Will the Committee have the cooperation of Coalition nations via the U.S. Department of State, including cooperation for obtaining classified data?
6. Will the Committee, and its final report, be independent of all DoD, VA, and HHS pre-review and control?
7. Will the Committee review both classified and unclassified data the DoD, VA, and HHS have, to date, refused to release to Congress or the public?
8. Since a number of veterans of the Gulf War, both well and ill, have apparently been rejected for membership on the Committee, what is the criteria for the "lay" position to be held by a veteran of the Gulf War?

President Clinton, Page Two, April 21, 1995

9. We oppose the presence of a flag officer whose unit(s) may have participated in (or had involvement with) a chemical (or other exposure) event as a "lay" veteran member of the Committee. Our opposition does not preclude the participation of those of flag rank, so long as the proposed Committee member is independent to review evidence, free from retribution from the DoD, and open minded on the issue. Gulf War veterans seek someone who has a reputation for trust and honesty to serve as the "lay" veteran member.

10. We oppose the presence of past or present DoD, VA, and HHS employees (or contract workers or researchers) on the Committee. This opposition includes those who participated in earlier executive branch reviews of the issue.

11. Will the Committee have subpoena powers for evidence and witnesses?

12. Will the Committee obtain testimony and evidence at several different locations throughout America, or will it meet only in Washington?

13. When should we begin submitting names of individuals and groups for testimony regarding illnesses and exposures to the Committee?

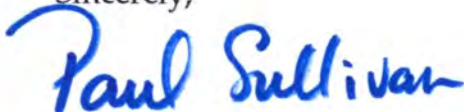
14. When should we begin to send evidence regarding the illnesses and various exposures to the Committee?

15. Given that two-thirds of those who served in the Gulf War remain on active duty, what protections can be offered or guaranteed for those on active duty for stepping forward with relevant testimony or evidence?

16. What precautions are there for the safeguard of evidence presented to the Committee for independent review and analysis? For instance, according to the Department of the Army Forces Command, in a letter dated March 13, 1995, FORSCOM wrote that the Nuclear, Biological, and Chemical and other duty logs were "destroyed" by Army Central Command and the Army's 1st Cavalry Division. This may have been caused by negligence or with criminal intent.

We hope to hear from you soon regarding these important questions.

Sincerely,



Paul Sullivan
President

WH and DOD Response to 60 Minutes

It's unfortunate that, because of the lag time between taping these segments and broadcasting them, that the 60 Minutes story is already out of date. Just last Monday, the President announced several new initiatives that address many of the concerns that were raised in the 60 Minutes piece.

For example, the Pentagon will soon be making public (upon request) explanations regarding reports of detections of chemical weapons, such as the one(s) described on the program. And, although we still have no **conclusive** evidence of the use of chemical weapons in the Gulf, we are now examining and re-examining every credible report to see if we missed anything in previous reviews.

[We acknowledged the validity of the Czech report of a chemical agent, but that was located far from any likely target and there was no evidence of any weapon.] But, when veterans tell us that they believe they were exposed to chemical weapons in the Gulf, and the Pentagon tells us that there is no conclusive evidence, it's important to reconcile those differences. We owe it to veterans to find out whether they were exposed or not. The President was referring to this on Monday when he said "We must listen to what veterans are telling us and respond to their concerns. Just as we relied on these men and women to fight for our country, they must now be able to rely on us to try to determine what happened to them in the Gulf and to help restore them to full health." That's why the President has created an Advisory Committee that will review everything that the Administration is doing and make recommendations about what else is needed.

?Should we continue saying that Deutch's comment was a mistake, or that it reflects his openness to examining any and all evidence?

Melanne Verveer

Melanne -
do you need to
copy this for HRC

Withdrawal/Redaction Marker

Clinton Library

DOCUMENT NO. AND TYPE	SUBJECT/TITLE	DATE	RESTRICTION
001, memo	Elisa D. Harris to Samuel R. Berger re: non-selection [partial] (2 pages)	03/07/1995	b(6)

COLLECTION:

Clinton Presidential Records
First Lady's Office
Melanne Verveer
OA/Box Number: 20033

FOLDER TITLE:

Gulf War Illness - Gulf War Illnesses

2013-0534-S

rc1867

RESTRICTION CODES

Presidential Records Act - [44 U.S.C. 2204(a)]

- P1 National Security Classified Information [(a)(1) of the PRA]
- P2 Relating to the appointment to Federal office [(a)(2) of the PRA]
- P3 Release would violate a Federal statute [(a)(3) of the PRA]
- P4 Release would disclose trade secrets or confidential commercial or financial information [(a)(4) of the PRA]
- P5 Release would disclose confidential advice between the President and his advisors, or between such advisors [(a)(5) of the PRA]
- P6 Release would constitute a clearly unwarranted invasion of personal privacy [(a)(6) of the PRA]

C. Closed in accordance with restrictions contained in donor's deed of gift.

PRM. Personal record misfile defined in accordance with 44 U.S.C. 2201(3).

RR. Document will be reviewed upon request.

Freedom of Information Act - [5 U.S.C. 552(b)]

- b(1) National security classified information [(b)(1) of the FOIA]
- b(2) Release would disclose internal personnel rules and practices of an agency [(b)(2) of the FOIA]
- b(3) Release would violate a Federal statute [(b)(3) of the FOIA]
- b(4) Release would disclose trade secrets or confidential or financial information [(b)(4) of the FOIA]
- b(6) Release would constitute a clearly unwarranted invasion of personal privacy [(b)(6) of the FOIA]
- b(7) Release would disclose information compiled for law enforcement purposes [(b)(7) of the FOIA]
- b(8) Release would disclose information concerning the regulation of financial institutions [(b)(8) of the FOIA]
- b(9) Release would disclose geological or geophysical information concerning wells [(b)(9) of the FOIA]

NATIONAL SECURITY COUNCIL
WASHINGTON, D.C. 20506

March 7, 1995

INFORMATION

MEMORANDUM FOR SAMUEL R. BERGER

THROUGH: ROBERT BELL ^{SH/O} / DANIEL PONEMAN ^{SH/O}FROM: ELISA D. HARRIS ^{EDH}

SUBJECT: Gulf War Veterans' Illnesses -- Next Steps

Now that the President has approved the establishment of a Presidential Advisory Committee on Gulf War veterans' illnesses, a number of outstanding issues need to be resolved related to the Committee.

Committee Members

(b)(6)

[001]

As far as the other Committee members are concerned, we believe that it would be useful to establish a formal nominating and screening process prior to submitting a list of proposed names to the President. Specifically, we recommend that candidates first be solicited from the three agencies (DOD, VA and HHS), veterans service organizations (VFW, American Legion and others), the chairpersons of previous review panels (Lederberg, the Institute of Medicine and the National Institutes of Health) and Members of Congress with jurisdiction over or a particular interest in this issue. Once we have a list of candidates, a screening committee comprised of representatives from OSD, VA, HHS, NSC, Cabinet Affairs, the First Lady's office and OSTP would then review the list and recommend 15-20 candidates for the President to choose from. Although complicated, such an approach would ensure that all of the interested parties have the opportunity to have their candidates considered.

Committee Budget and Staff

DOD has agreed to fund the Advisory Committee. This makes sense, given the resources that are likely to be required. Nevertheless, we need to ensure that the Committee is and is seen as fully independent. We recommend that DOD be asked to set aside funds for securing office space from GSA and for hiring an outside staff.

As far as the actual staff is concerned, we recommend that we ask DOD, VA and HHS to suggest candidates for the **Staff Director's** position. Once we have a list, we should ask [redacted] to approve 2 or 3 of the candidates, including anyone of his choosing not on the agency list. These candidates should then be interviewed by senior White House officials (preferably you, Kitty Higgins and Melanne Verveer), and a Staff Director chosen. (b)(6)

(b)(6)

One further staff-related issue you will want to consider is whether the **Advisory Committee** should include someone responsible **for public liaison** (especially with veterans groups), press and Congressional relations. Given our desire to reach out to such groups, we recommend that the Committee be encouraged to hire someone to develop strategies for interacting with veterans, the press and Congress, in close coordination with the White House.

White House Role

A further issue that needs to be resolved is who will be the **White House point of contact for the Committee**. Cabinet Affairs' experience with the Human Radiation Committee suggests that this will be a full-time job during the first 3-4 months that the Committee is being established and beginning its activities. This individual will also need to oversee implementation of the 6-month public affairs strategy that we are putting together. Bob, Dan and I do not believe that this job can be done by the NSC. Accordingly, we recommend that you try to secure agreement from either Cabinet Affairs or OSTP that they will make someone available for this position.

On a related note, veterans and others are calling VA looking for someone at the White House to talk with about the Committee. We recommend that you ask Alexis Herman if her office can take these calls, at least until the official White House point of contact has been determined.

Documents Related to the Advisory Committee

Four documents related to the Advisory Committee are in various stages of preparation. In order to establish the Advisory Committee, we need an Executive Order, a Charter (which must be filed with GSA), and agreed Terms of Reference. I have drafted all three of these documents (copies at Tabs A-C), and am in the process of securing agency clearances. We hope to have them finalized by the end of the week. The fourth document, which you tasked out of one of your earlier meetings, is a 6-month public affairs strategy. We are still working on this, and hope to have a draft ready to circulate to agencies by early next week.

Attachments

Tab A Draft Executive Order
Tab B Draft Charter
Tab C Draft Terms of Reference

DRAFT

EXECUTIVE ORDER

PRESIDENTIAL ADVISORY COMMITTEE ON
GULF WAR VETERANS' ILLNESSES

By the authority vested in me as President by the Constitution and the laws of the United States of America, it is hereby ordered as follows:

Section 1. Establishment. (a) There shall be established a Presidential Advisory Committee on Gulf War Veterans' Illnesses (the "Committee"). The Committee shall be composed of not more than 10 members to be appointed or designated by the President. The members of the Advisory Committee shall have expertise relevant to the functions of the Committee and shall not be full-time federal officials or employees of the Executive Branch of the federal government. The Committee shall comply with the Federal Advisory Committee Act, as amended, 5 U.S.C. App. 2.

(b) The President shall designate a Chairperson from among the members of the Committee.

Section 2. Functions. (a) The Advisory Committee shall report to the President through the Secretary of Defense, the Secretary of Veterans Affairs and the Secretary of Health and Human Services.

(b) The Committee shall provide advice and recommendations based on its review of the following matters:

(1) Research: specifically, epidemiological, clinical and other research concerning Gulf War veterans' illnesses.

(2) Coordinating efforts: specifically, the activities of the Persian Gulf Veterans Coordinating Board, including the Research Coordinating Council, the Clinical Coordinating Council, and the Disability/Compensation Coordinating Council.

(3) Medical treatment: specifically, medical examinations and treatment in connection with Gulf War veterans' illnesses, including the Comprehensive Clinical Evaluation Program and the Persian Gulf Registry Medical Examination Program.

(4) Outreach: specifically, government-sponsored outreach efforts such as hotlines and newsletters related to Gulf War veterans' illnesses.

(5) External reviews: specifically, the steps taken to implement recommendations in external reviews by the Institute of Medicine's Committee to Review the Health Consequences of Service During the Persian Gulf War, the Defense Science Board Task Force on Persian Gulf War Health Effects, the National Institutes of Health Technology Assessment Workshop on the Persian Gulf Experience and Health, the Persian Gulf Expert Scientific Committee, and other bodies.

(6) Risk factors: specifically, the possible risks associated with prophylactic drugs and vaccines; infectious

diseases; environmental chemicals, radiation and other toxic substances; smoke from oil well fires; depleted uranium; physical and psychological stress; and other factors applicable to the Gulf War.

(7) Chemical and Biological Weapons: specifically, information related to reports of the possible detection of chemical or biological weapons during the Gulf War.

(c) It shall not be a function of the Advisory Committee to provide advice or recommendations on any legal liability of the Federal Government for any claims or potential claims against the Federal Government.

(d) As used herein, "Gulf War Veterans' Illnesses" means the symptoms and illnesses reported by U.S. uniformed services personnel who served in the Gulf War.

(e) The Advisory Committee shall submit an interim report within six months of beginning its work and a final report by December 31, 1996, unless extended by the President.

Sec. 3. Administration. (a) The heads of executive departments and agencies shall, to the extent permitted by law, provide the Committee with such information as it may require for purposes of carrying out its functions.

(b) Members of the Committee shall be compensated in accordance with Federal law. Committee members may be allowed travel expenses, including per diem in lieu of subsistence, to

the extent permitted by law for persons serving intermittently in the government service (5 U.S.C. 5701-5707).

(c) To the extent permitted by law, and subject to the availability of appropriations, the Department of Defense shall provide the Committee with such funds as may be necessary for the performance of its functions.

Sec. 4. General Provisions. (a) Notwithstanding the provisions of any other Executive Order, the functions of the President under the Federal Advisory Committee Act that are applicable to the Advisory Committee, except that of reporting annually to the Congress, shall be performed by the Secretary of Defense, in accordance with the guidelines and procedures established by the Administrator of General Services.

(b) The Committee shall terminate 30 days after submitting its final report.

(c) This order is intended only to improve the internal management of the executive branch and it is not intended to create any right, benefit, trust, or responsibility, substantive or procedural, enforceable at law or equity by a party against the United States, its agencies its officers, or any person.

THE WHITE HOUSE
March _____, 1995

DRAFT
CHARTER

PRESIDENTIAL ADVISORY COMMITTEE ON
GULF WAR VETERANS' ILLNESSES

1. Official Designation: Presidential Advisory Committee on Gulf War Veterans' Illnesses ("Committee").
2. Objectives and Scope: The Committee shall review and provide advice and recommendations on U.S. government activities related to the symptoms and illnesses reported by U.S. uniformed services personnel who served in the Gulf War. Executive Order _____, of March __, 1995, governs the Committee's functions.
3. Description of Duties for Which Committee is Responsible:

The duties of the Committee are solely advisory. The Committee shall provide advice and recommendations based on its review of the following matters:

 - a. Research: specifically, epidemiological, clinical and other research concerning Gulf War veterans' illnesses.
 - b. Coordinating efforts: specifically, the activities of the Persian Gulf Veterans Coordinating Board, including the Research Coordinating Council, the Clinical Coordinating Council, and the Disability/Compensation Coordinating Council.
 - c. Medical treatment: specifically, medical examinations and treatment in connection with Gulf War veterans' illnesses,

including the Comprehensive Clinical Evaluation Program and the Persian Gulf Registry, Medical Examination Program.

- d. Outreach: specifically, government-sponsored outreach efforts such as hotlines and newsletters related to Gulf War veterans' illnesses.
 - e. External reviews: specifically, steps taken to implement recommendations in external reviews by the Institute of Medicine's Committee to Review the Health Consequences of Service during the Persian Gulf War, the Defense Science Board Task Force on Persian Gulf War Health Effects, the National Institutes of Health Technology Assessment Workshop on the Persian Gulf Experience and Health, the Persian Gulf Expert Scientific Committee, and other bodies.
 - f. Risk factors: specifically, the possible risks associated with prophylactic drugs and vaccines; infectious diseases; environmental chemicals, radiation and other toxic substances; smoke from oil well fires; depleted uranium; physical and psychological stress; and other factors applicable to the Gulf War.
 - g. Chemical and Biological Weapons: specifically, information related to reports of the possible detection of chemical or biological weapons during the Gulf War.
4. Period of Time Required: The Committee shall terminate thirty days after submission of its final report. The Committee shall submit its final report to the President

through the Secretary of Defense, the Secretary of Veterans Affairs and the Secretary of Health and Human Services by December 31, 1996, unless extended by the President.

5. Official to Whom the Committee Reports: The Committee shall report to the President through the Secretary of Defense, the Secretary of Veterans Affairs and the Secretary of Health and Human Services. The Committee shall submit an interim report within six months of beginning its work and a final report by December 31, 1996, unless extended by the President. The final report shall include advice and recommendations on U.S. government efforts in the areas identified in section 3 of this Charter, including means of enhancing the U.S. government response to Gulf War veterans' illnesses and for future planning.
6. The Agency Responsible for Providing Necessary Support: Financial and administrative support shall be provided by the Department of Defense.
7. Membership: The President shall appoint up to ten (10) members to the Committee from the private sector with the stature and expertise related to matters outlined in sections 2 and 3 above. The President shall designate a Chairperson from among the members of the Committee.
8. Estimated Annual Operating Costs and Staff Support Years: It is estimated that the total annual costs of operations for travel, per diem, consultant fees, staff support and

other incidental costs will not exceed \$ _____. Full time equivalent staff support years are anticipated to be _____ years of effort.

9. Number of Meetings: The Committee shall meet as it deems necessary to complete its functions.
10. Subcommittees: To facilitate functioning of the Committee, subcommittee(s) may be formed. The objectives of the subcommittee(s) are to make recommendations to the Committee related to the responsibilities of the Committee.
Subcommittees shall meet as the Committee deems appropriate.
11. Termination Date: The Committee shall terminate 30 days after submitting its final report. This charter must be renewed two years from the date filed below.
12. Date Charter Filed:

Terms of Reference
for the
Presidential Advisory Committee on
Gulf War Veterans' Illnesses

Statement of Mission: The Committee shall review and provide advice and recommendations on U.S. government activities related to the symptoms and illnesses reported by U.S. uniformed services personnel who served in the Gulf War (hereinafter referred to as "Gulf War veterans' illnesses").

This committee draws its authority from Executive Order (add number).

Areas to be Studied:

Research: specifically, epidemiological, clinical and other research concerning Gulf War veterans' illnesses.

Coordinating Efforts: specifically, the activities of the Persian Gulf Veterans Coordinating Board, including the Research Coordinating Council, the Clinical Coordinating Council, and the Disability/Compensation Coordinating Council.

Medical Treatment: specifically, medical examinations and treatment in connection with Gulf War veterans' illnesses, including the Comprehensive Clinical Evaluation Program and the Persian Gulf Registry Medical Examination Program.

Outreach: specifically, government-sponsored outreach efforts such as hotlines and newsletters related to Gulf War veterans' illnesses.

External Reviews: specifically, steps taken to implement recommendations in external reviews by the Institute of Medicine's Committee to Review the Health Consequences of Service During the Persian Gulf War, the Defense Science Board Task Force on Persian Gulf War Health Effects, the National Institutes of Health Technology Assessment Workshop on the Persian Gulf Experience and Health, the Persian Gulf Expert Scientific Committee, and other bodies.

Risk Factors: specifically, the possible risks associated with prophylactic drugs and vaccines; infectious diseases; environmental chemicals, radiation and other toxic substances; smoke from oil well fires; depleted uranium;

physical and psychological stress; and other factors applicable to the Gulf War.

Chemical and Biological Weapons: specifically, information related to reports of the possible detection of chemical or biological weapons during the Gulf War.

Work Products: The Committee shall report to the President through the Secretary of Defense, the Secretary of Veterans Affairs, and the Secretary of Health and Human Services. The Committee shall submit an interim report within six months of beginning its work and a final report by December 31, 1996, unless extended by the President. The final report shall include advice and recommendations on U.S. government efforts in the areas described above, including means of enhancing the U.S. government response to Gulf War veterans' illnesses and on future planning.

① USA Today

Sunday
~~Monday~~ strategy

60 mins - W.H. Response to?

②

USA Today

③

Hamburg,

W.H. mly

④

Liana

In DC or see the Tues
feel me a little.

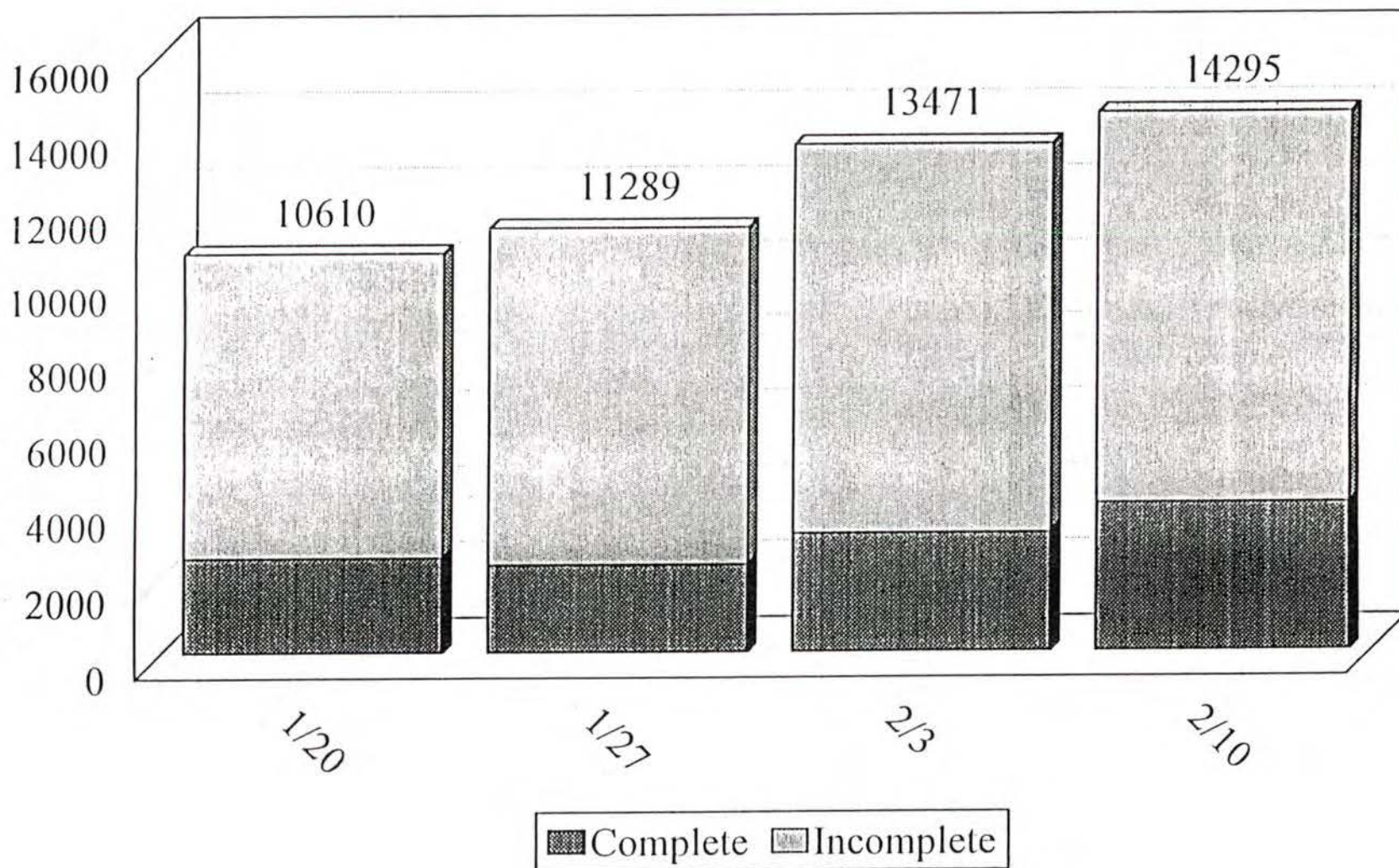
Unfortunate
60 mins
out of date
already
BoTos loss
w/c
Lag time

Phil Lee -

• uninf
• Kemler - HBA

CCEP STATUS

Since 17 January 1995



diagnoses for these individuals have been grouped into related categories. Table 1 presents the frequency of diagnostic categories from completed CCEP evaluations.

Table 1: Frequency Distribution of Primary Diagnoses Among 1,019 Completed CCEP Evaluations

Categories	Primary Diagnosis
Circulatory System	3%
Digestive System	7%
Endocrine	3%
Genitourinary System	2%
Healthy ¹	8%
Infectious Disease	4%
Injury and Poisoning	0%
Musculoskeletal	16%
Neoplasm	1%
Nervous System	6%
Other ²	
Headache/CNS/Memory Loss	3%
Fatigue	3%
Sleep Disorders	3%
Respiratory Related	2%
GI / Abdomen	1%
Misc.	2%
Psychological Conditions	
Depression	8%
Tension Headache	5%
Post Traumatic Stress Disorder	4%
Somatization	2%
Anxiety	2%
Misc.	3%
Respiratory System	6%
Skin & Subcutaneous	5%

1 Persons seeking consultation without complaint or sickness.

2 Includes "Symptoms, Signs & Other Ill Defined Conditions" as categorized according to International Classification of Diseases (ICD-9) nomenclature consisting of cases for which no diagnosis is classifiable elsewhere; no more specific diagnosis can be made; signs or symptoms that prove to be transient; and, cases in which a more precise diagnosis was not available for any other reason.

DISTRIBUTION OF CCEP DIAGNOSES

Primary and First Three Diagnoses

n = 203

<u>ICD-CODE CATEGORY</u>	<u>1ST DX (%)</u>	<u>3 DX (%)</u>
Mental Disorders (290-319)	39	89
Musculoskeletal/Connective (710-739)	13	41
Ill-defined conditions (780-799)	12	37
Nervous System (320-389)	8	23
Respiratory System (460-519)	7	20
Digestive System (520-579)	6	22
Skin and Subcutaneous (680-709)	3	12
Infectious Diseases (001-139)	3	8
Circulatory System (390-459)	2	5
Injury and Poisoning (800-999)	1	3
Genitourinary System (580-629)	1	3
Endocrine (240-289)	1	6
Neoplasm (140-239)	1	3
Healthy Person	< 1	< 1

MEDICAL DIAGNOSES

(all joint pains are not equal)

Rheum

Derm

GI

Osteoarthritis

Tinea

IBS

Focal Trauma

Eczema

H. pylori

Fibromyalgia

Pityriasis

GERD

Sjogren's

Contact Derm

H. hernia

Lupus

HSV

Hepatitis

Rheum Arthritis

Xerosis

MEDICAL DIAGNOSES

Pulm

Asthma

Lar. Dyskin.

Decondition

COPD

HS Pneumon.

Neuro

Migraines

Tension HAs

Neuropathy

Carpal Tunnel

Other

Diabetes

Thyroid

Sleep Apnea

Restless Leg

Iron Def.

PSYCHIATRIC DIAGNOSES

most common

- Depressive Disorders
- Somatoform Disorders
- Anxiety Disorders
- Post-Traumatic Stress

PSYCHIATRIC DIAGNOSES

less common

- Adjustment Disorder
- Alcohol/Substance Abuse
- Obsessive-Compulsive
- Eating Disorder
- Conversion Disorder

SUMMARY OF CCEP FINDINGS

- **Some patients are in good health**
- **An intensive medical and psychiatric evaluation can make a diagnosis in nearly all symptomatic patients**
- **Distribution of diagnoses is what one would expect given presenting symptoms**
- **Diagnoses related to Gulf deployment include leishmania, asthma, and PTSD**

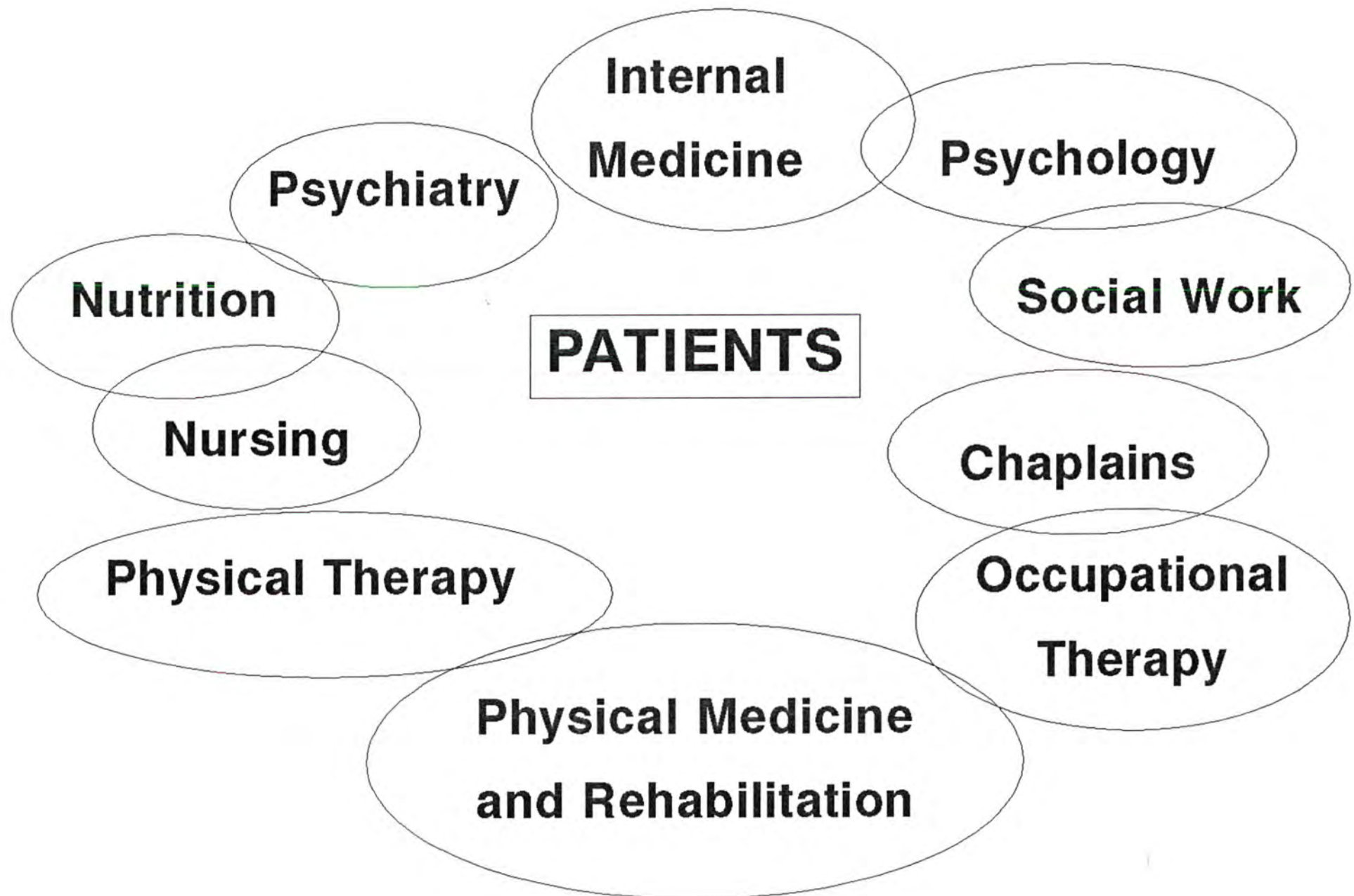
IMPLICATIONS OF CCEP FINDINGS

- **Many patients require treatment of diagnoses made in CCEP**
- **Most will do best with longitudinal care from a primary care provider or mental health professional**
- **Some may benefit from intensive short-term program to re-focus from illness to wellness**

SPECIALIZED CARE

- **Eight–bed inpatient unit**
- **Multidisciplinary team of pro**
- **Comprehensive intake evalu
design individualized treatm**
- **Three–week intensive progr**
- **Final Assessment with recoi
for continuing treatment**
- **Six–month follow–up evalu**

SPECIALIZED CARE CENTER



ELEMENTS OF THE SCC

MIND &

Group Therapy

Individual Therapy

Relaxation Techniques

Stress Management

Coping Skills

Role Play

Recreational Activities

BODY

Physical Therapy

Occupational Therapy

Daily Exercise

Pain Management

Smoking Cessation

Nutrition Counseling

Pain Management