# Protein Binding of Isofluorophate *in Vivo* after Coexposure to Multiple Chemicals

***John S. Vogel, Garrett A. Keating II, and Bruce A. Buchholz***

University of California, Lawrence Livermore National Laboratory, Livermore, California, USA

**Full toxicologic profiles of chemical mixtures, including dose–response extrapolations to realistic exposures, is a prohibitive analytical problem, even for a restricted class of chemicals. We present an approach to probing *in vivo* interactions of pesticide mixtures at relevant low doses using a monitor compound to report the response of biochemical pathways shared by mixture components. We use accelerator mass spectrometry (AMS) to quantify [$^{14}$C]-diisopropylfluorophosphate as a tracer at attomole levels with 1–5% precision after coexposures to parathion (PTN), permethrin (PER), and pyridostigmine bromide separately and in conjunction. Pyridostigmine shows an overall protective effect against tracer binding in plasma, red blood cells, muscle, and brain that is not explained as competitive protein binding. PTN and PER induce a significant 25–30% increase in the amount of tracer reaching the brain with or without pyridostigmine. The sensitivity of AMS for isotope-labeled tracer compounds can be used to probe the physiologic responses of specific biochemical pathways to multiple compound exposures. *Key words:* accelerator mass spectrometry, chemical mixture, diisopropylfluorophosphate, esterase, organophosphate, parathion, permethrin, pyrethroid, radioisotope. *Environ Health Perspect* 110(suppl 6):1031–1036 (2002). *http://ehpnet1.niehs.nih.gov/docs/2002/suppl-6/1031-1036vogel/abstract.html***

People are exposed to many unnatural chemical combinations in workplaces, surroundings, diet, or medications. Most of these exposures occur at very low doses for which individual chemical toxicity is not expected, but unrecognized interactions could result. Environmental toxins occur at exposures (micrograms per kilogram body mass) for which studies of high-dose mechanisms may no longer apply. Full toxicologic profiles of all chemical mixtures, including dose–response extrapolations to realistic exposures, is a prohibitive analytical problem, even for a restricted class of chemicals such as pesticide residues on food. We present an approach to probing *in vivo* interactions of toxin mixtures at relevant low doses, using a monitor compound to report the response of a biochemical pathway shared by mixture components. This approach has the potential for revealing unexpected interactions arising from physiochemical effects throughout a living test organism.

We tested this approach by measuring modifications of tissue binding using common organophosphate (OP) and pyrethroid pesticides. The toxic mechanism of OPs is primarily disruption of the cholinergic pathway in nerve transmissions. Cholinesterase inhibition is seen in high-dose exposures of most OPs, with a wide variance in effective toxicity (*1,2*). Serine hydrolases are found in many biochemical pathways, including immunity and respiration, and these proteins are also classic OP targets with potential toxic consequences (*3*). We chose diisopropylfluorophosphate (DFP) as a tracer compound because it is an anticholinesterase pharmaceutical, a binder to serine hydrolases, and a simulant of offensive nerve agents that inhibits target proteins through covalent attachment. This study included parathion (PTN) and permethrin (PER), two of the most commonly used insecticides of the OP and pyrethroid classes, respectively. An additional quaternary cholinesterase inhibitor, pyridostigmine bromide (PYB), was added to the group to represent natural cholinergics or protective pharmaceutical intake. The chemical combination has minor relevance to exposures believed related to Gulf War syndrome (*4*) but is also relevant to domestic exposures of insecticides and pediculocides.

OP metabolites are found in human urine at levels of 0.1 μmol/kg, even for children (*5*). These trace concentrations are insufficient for quantifiable inhibition of enzymes through activity assays, the standard technique for quantification. This study used chemical exposures of < 0.01 μmol/kg/day to maintain undisturbed enzymatic activities. Highly sensitive quantitation of the monitor compound is required so all exposures remain at environmental concentrations or less.

Sensitive quantitation is possible using isotope-labeled compounds whose labels have very low natural concentrations, as do radioisotopes. Isotope decay detection is inefficient, however, so we used accelerator mass spectrometry (AMS) to quantify a $^{14}$C isotopic label on tracer compounds at attomole levels with 1–5% precision (*6*). This sensitivity quantitates even tracer doses in highly fractionated tissue samples from small animal models such as mice. A 30-g mouse contains about 1.4 becquerels (Bq) (37 pCi) of natural $^{14}$C, and AMS easily quantifies tracer $^{14}$C at 5% above natural (75 milliBq or 2 pCi tracer $^{14}$C/mouse). A dynamic range of 1,000 in a tracing experiment thus needs no more than 2 nCi or 75 Bq tracer/animal. The Nuclear Regulatory Commission has long ruled that a yearly nonradioactive disposal of up to 1 μCi of $^{14}$C-containing biologic material with concentrations <50 nCi/g is acceptable as nonradioactive waste (*7*). With a total of <2 nCi/30-g mouse, this disposal limit is well above the levels in AMS tracing experiments. The typical AMS sample contains 1 mg carbon, obtainable from 25 μL plasma, 7 mg brain tissue, or the red blood cells (RBCs) from 15 μL whole blood. These small sample sizes are readily available from mice. DFP is available at high specific activity (~2 $^{14}$C/molecule), making it a particularly sensitive probe of delivered protein inhibition. DFP retains this $^{14}$C label when bound to enzymes and is easily dosed orally in food.

The reporter DFP binds rapidly and irreversibly to serine hydrolases in the brain, in other nerve tissue, on RBC membranes, and in skeletal muscle, including membrane esterases. It also binds to soluble plasma hydrolases, including coagulation factors, carboxylesterase, and butyrylcholinesterase (BChE), and to cytosolic serine proteases, but is hydrolyzed to the nonbinding diisopropylphosphoric acid (DPA) by circulating and hepatic A-esterases (*8,9*). Although DFP is frequently used as an enzyme inhibitor to identify the targets of serine-based hydrolysis (*10*), the complement and relative affinities of DFP-binding proteins for mammals *in vivo* is unknown. Two primary protein targets in homogenized rat brain *in vitro* are OP-reactive sites, along with six or more minor targets (*11*); similar numbers of both

This article is part of the monograph *Application of Technology to Chemical Mixture Research.*

Address correspondence to J.S. Vogel, Center for Accelerator Mass Spectrometry, Lawrence Livermore National Laboratory, 7000 East Ave., L-397, Livermore, CA 94551 USA. Telephone: (925) 423-4232. Fax: (925) 423-7884. E-mail: jsvogel@llnl.gov

This research was supported by National Institute of Environmental Health Sciences ES 09690, National Center for Research Resources RR13461, and University of California-Campus Laboratory Collaboration 95-103. One reviewer made significant contributions in conceptual clarification. This work was performed in part under the auspices of the U.S. Department of Energy by University of California Lawrence Livermore National Laboratory under contract W-7405-Eng-48.

Received 18 December 2001; accepted 4 October 2002.

membrane and cytosol proteins are found in homogenized chicken brain *in vitro* (*12*). Neither of these examples addresses the likely targets or extent of DFP binding that occurs through natural distribution and metabolism in living animals at low doses. The hydrophobic DFP will primarily report levels of dissolution within lipids, binding to cell membrane proteins, or to plasma-soluble proteins. Our procedure attempted to prevent postdissection binding to cytosol proteases during sample acquisition. A follow-up of this study will use remaining tissues to identify DFP-binding proteins in the living mouse brain and plasma, along with their binding probabilities.

Only trace amounts of DFP will be bound to proteins at very low doses, and any unbound compound is rapidly cleared (*13*). Interanimal and even intratissue variations in hydrolase concentrations are expected to be much greater than the trace concentration of the bound reporter compound. Traditional enzyme activity assays respond to these animal and tissue variations, which mask physiologically induced changes at tracer levels. Our reporter fluctuations are instead expected to reflect physiologic or physiochemical changes brought about by coexposed chemicals that modulate the amount of reporter reaching a tissue or binding to enzymes. Stress can affect cerebral-vascular transmission (*14*), requiring experimental design that either quantifies or minimizes stress effects that might mask those resulting from chemical coexposures. We designed our experiment to eliminate stress by avoiding handling of the mice throughout the procedures until euthanasia.

The pesticides in our chemical mixtures are known at high doses to perturb numerous biologic pathways, as they possess hormonal activities (*15*), affect enzymatic regulation (*16*), induce membrane permeabilities (*17*), and produce cytotoxicity *in vitro* (*18*), among other physiologic effects not directly related to their esterase interactions. Chronic low-level exposures are implicated in cognitive dysfunctions (*19*), but carefully controlled animal studies have shown no detrimental cognitive effects at chronic daily doses ranging up to 500 µg/kg. However, direct acetylcholinesterase (AChE) inhibition was measurable at the highest doses (*20*). Thus, enzyme-binding compounds at doses of 1 µg/kg are suitable probes of how chemical mixtures of pesticides might affect physiology and chemistry but should have little effect on mouse health and behavior. The goal of our work was to quantify any changes in tissue exposures of DFP that could be ascribed to subchronic preexposure to other anthropogenic esters at the very low doses obtained from household or occupational exposure.

## Materials and Methods

***Chemicals.*** [$^{14}$C]-DFP (specific activity = 160 mCi/mmol) was purchased from New England Nuclear Life Science Products, Inc. (NEC-378; Boston, MA, USA). Uniform ring label[$^{14}$C]-PTN (9.2 mCi/mmol) and [$^{14}$C]-PER (9.8 mCi/mmol) were purchased from Sigma Chemical Co. (31396-3, and Z9999; St. Louis, MO, USA). Unlabeled PTN and PER were purchased from Chem Service (PS-95 and PS-758; West Chester, PA, USA) and assayed > 98.5% purity. PYB and unlabeled DFP were purchased from Sigma (P9797 and D0879). [$^{14}$C]-DFP was diluted in unlabeled DFP to adjust specific isotope activities to desired levels. Purina 5K92 Pico-Vac Rodent Soft Mix food came from PMI Nutrition International (Richmond, IN, USA). All other chemicals and solvents were of the highest grade readily available from commercial suppliers.

***Test animals.*** CD2/F1 male mice were obtained from Charles River (Wilmington, MA, USA) at 6–8 weeks of age and quarantined for 5 days for acclimatization and to ascertain health before moving to an Association for Assessment and Accreditation of Laboratory Animal Care–approved animal care facility for experiments. Protocols were approved by the Lawrence Livermore National Laboratory Institutional Animal Care and Use Committee, and all care and treatment conformed to the U.S. Public Health Service policy on the humane care and use of laboratory animals. Mice were housed individually in plexiglass containers with wire cage tops and lined with shaved wood animal bedding. Food and water were provided *ad libitum*, except as noted below, during a 12-hr light/dark cycle at 20°C. Mice were either not, or minimally, handled throughout the experimental procedures to minimize stress-induced differences among animals.

***Pharmacokinetic study.*** Mice received intraperitoneal (ip) DFP doses of 100 ng/kg (13.6 pmol, 2.2 nCi/mouse) and were sampled for tissue kinetics at 1, 12, 24, 48, and 168 hr. DFP was dissolved in hexane/polyethylene glycol and added to peanut oil for dosing. Triplicate mice were weighed and sacrificed by $CO_2$ asphyxiation at appropriate times. Blood (0.25–0.5 mL) was immediately obtained by cardiac puncture with syringes prewetted with 100 mM EDTA, transferred to plastic tubes containing 2–3 drops of EDTA, mixed, and stored on ice. The following tissues were then obtained and stored on ice: a piece of the right femur muscle, liver, spleen, and brain. The brain was cut in half sagitally, the left hemisphere placed in a homogenization tube (see below), the right half hemisphere in a plastic tube, and both tubes stored on ice. The blood was centrifuged at 1,000 rpm for 5 min to separate plasma and RBCs. Samples were either processed immediately for AMS analysis or stored at –70°C.

***Chemical dosing.*** Chemicals for dose–response and chemical mixture studies were presented in food to reduce stress to the animals. Mice were placed on a fast/feed regimen that removed food in the evening, presented 300–500 mg of moist food in the morning containing the particular chemical dose, and resumed availability of dry food *ad libitum* until the next evening. The moist food consisted of a 1/1.6 mixture of Purina 5K92 Pico-Vac Rodent Soft Mix (PMI Nutrition International)/distilled water prepared each morning. This food/water consistency prevented the mice from handling the food during consumption. Aliquots of the food were transferred by spatula into the bulb of a plastic disposable transfer pipette that was cut with scissors so food retained in the bulb was accessible to the mice. Chemicals were added to the food in 10–40 µL vegetable oil or ethanol diluent vehicles. The modified pipettes containing the food were wedged between the bars of the wire cage top so the food was suspended approximately 3 cm from the cage bottom. Mice consumed the food within 15–30 min, after which pipettes were removed from the cage. More than 80%, >95% in most cases, of the moist food was consumed by the mice.

***Dose–response study.*** Five mice per dose group were acclimated to the fast/feed regimen for 5 days prior to dosing. The moist food was amended with the $^{14}$C chemicals immediately prior to dosing by pipetting 10–40 µL oil or alcohol vehicle containing the radiolabel. Doses of $^{14}$C-labeled PER, PTN, and DFP ranged from 500 ng to 500 µg kg body weight, except for the PTN, which extended to 2 mg/kg. Actual doses were calculated from body mass at euthanasia and percentage of consumed carrier food. $^{14}$C-labeled compounds were diluted with unlabeled equivalents to maintain the optimal tissue content of $^{14}$C for accurate AMS quantitation (approximately 2 nCi/mouse, producing an animal-averaged concentration 50–100 times the natural $^{14}$C). Control mice were simultaneously fed moist food mixed with an equal volume of vehicle. Dry food and water were available *ad libitum* until euthanasia at 48 hr postdosing. Blood and tissues were harvested as described above. Two mice per PTN dose group were transferred to clean animal bedding immediately after dosing. Fecal pellets were sieved from the cage bedding of these mice to determine bioavailability by quantifying unabsorbed [$^{14}$C]-PTN in the pellets. Pellets were homogenized and combusted using an OX300 Biological Oxidizer (Harvey Instrument Corporation,

Hillsdale, NJ, USA) for liquid scintillation counting. Recovery efficiency of the oxidizer was measured at 99.2% using spiked controls.

***Chemical mixture study.*** Moist food in presentation pipettes was supplemented with 1 µg/kg PTN, PER, PTN plus PER, or blank solvent, all with and without 50 µg/kg PYB through a 5-day fast/feed cycle. Four mice per each mixture group were then dosed with 1 µg/kg [$^{14}$C]-DFP in a final moist food presentation. Dry food and water were available *ad libitum* until euthanasia at 48 hr postdosing. Blood and tissues were harvested as described above.

***Tissue preparation.*** The left hemisphere of each brain was placed in a 2.0-mL plastic tube with 1.0 mL water prior to violent agitation in a Biospec Mini-Beadbeater (Biospec Products, Bartlesville, OK, USA) for 10 sec to disrupt the cells and solubilize residual blood. Unbound [$^{14}$C]-DFP and soluble proteins were removed by three sequential centrifugations at 10,000×*g* in 0.5 mL water for 8 min each, with immediate supernatant removal to avoid cell membranes refloating from the pellet. Sequential washes indicated more than 99% of the soluble isotope (bound or unbound) was removed from the tissue after three rinses. Lipid membrane material was not separated from remaining cellular components, and all bound isotope was assumed to represent primarily membrane binding.

RBCs were recovered from whole blood by centrifugation and removal of the plasma. Aliquots of muscle, liver, spleen, RBC, plasma, and rinsed brain containing 1–5 mg carbon were packaged for AMS sample preparation. All samples were dried in a vacuum centrifuge and converted to graphite for introduction into the LLNL AMS ion source.

***Statistics.*** Three to five mice provided data for each experimental condition. All chemical mixture determinations included four mice. Kinetic data were analyzed by a linear compartment model developed in Berkeley Madonna software (University of California, Berkeley, CA, USA). Mixture exposure effects were analyzed for significance ($p \leq 0.05$) using unpaired, 2-tailed *t*-test with InStat software (Graphpad Software Inc., San Diego, CA, USA) and expressed as medians with the standard error in the median. Very broad data distributions were prevalent in the sampled muscle tissue, which was not homogenized, and in short-time kinetic data with rapid changes in tissue concentrations. Homogenized brain and well-mixed plasma showed no difference between means and medians within the calculated errors, confirming near-normal distributions.

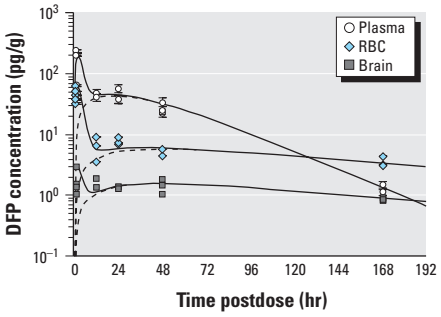


**Figure 1.** Temporal behavior of protein-bound [$^{14}$C]-DFP is shown for plasma (circle), RBC (diamond), and brain (square) after a 100-ng/kg ip dose. Model concentrations are given for both bound (dotted lines) and total components of DFP in the sampled compartment.

## Results and Discussion

The bioavailability of PTN delivered in the moist food ranged from 98.7 to 99.7%, based on the measured $^{14}$C content of the feces collected in the dose–response study. The dose–response data for PTN showed low (<20%) retention of the PTN isotope label at 48 hr, indicating that the majority of the label was hydrolyzed and excreted as *p*-nitrophenol in urine between dosing and euthanasia. Any urine contamination of the recovered fecal pellets would only decrease the quantified bioavailability, an effect that must be minimal given our measured results. We concluded that the chemical delivery in food was efficient and avoided the confounding physical factor of stress on the study animals from handling or from living in metabolism cages.

The kinetic responses of [$^{14}$C]-DFP in plasma, RBC, and brain are shown in Figure 1 for times between 1 hr and 1 week, along with fitted curves from a simple compartmental model of protein-bound DFP in Figure 2. A-esterases hydrolyze DFP to DPA with a maximum velocity of more than 1 mol/hr, rapidly eliminating most of the maximum concentration of 11 nM DFP. This rapid elimination is seen in the first 12 hr. The 1-hr data for RBC and brain imply inefficiencies of removing plasma from these samples at 10 and 0.5%, respectively. Intact DFP has a higher reaction rate with plasma protein, including BChE (350/µM-hr), than with the bound proteins of the RBCs or the brain (e.g., AChE 14/µM-hr), resulting in an expectation of RBC binding that is lower than plasma-bound levels and very low brain labeling in the early exposure. The long-term plasma clearance time (48 hr) matches that found by Martin (*13*), but the brain binding relative to plasma is a factor of 10 lower than that found by Martin using a 10,000-fold higher dose. The brain clearance is more than a factor of 2 slower than found in that high-dose study. The 100 ng/kg DFP dose presents 16 pmol to a 30-g mouse whose blood volume is 1.3–1.6 mL (*21*), for a maximum blood concentration of 11 nM. This represents just 0.001% of the Michaelis-Menten concentration of DFP metabolism in mouse blood and tissue (*22*), allowing linear modeling that assumes the concentrations of metabolic enzymes remain essentially unchanged by this dose throughout the exposure and clearance. The measurements and model represent the regeneration and elimination of the enzyme-substrate pair, as neither the metabolized free DPA nor the recycled protein components are able to further label proteins. Therefore, the model does not return resuspended components containing tracer $^{14}$C to the plasma pool.

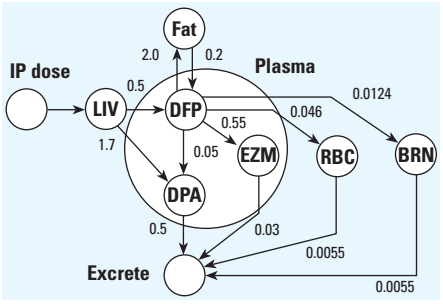


**Figure 2.** The compartmental model used to fit the measured kinetics of the ip dose of DFP shows the transfer coefficients of the fit plotted in Figure 1. Three compartments make up the measured plasma pool: circulating free DFP, the metabolized phosphoric acid of DFP (DPA), and the compound bound to soluble enzymes (EZM). Other compartments include the liver (LIV), RBCs, and brain (BRN). Clearance from bound components to excreta (XCR) is assumed rapid compared with plasma enzyme turnover. Transfer coefficients have units of per hr.

The fitted model and its transport parameters (in units per hour) are shown in Figure 2. A storage and release compartment, assumed to be lipid, is required to correctly model the retention in plasma binding at 24 hr. We have seen similar kinetic behavior for other lipophilic pesticides in mice (*23*), and the Martin's kinetic profiles of DFP show the same delayed peak (*13*). Peak AChE inhibition occurs 24–48 hr after PTN and chlorpyrifos exposures (*24*). Anticholinesterases can have differing peak binding times to esterase *in vitro* (*25*), casting some doubt on the need for this purely physical delay compartment. However, the rapid metabolism of DFP argues for some sort of chemical or physical protection of the fluorine during this delay. The lipid partition coefficient for DFP is more than 10 times that for liver, kidney, and perfused tissue (*22*), so the assumed model of lipid storage delay is plausible; however, the compartment may also represent a delayed chemical interaction.

The model and data show that isotope retention is achieved primarily through protein binding by the 48-hr point for both RBC and brain, which forms the basis of

sampling the mixture-exposed animals at that time. Buchholz et al. found a 15-hr mean lifetime for unbound PER, implying that the 48-hr delay is sufficient in clearing that unbound compound also (*26*). Enzyme regeneration or cell turnover removing the tracer label from both RBC and brain is modeled at 7.5 days mean lifetime. The modeled mean life of plasma-bound DFP (48 hr) is similar to measured lifetimes of other plasma proteins, such as albumin (*27*).

***Dose–response study.*** The dose responses of DFP, PTN, and PER were measured for assurance that differences detected in multichemical exposures were mixture effects rather than unrecognized threshold effects of a single compound. Plots of dose response from dietary intake in Figure 3 showed low survival from first-pass metabolism for DFP and PER, with plasma concentrations only 5–10% the averaged body dose. Metabolism and clearance of high-dose PTN was sufficient to reduce the plasma concentration of intact PTN or its metabolized leaving group to 0.1% of the exposure concentration at 48 hr. Plasma partitioning to RBC was similar for DFP and PER. PTN, or the labeled metabolite *p*-nitrophenol, showed preferential uptake, perhaps lipophilic, to RBC over remaining in plasma. PER partitioned into the brain at only 10% the rate of DFP for equivalent body dose (Figure 3C), despite its high lipophilicity. Retained PTN label had significantly nonlinear dose response in the plasma and brain, with relatively greater loss of the labeled fraction at higher doses. PER showed significant nonlinearity in plasma, with greater retention at higher doses. Dose responses were linear within measurement and fitting uncertainties for all other compounds and tissues. No threshold effects were noted near the planned mixture dose of 1 g/kg.

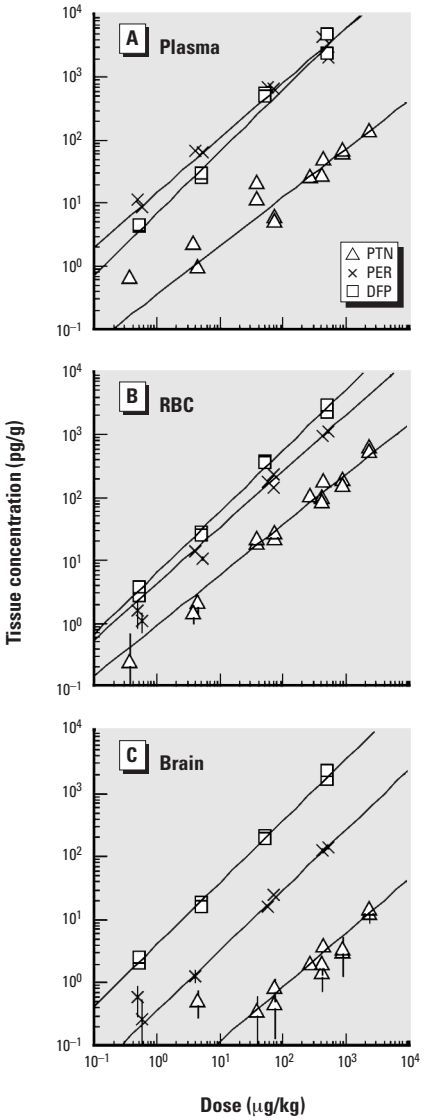


**Figure 3.** Tissue concentrations of [$^{14}$C]-PTN, [$^{14}$C]-PER, and [$^{14}$C]-DFP in plasma, RBC, and brain are shown as functions of the orally administered dose corrected for mouse body mass and dose consumption. Power fits to the data are shown. The only significantly nonlinear data are for PTN residues in plasma and brain and PER in plasma. No threshold effects are found near 1-μg/kg doses. Measurement uncertainties are present on all data.

DFP tissue concentrations from oral exposures differ markedly from those of the ip exposures used in determining kinetics. RBC and brain concentrations of DFP are 79 ± 16% and 53 ± 13%, respectively, of that of plasma concentration, as opposed to the 20 and 5.4% levels observed at 48 hr in the ip kinetic data. The extrapolated response for an oral 100-ng/kg dose predicts a brain concentration of 0.5 pg/g, a factor of 2 lower than the concentration measured 48 hr after the ip dose. In contrast, the plasma and RBC concentrations for oral exposure were a factor of 30 and 10, respectively, lower than those from ip exposure at equivalent extrapolated doses. Digestive hydrolysis of DFP to a nonbinding DPA may be significant in oral exposures, greatly reducing the active component available for binding to circulating protein and cells, but that low plasma concentration, perhaps distributed on lipoproteins, remained nearly as effective in entering the brain and binding to RBC.

***Chemical mixture study.*** Table 1 gives the median tissue concentrations of [$^{14}$C]-DFP, with and without subchronic PYB, at ppt for groups of animals unexposed (DFP only) and preexposed to PTN, PER, and PTN and PER together. Control data for animal handling, tissue isolation, and measurement were obtained from animals receiving equivalent treatments with moist food containing only vehicle solvents. These control tissues all contained within 2% of the expected natural $^{14}$C contents (108 amol $^{14}$C/mg carbon). This natural $^{14}$C was subtracted from all tissue measurements before converting the remaining $^{14}$C signal to equivalent [$^{14}$C]-DFP concentrations. The uncertainty in controls represents <0.03 pg DFP equivalents/g tissue and is incorporated into the uncertainties stated in the table and shown in figures. Liver and spleen tissues were not measured from the PYB groups. These highly perfused tissues retained high levels of bound DFP even after 48 hr, up to 10 times those found in plasma. Muscle also retained more than twice the concentration of DFP tracer as plasma, RBC, and brain. Uncertainties in these higher retention tissues arise from sampling and circulatory variations but also from the measurement procedure. AMS is highly sensitive for low levels of isotope, but instrumental corrections and uncertainties increase at the high concentrations found in these tissues.

Figure 4 summarizes the [$^{14}$C]-DFP binding in tissues, without (Figure 4A) and with (Figure 4B) PYB dosing. Chemical coexposures did not affect absorption or distribution of the reporter DFP, as seen by the constant plasma, muscle, liver, and spleen concentrations. Only brain samples have consistent changes in DFP binding for all preexposures, with DFP increasing by 32, 27, and 43% for PTN, PER, and PTN-PER exposures, respectively. The increases persist with the coadministered dose of PYB (25, 24, and 38%). The combined effect of PTN with PER was the pythagorean sum (the square root of the sum of the squares) of the individual changes.

The increased level of brain-bound DFP could arise through greater absorption or

**Table 1.** The median values of [$^{14}$C]-DFP concentrations are expressed in picograms DFP per gram tissue (± the standard error in the median) for the blood and four tissues from mice coexposed to pesticides and PYB.

| Tissue | | DFP only | | + PTN | | + PER | | + PTN + PER | |
|---|---|---|---|---|---|---|---|---|---|
| Plasma | | 9.01 | 1.38 | 7.84 | 0.90 | 8.73 | 0.99 | 7.74 | 0.88 |
| | + PYB | 7.15 | 1.15 | 7.23 | 0.44 | 7.70 | 1.15 | 7.19 | 0.81 |
| RBC | | 7.68 | 1.63 | 6.77 | 2.95 | 9.11 | 0.93 | 12.7 | 2.90 |
| | + PYB | 5.54 | 0.81 | 6.46 | 1.18 | 7.67 | 2.04 | 8.50 | 2.91 |
| Brain | | 4.09 | 0.50 | 5.42 | 0.19 | 5.19 | 0.14 | 5.85 | 0.47 |
| | + PYB | 3.60 | 0.28 | 4.49 | 0.24 | 4.48 | 0.22 | 4.97 | 0.49 |
| Muscle | | 13.5 | 4.3 | 13.1 | 2.9 | 11 | 14 | 18.6 | 5.9 |
| | + PYB | 6.3 | 4.4 | 12.3 | 2.5 | 7.7 | 3.0 | 8.3 | 7.1 |
| Liver | | 78 | 16 | 57 | 18 | 90.1 | 5.7 | 87 | 12 |
| Spleen | | 25.2 | 4.7 | 21.3 | 2.0 | 36.0 | 7.5 | 22.5 | 3.6 |

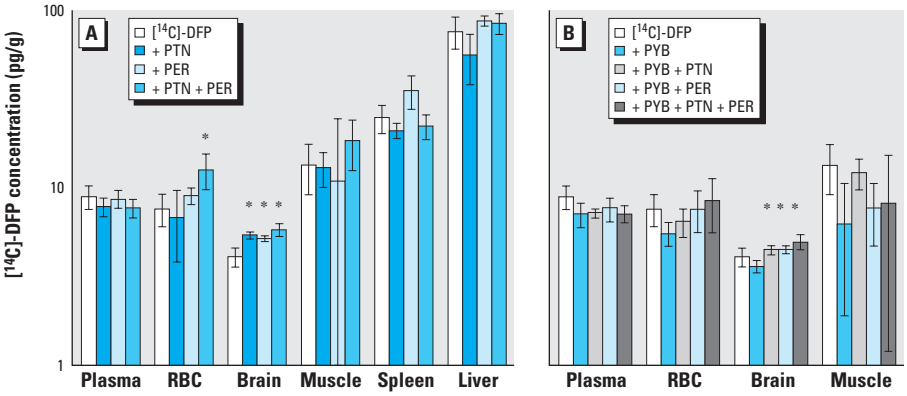


**Figure 4.** Concentrations of [$^{14}$C]-DFP bound in plasma, RBC, brain, muscle, liver, and spleen due to preexposure of mice to pesticides at 1-µg/kg doses, without (*A*) and with (*B*) preexposure to 50 µg/kg PYB. Uncertainties are propagated standard errors in the medians of groups of four or five mice. Asterisk (*) indicates significant differences from the case of no added pesticides.

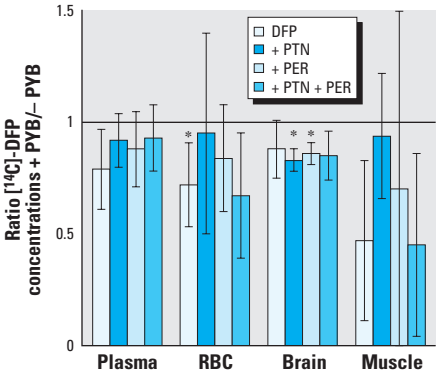


**Figure 5.** Percentage change of DFP binding in plasma, RBC, brain, and muscle due to subchronic coadministration of PYB at 50 µg/kg prior to DFP exposure along with other chemical exposures. PYB shows an overall protective effect that is significant in data for the brain and pesticide-free RBC. Asterisk (*) indicates significant differences from the case of no added pesticides.

greater retention. Greater retention implies increased brain protein binding by DFP influenced by the presence of the other compounds. AChE activity in rat brain significantly increased after 60 mg/kg PER dosed both subchronically for 7 days and acutely (*28*), along with modifying other enzyme-based membrane properties. Our PER exposure dose was 0.002% of that level and was unlikely to cause upregulation of serine hydrolases in the brain. Synergistic effects such as multisite binding that effectively increases binding affinity are possible (*29,30*). The DFP delivered to the brain is approximately 4 pg/g, or 20 pM. AChE, only one of the DFP-binding proteins, is present in mammal brain at many tens to hundreds nanomolar (*28*), providing binding proteins already in large excess of the DFP and minimizing the probability of coincident binding on a given protein. Thus, induction of enzymatic production by pesticides should not change retention.

The sublinear pythagorean additivity might indicate an overall process of circulation or brain absorption nearing saturation, or the interaction of at least two DFP absorption processes sensitive to the pesticides in varying degrees. The brain absorption of DFP is lipophilic, which argues against specifically mediated absorption required for the latter interpretation. Pesticide disturbance of the hypothesized lipid storage (Figure 2) and reemergence of the DFP preferentially in the lipid-filled brain could contribute to the observed effect, but tissue specificity would have to be high for the brain signal to be so unlike the RBC or muscle, which show no pesticide-induced increases. Instead, the brain level most likely reflects the concentration of active tracer compound reaching the brain, suggesting that a 25–45% increase in cerebral-vascular DFP transmission arose from pesticide exposures at 1 µg/kg.

The effect of PYB exposure is summarized in Figure 5, where the percentage changes in [$^{14}$C]-DFP tissue concentrations due to PYB are shown for all comparable experimental conditions. The brain data show some significant differences consistent among all pesticide exposures. PYB does not easily pass the rodent blood–brain barrier, even under stressed conditions (*31,32*), although one study suggests otherwise (*33*). The compounds, even in total, remain at tracer concentrations (~100 nM maximum whole-body average for PYB) that can cause only minor competitive inhibition of proteins. Abou-Donia et al. see no significant change in brain enzyme activities for AChE, choline acetyltransferase, m2 muscarinic acetylcholine (ACh) receptor, and nicotinic ACh receptor at PYB doses 25 times greater than those used here, despite significant changes in a measure of locomotor skills most affected by PYB alone (*34*). Our observed 10–15% reduction in brain DFP binding does not fit a dose-dependent extrapolation of those higher-dose studies. An induction of protective esterase activity elsewhere in the body leading to lower DFP plasma concentration is consistent with our observation, and we do see a decrease of DFP in plasma under PYB exposure with and without pesticides. Any such induction of enzyme activity was specific to elimination of active DFP, however, leaving the PTN and PER enhancement of brain DFP unaffected by PYB. PYB may possess a protective mechanism of action unrelated to the minimal competitive enzyme binding possible at these doses. This PYB dose is only 4% of the prophylactic dose used by service people in the Persian Gulf War to protect against nerve agent exposure. That dose was chosen to temporarily shield about 30% of nerve esterase from OP binding (*34*). The 10% protection observed here is a factor of 10 greater than a linear extrapolation of that effect.

## Conclusions

We demonstrated a method for detecting tissue-specific effects due to low-dose exposures to multiple compounds. The concentrations of bound DFP in all tissues reflect the level of active compound reaching the tissue shortly after the oral [$^{14}$C]-DFP exposure, with possible modulation of uptake, distribution, and binding due to the preexposed compounds. These data showed that common pesticides significantly change the amount of toxin that reaches the brain at concentrations that would not have measurable effects on traditional enzyme activity assays. These pesticides were provided by oral exposure doses that are commensurate with doses available through normal ingestion of sprayed foods, drinking of surface waters, or use of home pesticides. The mechanism appears to be an increase in cerebral-vascular transmission of DFP that is independent of protective effects from transient binding by PYB. If the increase in brain DFP concentrations is due to greater barrier permeability, other toxins and even pathogens may also obtain greater access to brain tissue during low-level pesticide exposure. This would represent an increase in neural toxicity that results from alternative mechanisms from the primary decrease in enzyme activity, a usual measure of toxic end points. The sensitivity of AMS for isotope-labeled tracer compounds can be used to probe the physiologic responses of specific biochemical pathways to multiple compound exposures.

### References and Notes

1. Storm JE, Rozman KK, Doull J. Occupational exposure limits for 30 organophosphate pesticides based on inhibition of red blood cell acetylcholinesterase. Toxicology 50:1–29 (2000).
2. Pope CN. Organophosphorus pesticides: do they all have the same mechanism of toxicity? J Toxicol Environ Health B Crit Rev 2:161–181 (1999).

3. O'Neill JJ. Non-cholinesterase effects of anticholinesterases. Fundam Appl Toxicol 1:154–160 (1981).
4. Haley RW, Kurt TL. Self-reported exposure to neurotoxic chemical combinations in the Gulf War. A cross-sectional epidemiologic study. JAMA 277(3):231–237 (1997).
5. Lu C, Knutson DE, Fisker-Andersen J, Fenske RA. Biological monitoring survey of organophosphorus pesticide exposure among pre-school children in the Seattle metropolitan area. Environ Health Perspect 109:299–303 (2001).
6. Vogel JS, Turteltaub KW, Finkel R, Nelson DE. Accelerator mass spectrometry. Anal Chem 67:353A–359A (1995).
7. U.S. Nuclear Regulatory Commission. 10 CFR 20.2005, 56. Fed Reg 98:23403 (1991).
8. Mazur A. An enzyme in animal tissue capable of hydrolyzing the phosphorus-fluorine bond of alkylfluorophosphates. J Biol Chem 164:271–289 (1946).
9. Billecke SS, Primo-Parmo SL, Dunlop CS, Doorn JA, La Du BN, Broomfield CA. Characterization of a soluble mouse liver enzyme capable of hydrolyzing diisopropyl phosphorofluoridate. Chem Biol Interact 119-120:251–256 (1999).
10. Kidd D, Liu Y, Cravatt BF. Profiling serine hydrolase activities in complex proteomes. Biochemistry 40:4005–4015 (2001).
11. Richards P, Johnson M, Ray D, Walker C. Novel protein targets for organophosphorus compounds. Chem Biol Interact 119–120:503–511 (1999).
12. Carrington CD, Abou-Donia MB. Characterization of [$^3$H]di-isopropyl phosphorofluoridate-binding proteins in hen brain. Rates of phosphorylation and sensitivity to neurotoxic and non-neurotoxic organophosphorus compounds. Biochem J 228:537–544 (1985).
13. Martin BR. Biodisposition of [3H]-diisopropylfluorophosphate in mice. Toxicol Appl Pharmacol 77:275–284 (1985).
14. Sharma HS, Cervos-Navarro J, Dey PK. Increased blood-brain barrier permeability following acute short-term swimming exercise in conscious normotensive young rats. Neurosci Res 10:211–221 (1991).
15. Garey J, Wolff MS. Estrogenic and antiprogestagenic activities of pyrethroid insecticides. Biochem Biophys Res Commun 251:855–859 (1998).
16. Jett DA, Hill EF, Fernando JC, Eldefrawi ME, Eldefrawi AT. Down-regulation of muscarinic receptors and the m3 subtype in white-footed mice by dietary exposure to parathion. J Toxicol Environ Health 39:395–415 (1993).
17. Antunes-Madeira M, Videira RA, Lopes V, Vitor MC. Toxicity of organophosphate insecticides: alteration of membrane fluidity. Med Sci Res 24:753–756 (1996).
18. Cao CJ, Mioduszewski RJ, Menking DE, Valdes JJ, Katz EJ, Eldefrawi ME, Eldefrawi AT. Cytotoxicity of organophosphate anticholinesterases. In Vitro Cell Dev Biol Anim 35:493–500 (1999).
19. D'Mello GD. Behavioural toxicity of anticholinesterases in humans and animals—a review. Hum Exp Toxicol 12:3–7 (1993).
20. Ivens IA, Schmuck G, Machemer L. Learning and memory of rats after long-term administration of low doses of parathion. Toxicol Sci 46:101–111 (1998).
21. Krivitski NM, Starostin D, Smith TL. Extracorporeal recording of mouse hemodynamic parameters by ultrasound velocity dilution. Asaio J 45:32–36 (1999).
22. Gearhart JM, Jepson GW, Clewell HJ, Andersen ME, Conolly RB. Physiologically based pharmacokinetic model for the inhibition of acetylcholinesterase by organophosphate esters. Environ Health Perspect 102(suppl 11):51–60 (1994).
23. Barnett J, Schafer R. Unpublished data.
24. Tang J, Chambers JE. Detoxication of paraoxon by rat liver homogenate and serum carboxylesterases and A-esterases. J Biochem Mol Toxicol 13:261–268 (1999).
25. Ogura H, Kosasa T, Kuriya Y, Yamanishi Y. Comparison of inhibitory activities of donepezil and other cholinesterase inhibitors on acetylcholinesterase and butyrylcholinesterase *in vitro*. Methods Find Exp Clin Pharmacol 22:609–613 (2000).
26. Buchholz BA, Pawley NH, Vogel JS, Mauthe RJ. Pyrethroid decrease in central nervous system from nerve agent pretreatment. J Appl Toxicol 17:231–234 (1997).
27. Dingley KH, Curtis KD, Nowell S, Felton JS, Lang NP, Turteltaub KW. DNA and protein adduct formation in the colon and blood of humans after exposure to a dietary-relevant dose of 2-amino-1-methyl-6- phenylimidazo[4,5-b]pyridine. Cancer Epidemiol Biomarkers Prev 8:507–512 (1999).
28. Blomqvist G, Tavitian B, Pappata S, Crouzel C, Jobert A, Doignon I, Di Giamberardino L. Quantitative measurement of cerebral acetylcholinesterase using positron emission tomography. J Cereb Blood Flow Metab 21:114–131 (2001).
29. Bhatnagar R, Kataria M. Influence of permethrin on certain biochemical parameters in the rat brain. Med Sci Res 25:327–328 (1997).
30. Rao GV, Rao KS. Modulation in acetylcholinesterase of rat brain by pyrethroids *in vivo* and an *in vitro* kinetic study. J Neurochem 65:2259–2266 (1995).
31. Grauer E, Alkalai D, Kapon J, Cohen G, Raveh L. Stress does not enable pyridostigmine to inhibit brain cholinesterase after parenteral administration. Toxicol Appl Pharmacol 164:301–304 (2000).
32. Song X, Tian H, Bressler J, Pruett S, Pope C. Acute and repeated restraint stress have little effect on pyridostigmine toxicity or brain regional cholinesterase inhibition in rats. Toxicol Sci 69:157–164 (2002).
33. Friedman A, Kaufer D, Shemer J, Hendler I, Soreq H, Tur-Kaspa I. Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. Nat Med 2:1382–1385 (1996).
34. Abou-Donia MB, Goldstein LB, Jones KH, Abdel-Rahman AA, Damodaran TV, Dechkovskaia AM, Bullman SL, Amir BE, Khan WA. Locomotor and sensorimotor performance deficit in rats following exposure to pyridostigmine bromide, DEET, and permethrin, alone and in combination. Toxicol Sci 60:305–314 (2001).