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# Development of diagnostics in the search for an explanation of aerotoxic syndrome

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#### ABSTRACT

Aerotoxic syndrome is assumed to be caused by exposure to tricresyl phosphate, an additive in engine lubricants and hydraulic fluids that is activated to the toxic 2-(ortho-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one (CBDP). Currently, there is no laboratory evidence to support intoxication of airline crew members by CBDP. Our goal was to develop methods for testing in vivo exposure by identifying and characterizing biomarkers. Mass spectrometry was used to study the reaction of CBDP with human albumin, free tyrosine, and human butyrylcholinesterase. Human albumin made a covalent bond with CBDP, adding a mass of 170 amu to Tyr411 to yield the o-cresyl phosphotyrosine derivative. Human butyrylcholinesterase made a covalent bond with CBDP on Ser198 to yield five adducts with added masses of 80, 108, 156, 170, and 186 amu. The most abundant adduct had an added mass of 80 amu from phosphate (HPO<sub>3</sub>), a surprising result given that no pesticide or nerve agent is known to yield phosphorylated serine with an added mass of 80 amu. The next most abundant adduct had an added mass of 170 amu to form o-cresyl phosphoserine. It is concluded that toxic gases or oil mists in cabin air may form adducts on plasma butyrylcholinesterase and albumin, detectable by mass spectrometry.

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Over the past 20 years, air crew members (both commercial and military) have reported symptoms such as dizziness, nausea, disorientation, blurred vision, short-term memory issues, and tingling legs that have been associated with smoke or fumes from the jet engines that have entered the cabin area. A prime candidate for the causative agent in these exposures is tricresyl phosphate (TCP), a common additive in engine lubricants and hydraulic fluids [1–3]. TCP is a mixture of various positional cresyl isomers (*ortho*, *meta*, and *para*-TCP). Tri-*ortho*-cresyl phosphate (TOCP) is more toxic than the *meta* and *para* forms, which are considered to be nontoxic [1,4]. Isomers containing mono-*ortho*-cresyl phosphate are considered to be the most toxic, followed by the di-*ortho* and tri-*ortho* compounds [5].

TCP is infamously associated with "ginger jake paralysis," a condition that afflicted 20,000–50,000 people in the United States in 1930. The paralysis was caused by the use of TCP (containing principally TOCP) as an adulterant in Jamaica ginger, a medicinal alco-

hol extract of ginger that was used for stomach problems and commonly abused as an illicit source of alcohol during Prohibition [6,7]. Another major outbreak of TCP poisoning, involving 10,000 victims, occurred in 1959 in Morocco. This one was caused by cooking oil adulterated with aircraft hydraulic oil [7,8]. Other outbreaks have been reported [7], with the latest occurring in China in 1995 [9].

Paralysis in these cases involved the extremities, principally the legs, and appeared 1–2 weeks after consumption of the TCP [6]. All of these outbreaks involved oral consumption of high doses of TOCP. A high oral dose for humans is considered to be approximately 6.6 mg of TOCP/kg body weight [10]. As a consequence of these poisonings, manufacturers of TCP reduced the level of *o*-cresyl phosphate isomers in their products from 25% to 40% during the 1930s/1940s to 0.1% to 1.0% during the 1990s [1,2]. Reduction in TOCP levels was the primary focus; however, the content of monoand di-*o*-cresyl phosphate remain a point of concern.

TCP currently is used as an anti-wear and extreme pressure additive in lubricants and hydraulic fluids [1,2]. It has been used as a plasticizer in lacquers and varnishes, as a flame retardant in plastics and rubbers, as a lead scavenger in gasoline, and as a hydrophobic additive in waterproofing materials [2,11]. With the exception of its use as an additive in engine lubricants and hydraulic fluids, most commercial applications of TCP were discontinued by 2002 [2].

Assuming that *ortho* isomers of TCP are the causative agents in airline incidents, symptoms appear after relatively low dose expo-

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 $<sup>^1</sup>$  Abbreviations used: TCP, tricresyl phosphate; TOCP, tri-ortho-cresyl phosphate; BChE, butyrylcholinesterase; OP, organophosphorus agent; CBDP, 2-(o-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one; OPIDN, organophosphorus ester-induced delayed neuropathy; TPCK, tosyl-t-phenylalanine chloromethyl ketone; CHCA,  $\alpha$ -cyano-4-hydroxycinnamic acid; MALDI, matrix-assisted laser desorption/ionization; LC-MS/MS, liquid chromatography-tandem mass spectrometry; TOF/TOF, tandem time-of-flight; ACTH, adrenocorticotropic hormone; MS/MS, fragmentation induced in a mass spectrometer.

sure. Exposure is presumed to occur from breathing contaminated cabin air or by absorption through the skin from deposits of contaminated particulates coming from the cabin air [1-3]. Lack of accurate data makes it difficult to assess the actual level of exposure that may have been involved [3], but the absence of paralysis argues that exposure levels were not high. The range of potential exposure covers four orders of magnitude. High dose exposure would correspond to breathing an atmosphere containing 1300 mg of TOCP per cubic meter, for 30 min, by a 70-kg individual. This corresponds to oral consumption of 6.6 mg of TOCP per kilogram, which is sufficient to cause serious paralysis. The recommended safe exposure limit is considered to be breathing 0.1 mg of TOCP per cubic meter, for 30 min, by a 70-kg individual [10]. Neither of these estimates takes into consideration the variability in P450 enzyme levels among individuals or the higher toxicity of the mono-o isomer of TOCP.

The increasing number of reports from air crew members complaining of ill health following incidents of cabin air contamination by engine fumes [2,3] prompted us to explore ways to test for exposure to TCP. Our plan was to use adducts formed on selected peptides from human serum albumin and human butyrylcholinesterase (BChE) as biomarkers of exposure. Previous studies with other organophosphorus agents (OPs) have identified Tyr411 as the most reactive residue on serum albumin [12], and it is generally accepted that serine-198 is the only residue in BChE that reacts with OPs. Peptides associated with these residues have already been successfully used for determination of in vivo exposure to other OPs [13-15]. We anticipated that adducts formed on exposure to TOCP would appear on these same peptides. However, to properly apply these biomarkers to TOCP exposure, we first needed to more fully understand the reactions that might be involved. To that end, we have examined the reactions of human BChE and human serum albumin with the principal toxic metabolite of TOCP, 2-(o-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one namely (CBDP) [16].

We chose to work with CBDP, rather than with TOCP, because it is accepted that TOCP is activated to CBDP in vivo [16,17]. Activation involves oxidation of TOCP to di-o-cresyl-o-( $\alpha$ -hydroxy)tolyl phosphate in the liver [16] followed by cyclization, with the aid of albumin, to yield CBDP in the serum (Scheme 1). Cyclization will occur spontaneously, but at 1/10 the rate of that catalyzed by albumin [18].

The toxic effects of TOCP are generally attributed to CBDP. CBDP is known to react with the active site serine in serine esterases, lipases, and proteases, forming a covalent o-cresyl phosphate adduct and causing inhibition of catalytic activity [16,19]. Such a reaction with neurotoxic esterase has been proposed as the causative event in organophosphorus ester-induced delayed neuropathy (OPIDN) [20]. OPIDN is the formal description for ginger jake paralysis.

We performed four studies for this article. First, we examined the mass spectrum of CBDP to determine its purity, and we annotated the mass spectral fragmentation pattern of CBDP so that we would have a better understanding of the mass spectral properties of the free compound. CBDP was found to be pure. Second, we used mass spectrometry to test whether or not CBDP could react with human serum albumin. It was found that an *o*-cresyl phosphotyrosine adduct was formed on Tyr411. Third, we reacted CBDP with free tyrosine in solution to test the general reactivity of CDBP with tyrosine. *o*-Cresyl phosphotyrosine was formed in a two-step process involving a transient intermediate (a ring-opened form of the cyclic saligenin portion of CBDP-tyrosine). Fourth, we reacted CBDP with human BChE and analyzed the tryptic peptides using mass spectrometry to confirm that the reaction of CBDP was with the active site serine in this serine hydrolase and that the reaction gave an *o*-cresyl-phosphate serine adduct. We found that the active site serine (Ser198) formed several CBDP-related adducts, the major adduct being phosphoserine with *o*-cresyl-phosphoserine being a minor species.

#### Materials and methods

CBDP was obtained as a generous gift from W. D. Dettbarn. The CBDP (99.5% pure) had been custom synthesized by Starks Associates (Buffalo, NY, USA). CBDP was dissolved in acetonitrile to 100 mM and stored at -80 °C. Tyrosine (cat. no. T3754), acetonitrile (ACS reagent grade, cat. no. A6914), and porcine pepsin (cat. no. P6887) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human serum albumin (essentially fatty acid free, cat. no. 05418) and formic acid (puriss p.a. for mass spectrometry, cat. no. 94318) were obtained from Fluka (Buchs, Switzer-Sequencing-grade trypsin (porcine, reductively methylated, tosyl-L-phenylalanine chloromethyl ketone [TPCK] treated, cat. no. V5113, Promega, Madison, WI, USA) was dissolved at  $0.4 \,\mu g/\mu l$  in 50 mM acetic acid and stored at  $-80 \,^{\circ}$ C. α-Cyano-4-hydroxycinnamic acid (CHCA, cat. no. 70990, Fluka) was recrystallized before use and then suspended to 10 mg/ml (saturated solution) in 50% acetonitrile/50% water/0.1% trifluoroacetic acid. Trifluoroacetic acid (cat. no. 13972) was obtained from Acros (Geel, Belgium). Methanol (HPLC grade, cat. no. MX0475) was obtained from EMD (Gibbstown, NJ, USA). Acetic acid (glacial, ACS reagent grade, cat. no. A38C-212) was purchased from Fisher (Pittsburgh, PA, USA). BChE was purified from human plasma using ion exchange chromatography on Q Sepharose (cat. no. 17-0510-04, Amersham Biosciences, Piscataway, NJ, USA) and affinity chromatography on procainamide Sepharose 4B, as described previously [21]. BChE was stored at 4 °C in the presence of 0.02% azide. All other chemicals were of ACS quality and used without further purification.

Sample preparation

BChE

Stock BChE was purified to 54% purity, as described by Lockridge and coworkers [21]. It had an activity of 2620 U/ml, where 1 U is defined as the amount of enzyme that hydrolyzes 1  $\mu$ mol of butyrylthiocholine per minute. Here 55  $\mu$ l of stock BChE (0.2 mg or 2.34 nmol) in 10 mM ammonium bicarbonate (pH 8.0)

**Scheme 1.** Activation of *tri-ortho*-cresyl phosphate to CBDP.

plus 0.02% sodium azide was mixed with 1 µl of 100 mM CBDP (100 nmol) and incubated for 0.5–10 min at room temperature. These conditions produce a 40:1 molar ratio of CBDP to protein. Although this ratio is much higher than would be expected for an in vivo exposure, we chose it to ensure that the active site serine (Ser198) would be labeled extensively in these in vitro studies. This treatment inhibited all of the activity of BChE. The mixture was then either boiled for 10 min or mixed 1:1 with acetonitrile to denature the protein. Acetonitrile was used in place of boiling to test the effect of a milder denaturant on the nature of the labeled products. The method of denaturation had no effect on the nature of the final labeled product. When boiling was used to denature, 1 μl of 1 M ammonium bicarbonate and 8 μl of trypsin (0.4 μg/ μl) were added to the reaction mixture (to give a 60:1 molar ratio of BChE to trypsin) and the mixture was incubated overnight at 37 °C. When acetonitrile was used to denature, the reaction mixture was dried by vacuum centrifugation and resuspended in 50 μl of water plus 1 μl of 1 M ammonium bicarbonate before adding the 8 µl of trypsin. Drying was done to remove the acetonitrile so that the trypsin would remain active. In neither procedure was the sample reduced or alkylated.

For matrix-assisted laser desorption/ionization (MALDI) analysis, two methods for handling the tryptic peptides were used. In the first, a 1- $\mu$ l aliquot of the digest was diluted 1:10 with 50% acetonitrile plus 0.1% trifluoroacetic acid and used directly. In the second method, the entire 0.2-mg digest was separated via offline HPLC. Fractions were dried in a vacuum centrifuge and redissolved in 5  $\mu$ l of 50% acetonitrile plus 0.1% trifluoroacetic acid. (For HPLC details, see section titled "Offline HPLC" below.)

For liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis with electrospray ionization, two methods for handling the tryptic peptides were used. In the first, an aliquot of the entire tryptic digest was simply dried in a vacuum centrifuge and redissolved in 5% acetonitrile plus 0.1% formic acid to a final concentration of 2 pmol/µl (assuming no losses during preparation). In the second method, the entire 0.2-mg digest was separated via offline HPLC. Selected fractions were redried in a vacuum centrifuge and redissolved in 5% acetonitrile plus 0.1% formic acid to a final concentration of 2 pmol/µl (assuming no losses).

For mass spectral analysis using static infusion and electrospray ionization to introduce the sample into the mass spectrometer, off-line HPLC was used to reduce the complexity of the sample. Selected fractions were redried in a vacuum centrifuge and redissolved in 50% acetonitrile, 25% methanol, and 1% acetic acid.

#### Serum albumin

Here 10 ml of human serum albumin (1 mg/ml or 150 nmol) in 10 mM Tris–Cl (pH 8.0) containing 0.1% sodium azide was mixed with 0.06 ml of 100 mM CBDP (6000 nmol) and incubated at 37 °C for 43 h. These conditions produce a 40:1 molar ratio of CBDP to protein. Although this ratio is much higher than would be expected for an in vivo exposure, we chose it to ensure that the reactive residue (Tyr411) would be labeled extensively in these in vitro studies. A 10- $\mu$ l aliquot (0.01 mg or 1.5 nmol of albumin) was mixed with 10  $\mu$ l of 1% trifluoroacetic acid to stop the reaction, denature the albumin, and prepare the mixture for peptic digestion. Then 2  $\mu$ l of pepsin (1 mg/ml in 10 mM HCl) was added and the preparation was digested for 4 h at 37 °C. The remainder of the reaction mixture was divided into aliquots and frozen to -80 °C. CBDP-treated human serum albumin was used without reduction or alkylation.

For MALDI analysis, two methods of sample preparation were used. In the first, an aliquot of the digest was diluted 1:10 with 50% acetonitrile plus 0.1% trifluoroacetic acid and used directly. In the second method, peptides from 0.01 mg of the albumin digest

were separated by offline HPLC. Fractions were dried by vacuum centrifugation and redissolved in  $5\,\mu l$  of 50% acetonitrile plus 0.1% trifluoroacetic acid.

#### **Tyrosine**

Here 1 mM L-tyrosine was reacted with 1 mM CBDP in 1 ml of 25 mM ammonium bicarbonate buffer (pH 7.8) at 37 °C. The reaction mixture was kept in a sealed microfuge tube. At intervals, 2  $\mu$ l of the reaction mixture was mixed with 2  $\mu$ l of a saturated solution of CHCA in 50% acetonitrile plus 0.1% trifluoroacetic acid to stop the reaction and to prepare the sample for analysis on the MALDI mass spectrometer.

## Offline HPLC

Offline HPLC was performed on proteolytic digests. Each sample was centrifuged to remove particulate material before injecting it into a Phenomenex C18 column (100  $\times$  4.6 mm) on a Waters 625 LC system (Waters, Milford, MA, USA). Peptides were eluted at a flow rate of 1 ml/min with a 60-min gradient starting at 100% buffer A (0.1% trifluoroacetic acid in water) and ending with 60% buffer B (acetonitrile containing 0.09% trifluoroacetic acid) and 40% buffer A. Here 1-ml fractions were collected, evaporated to dryness in a vacuum centrifuge, and resuspended in 100  $\mu$ l of 50% acetonitrile plus 0.1% trifluoroacetic acid. Each fraction was examined in the MALDI-tandem time-of-flight (TOF/TOF) mass spectrometer to locate the peptides of interest.

#### MALDI-TOF/TOF mass spectrometry

Here 1 µl of sample was air-dried onto a 384-well Opti-TOF sample plate (cat. no. 1016491, Applied Biosystems, Foster City, CA, USA). If the sample did not already contain the CHCA matrix, it was overlaid with 1 µl of CHCA. MALDI mass spectra were taken using a MALDI-TOF/TOF 4800 mass spectrometer (Applied Biosystems, Framingham, MA, USA), Data collection was controlled by 4000 Series Explorer software (version 3.5). Simple mass spectra were acquired in reflector mode using delayed extraction and default calibration. Mass spectra calibration was made with Cal Mix 5 (bradykinin, 2-9 clip; angiotensin I; Glu-fibrinopeptide B; adrenocorticotropic hormone [ACTH], 1-17 clip; ACTH, 18-39 clip; and ACTH, 7-38 clip, Applied Biosystems). Mass spectra consisted of 500 laser pulses taken with the laser energy adjusted to yield optimal signal to noise. MS/MS fragmentation spectra were taken using postsource decay in either positive or negative ion mode at 1 V collision energy in the absence of collision gas and with metastable ion suppression on. Each spectrum consisted of 500 laser pulses taken with the laser energy adjusted to yield optimal signal to noise. MS/MS calibration was made on the fragmentation spectrum of angiotensin I.

Mass spectra were examined manually for the presence of masses that were not present in unlabeled controls or theoretical digests (tryptic digest of BChE or peptic digest of serum albumin). Theoretical digests were generated by the MS-Digest algorithm from Protein Prospector (version 5.3.2) from the University of California Mass Spectrometry Facility (http://prospector.ucsf.edu/prospector). The amino acid sequences of peptides were determined by manual inspection of MS/MS fragmentation spectra with the aid of the MS-Product algorithm from Protein Prospector and the Proteomics Toolkit from DB Systems Biology (http://db.systemsbiology.net:8080/proteomicsToolkit/FraglonServlet.html).

<sup>&</sup>lt;sup>2</sup> Mass spectra were obtained with the support of the Mass Spectrometry and Proteomics core facility at the University of Nebraska Medical Center.

Tandem quadrupole ion trap electrospray ionization mass spectrometry

A QTRAP 4000 tandem quadrupole, linear ion trap mass spectrometer (Applied Biosystems) was used to collect electrospray ionization mass spectra.<sup>3</sup> Data collection was controlled by Analyst software (version 1.5).

Static infusion with electrospray ionization was performed on 5–10  $\mu$ l of sample dissolved in 50% acetonitrile, 25% methanol, and 1% acetic acid using an EconoTip emitter (1  $\mu$ m orifice, Econo12, New Objective, Woburn, MA, USA). The mass spectrometer was run in positive mode with an ion spray voltage of 1850 V, an interface heater temperature of 70 °C, a declustering potential of 70 V, Qo trapping on, a linear ion trap filling time of 20 ms, and a scan rate of 1000 Da/s. Product ion fragmentation spectra were taken at a collision energy of 30 V with 40  $\mu$ Torr of pure nitrogen in the collision cell. In total, 500 spectra were summed. The mass spectrometer was calibrated against fragments of Glufibrinopeptide B and Agilent electrospray calibrant solution (cat. no. G2421-6001, Agilent Technologies, Santa Clara, CA, USA).

LC-MS/MS was performed on 4 pmol of sample in a 2-µl volume. The sample was injected onto an HPLC nanocolumn (Vydac C18 polymeric reverse phase, 75  $\mu$ m i.d.  $\times$  150 mm long, cat. no. 218MS3.07515, P. J. Cobert Associates, St. Louis, MO, USA). Peptides were separated with a 90-min linear gradient from 0% to 60% acetonitrile containing 0.1% formic acid at a flow rate of 0.3 µl/min and electrosprayed through a nanospray fused silica emitter (360 μm o.d., 75 μm i.d., 15 μm taper, New Objective) directly into the QTRAP 4000 mass spectrometer. Data were collected using information-dependent acquisition, which took a simple mass spectrum and then triggered the collection of an enhanced highresolution spectrum and four enhanced product ion spectra on the four most intense ions entering the mass spectrometer having an m/z between 200 and 1500, a charge state of +2 to +4, and an intensity greater than 500,000 cps. After an ion was analyzed twice, it was excluded from analysis for 60 s. Collision energy was determined by the mass spectrometer based on mass and charge state of the ion. Collision gas was pure nitrogen (40 µTorr). and the scan rate was 4000 Da/s. An ion spray voltage of 1900 V was maintained between the emitter and the mass spectrometer. The mass spectrometer was calibrated using MS/MS fragments of Glu-fibrinopeptide B and Agilent electrospray calibrant solution.

Mass spectra of the tryptic digest of BChE were examined manually for the presence of multiply charged masses consistent with singly charged peptides: 2910, 2928, 3008, 3036, 3084, 3098, and 3114 amu. These masses were identified in earlier MALDI mass spectrometry analyses as forms of the CBDP-labeled BChE active site peptide. MS/MS spectra were examined manually to determine peptide sequences with the aid of the MS-Product algorithm from Protein Prospector and the Proteomics Toolkit.

#### Results

Adducts formed from the reaction of CBDP with human serum albumin

We recently found that organophosphates react with Tyr411 on human serum albumin as well as with tyrosines on a variety of other proteins [22]. To determine whether CBDP covalently modifies albumin, we treated human serum albumin with CBDP and digested the mixture with pepsin.

The MALDI mass spectrum of the peptic digest in Fig. 1 yielded four masses of interest: the unlabeled Tyr411 peptide VRYTKKVPQVSTPTL ([M + H]<sup>+1</sup> of 1717.0 amu), a missed cleavage

form of the same peptide, LVRYTKKVPQVSTPTL ( $[M+H]^{+1}$  of 1830.1 amu), a mass at 1887.0 amu (+170 from 1717.0 amu), and another mass at 2000.1 amu (+170 from 1830.1 amu). The latter two masses were not present in untreated albumin.

The 170-amu added mass indicates that the cyclic saligenin moiety was displaced from CBDP during this reaction (see Scheme 2). Note that the masses in Scheme 2 are for the neutral species. If the o-cresyl moiety had been eliminated from CBDP, rather than the saligenin, the added mass would have been 168 amu.

For saligenin to have been eliminated, two phosphorus-oxygen bonds must have been broken. To accomplish this, sequential reactions would be expected with an intermediate in which only one of the cyclic saligenin bonds to the phosphorus was hydrolyzed (see Scheme 3). In Scheme 3, note that the ring-opened structure depicted is only one of two possible forms. The choice of this depiction is for illustrative purposes and is not intended to indicate the actual chemical mechanism. Also note that the masses are for the neutral forms. The added mass from CBDP for such an intermediate be 276 amu. Peptides VRYTKKVPQVSTPTL LVRYTKKVPQVSTPTL with an added mass of 276 amu would apat 1993 amu (1717.0 + 276 amu) and 2106 amu (1830.1 + 276 amu), respectively. Careful examination of the MAL-DI spectrum revealed peaks at these masses, but their intensities were barely 2-fold above background. More convincing evidence for this ring-opened intermediate was found when CBDP was reacted with free tyrosine (see section titled "Adducts formed from the reaction of CBDP with free tyrosine" below).

Fragmentation of the 2000-amu mass (LVRYTKKVPQVSTP-TL+170 amu) in the MALDI mass spectrometer yielded an extensive b-ion series (b2-b12) that was characteristic of this peptide (see Fig. 2). The sequence included the interval for the sequence ion of CBDP-labeled tyrosine (b3 to b4 = 163 + 170 = 333 amu). The majority of the unannotated peaks correspond to a-ions, c-ions, ions that have lost NH $_3$  or H $_2$ O, an internal fragment (Pro-Gln-Val), and immonium ions for amino acids Val, Leu, and Arg. These peaks were left unlabeled in Fig. 2 for the sake of clarity, but a complete list of the annotated masses is given in Table 1 in the supplemental material.

In addition to the above-mentioned masses, the MS/MS spectrum contains a prominent mass at 306.0 amu that corresponds to the tyrosine immonium ion plus the 170-amu added mass from CBDP (136 + 170 = 306 amu). We have found that MS/MS spectra from organophosphate-labeled tyrosine-containing peptides typically yield masses equal to the tyrosine immonium ion plus the added mass from the organophosphate [22].

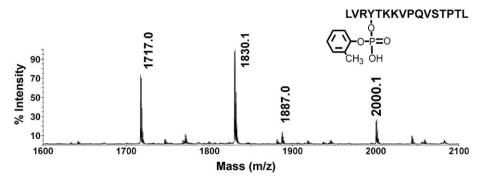
The most intense fragment in the MS/MS spectrum in Fig. 2 appeared at 1830.2 amu, which is 170 amu less than the parent ion 2000.2 amu. This is consistent with facile elimination of the organophosphorus adduct from the parent ion. The next most intense fragment in the MS/MS spectrum appeared at 1887.1 amu (-113.0 amu), consistent with release of leucine from the N terminus of the parent ion to produce the y15 ion.

Observation of an added mass of 170 amu on the parent peptide plus release of 170 amu from the parent ion during MS/MS is indicative of the presence of an o-cresyl phosphate adduct on the peptide. Observation of the tyrosine immonium +170 amu ion (306 amu) and the sequence ion for tyrosine +170 amu (702.4 amu) strongly indicates that the o-cresyl phosphate is present on tyrosine (Tyr411).

A similar MALDI fragmentation spectrum was obtained from the 1887-amu peptide, VRYTKKVPQVSTPTL plus 170 amu (data not shown).

The MALDI mass spectrum from a total peptic digest of human serum albumin (36% sequence coverage) was examined manually for other peptides that might have received 170 amu added mass

<sup>&</sup>lt;sup>3</sup> See note 2.



**Fig. 1.** Portion of a MALDI mass spectrum taken from a complete peptic digest of CBDP-modified human serum albumin. An aliquot of the complete digest was diluted 10-fold with 50% acetonitrile plus 0.1% trifluoroacetic acid, and 1 µl was applied to a MALDI target plate. The values shown indicate the monoisotopic masses for VRYTKKVPQVSTPTL at 1717 amu, LVRYTKKVPQVSTPTL at 1830.1 amu, VRYTKKVPQVSTPTL with an added mass of 170 amu on Tyr411 to give 1887 amu, and LVRYTKKVPQVSTPTL with an added mass of 170 amu on Tyr411 to give 2000.1 amu. The structure is for the 2000.1-amu peptide that carries *o*-cresyl phosphate attached to Tyr411. The accession number for human albumin in the NCBI database is gi:122920512.

Scheme 2. Origin of the 170 amu mass in the reaction of serum albumin with CBDP.

Scheme 3. Reaction of tyrosine with CBDP.

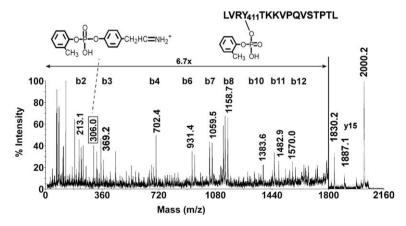


Fig. 2. MALDI postsource decay fragmentation spectrum of the CBDP-labeled human serum albumin peptide LVRYTKKVPQVSTPTL ( $[M + H]^{+1} = 2000.2$  amu). The spectrum was for an HPLC-purified fraction of a peptic digest. The masses are centered over the peaks to which they apply. The *y* axis is expanded 6.7-fold for peaks between m/z 0 and 1800. The 306.0-amu mass, enclosed in a box, indicates the Tyr immonium ion derived from *o*-cresyl phosphate, whose structure is shown. The 1830.2-amu mass is the parent ion minus the OP.

from CBDP. None was found. The spectrum was also examined for the presence of a phosphate adduct on the Tyr411-containing peptides (+80 amu). This was done because the +80-amu adduct was

the dominant modification arising from the reaction of CBDP with BChE (see section titled "Adducts formed from the reaction of CBDP with human BChE" below). No evidence for phosphate was found.

The absence of this phosphate adduct marks a noteworthy difference between the reactions of CDBP with albumin and BChE.

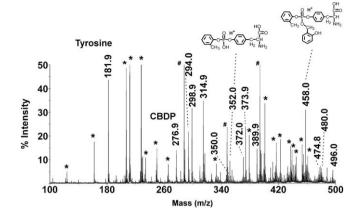
Adducts formed from the reaction of CBDP with free tyrosine

To further test the reaction of CBDP with tyrosine and to reexamine the issue of the chemical nature of the adducts formed, 1 mM CBDP was reacted with 1 mM free tyrosine at pH 7.8 and samples were withdrawn at intervals. Simple MALDI mass spectra of the samples showed prominent masses, consistent with the presence of the protonated forms of tyrosine ( $[M + H]^{+1} = 181.9$  amu), CBDP ( $[M + H]^{+1} = 276.9$  amu), a tyrosine–CBDP ring-opened adduct ( $[M + H]^{+1} = 458.0$  amu), and o-cresyl phosphotyrosine ( $[M + H]^{+1} = 352.0$  amu) (see Scheme 4). Note that the masses given in Scheme 4 are for the neutral charge state. No phosphorylated tyrosine with an added mass of 80 amu from HPO<sub>3</sub> was seen. This is noted because the +80 adduct is the major form found in the reaction of CBDP with the active site serine of BChE.

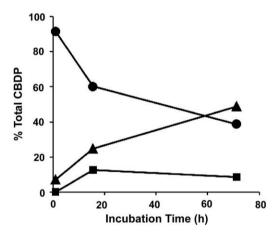
Fig. 3 shows the mass spectrum for the 15.5-h time point, at which time each of the major species was represented. In addition to the protonated form of each CBDP species, there were significant signals corresponding to the NH<sub>4</sub><sup>+1</sup>, Na<sup>+1</sup>, and K<sup>+1</sup> forms (plus 17, 22, and 38 amu, respectively). These alternate forms created a group of four peaks for CBDP (276.9, 294.0, 298.9, and 314.9 amu) and four peaks for the tyrosine-CBDP ring-opened adduct (458.0, 474.8, 480.0, and 496.0 amu). For the o-cresyl phosphotyrosine, the NH<sub>4</sub><sup>+1</sup> form was not seen, leaving a three-peak group (352.0, 373.9, and 389.9 amu). Small signals were also detected at  $[M + H]^{+1}$  = 350 and 372 (see Fig. 3), consistent with cyclic saligenin phosphotyrosine (protonated and Na<sup>+</sup> forms). These signals suggest that a small portion of the reaction of CBDP with tyrosine proceeds through displacement of the cresyl moiety. The cyclic saligenin phosphotyrosine signals amounted to no more than 4% of the total CBDP signal. Most of the other major peaks in Fig. 3 were present in the buffer/matrix blank; these are marked by asterisks (\*). In addition, there were three prominent masses (at 288.0, 348.0, and 394.0 amu) that could not be assigned; these are marked by pound signs (#). They were not present in the 1-h time point and had decayed significantly by the 72-h time point, suggesting that they were products of the reaction.

The time dependence for the reaction of CBDP with tyrosine is shown in Fig. 4. There is a progressive fall in the amount of CBDP (circles) with a concomitant rise in the amount of *o*-cresyl phosphotyrosine (triangles). The fraction of the tyrosine–CBDP ring-opened adduct (squares) rises initially but then falls. This pattern is characteristic of a reaction sequence that proceeds through the tyrosine–CBDP ring-opened adduct as an intermediate on the way to formation of *o*-cresyl phosphotyrosine.

The percentage total CBDP for each species at each time point was calculated by summing the cluster areas for all forms of that species (i.e., protonated,  $\mathrm{NH_4}^+$ ,  $\mathrm{Na}^+$ , and  $\mathrm{K}^+$  forms) at that time point



**Fig. 3.** Mass spectrum from a mixture of tyrosine and CBDP after a 15.5-h reaction. Peaks marked by masses represent the reactants and reaction products. The inserted structures are consistent with the indicated masses. Masses found in a mass spectrum of the buffer/matrix alone are marked by an asterisk (\*). Three prominent unassigned masses (at 288.0, 348.0, and 394.0 amu) are marked by a pound sign (#).



**Fig. 4.** Time course for the reaction of CBDP with tyrosine at pH 7.8. The circles indicate CBDP, the squares indicate a tyrosine–CBDP ring-opened adduct, and the triangles indicate *o*-cresyl phosphotyrosine.

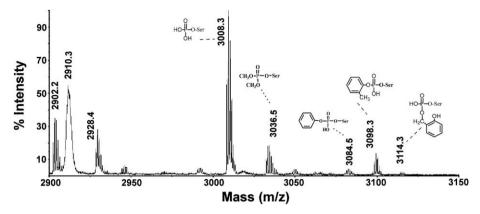
and then dividing that value by the sum of the cluster areas for all species at that time point.

Adducts formed from the reaction of CBDP with human BChE

The MALDI mass spectrum in Fig. 5 is from a tryptic digest of CBDP-treated BChE. The 29-residue active site peptide SVTLF-GES $_{198}$ AGAASVSLHLLSPGSHSLFTR has a mass of 2928.4 amu.

**Scheme 4.** Reaction of CBDP with tyrosine.

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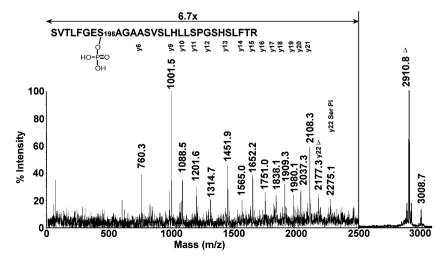
**Fig. 5.** Portion of a MALDI mass spectrum taken on a complete tryptic digest of CBDP-inhibited human BChE. BChE was reacted with a 40-fold excess of CBDP for 30 s, and the reaction was stopped by the addition of an equal volume of acetonitrile. The sample was digested with trypsin, and the complete digest was diluted 10-fold in 50% acetonitrile plus 0.1% trifluoroacetic acid before applying 1  $\mu$ l to the MALDI target plate. The values shown indicate the monoisotopic masses. The active site peptide SVTLFGES<sub>198</sub>AGAASVSLHLLSPGSHSLFTR has a mass of 2928.4 amu. Five adducts on Ser198 (Ser8 in the peptide) were found. They have masses of 3008.3, 3036.5, 3084.5, 3098.3, and 3114.3 amu. Adduct structures are associated with the indicated masses. The accession number for human BChE in the NCBI database is gi:116353.

Isotopic clusters at 3008.3, 3036.5, 3084.5, 3098.3, and 3114.3 amu (monoisotopic masses) are present in the CBDP-treated BChE. These clusters were not present in the spectrum of untreated BChE. Three of these can be related to adducts of CBDP. The new masses are consistent with the active site peptide from BChE (SVTLFGESA-GAASVSLHLLSPGSHSLFTR, 2928.4 amu) plus masses for phosphate (3008.3 = 2928.4 + 80 amu), o-cresyl phosphate (3098.3 = 2928.4 + 170 amu), and a ring-opened form of CBDP lacking the cresyl moiety (3114.3 = 2928.4 + 186 amu). The identities of these masses were confirmed by MS/MS.

The MALDI MS/MS spectrum of the 3008-amu mass (+80-amu adduct) is shown in Fig. 6. A *y*-ion series extending from *y*7 to *y*22 defines the active site peptide for human BChE. The series encompasses the position of the active site serine (*y*22) that appears in two forms. The interval between 2108.3 amu (*y*21) and 2177.3 amu (*y*22) is 69.0 amu, consistent with the presence of dehydroalanine in the position expected for Ser198. This strongly suggests that the serine was modified and that a portion of the modification was lost during the fragmentation process. The interval between 2108.3 amu (*y*21) and 2275.1 amu (*y*22) is 166.9 amu, consistent with the presence of phosphoserine at position *y*22

(mass for dehydroserine [87 amu] plus mass for phosphate [80 amu]). Taken together, these observations indicate that the active site serine (Ser198) was modified by a phosphate in this peptide. The most intense fragment mass was observed at 2910.8 amu, which is 97.9 amu smaller than the parent ion at 3008.7 amu. We typically find that the major mass fragment in MALDI MS/MS spectra of organophosphopeptides corresponds to loss of the phosphomodification from the parent ion. When the adduct is on a tyrosine, this loss is equivalent to the original added mass (170 amu in the case of the *o*-cresyl phosphate-modified albumin peptide in Fig. 2). When the modified residue is a serine, the loss is equivalent to the added mass plus loss of water, thereby converting the active site serine to dehydroalanine. In Fig. 6, the 98-amu mass loss from the parent ion is consistent with the loss of phosphate (added mass = 80 plus 18 amu for water).

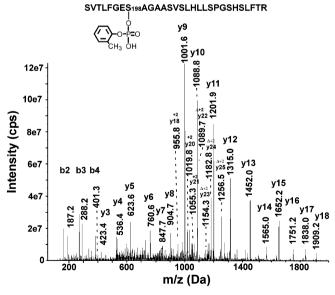
A similar fragmentation pattern was obtained from a triply charged peptide ( $[M+3H]^{+3} = m/z \ 1003.8$ ) in an LC-MS/MS experiment on the complete tryptic digest of CBDP-modified BChE. These data were taken in the QTRAP mass spectrometer using electrospray ionization and low-energy collision-induced dissociation (data not shown).



**Fig. 6.** MALDI postsource decay fragmentation spectrum of the human BChE active site peptide labeled at Ser198 with phosphate (monoisotopic mass of 3008.7 amu). The spectrum was taken from an offline HPLC-purified fraction of the tryptic digest. The masses are centered over the peaks to which they apply. The y axis is expanded 6.7-fold for peaks between m/z 0 and 2500. The triangle ( $\Delta$ ) indicates ions that contain dehydroalanine in place of Ser198 due to loss of phosphate plus a molecule of water. The y22 ion at 2275.1 amu retains the phosphate on Ser198.

MS/MS spectra were obtained by infusing the sample into the QTRAP 4000 mass spectrometer and averaging 500 scans. Fig. 7 shows the collision-induced fragmentation spectrum of the 3098amu mass (observed mass for the triply charged parent ion  $[M + 3H]^{+3} = m/z$  1034.2). A singly charged y-ion series from y3 to y18 and a singly charged b-ion series from b2 to b4 establish this peptide as the active site tryptic peptide from human BChE. A doubly charged y-ion series from y18 to y25 defines the region of the peptide around the active site serine (Ser198). The sequence interval between m/z 1055.3 and 1089.7 (y21-y22) is 68.8/2, which identifies the presence of dehydroalanine at the position expected for Ser198. During collision-induced fragmentation, we routinely observe loss of the organophosphate adduct from serine to yield dehydroalanine. This observation is analogous to the well-established loss of phosphate from phosphoserine during collision-induced dissociation. The presence of dehydroalanine at the sequence location expected for serine strongly argues that this serine was modified by the organophosphate. The added mass on the parent ion (170 amu) strongly indicates that the organophosphate modification was o-cresyl phosphate.

The 3114.3-amu mass (+186-amu adduct) in Fig. 5 is present in low yield. MALDI MS/MS of an HPLC-purified fraction containing this peptide showed release of the 186-amu adduct mass plus water that is expected from a modified serine. Infusion of this HPLC fraction into the QTRAP 4000 mass spectrometer enabled the accumulation of 500 collision-induced fragmentation spectra. This revealed a short *y*-ion series consisting of the five most intense fragments from the BChE active site peptide (data not shown), confirming that this modified peptide was the active site peptide from human BChE. Taken together, the parent ion mass, the MALDI MS/MS spectrum, and the QTRAP fragmentation spectrum indicate that the 3114.3-amu mass consisted of the active site peptide from human BChE modified on Ser198 by an added mass of 186 amu. An added mass of 186 amu is consistent with the presence of a ring-opened phospho-saligenin without the cresyl moiety.



**Fig. 7.** Low-energy, collision-induced fragmentation spectrum of the active site peptide from BChE labeled with *o*-cresyl phosphate (monoisotopic mass of 3098.3 amu). The tryptic digest of CBDP-modified BChE was separated offline by HPLC, and the fraction containing the 3098-amu mass was infused into the QTRAP 4000 mass spectrometer, using static infusion. The spectrum is the sum of 500 scans. Annotated masses include a singly charged *b*-ion series (*b*2–*b*4), a singly charged *y*-ion series (*y*3–*y*18), and a doubly charged *y*-ion series (*y*18–*y*25). The triangle (Δ) indicates ions that have dehydroalanine in place of serine due to β-elimination of the OP (mass of OP plus mass of a molecule of water). All other unannotated major peaks could be accounted for as *b*- or *y*-ions minus water or minus amine, as *a*-ions, or as internal fragments.

Two additional masses could be ascribed to modified active site peptides. One was at 3036.5 amu (added mass of 108 amu). Fragmentation of this mass using infusion and summation of 500 scans showed peaks for the six most intense fragments from the active site peptide of human BChE (data not shown), thereby establishing the identity of this modified peptide. Fragmentation on the MAL-DI-TOF/TOF mass spectrometer yielded an intense peak with a mass of 2910.5 amu. This mass is the dehydroalanine form of the active site peptide and supports the conclusion that the label is on serine. An added mass of 108 amu is consistent with a dimethoxyphosphate adduct or a monoethoxyphosphate adduct. It is not clear how such adducts would arise from CBDP. The second peptide was at 3084.5 amu (added mass of 156 amu). Fragmentation of this peptide was also performed after infusing the sample into the mass spectrometer. The MS/MS spectrum from 500 scans showed an 8-residue y-ion series (singly charged) stretching from v8 to v16 and ions for b2, a2, and b3. These masses established the 3084.5-amu peptide to be the active site tryptic peptide from human BChE. All masses for fragments from a doubly charged y-ion series stretching from y22 to y25 included the mass of dehydroalanine. This is consistent with conversion of Ser198 (residue y22) into dehydroalanine during fragmentation and argues that this serine was modified. The added mass of 156 amu is consistent with the addition of o-phenyl phosphoserine to the peptide. The origin of this adduct is not clear, although it could have arisen from the presence of a minor amount of o-phenyl phospho-saligenin.

The 2910.3-amu mass in Fig. 5 is another noteworthy peak that did not appear in the unlabeled spectrum. The expected mass for the active site peptide minus one water is 2910 amu. Conversion of the organophosphorus-modified active site serine into dehydroalanine would yield this mass. Because this mass appears in the mass spectrum, we initially considered it to be formed by loss of the organophosphorus modification before the preparation was introduced into the mass spectrometer. However, this mass spectrum peak lacks isotopic resolution. This argues that it is generated by fragmentation occurring in the MALDI mass spectrometer, probably after the reflector. Continuous fragmentation of labile masses in the portion of the mass spectrometer after the reflector leads to slight changes in the speed of the newly formed fragments, causing them to arrive at the detector at slightly different times and, thereby, resulting in loss of resolution. We have observed this type of behavior for BChE modified with other OP compounds. MS/MS analysis of this mass has confirmed that it was the active site peptide.

The MALDI mass spectrum and the electrospray LC-MS/MS spectrum from a total tryptic digest of human serum albumin (47% and 25% sequence coverage, respectively) were examined manually for peptides other than the active site peptide that might have received 80 or 170 amu added mass from CBDP. None was found.

Analysis of mass spectral fragmentation spectrum of CBDP

Understanding the nature of the fragmentation of CBDP in the mass spectrometer would be a valuable asset to the study of the mass spectrometry of CBDP. Therefore, we annotated the fragmentation pattern of CBDP from the MALDI-TOF/TOF mass spectrometer (obtained by postsource decay using 1 kV of collision energy in the absence of collision gas). The results are presented in the supplementary material.

#### Discussion

Reaction of CBDP with albumin and tyrosine

Reaction of CBDP with human serum albumin resulted in a 170amu mass being added to Tyr411. The 170-amu added mass indi-

cates that the saligenin moiety was displaced from CBDP, leaving an o-cresyl phosphotyrosine adduct. For saligenin to have been displaced, two phosphorus—oxygen bonds must have been broken. Although it is reasonable to expect that the two bonds were broken sequentially, convincing evidence for an intermediate was not detected with albumin but was detected with free tyrosine.

Casida and coworkers showed that reaction of CBDP with trypsin and chymotrypsin also resulted in the displacement of saligenin. They also argued that the two bonds were broken in sequential steps, with the first being displaced on initial reaction with the nucleophilic active site serine and the second being hydrolyzed in a rapid "aging" reaction [16,17,19] (see Scheme 5). Aging is a well-established phenomenon exhibited by serine hydrolases. It is defined as loss of the alcohol group from the organophosphorylated serine adduct. It is catalyzed by groups in the active site of the OP-inhibited enzyme [23]. Aging is generally considered to be a unique function of serine hydrolases. It is noteworthy to mention here that OP-albumin does not age. Organophosphorus adducts that age rapidly on BChE or acetylcholinesterase are stable on albumin [24]. Because displacement of saligenin occurs on reaction of CBDP with Tyr411 on serum albumin, we suggest that the second step does not require enzymatic intervention such as occurs in aging and, therefore, should be called dealkylation rather than aging. This conclusion is supported by the results from the reaction of CBDP with free tyrosine, where release of saligenin also occurs. That reaction can be seen to proceed via the expected two-step mechanism without any apparent assistance for the second step.

CBDP appears to be more reactive toward tyrosine than does paraoxon. At pH 7.8 and 37 °C, 60% of a 1-mM preparation of CBDP had reacted with 1 mM tyrosine in 60 h. Under the same conditions, there was no reaction of paraoxon with tyrosine, in agreement with Ashbolt and Rydon [25].

#### Reaction of CBDP with human BChE

Reaction of CBDP with BChE yields at least five different adducts: a phosphoserine (+80 amu), an o-cresyl phosphoserine (+170 amu), a ring-opened phospho-saligenin adduct without cresyl (+186 amu), an o-phenyl phosphoserine (+156 amu), and a +108-amu adduct. These modifications are not artifacts generated in the mass spectrometer given that all five adducts can be separated by offline HPLC before being exposed to the mass spectrometer. Although we boiled the samples in some cases to stop the reaction, these modifications are not the result of boiling given that

$$\begin{array}{c|c} & \text{Dealkylation} \\ & \text{Attack} \\ & \text{CBDP} \\ & & \text{Saligenin} \\ & & \text{Dealkylation} \\ & & \text{CBDP} \\ & & \text{Saligenin} \\ & & \text{Saligenin} \\ & & \text{CBDP} \\ & &$$

Scheme 5. Reaction of serine hydrolyases with CBDP.

Scheme 6. Reaction of butyrylcholine esterase with CBDP, aging and hydrolysis of the adduct.

they were observed when the reaction was stopped by diluting the reaction with an equal volume of acetonitrile.

Formation of the phospho-, o-cresyl phospho-, and ring-opened saligenin minus cresyl adducts can be rationalized by a logical sequence of reactions, as shown in Scheme 6. Initial reaction of CBDP with BChE would be expected to yield a ring-opened derivative by analogy with the tyrosine-CBDP reaction (see Scheme 4). Subsequent hydrolysis of the ring-opened species could remove either the cresyl moiety or the saligenin moiety. Loss of the cresyl would yield the +186-amu species, and loss of the saligenin would yield the +170-amu species. The existence of both of these species strongly implies the transient existence of the ring-opened derivative. Failure to observe the ring-opened species argues that it is hydrolyzed more rapidly than it is formed. Finally, hydrolysis of either the +170- or +186-amu adduct would yield the phosphoserine adduct (+80 amu).

On the other hand, one might argue that each of these modifications arose independently from contaminations in the CBDP preparation. However, this alternative is not supported by observation. There was no observable contamination in the MALDI mass spectrum of the CBDP preparation or in the electrospray ionization mass spectrum. Furthermore, based on the relative intensities of the adduct masses in the MALDI mass spectrum for the tryptic digest from the CBDP–BChE reaction (Fig. 5), the phosphoserine adduct is the most abundant form present. This observation makes it highly unlikely that the phosphoserine adduct was formed by a contamination in the CBDP preparation.

Although there is a well-established precedent for BChE-catalyzed hydrolysis of OP adducts via the process commonly referred to as aging [23], it is unclear whether or not any of the hydrolysis reactions proposed in Scheme 6 were promoted by groups in the active site of BChE. However, it is abundantly clear from the data on the reaction of CBDP with albumin and tyrosine that formation of the *o*-cresyl phosphoserine adduct could have occurred spontaneously. By analogy, formation of the ring-opened saligenin phosphoserine adduct minus cresyl also could have occurred spontaneously. Although there is no evidence for or against an aging mechanism being responsible for the formation of the phosphoserine, the fact that neither albumin nor tyrosine promotes formation of the simple phosphoderivative makes it appealing to speculate that some form of aging is responsible for the formation of phosphoserine in BChE.

An interesting note is that, to our knowledge, a phosphoserine adduct on BChE with an added mass of 80 amu has never been reported previously. We reexamined our mass spectral data from reactions between BChE and a variety of OPs looking for phosphoserine adducts, but we found no evidence for the presence of phosphorylated serine adducts (added mass of 80 amu) from reaction with any OPs other than CBDP.

The discovery of phosphoserine as the predominant adduct formed on reaction of BChE with CBDP validates the importance of the experiments described in this article. None of our experience on OP reactions with BChE or albumin would have induced us to predict the formation of this phosphoserine adduct a priori. Without prior knowledge of the adducts formed by reaction with CBDP, designing a diagnostic protocol would be fruitless.

#### Aerotoxic syndrome

Exposure to TCP isomers through leaks of engine gases into the cabin area of aircraft is currently the leading scenario for the cause of aerotoxic syndrome. The active compound derived from *ortho* isomers of TCP is CBDP. Historically, diagnoses of incidences of poisoning by CBDP have relied on clinical symptomatology and epidemiology. Successful diagnoses generally have been made only after severe, widespread, high-dose exposure. If aerotoxic syndrome is

in fact caused by CBDP, the levels of exposure are probably relatively low because the symptomatology is different from the paralysis observed on high-dose exposure.

There are exceptions to the generalization that aerotoxic syndrome produces only mild symptoms. Recent incidents indicate that some individuals are significantly more sensitive than others to cabin air oil exposure. We hypothesize that this is most likely due to the well-known interindividual difference in OP metabolism by cytochrome P450 [26]. Cytochrome P450 catalyzes the first step in the conversion of TOCP into the toxic CBDP. Still, the levels of exposure are likely to have been relatively low compared with the cases of TCP ingestion that caused paralysis.

Investigation into low-dose exposure calls for a sensitive means of diagnosis. The experiments described in this article position our laboratories to investigate samples from candidates who may have been exposed to low doses of TCP isomers. Identification of phosphoserine adducts on Ser198 of human BChE and/or o-cresyl phosphotyrosine adducts on Tyr411 of serum albumin from airline passengers and crew members would strongly support the proposal that low-level exposure to ortho-containing TCP isomers occurs on aircraft. Such a diagnostic for TCP exposure would contribute greatly to epidemiological studies on aerotoxic syndrome.

The neurological symptoms characteristic of aerotoxic syndrome are not likely to be caused by reaction of CBDP with either serum albumin or BChE. This suggests that other proteins must also be sensitive to CBDP. As support for this proposal, we have demonstrated that an array of proteins can be labeled by a variety of OPs in vitro [22]. In addition, we recently found that tubulin from mouse brain can be labeled in vivo by sublethal doses of chlorpyrifos or chlorpyrifos oxon [27].

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.04.032.

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