INTERNATIONAL AEROSPACE CONFERENCE

AIRCRAFT CABIN AIR CONFERENCE

Flight Safety and Cabin Air Quality

IMPERIAL COLLEGE, LONDON September 19-20, 2017

Have You Been Exposed to Aircraft Engine Oil? - Candidate Biomarkers of Exposure

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Chris Winder, editor

BALPA Contaminated Air Protection: Air Safety and Cabin Air Quality International Aero Industry Conference

Proceedings of the BALPA Air Safety and Cabin Air Quality International Aero Industry Conference. Held at Imperial College, London, 20-21 April 2005

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WWW.AOPIS.ORG

Reports in Safety and Environmental Science
School of Safety Science, The University of New South Wales
August 2005

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Discussions at two conferences on cabin air quality (London - 2005; Boeing, Everett, WA - 2004) pointed to the **urgent need** for developing molecular methods to determine whether or not an individual has been exposed to toxic organophosphorus (OP) compounds (e.g.,TCP) during a fume event

We've been working on this issue since then.

Continued exposures emphasize the urgent need for documentation of exposures, the development of safer engine oil anti-wear additives and retrofitting air filtration systems.

1930 Identification of *ToCP* as Cause for Jake Paralysis

Maurice Smith

National Hygienic Laboratories

http://www.thejakewalk.com/the-jakewalk-in-history.html

Tricresyl Phosphates and Cholinesterase

By W. N. ALDRIDGE
Medical Research Council Unit for Research in Toxicology, Serum Institute, Carshalton, Surrey

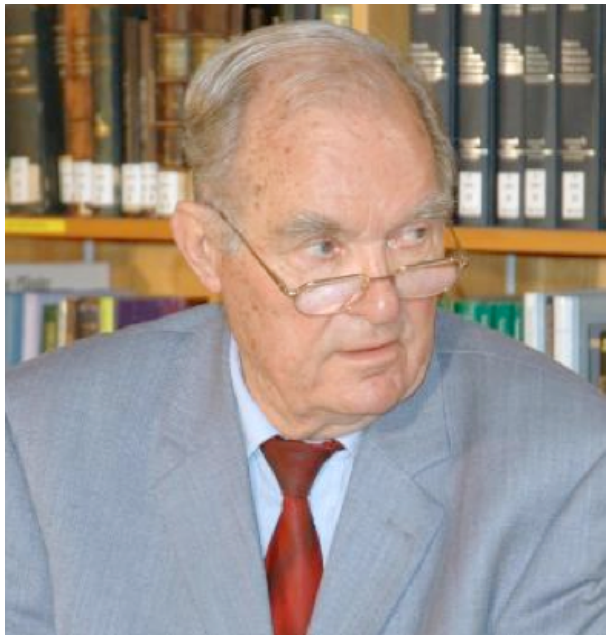
(*Received* 30 *May* 1953) Biochem J. 1954 Feb;56(2):185-9.

Table 4. *Effect of oral administration of tricresyl phosphates on the cholinesterase of chicken serum*

(5 ml. of a 10% (w/v) solution of the tricresyl phosphate in arachis oil was given daily by mouth to each of two chickens. Samples of blood were removed 24 hr. after such a dose for the determination of cholinesterase activity using acetylcholine (0·015 M) as substrate.)

Daily dose (mg./kg.)	No. of daily doses	Time blood sample taken (day)	Cholinesterase activity of serum (ml. CO_2/ml./min.) (1)	(2)	Remarks
Tri-*o*-cresyl phosphate					
0·2	1	0	27·6	—	Ataxia at 14 days
		1	5·8	—	Histological examination at 21 days showed extensive demyelination in the spinal chord
		4	9·7	—	
Tri-*m*-cresyl phosphate					
0·21	20	0	20·5	21·6	No paralysis or ataxia 27 days after last dose
		5	20·3	20·9	
		17	21·6	18·8	Histological examination at this time showed traces of demyelination in the spinal cord
Tri-*p*-cresyl phosphate					
0·26	18	0	—	—	No paralysis or ataxia 43 days after last dose
		16	20·8	—	
		21	20·1	19·2	Histological examination showed traces of demyelination in the spinal cord

Professor Dietrich Henschler (1924-2014)



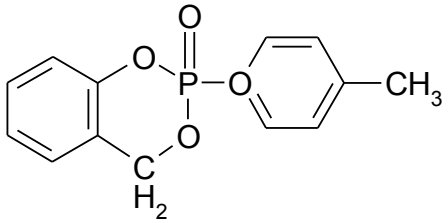
1975 Henschler was honored for his achievements with the large Federal Cross of Merit.

"I was called to Morocco, as in 1959 was a mass poisoning there… This was caused by engine oil. This has been used on an American military base for the local jet fighters…There were 10,000 victims who were paralyzed for the rest of their lives… One of the best solutions is certainly filtering the air. Why this has not happened, I can not understand…As far as I know, there is just no threshold for TCP. A limit for TOCP was set once by the American AGHG (American Governmental Hygienist Group). The value is 0.1 milligrams per cubic meter of air. But this value is based on a very vague data base. (Google translation)

1958
Henschler noted mono- and di-ortho isomers > toxic than T*o*CP

From an Interview with Tim van Beveren: http://www.timvanbeveren.de/?page_id=57

Professor John Casida UC Berkeley



Structure of CBDP
Nature. 1961-191:1396-7

Professor Casida has also identified a number of potential biomarker proteins for OP exposures

Types of TCP "isomers" in Engine Oil Fumes

Table 9. Percent of TCP isomers per unit weight in oils and fluids as well as relative percent of each isomer to the total.

Sample ID	% TCP total	Rel. % ooo TCP	Rel. % mmm TCP	Rel. % mmp TCP	Rel.% mpp TCP	Rel % ppp TCP
Aeroshell 560	2.23	0.02	29.53	49.05	21.34	0.09
BP 2389	2.80	0.01	15.68	49.63	34.39	0.30
BP 2197	2.85	0.01	29.73	48.45	21.73	0.09
Mobil II	5.23	<0.01	31.48	47.04	21.37	0.11
Used BP 2380	5.10	<0.01	29.81	47.67	22.40	0.12
Bulk BP 2380	4.70	<0.01	32.22	47.64	20.04	0.10
Chevron Hyjet	0.00	<0.01	<0.01	<0.01	<0.01	<0.01
Mobil 291	5.59	<0.01	24.83	47.22	27.76	0.19
Skydrol LD-4	0.00	<0.01	<0.01	<0.01	<0.01	<0.01
Skydrol 500B-4	0.00	<0.01	<0.01	<0.01	<0.01	<0.01
Exxon O-156	4.48	<0.01	29.11	48.19	22.59	0.12
Mobil 254	4.99	<0.01	33.58	46.50	19.85	0.09

Hecker, S; Kincl; L; McNeely, E; et al. (2014) "Cabin Air Quality Incidents Project Report," Submitted to the Federal Aviation Administration as part of a collaborative project between the Occupational Health Research Consortium in Aviation (OHRCA) and the Airliner Cabin Environment Research (ACER) Center of Excellence, Washington, DC.

Issues to Discuss

- Health and flight safety implications of breathing oil fumes onboard
- Overview of process to develop “biomarker” (blood test) specific to types of TCPs in av. engine oils
  - Select the best candidate blood protein
  - Develop protocols to isolate and purify the protein from the rest of the blood
  - Identify and measure the structural modifications to the protein after exposure to Durad 125 in test tube
  - Test that method on blood samples of people exposed to oil fumes on aircraft

Issues to Discuss, continued

- Are safer anti-wear additives possible?
- Why are some people more sensitive to organophosphate chemicals than others?
- Can anything be done to mitigate the health effects of exposure to TCPs after-the-fact?

Health and Safety Issues Related to Oil Leak Events

US Airways Boeing 767-200, registration N251AY performing flight US-1041 from Saint Thomas (US Virgin Islands) to Charlotte,NC (USA) with 174 passengers. In March 2010 US Airways confirmed, that engine oil had leaked through a faulty seal into the bleed air supplying the air conditioning system. "…both flight crew received permanent injuries.
http://avherald.com/h?article=425f6a41&opt=0

British Airways flight attendants on board an Airbus A380 “superjumbo” vomited, became “spaced out” and had to use emergency oxygen after suspected “toxic fumes” were detected in the cabin during a long-haul flight, a leaked internal report reveals.

At least one crew member became so ill that he curled up on the floor and put a blanket over his head. Others displayed bizarre behaviour including “stuffing” food into their mouths while using oxygen masks and wandering around “lost” in the cabin.

https://www.thetimes.co.uk/article/flight-crew-spaced-out-on-fumes-bzf7sxl65

How frequent are cabin air fume events?

Indoor Air. 2016 Jun;26(3):478-88. doi: 10.1111/ina.12211. Epub 2015 Apr 25.

Characterization of the frequency and nature of bleed air contamination events in commercial aircraft.

Shehadi M[1], Jones B[1], Hosni M[1].

Mechanical and Nuclear Engineering Department, Kansas State University, Manhattan, KS, USA.

The authors reviewed online FAA smoke/fume incident databases and reported US airline documentation for an industry average of 0.2 fume events per 1000 flights during an six-year period (Jan. 1, 2007- Dec. 31, 2012); see p.9. The fume events under study are described as being specific to either oil or hydraulic fluid fumes in the bleed air, and may or may not include smoke/haze.

During that time, according to this site (selecting US carriers only), there were 58,638,558 flights on US carriers that would have been subject to the FAA smoke/fume reporting rules during that time period. Thus, 0.2 incidents per 1,000 flights equates to 11,728 incidents during that six year period, or 1,955 fume incidents in one year or 5.3 per day. (Judith Anderson)

Conclusion – cabin air fume events are an ongoing problem.

How can an exposure be documented?

Potential Biomarkers of OP Exposure

Butyrylcholinesterase (BChE)

- Plasma soluble protein
- ~11 day half-life

Acetylcholinesterase (AChE)

- In erythrocyte membranes
- ~33 day half-life

Acylpeptide hydrolase (APH)

- In erythrocytes
- ~33 day half-life

Carboxylesterase (CES)

- In monocytes
- Short half-life (hours)

Neuropathy target esterase (NTE)

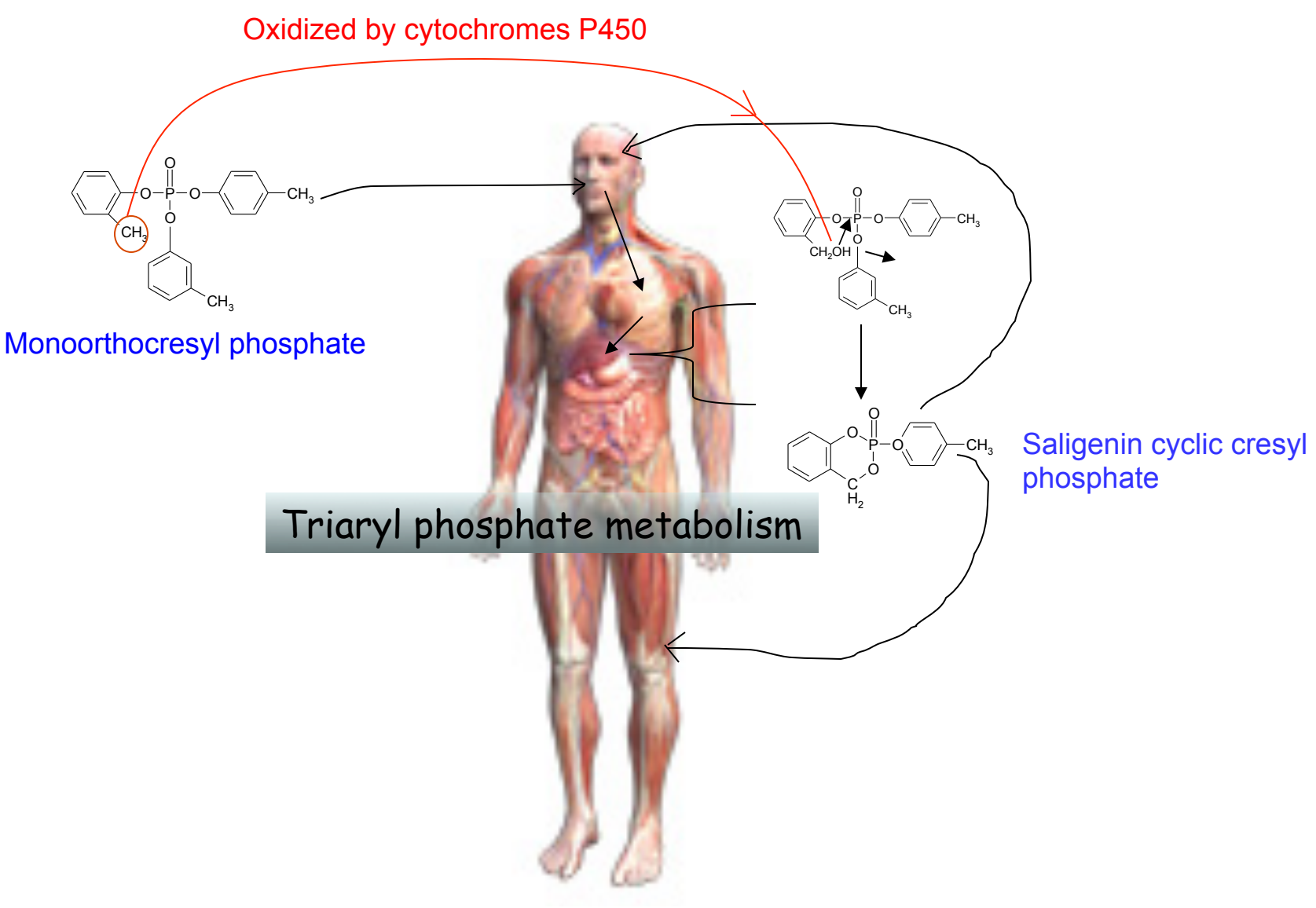
- In lymphocytes
- Delayed neuropathy

Albumin

- Plasma soluble protein
- ~20 day half-life

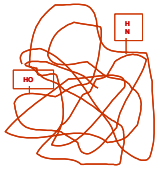
The next slide shows the generation of a highly toxic metabolite from Tri-ortho-cresyl phosphate. Other isomers will generate different metabolites when bioactivated as well as interacting directly with other blood proteins without being bioactivated.

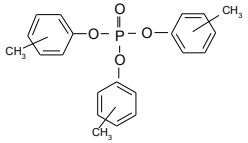
The Problem

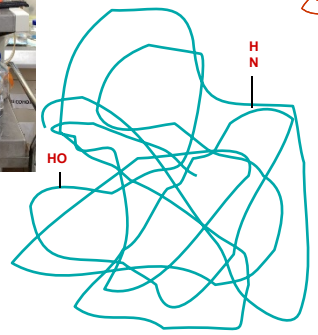

Oxidized by cytochromes P450
Monoorthocresyl phosphate
Triaryl phosphate metabolism
Saligenin cyclic cresyl phosphate

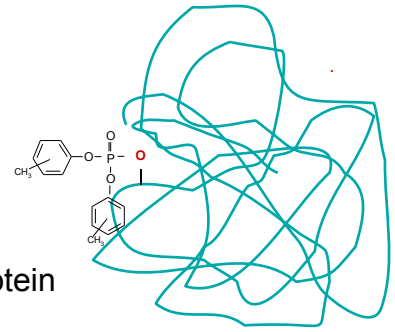
Following modification by an OP molecule, a protein can be chopped into small pieces by specific enzymes and the modification then identified by mass spectrometry. Modification by TCP or TCP metabolites is unique in that the methyl phenyl group remains attached to the active site peptide (piece of the protein). Insecticides leave only an ethyl or methyl phosphate attached to the peptide. Heavy isotope labeled protein is generated by cloning the gene encoding the protein into *E. coli* which are grown in a fermentor on 15-nitrogen generating an internal MS standard.

Modified Protein Biomarkers of Exposure





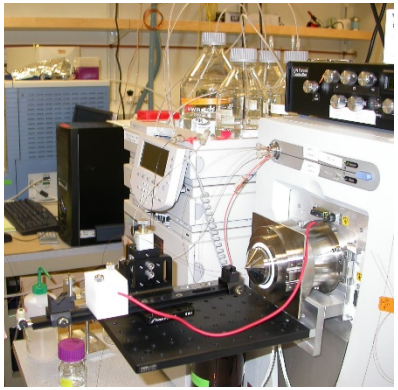




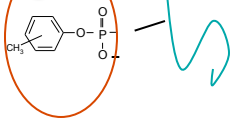
Antibody to active site part of protein

Protein

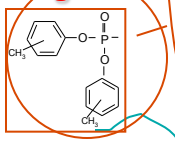
Modified Protein



Aged residue

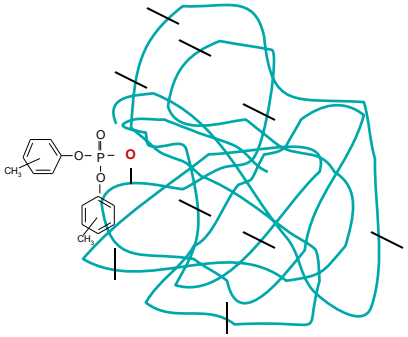


Un-Aged residue



Digest with specific proteases

Mass spectrometer



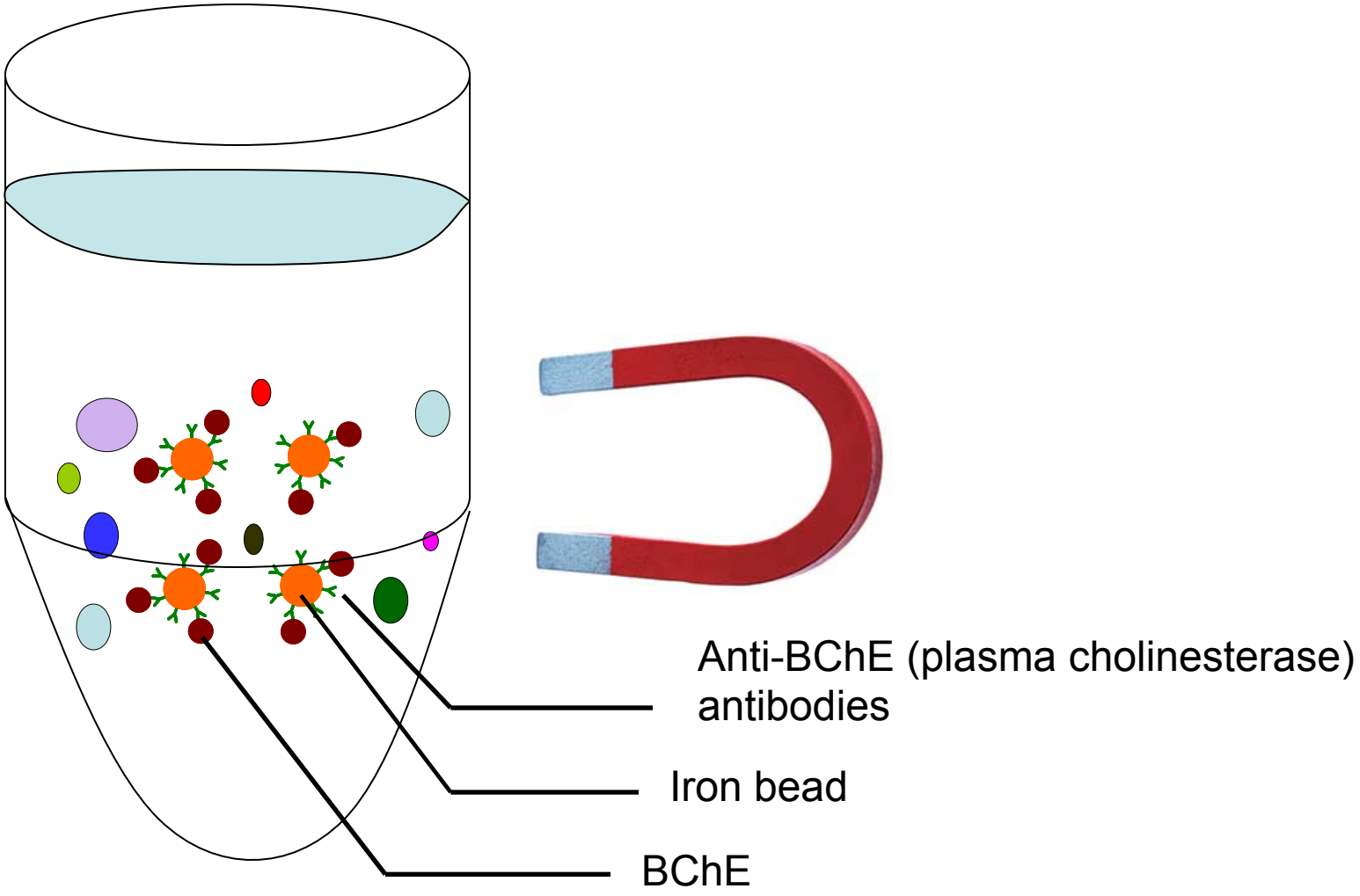
Separate Fragments

To mass spectrometer (MacCoss, Hoofnagle, Marsillach, Henderson)

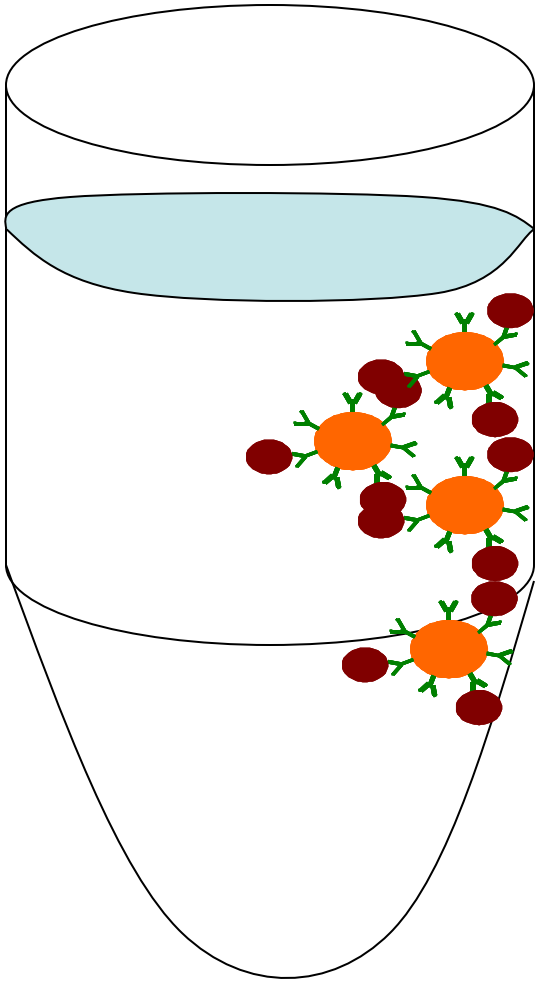
Anti-Target Protein Antibodies

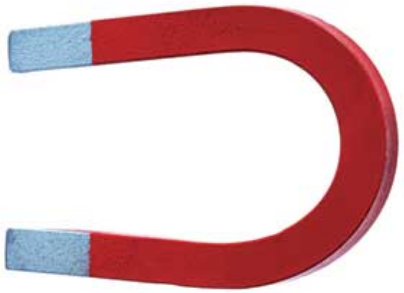
- For Each of the target proteins it is useful to have specific antibodies for rapid isolation of the target protein.
- Even more useful is to have in addition an antibody that is specific for the part of the protein that contains the active site that is modified by OP exposure. Examples follow.

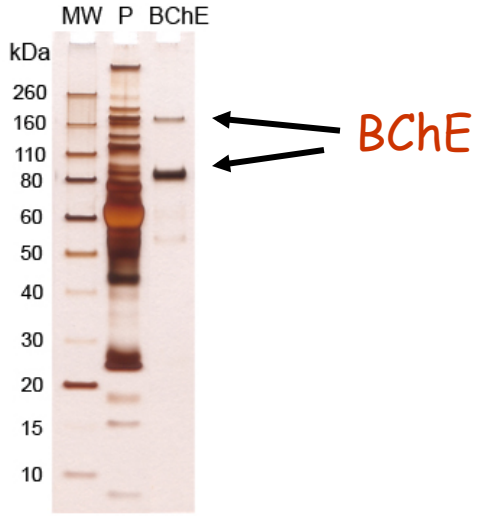
Immuno Magnetic Bead Separation (IMS)



Immuno Magnetic Bead Separation (IMS)





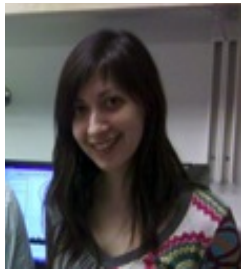


This simple protocol purifies plasma ChE (BChE) from all of the other serum proteins in a single step.

Inhibition of butyrylcholinesterase activity has long served as a biomarker for exposure to organophosphorus (OP) compounds (insecticides and nerve agents, for example). However, activity measurements are inaccurate and require a baseline measurement for each individual. Mass spectrometric analysis is much more accurate and does not require a baseline measurement of activity since the analysis provides the percentage of the protein modified by an OP exposure.

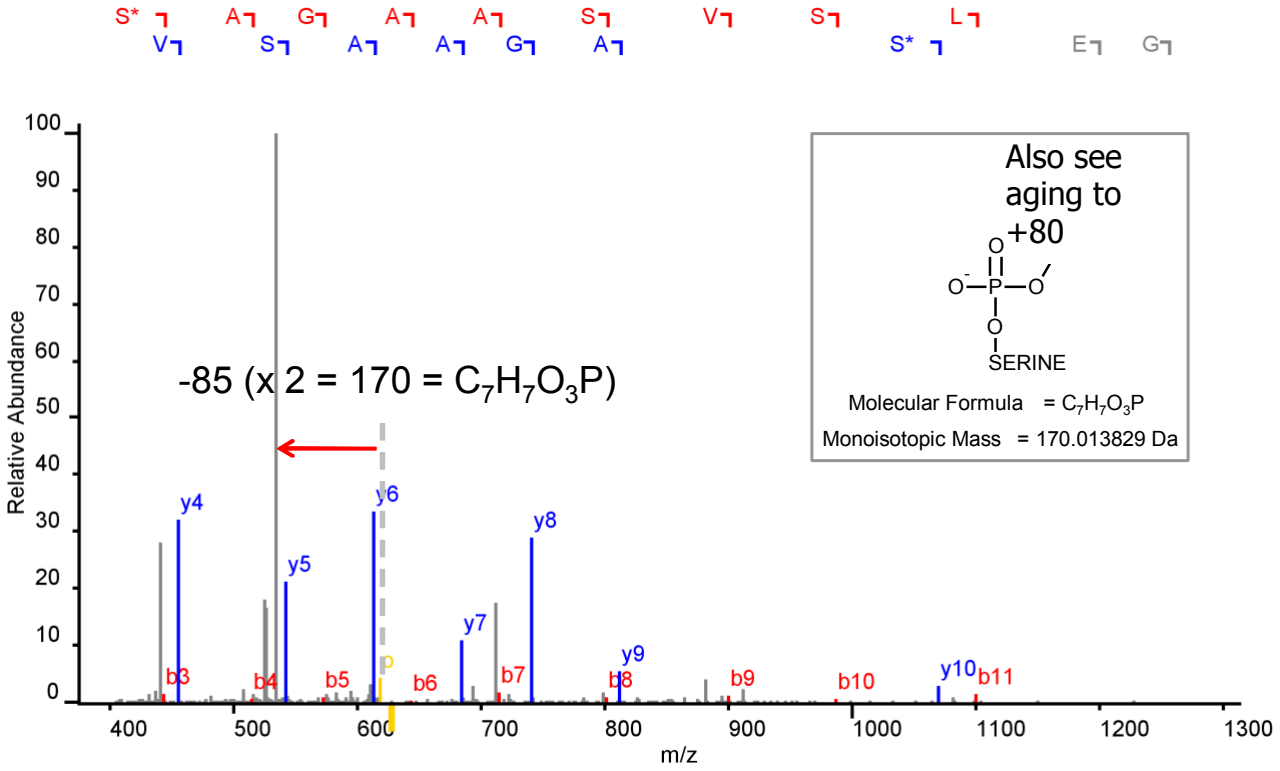
The problem with exposure to the triaryl phosphates is that the initial adduct to the protein, a cresyl phosphoserine, “ages” to leave only phosphate attached to the active site serine. Our current efforts are aimed at characterizing other biomarker proteins that retain the cresyl phosphoserine indicative of exposure.

Mass Spec Analysis of OP Modified Protein (Butyrylcholinesterase)



Judit Marsillach

Chymotrypsin digestion
F.GES*AGAASVSLH.L
*: CBDP / +170



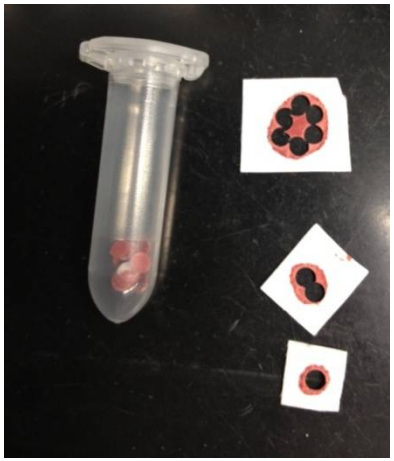
Mike MacCoss & Daniela Tomazela

Schopfer et al. Anal Biochem 2010

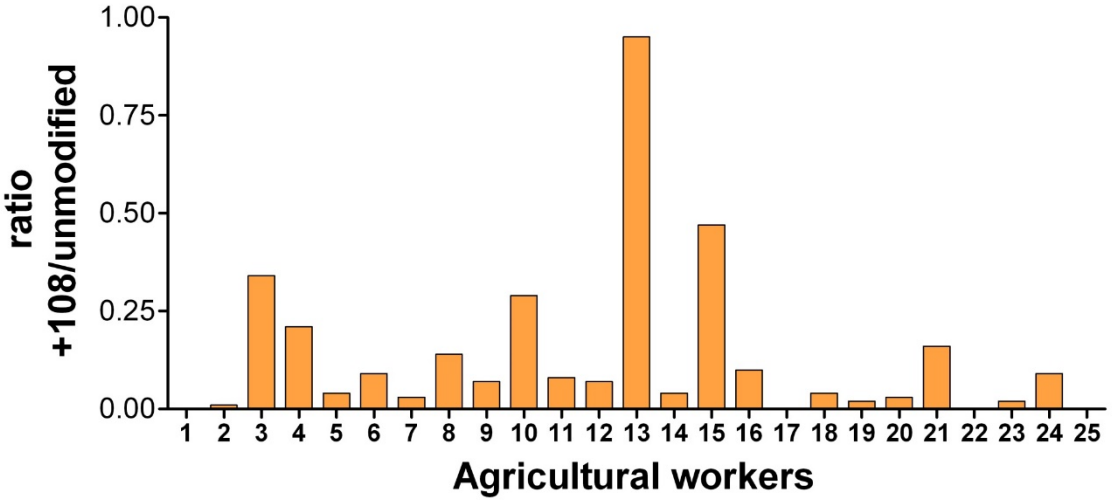
Analysis of OP decoration of the active site of BChE by agricultural insecticides is so sensitive that exposure can be determined by analysis of blood spots dried on filter paper which can be inexpensively shipped through standard mail.

The next slide shows the degree of modification of the active site serine in dried blood spots from agricultural workers. The bar graph shows the ratio of the modified BChE/unmodified BChE.

Determining the Percentage Modification of the BChE Active Site by OP Insecticide Exposure



Samples can be extracted from dried blood spots



This analysis resulted from the cabin air research

We can also determine an individual's sensitivity to some OP insecticides from a drop of blood.

Dr. Judit Marsillach

Human and Mouse* AChE does not age with a loss of the cresyl group.

Our efforts are focused on biomarker proteins that do not lose the hallmark cresyl group though the "aging" process.

*Carletti E[1], Colletier JP, Schopfer LM, Santoni G, Masson P, Lockridge O, Nachon F, Weik M. **Inhibition pathways of the potent organophosphate CBDP with cholinesterases revealed by X-ray crystallographic snapshots and mass spectrometry.** Chem Res Toxicol. 2013 Feb 18;26(2):280-9. doi: 10.1021/tx3004505. Epub 2013 Feb 5.

Searching for a Safer OP Anti-wear Additive
(Collaboration with NYCO Oil, Paris)

The following table shows the amount of triaryl phosphate (TAP) required to inhibit 50% of BChE activity when the TAP is bioactivated with rat liver microsomes. A high value indicates a potential safer oil additive.

In vitro Analysis of BChE Inhibition by Bioactivated TAPs

Table 1
IC_{50} values of TAPs bioactivated with rat liver microsomes, *in vitro*.

Triaryl phosphate	Referred to as:	ID No.	Source	% Purity[a]	IC_{50}[b] (μg/ml)
Tri-(*o*-cresyl) PO_4	ToCP	433-89B	Chem Service[c]	98.8	0.12
Tri-(*o*-cresyl) PO_4	ToCP	60M108	City Chem[d]	Unknown	0.15
Syn-O-Add 8484	N/A	E10-234	Supresta[e]	Mixed Isomers	0.32
Durad 125	D125	E09-652	Chemtura[f]	Mixed Isomers	0.36
Mono-cresyl diphenyl PO_4	N/A	106-83A	Chem Service	Unknown	0.41
Mono-(*p*-isopropyl phenyl) diphenyl PO_4	N/A	E08-338	NYCO[g]	99.8	0.51
p-isopropyl phenyl PO_4 [mixture of tri-, di-, and mono-(*p*-isopropyl phenyl) PO_4]	N/A	E08-337	NYCO	99.9	0.56
Mono-(*t*-butyl phenyl) diphenyl PO_4	N/A	E10-225	NYCO	>99.9	0.63
Mono-(dodecyl phenyl) diphenyl PO_4	N/A	E10-228	NYCO	99.3	1.22
Tri-(*m*-cresyl) PO_4	N/A	AO1090 99	ACROS[h]	97	2.52
Mono-(1-methyl-nonyl phenyl) diphenyl PO_4 (mixture of isomers)	N/A	E10-227	NYCO	Mixed Isomers	4.23
Tri-(*p*-cresyl) PO_4	TpCP	60M107	City Chem	Unknown	>20.0
Tri-(*p*-cresyl) PO_4	TpCP	E08-292	NYCO	99.8	>20.0
Di-(isopropyl phenyl) phenyl PO_4	N/A	E10-229	NYCO	99.7	>20.0
Tri-(*o*-isopropyl-phenyl) PO_4	N/A	E08-293	NYCO	99.8	>20.0
Tri-(*p*-isopropyl-phenyl) PO_4	N/A	E08-292	NYCO	99.8	>20.0
Di-(*tert*-butyl phenyl) phenyl PO_4	N/A	E10-226	NYCO	99.5	>20.0
Tri-butyl PO_4	N/A	90818	Fluka[i]	>98	>20.0
Tri-(*p*-*tert*-butyl phenyl) PO_4	TpBP	E09-649	NYCO	>99.9	>20.0
Tri-(*o*-*tert*-butyl phenyl) PO_4	ToBP	E09-650	NYCO	>99.9	>20.0
Tri-(*m*-*tert*-butyl phenyl) PO_4	TmBP	E10-036	NYCO	>99.9	>20.0

[a] Percent purity was determined by gas chromatography.
[b] IC_{50} based on *in vitro* bioactivation using rat liver microsomes. When TAPs were assessed repeatedly, the lowest value is reported.
[c] Chemical Service, West Chester, PA.
[d] City Chemical, West Haven, CT.
[e] Supresta, c/o Clearon Corporation, Charleston, WV.
[f] Chemtura Corporation, Middlebury, CT.
[g] NYCO S.A., Paris.
[h] ACROS Organics, Geel, Belgium.
[i] Fluka/Sigma–Aldridge, Buchs, Switzerland.

Baker et al. Chemico-Biological Interactions 203 (2013) 257–264

The next slide examines the *in vivo* inhibition of three blood enzymes [red cell acylpeptide hydrolase, carboxylesterase, and butyrylcholinesterase (BChE)] and two liver enzymes (acylpeptide hydrolase and carboxylesterase) when mice were fed the indicated triaryl phosphates.

In vivo analysis of D125, Tri-*p*-cresyl phosphate & Tri-*tert*-butyl phenyl phosphates

Table 2
ED_{50} values from blood and liver of mice exposed to D125, TpCP or TpBP.[a]

		Enzyme assayed				
		Blood enzymes			Liver enzymes	
		BChE	APH	CES	APH	CES
Assay substrate	BTC[b]	αNB	PNV	MUA	AcAla	MUA
Triaryl phosphate						
D125	63	>240	19	19	33	85
TpCP	>240	>240	27	17	14	52
TpBP	>240	>240	93	86	>240	>240

[a] ED_{50} values (mg/kg body weight) were based on enzyme activity from blood plasma (for BChE and CES) or RBC homogenates (for APH) and liver homogenates (for liver APH and CES) 24 h after exposure to TAPs (TpCP and TpBP) or TAP-containing D125.
[b] S-butyrylthiocholine iodide (BTC).

The tri-tert butyl phenyl phosphate was significantly less inhibitory than D125 or tri-*p*-cresyl phosphate

Since the commercial TAPs have very low concentrations of ortho isomers, It is important to know what an additive from D125 would look like.

Baker et al. Chemico-Biological Interactions 203 (2013) 257–264

It is important to characterize a specific biomarker enzyme(s) inhibited with a specific OP adduct that can serve as a biomarker of exposure to TAPs.

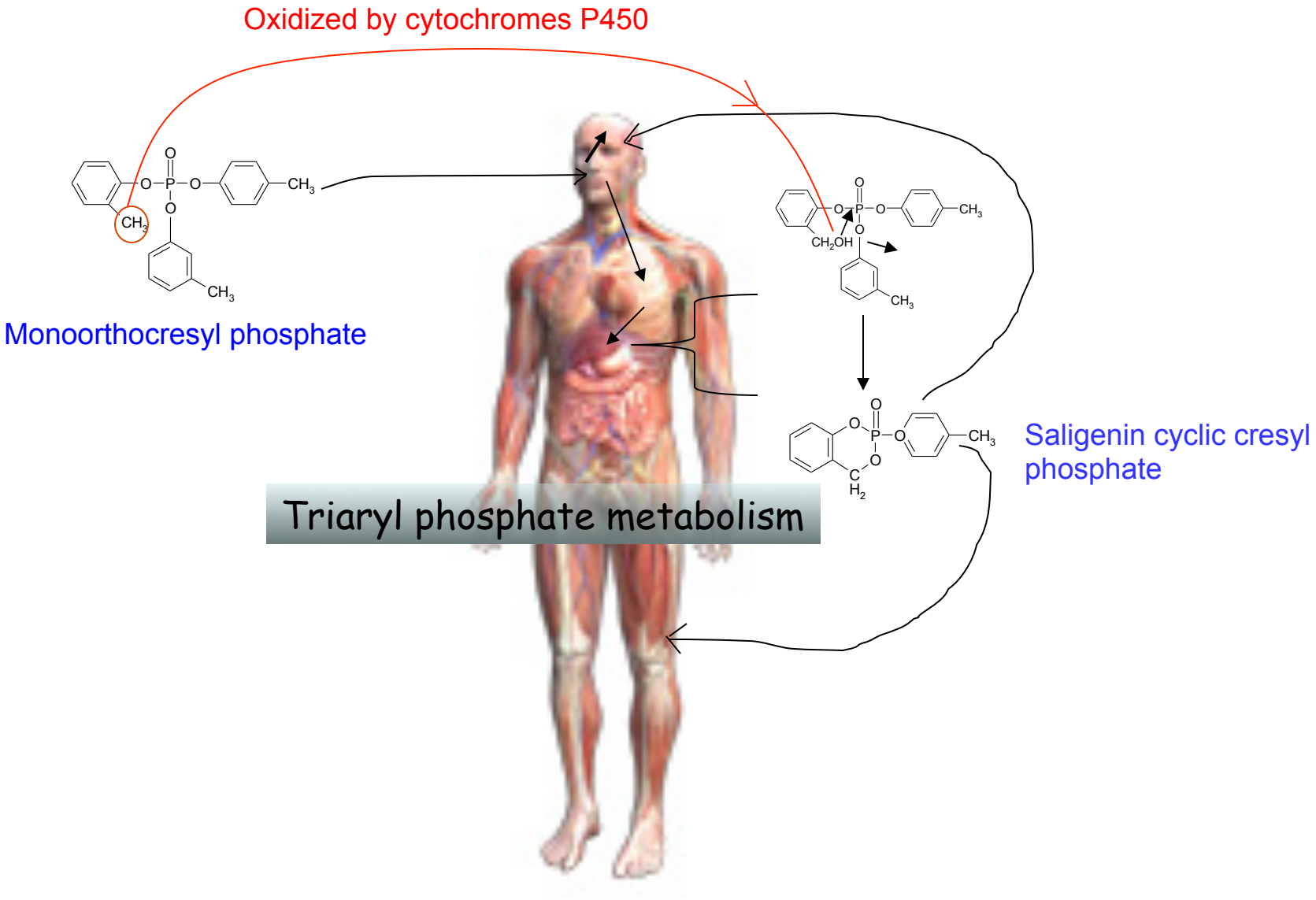
To summarize our findings to date: different TAPs generate different metabolites which differentially inhibit specific enzymes.

Different TAPs generate different metabolites which differentially inhibit specific enzymes.

It is important to characterize a specific biomarker enzyme(s) with a specific adduct that can serve as a biomarker of exposure to TAPs.

Why are some individuals more sensitive to TAP exposures than others?

The cytochromes P450 vary in levels and structure among individuals



Is it possible to mitigate a TAP exposure?

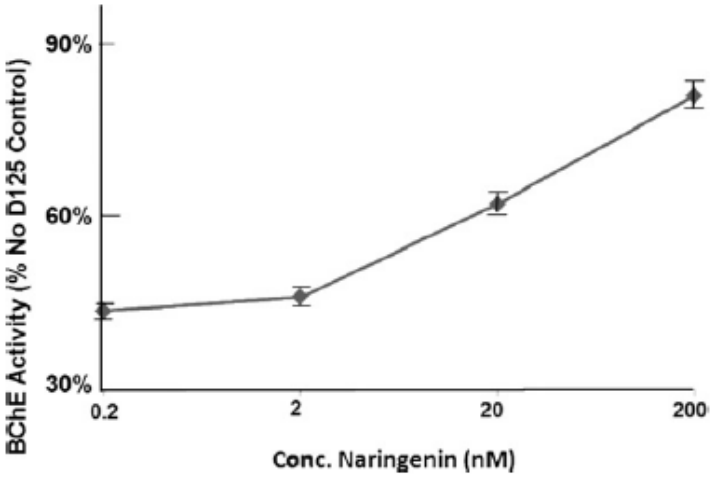


Fig. 3. Concentration dependence of naringenin inhibition of D125 bioactivation *in vitro*. Triplicate samples of RLMs containing NADPH were treated in the following order: (1) naringenin incubation (concentration on *x* axis); (2) incubation with D125 at its IC_{50} level for BChE inhibition, 0.36 μg/ml; (3) incubation with purified human BChE; (4) assay for BChE activity. One triplicate set of assays contained no D125 to serve as a control value. An additional triplicate set of assays contained D125 but no naringenin (not shown). Data points represent percent of the no-D125 control (mean ± SD).

In the case of BChE, it is clear that it is necessary to metabolize TAPs before they become potent inhibitors of activity. We examined the ability of a naturally occurring compound found in grapefruit to block the metabolism of a TAP into a potent inhibitor. As shown, physiological levels of naringenin were able to reduce the conversion of D125 into a potent inhibitor(s) of BChE;

Much more needs to be learned about the *in vivo* effects of inhibiting P450s in preventing the consequences of exposure.

Baker et al. Chemico-Biological Interactions 203 (2013) 257–264

Issues Related to Acquiring and Analyzing Blood Samples

- It is important to establish a chain of custody of samples
- It is best if the samples are analyzed by a CLIA-certified* Lab

*The Clinical Laboratory Improvement Amendments (**CLIA**) regulate laboratory testing and require clinical laboratories to be certificated by their state as well as the Center for Medicare and Medicaid Services (CMS) before they can accept human samples for diagnostic testing. Apr 16, 2014

Summary

- There is abundant evidence that a problem related to fume events exists and is ongoing
- The research described in this presentation is aimed at:
  - Developing protocols for documenting exposure
  - Understanding the physiology of TAP exposures
  - Understanding the basis of variability of sensitivity to exposure
  - Assisting in identifying safer TAP additives
  - Examining possible treatments to prevent consequences of exposure
  - Assisting with the organization of clinical assays

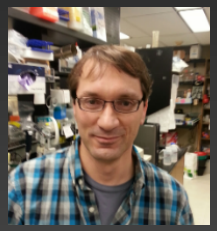
Thank you for your attention

Many thanks for support from:
The pilot and crew unions,
The Royal Australian Air Force and
Early support from NIEHS and

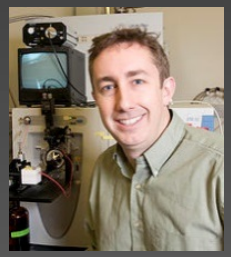
clem@uw.edu

Team Expertise

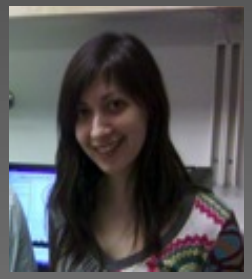
Prof. Allan Rettie
>160 Publications
P450 Expert
Metabolism of small molecules
5%



Dr. Matthew McDonald, Research Scientist III
Mass Spec Expert for small molecules (TAPS)
Chemist, Specialty Organic Synthesis
10 Publications
15%



Prof. Mike MacCoss
Mass Spec Expert for protein adducts
>140 Publications
(no cost to project)Mass Spec Expert for protein adducts



Dr. Judit Marsillach, Acting Lecturer
60 publications
TAP metabolism for generating adducts
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Mass Spectrometric Analysis of Protein Adducts
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