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WORKSHOP ON
PROBLEMS IN CHEMICAL TOXICOLOGY

FINAL
AFOSR-80-0248
June 19-20, 1980

The University of Connecticut
School of Pharmacy
Storrs, CT 06268

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FOREWORD

The bulk of the material which follows was transcribed from the proceedings of a two-day conference on biological aspects of chemical defense. In the meeting, six representatives from the Department of Defense exchanged views on the subject with an equal number of civilian scientists whose scholarly interests touch on various aspects of the overall chemical problem.

The exchange between military and civilian, as well as between the civilians themselves, was both heartening and productive. Attached letters from the military participants speak to the utility of such meetings.

Thanks are due the Air Force Office of Scientific Research, especially Colonel Robert Sigethy, Commander, and Dr. Donald Ulrich, Program Manager, for their interest in and support for the meeting.

Special thanks to Dr. Philip Rosenberg for editing the transcript and to Sybil Rosenberg and Laura Lucier for transcribing and typing.

Topics to be discussed: chemical warfare, chemical defense

Topics: physiological effects, immunology, etc.

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PROBLEMS IN CHEMICAL TOXICOLOGY
THE UNIVERSITY OF CONNECTICUT

A G E N D A

Thursday, June 19th - Morning Session

Opening Remarks

Dr. Richard E. Lindstrom, Pharmaceuticals

DOD Briefing

Major Wallace Deen
Medical Intelligence and Information Agency (U.S. Army)

Lt. Col. Thomas Butler,
Aerospace Medical Division (U.S. Air Force)

Col. Craig Llewellyn,
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Civilian Briefings

Dr. Steven Cohen, Toxicologist
Dr. James Henkel, Medicinal Chemist
Dr. Ezio Giacobini, Neuropharmacologist
Dr. Philip Rosenberg, Toxicologist
Dr. Wolf-Dietrich Dettbarn, Neurophysiologist
Dr. Enrico Mugnaini, Neuromorphologist

Thursday, June 19th - LUNCH - Faculty Alumni Center

Thursday, June 19th - Afternoon

Working session - School of Pharmacy

Thursday, June 19th - Evening - Dinner/Discussion Session

Friday, June 20th - Morning

Working session - School of Pharmacy

Friday, June 20th - LUNCH - Faculty Alumni Center

ABBREVIATIONS AND CHEMICAL FORMULAE

ACHE	Acetylcholinesterase
BUCH	Butyrylcholinesterase
DFP	Dilsopropyl fluorophosphate (Isofluorophate)
GB	Sarin (O-isopropyl methylphosphono-fluoridate)
GD	Soman (O-1,2,2-trimethylpropyl-methylphosphonofluoridate)
Mustard Gas	Bis(2-chloroethyl)sulfide
2-PAM	2-Pyridine aldoxime methiodide
Parathion	Diethyl-p-nitrophenyl monothiophosphate
Paraoxon	Diethyl-p-nitrophenyl phosphate
Phospholine	Echothiophate (2-Mercaptoethyl trimethylammonium iodide)
TEPP	Tetraethyl pyrophosphate
TOCP	TOTP, TCP (Triortho cresyl [Toyl] phosphate)
VS	O-ethyl S-2-diisopropylaminoethyl methylphosphonothiolate

LINDSTROM:

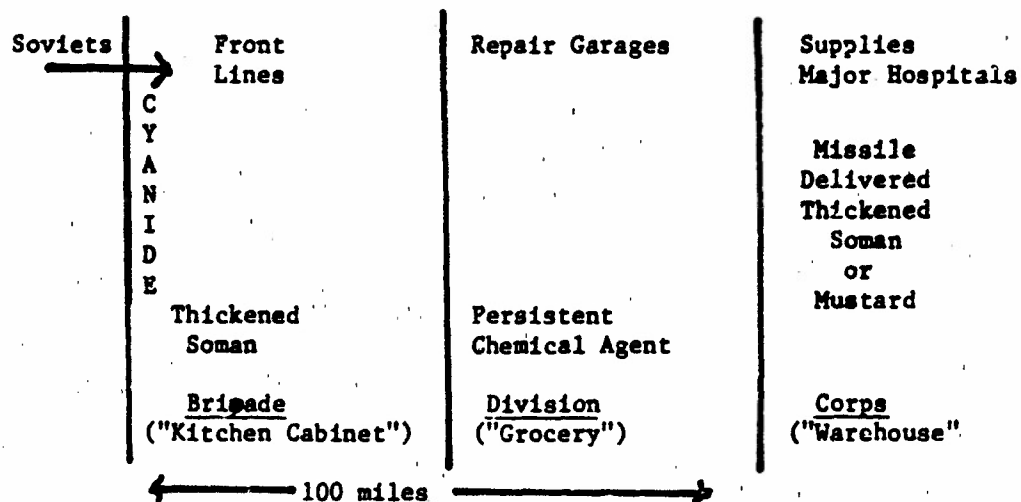
Welcome to this Workshop on Problems in Chemical Toxicology. I am pleased to introduce Major Wallace Deen from the Medical Intelligence and Information Agency, (U.S. Army) who will describe their responsibilities and findings.

DEEN:

The Medical Intelligence and Information Agency has 26 people. In recent years with the chemical warfare threat we are getting resources to try and find out more about what Soviets are doing with chemicals. I would like to review some of the known chemical warfare activities going on. To give you what we have, not as official policy, on the Soviet threat and the way we see the Soviets equipped, how they might employ their weapons and a little bit on the Soviet R and D effort. Just recently, we had four very significant reports of chemical use; use in Laos and Cambodia are well substantiated and in Afghanistan there is increasing evidence that it is being used. The Laos and Cambodian use is very similar to what the way the Egyptians used chemicals in Yemen, and there are some allegations of chemical use in Yemen, that is mustard gas, however, there is no proof of that. In Laos and Cambodia there seems to be three agents that they are using, tear-gas or CS which is non-lethal for most normal and healthy individuals.

Then they are using a nerve agent of unknown origin probably an organophosphate, but that is just a guess. Then a third unknown entity, that causes very rapid hemorrhage of the upper respiratory tract and death in a period of ten minutes to 24 hours. In Afghanistan there has also been reported some use of chemical warfare agents according to the refugees coming out. The military people say, "If I were a Soviet soldier in Afghanistan and wanted to get these people out of the mountains, what is the best way to do it?" The Soviets had a terrible experience in World War I where half of their casualties were from chemicals and they have not forgotten this. Since that time they have had an attachment to gases, and they have maintained some effort in the chemical warfare program ever since. The Soviets are well equipped and most important carry out extensive practice. Every major Soviet base has a chemical warfare training group.

The Soviets do train with live agents. For those of you that are not militarily inclined, let me explain how the Soviets might use chemical attacks in an area with or without nuclear weapons against troops.



We basically have our forward battle positions while militarily we are behind this brigade. If the Soviets were to attack first it would be a surprise and it would be about six o'clock in the morning. What you would most likely see them do is to attack in the brigade area; they are not great on development - they would just hit head on. They have capabilities of putting down a lethal cyanide barrage in about 30 seconds and of holding it there for 30 minutes. They will do this with artillery and rockets. In order to keep you from supplying into the brigade area from the side they would use some persistent agent such as "thickened" Soman or you could use mustard gas just as well. You would probably figure that they would move about 100 kilometers the first day. The big problem you have using persistent chemicals is that if you are successful what you end up with is contamination of your own equipment and zones. You need something that you can get out to do its business and then get rid of it. Cyanide works very good with the Soviets. They can get their people moving, lay down their cyanide barrage, have it do its work and when the first Soviet troops arrive they don't even need their masks. Our people on the other hand are suited with their protective gear and have more difficulty maneuvering. Then back in the area here they would use missile delivered thickened Soman. Basically if you want to talk about a quick agent. If this area has some terrain they do not have any use for. You got a multiple threat you also have a propensity for taking their literature about phosgene. They do not like to fight in a persistent chemical environment. Soviets have spent a great deal of money on chemical reconnaissance teams so that they can delineate this area of persistence and tell their troops to avoid it. It seems to indicate that most of their chemical protection is directed towards getting across these contaminated areas not fighting in them. This is a little different from the U.S. policy, we say we can fight in a contaminated area. We would have no choice.

I see two very interesting things in the Soviet R & D. One of the most fascinating is their open literature reporting all chemical poisoning incidents. They seem to have Toxin decontamination teams to clear out and investigate accidents.

This is one thing they have been able to do to put together a central toxin - monitoring so that they can take advantage of fortuitous accidents civilians suffer. They manage to put it together so they can exploit data from real cases. This is based on about 150 papers this group has published over a 10 year period. They are very conscious of the antidote problem. Apparently Czechoslovakia among the satellites is doing a great deal of work while Rumania is pursuing a more independent path. The Soviets themselves have one set of antidotes for the military and one for the civilians.

The Soviets have been working this chemical effort with the organic phosphorus compound since the end of World War II at high speed. They are terribly conscious of the risks to unprotected troops and they've done all they can to protect their troops. Probably the other point I wouldn't restrict ourselves to looking only at primary threat agents. They have all capability for example to exploit the carbamates. I know we haven't solved the phosphorus problems yet there may be other problems down the road.

DISCUSSION:

But what is the situation if a war should start. Would the Russians start using their war gases immediately. There are a half a dozen different scenarios one of which says that the Russian conditional capability is so good they don't need chemicals. You are looking at a six-day European war from East Germany to the Channel.

Nerve gas is organic to the philosophy of conventional war. Every conventional weapon should be made to work in a nerve gas environment. Out tanks suck air, dust, stones: theirs are positive pressure. Their mobile aircraft artillery are all positive pressure. Tank warfare these days is reduced down to where the first shot wins the engagement. Whoever sees the other tank first then fires is the victor. This means that the tank commander has to travel head up. You don't put the hatch down anymore. Head up on a Russian tank you have enough air flowing by your head that you can survive the chemical attack. An American tank head up you are sucking air past your head, and if you button up of course you lose the tank engagement. In terms of conventional warfare they have been fully equipped for 20 years and I think they are now showing us that they are willing to use this. Why not: - it is a beautiful weapon. Why limit yourself.

A well-debated question is that is the inter-relationship between nuclear, chemical and biological warfare. Are they lined or are they lined? Should war break out, would the use of chemical by either side bring nuclear retaliation or would the use of nuclear warfare lead to chemicals. I think that the question is still open. Both sides in

the Second World War had gases which have not been used widely but they may have been used somewhere else. That is a very complex situation, however. Germany thought we had had nerve gas and never had chemical agents been used against a protected adversary. It has been always used on unprotected. Japan used it beautifully against China in the war against unprotected people. The Russians are fully protected while we are not. A recent Scientific American article claims that our troops are very well protected; for example, that our clothing is better than the Russian clothing. There are a few points not made in the article, about cyanide for instance. Cyanide breaks your mask down so it is rendered useless.

LINDSTROM:

Our next speaker is Lt. Col. Tom Butler. Tom is in the Air Force in the Aerospace Medical Division.

BUTLER:

I want to tell you about the Aerospace Medical Division. There is not a manager in any bureaucratic type agency that doesn't like to have organizational charts and talk about missions. The Aerospace Medical Division is responsible for several basic missions, clinical medicine, education and research. I don't want to lead you to think that the research we do even though we call it medical research is what you think of as medical research. We are not in communicable diseases, we are not developing drugs for use in hospitals. Our work is more man-centered. It is more in capability enhancement and safety, health. How does man work in an environment, as well as how to perform better in a high-performance airplane. That is the type of work that we do. When you are subjected to a certain amount of g forces, vibration, noise, etc., that is the general type of work we do.

The Army is a primary agency for doing the jungle defense type work. The Air Force is concerned that the army does what we want them to do. Secondly, is for us to look at Air Force specific problems. Talking about pilots I want to show you what a pilot wears. A pilot puts on some cotton long johns, that is what he essentially starts off with. The next thing he puts on is a charcoal impregnated undergarment on top of the cotton johns. This is what we call the protective ensemble. The next thing he does is to put on his flight suit. As far as gloves, he has a cotton liner, his rubber gloves and then he has his flight gloves on top on that. You see, he is dealing with three layers all way around; same thing except socks and boots. As far as eye and respiratory protection, he has his mask, then his helmet on top of that and he has a ventilation pack in front. Of course, he has to put on his parachute, his g suit and everything else. You can see what kind of trouble he would have. He might have some trouble with vision and with those layers of gloves he might have a

little trouble working his controls. In high performance airplanes, how well they can do their job is something we are all interested in. Some of the pilots cut the ends of their gloves in order to be able to feel the switches and controls. We are now attempting to come up with our third generation of flight gear. The idea is to come up with lighter weight materials, cut down the layers, cut down the heat, cut down the breathing resistance that the filters and mask gives, and improve their dexterity. So if somebody can come up with better material, better filters, better masks, etc., that is what we need to do. The areas of research we do are broken down into four categories: a) individual protection, b) detection and warning, c) medical, and d) performance. As far as individual protection, these are the areas we are interested in. We need to know when to button up in those suits; we need to know when the area is all clear. We need detectors and all clear devices. We need service life indicators, things like filters.

Detection and Warning:

Need to know when area is all clear - when protection is needed

All clear detectors/Dosimeters
Detection for waterborne agents

Joint Service Requirements:

1. Antidotes and Prophylactics
Effective without being incapacitating
2. Treatment in protective equipment
Drugs and supportive techniques
3. Simulants
Agent effects on aircrew performance and effectiveness of decontamination

Prophylaxis:

Oral Administration and effective for 12-24 hours (Based on time needed for cleanup)

Protection against moderate exposure without therapy

Compatible with therapy

No impact on mission performance

FDA approved

Immunization - permanent protection?

Therapy:

Effective in defined dosage

Minimal incapacity due to therapy

FDA approved

If you can get a pill or shot that is a prophylactic it has got to be one that doesn't interfere with performance in an airplane, or else what good is it. The other is, as far as an antidote goes, therapy is the same kind of criteria. If a pilot has an antidote and gives it to himself and he can't fly the airplane, the antidote is no good.

Here's another problem. For us to use a lot of live agents in the research situation does not make people too happy. We need simulants. There is no one single ideal simulant and we need a whole family.

SIMULANTS:

Needed for:

- Large scale field exercises;
- Crew equipment training;
- Duplication of miosis effect in lab performance studies;
- Tasting effectiveness of decontamination must be
FDA approved and detectable at agent threat levels

We may need one that simulates a thickened agent to learn how to decontaminate and see how effective the decontamination procedures are. So you need a whole family of simulants. Simulants from the Air Force standpoint is our biggest problem right now.

DISCUSSION:

The Army's current doctrine is that at the first sign of incoming artillery you put on the mask. If you are caught in a gas attack the question is do you use your antidote to put yourself out of commission or do you take a chance of having got enough gas to put yourself out of commission. Either way you are at a loss. In the medical area I want to point out another problem. If you take a soldier, if you will, or a pilot and he's in the protective assembly and you have to get him into a hospital to be treated, you've got to decontaminate that individual who may have various lesions. You can see the problem in taking simple things like pulse, blood pressure, checking vital signs, or trying to give him an IV injection. There are numerous problems in that area for us to concern ourselves with. I would like to add a couple of points: 1) The Air Force is not really heartless in caring only for pilots; I want to point out that the mass of people are generally the Army's bailiwick. The Air Force can only work on things that are peculiar to them and from the practical standpoint the fallow in all that gets in the air is a dead man. There is no way he could fight. I have a number of hours in technical aircraft; you spend time trying to develop a way to scan every degree available since to look is life. The pilot is in a difficult and complicated situation; he's got a dozen switches on his side stick and must wear the Nomex gloves because of the fire

hazard (Nomex gloves are fire retardant). They cut the fingers out of them because you can't find the switches with the gloves on. So you develop all the five protective gear then you short circuit the gear and lose its effectiveness by cutting all the fingers and increasing visibility on you do all kinds of things.

Keep in mind that the Russian personnel carrier is behind their soldiers and can get in there clean and he can ride in that thing through anything he wants to and we can't, because of the positive pressure which they have in their motorized units. It's not easy to change now that we've got billions of dollars worth of tanks around that suck up gravel into them. The Russians would go through the contaminated areas and then fight beyond it; who wants to fight in a contaminated area.

LINDSTROM:

I am now pleased to call on Dr. Clyde Replogle of the Aerospace Medical Research Lab who will continue this discussion.

REPLOGLE:

As a matter of fact in our analysis any pilot who goes into treatment we write off as a casualty of war and as far as I am concerned that is losing the war. We are interested in how a pilot does his job. The army definition of a casualty is maybe a guy who can't dig a fox hole anymore while the Air Force definition of a casualty may be a pilot flying at 100 feet off the deck at 250 knots and having to roll it over to lose altitude because he really doesn't want to bounce the top. That is an entirely different job. Our concepts are entirely different but what I am interested in are those sort of things related to air operations. What happens when you do put the chemical gear on, of course, and how do you equate dose vs. protection? If I am going to say I had a design factor of 10^4 , 10^5 , or whatever, I get less dose exposure but more encumbrance. Is there an optimization procedure that I can go through? When I talk about Air Force tailored antidotes what I mean is let's let us worry about morbidity more than mortality. If you give a premedication that ends up with a lot of very soft stool you can end up losing the war. A lot of our work is in visual performance, for obvious reasons.

Pretreatments:

TAB - Bad side effects

2-PAM - Must be injected subconjunctively to counteract atropine

TMB-4 - Taken subcutaneously it may be quite toxic.

These are some problems which I think I've already spoken of. We've been using TAB as an antidote. TAB is, is an oxime plus Benactazine which is an anticholinesterase protectant and atropine

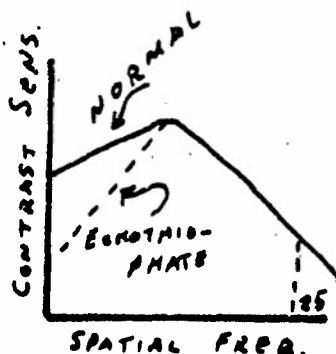
TMB-4 is the oxime. Once you've taken one of those then your going to have someone sitting next to you on the floor trying to convince you that the world is really alright and you are going to come out of the antidote. But you certainly are not going to fly the aircraft. The next generation of antidotes is unknown but it is being designed by the Army and will be accepted by the Air Force and it won't work. It may save a few lives but when they wake up they're going to be on the wrong side of the forward edge of the battle area. The Russians will be running the hospitals and they certainly won't be flying commissions in support of the Army, who developed the antidote. We are the only laboratory in the country that is actually dosing humans with organophosphorous compounds, and making measurements. Somebody mentioned that we might do something like stick 2-PAM on the eyes. You would actually have to use a syringe and stick it through your eye because 2-PAM won't penetrate the cornea. If you try to take enough oxime to counteract miosis systemically, your going to kill yourself. One of our biggest problems is this one: everybody says, "Good heavens what we've got to do is protect man from nerve gas," and you don't have to. Just protect the man a sufficient degree from nerve gas. A little spot of thickened Soman on your arm isn't going to hurt you; just wash it off. A little bit of Sarin vapor doesn't hurt you; just don't stay out in the rain, come in, take a shower and get the stuff off. But don't go to a physician and get treated because you'll be out of the war. Now, if you get a big dose go to a physician and get treated because you'll be out of the war either way.

Now we can't trade off this cumbersome gear that you've seen which was designed with a magic number in mind that was done on the back of an envelope in 1973. Nobody knows whether this degree of protection is necessary or not. In order to say whether it is necessary you have to say o.k. here are the effects of the ensemble, but how do you measure the effects of the ensemble? You do not measure the effects by rectal temperature, you measure them by mission effectiveness, because you're going to have to compare it to the effect of the agent and if you measure the rectal temperature for the agent you won't get anything. So you measure the mission effectiveness, how well do I fly an airplane, deliver ordinance and get back. What is my attrition rate, what is my kill rate. Given a dose of Serin, Soman or whatever, how well can I perform in the protective ensemble and where is the optimal designs point. For the last four years that I know of the Army has never submitted a protocol to do any human work because there is a stigma attached to doing a human research with drugs, certainly in the military. So we looked for a mimetic. In the work we did we used Phospholine iodine (Echothiophate). The reasons we used it was not because I thought it was a good mimetic, although its structure is very close to VX. It is a charged molecule and therefore doesn't penetrate the eye very well, but it was in current use in treatment of glaucoma. So I thought I could get approval to place Echothiophate in the pilot's eyes a lot faster than anything else. Sure enough I got approval so I put it in the eyes. Is it a good mimetic? I don't know. Up until the time we did our experimentation everybody

thought that the only visual problems with the organophosphorus agents was cycloplegia headaches, myosis. Why? Because it was the only thing they looked for. The best thing to use I think, as a mimetic is DFP. This was in common use for glaucoma before echothiophate came on the market. The only reasons it is not used now is because it is not water soluble and it doesn't have the shelf-life, so it has to be put out in oil. Then you get a lot more tearing and it is more difficult to measure eye pressure and so forth. Sarin went through clinical trials for glaucoma. It is a perfectly decent drug. Sarin is probably the safest thing we could use right now as a mimetic, but because it is a deadly nerve gas you would probably have to use DFP which is nearly as toxic as Sarin. All the tests of vision of interest were done in the 50's at Edgewood and at the chemical defense establishment at The United Kingdom. What they did was the standard optometric examinations.

Visual Function

Contrast sensitivity function - contrast varies sinusodally - cycles per degree increases with increasing distance. Get a spatial frequency.



peak of human response is 1-2 cycles/degree

Others testing chemicals on vision have done it above 25 cycles/degree

Twofold decrease in sensitivity at peak of vision.

Lose low frequency spatial vision.

I introduced those of you that don't know about contrast sensitivity functions or modulation transfer functions to the concepts very quickly. You look at a contrast grating whose, in terms, contrast varies sinusoidally. I can measure that gratings contrast in terms of cycles per degree and its amplitude of course in the ratio of the contrast. As I back away from the blackboard for instance the cycles/per degree increases, you've got to understand that or I'm not going to go any further. As I come closer the number of cycles in the particular angle goes down and as I back away it goes up. So I could draw little tiny, tiny marks and I'd have a very high spatial frequency, or very broad marks and I'd have very low spatial frequency.

Now, if I take contrast sensitivity versus spatial frequency that is I show you a whole bunch of these things where the bars are just barely visible that I increase the contrast between the background

and the bars until you can see them and I measure your contrast sensitivity versus spatial frequency it looks like this in humans. Now, in a good lens it looks like this. If I de-focus that view graph what I am doing is filtering out high frequency. I can use them as a high frequency filtering device. The peak of human response is about 1 to 2 cycles per degree. A Snellens lens chart measures nothing below 25 cycles per degree. If I take a picture of one of your faces and digitize it and filter out everything above 25 cycles per degree and put it back together again, you would not see any difference at all. Every important thing you do with your visual system occurs below 25 cycles per degree. Everything that was measured by anybody testing chemicals on vision was done above 25 cycles per degree. Now, if you want to change the correction for refraction in an eye you can do it any place you want, and it is very convenient to do it out here. So it works out optometrically, but it doesn't help you understand visual function. Visual function all happens below 25 cycles. So what do we find when we put echothiophate in the eyes. At high spatial frequencies we did not get any problem at all, remember measuring out here the British said there is no problem at all. All they saw was miosis. But we saw that right at the peak of vision there was a two-fold drop in contrast sensitivity, which is much more important than miosis. One of the other problems is that everybody says you have only two degrees of good vision. Well, you know that is one of the greatest lies that ever hit the textbook. What you have is two degrees in vision above 25 cycles per degree. By the time you get off to 45 or 50 degrees, you are down to 2 or 3 cycles per degree, which is very good vision out there. It just happens to be low frequency spatial vision. That means, of course, if you are losing low frequency spatial vision, not only are you losing the ability to separate low contrast targets, but you are also losing the ability for perfect processing. Now picture the pilot a hundred feet off the deck trying to fly an airplane not being able to cross his stated course. So we have a bunch of vision people who didn't know anything about chemistry and a bunch of chemists who didn't know about vision. On this slide you see a low contrast aircraft and here is a factor of two. Now if I had not told you that was an airplane and you knew exactly where it was you wouldn't see it. One guy is going to see that and one is not and that is with an extremely low dose of the anticholinesterase. What we are going to do, of course, is we are going to try some DFP. One of the things we like has been its extensive use on normal human eyes. Leopold in 40's and 50's did hundreds of experiments.

(Dettbarn) Have you tried pylocarpine?

With pylocarpine the effects are so fast you can't measure the major effects. Besides that you don't really know where the mechanisms are operating although they are obviously retinal. To go very far from an organophosphorous compound when I know I have an unknown mechanism in play is not appropriate. So what I would like to do is stick to an irreversible organophosphate and at least find out what I am doing. Now, I'm calling DFP a mimetic. DFP is just not a very good chemical agent. It is just not toxic enough to work in bombs and I

wouldn't call it terribly safe. It is the safest there is. It would be nice to have the action if we understood what was going on. Now there was work just finished by the Army using injections of benactazine, and they got the same results on low contrast visual changes (low frequency visual changes) as we saw. So here is a systemic cholinesterase inhibitor giving the same thing.

(Dettbarn) Is it as long lasting?

Well our effects are much longer. We did not see any symptoms at all after a couple of days. We were using doses by the way, that were only hitting one-tenth of our population. You have got to increase the numbers as there is a tremendous idiosyncratic response to organophosphates. We are using very very low doses which is difficult. Organophosphates are picked up, sort of attached to melanin so brown-eyed people don't have difficulties when you put echothiophate in their eyes. Blue-eyed people have all kinds of problems. Every one knows that, except that I did not know it, but it has been in the literature for 30 to 40 years. We find that the red hair, blue-eyed people we have tested (2) out of a population of 60 or 70 people (you don't have too many of them) are very sensitive. What does that mean? I don't know. Just what is the mechanism of action? I will tell you one thing, it's not a combination of spasm and miosis and headaches. That is there all the time but that probably isn't primary in an Air Force mission. If we're going to use not the real thing but a mimetic let's find something that penetrates FP.

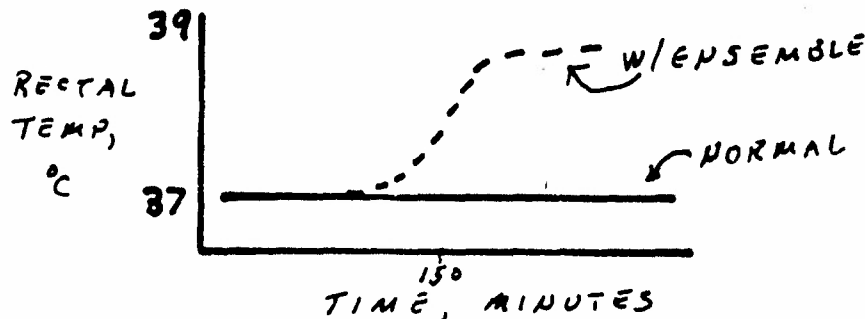
Protective Ensemble - Problems

Thermal burden

Amount of protection afforded

means of quantifying threat - Duration and Amount

How to take oral antidote



I'm just going to go through protective ensemble very quickly because I understand that there probably isn't interest in this group on protective ensemble but we still have a lot of troubles.

(Comment from audience): You know there is an implication about this protective ensemble, particularly in the mask. That is if you have an antidote that is taken orally you have to learn a good trick in order to get your mask off, swallow your tablets and get your mask back on without having a fatal error.

We did this on a centrifuge in a heat chamber that simulated sitting on a runway with a canopy down using the same temperature that we found. We simulated his flight his dropping bombs on the target and coming back and starting on the runway again. You see things like this on rectal temperature; here is an individual that is taking a certain kind of a burden. We also found people whose rectal temperature did not go up at all and came out with class 2 thermal problems being red like lobsters, stopped sweating and about ready to die. We lost one third of our subjects; it didn't kill them. The simulant problem and we've got to understand these suits; these are real tests with real man and in environmental conditions systems. This was a concentration put in the air and this is the cockpit concentration over a period of time. Here is the best simulant but you can see it goes the wrong way at the wrong time as contrasted to observations with man. One is going up while one is going down. Nothing we have for a simulant to test is any good and this is the finest one. Col. Butler mentioned methyl salicylate (oil of wintergreen) and it's the worst one. Everything we know about the efficacy of the equipment comes from these simulants; so we know nothing, absolutely nothing! If anybody thinks that you can measure rectal temperature or sweat rates or dehydration or just anything that you might think of and predict what primary task performance is you've got a thrill coming, because you can't do it. I wouldn't care about correlation with some physiological measure as long as I could predict primary task performance given some environment and we've gone to the extent of measuring brain tissues, CO₂ or brain tissue O₂, etc., to try to get at some of these problems. The other problem is that the primary task performance turns out to be the world's worst thing to measure. You give a pilot the directions that he is to go out and bomb the target and you suit him up in this stuff and you set him on the runway with a cockpit temperature 55°. You leave him there for a while and you let him soak that and then you take him off and he goes to some altitude where in some aircraft they actually spit ice crystals out of the air-conditioning systems. Then you have him go in for low level bombs while he's got this nice greenhouse over the top of him and he starts cooking in there and he delivers his bombs and comes back. You'll find that those bombs are on target every bit as well as in non-protective gear. He does his primary task just as well, until he dies. What he does is, for instance, tries to put up his landing gear while he's on the runway or he accidentally flies through his own ricochet patterns or gains two or three feet in altitude without knowing it or his air speed varies a couple of hundred knots when he doesn't expect it, a couple of hundred knots and these people are supposed to meet across at a certain point at exactly within fractions of seconds. They do everything wrong. All the secondary rules fall apart but you say make sure that you've got the bombs on target. So what happens usually, and as a matter of fact we have a contract with David Kleinman, at this University, to do

human control analysis. What they do, is give a very simple task to do and they find out that the stress doesn't affect it at all. Well, it doesn't, but if you had this myriad of cockpit tasks to perform you'd find out that all kinds of things would be falling apart that are extremely important to the overall mission. And we don't understand how to do it. That's all the problems I have. We have a program by the way that runs out to 1986 at the moment which the headquarters have only seen it to the 85's and they haven't got that yet, but we're talking about a baseline something like 9.9 million dollars. This is not a small problem and the basic research required is horrendous and we're not doing it and we don't intend to do it. We intend to brute force system answers so that we can get something back to the Air Force right now but we won't understand the basic processes.

(Comment) It's common belief that the Department of Defense can do research, can develop various agents and things of that nature and not have to worry about anything else but just do what we think is necessary. I'd like to point out that we have to have almost everything FDA approved and if we're using humans it has to go through very strict standards set up by HEW. Talking about methyl salicylate, how long has that been around? I mean how many years has it been prescribed? Yet we can't use it in a research and had to go through human use and it was ungodly to get that thing approved. I'm almost afraid if we developed another chemical warfare agent we would have to get it FDA approved.

(Comment, Cohen) I must have missed something, because when you were showing that bit about the cockpit, I don't understand why you can't just use the agent if you're just looking at penetration of a uniform.

They obviously used the agent and I just showed the comparisons. But that could only be done one place in the country and it has to be done inside of a chamber in Dugway proving ground and to pull an airplane into a chamber is a very interesting thing. The point I want to make is not on environmental conditioning systems in aircraft because one can actually pull the environmental condition systems from the aircraft and that is a "snake" that is 30 or 40 feet long and costs maybe \$200,000 to take one out and put it back in again. I'm talking about the efficacy of the suit. How long does a suit work? I don't mean a guy standing like this in a salt fog. I mean in combat conditions do you expect a ratio of 10^4 to 1, inside and out, or do you expect it to leak like a sieve? How are you going to do that without putting a man in there? Now we just put this stuff out (it was put out on the back of an envelope) saying we need 10^4 to 1 ratios and no one really knows whether it is optimal or sub-optimal. Now we've got it out and no one really knows how good it is. So I've got to take real live people. I have to do real live things including pulling G in an aircraft and I've got to subject them to real things. It's not only to get through it, but I have to be able to detect it. If he takes it up I have to be able to measure it. I have to know what dose he got of it. If I come out of my aircraft, I obviously have to hydrate myself, I might even have to go to the bathroom. So I go in and I take off all this stuff and then I put on something new, or something old. How much do I pick up? You also have to remember that for thirty days or so these doses are cumulative, so a little bit today and a little bit the next day and a little tomorrow. You've got to have the data, you've got to have a simulant.

(Comment - Rosenberg) But the problem as you've described it sounds almost insoluble. The type of simulant required but you're not looking at it by the performance of one organ system, you're looking at the performance of the entire individual.

No, this simulant, all I've got to do is measure its concentration in the person, I want to know how much of it got into the person from the outside, I don't care what it does to the guy. As a matter of fact, theoretically I'd want something that does nothing at all. All I want is a tracer, but I want it to act like the agent, that is, stick to things the way the agent does, track off of it the way the agent does, stick to the guy and go through, penetrate his clothing, the way the agent does. So I get vapor pressures and I can show all the vapor pressures and everything for all the various agents and thickened agents, but it's got to act physically not physiologically like the agent. That's what I call a simulant not a mimetic. All that I want the mimetic to do is do the important things physiologically and as soon as we know what those are we are going to be in good shape. But you know at least we've uncovered one a couple of years ago. Nobody bothered, however, and what you've got to do is to stick something into people or animals if you have that kind of measure. Now you can measure contrast sensitivity functions in animals, by using cortico-evoked potentials.

Research needed:

- New protective materials
- Means of measuring amount of dosage
- Means of detecting agents at distance
- Decontaminating agent - safe to equipment
- Good non-physiological simulant
- Correlate physiological stress with performance
(stress affects secondary performances)

Lindstrom - The academic colleagues will now give some background on themselves and describe their research interests.

Cohen - My background in the area of toxicology began at Harvard School of Public Health where I got my PhD training with Sheldon Murphy and my PhD work involved the studies of organophosphates but specifically insecticides and the role of esterases in toxicologic interactions of organic phosphates. My primary interest since I started has been the effect of toxic chemicals, trying to get into mechanisms where possible and principally focussing on how one agent in the environment can affect the toxicity of others, how environmental chemicals can effect the toxicity of drugs. The focus of most of the research that I have been doing since I have been here has been on the role not so much of cholinesterases which we were talking about earlier, but on other tissue esterases, even the pseudocholinesterases which is the non-critical one. The cholinesterases that are important for nerve tissue functions are called true cholinesterases. There are many other cholinesterases that fall in the category of pseudocholinesterases and you can pretty much inhibit these completely without any apparent adverse

effects on animals or humans as long as the nerve tissue cholinesterase does not drop below some critical level. That seems to vary with the compound and the species. What we have been able to do over the years is show that these other tissue esterases, since they can be inhibited by phosphorus compounds basically serve as a detoxification site, a place where phosphates can go where they can do no harm. They go to these sites and they are bound up where they are inactive. The problem with that is that exposure to low levels of phosphates, not sufficient to cause any signs or symptoms tie up these sites and it takes time for these sites to be regenerated. So subsequent exposure to another innocuous dose of a phosphate can put an individual in a situation where now they are faced with a situation where they have a decreased capacity to tie up these compounds in a harmless fashion. Their places of inactivation are lost to a degree, and we can show that in experimental animals certainly, and other wildlife, that if one inhibits these tissue esterases and then comes back with an organophosphate at a supposedly non toxic dose, you now have a crisis situation in the animal, greatly enhanced activity. Now this is not a dramatic earth-shattering finding from the point of view of metabolism, because if you block pathways of inactivation you are going to increase the amount of the material to higher than therapeutic levels. If you are talking about drugs you are going to get into toxicity, and with agents which are not therapeutic agents, such as most of the phosphates we have talked about which are insecticides, as soon as you build up to levels higher than those that can be tolerated you get into toxicity. Over the years our work has shown that for certain phosphates these esterases serve a very important role as sites of inactivation while for others they don't. We think that the reason they don't for the others is that for these so-called others there are other means by which they are inactivated. They may be hydrolyzed by plasma hydrolases that may not be the same as these other esterases. They may be metabolized by oxidative pathways. The areas that are most interesting to me, from the point of view of metabolism and structure activity relationships, are what is it about a compound that makes it more likely to go via an oxidative pathway or by an esterase binding pathway, etc. There has been relatively little from the point of studying structures and understanding what is involved in terms of reactivity of phosphates with these different tissue esterases or whether or not the phosphate compound may go through another pathway.

Another aspect of the work that I have been involved with in the phosphate area is in how organic phosphates alter drug action and metabolism, etc. Most of the work we have done in this area has been with pharmacological and therapeutic agents which are esters. So it makes sense that if you block esterases and if esterases are important from the point of view of drugs such as local anesthetics and if you block their metabolism you are going to alter their action and alter their toxicity. From the approaches we use in our research we have looked at what one of my colleagues used to call stuff them and count them toxicology looking at acute LD50 determinations to the other side of things where we get more sophisticated and do biochemical and pharmacological measurements of the effects of the agent. We also get into some kinetics studies of plasma transport (pharmacokinetics), how the drug moves in the body, so we not only do biochemical enzymatic determinations in my laboratory, but we also do GC analysis of plasma for drugs and so on. We are also identifying the drugs and showing the blood levels in the tissues and showing that the phosphate pretreatments can alter the levels and most importantly can alter levels under conditions where there is no toxicity of the phosphate per se.

Question: There are reports that oxims are poor for central nervous system protection because it cannot cross the blood brain barrier and then people found out as long as you had organophosphates present which were crossing the blood brain barrier that the oxims did also. So there is some suggestion that organophosphates were changing the modes of action of oximes.

Rosenberg: I guess I could say something on that because I was probably the first one working on this and to show that in fact under the influence of paraoxon there are sufficient amounts of PAM penetrating the blood brain barrier to reactivate the brain enzymes. It has been shown for some organophosphates, for example physostigmine decreases time for onset of sleep with barbituates. Organophosphates can apparently decrease the blood brain barrier and we would expect a decreased time for onset of anesthesia.

Dettbarn: On the other hand the anticholinesterases can get you out of sleep. If you are in a diazepam or alcoholic coma, physostigmine will wake you up at concentrations which cause no obvious cholinergic symptoms. They give 2 mg to old men so they do something in addition.

Cohen: There are many problems that have to be sorted out. You see elevated blood levels of procaine and while we didn't measure the brain levels of procaine in Japan they have done that and they show elevated levels of procaine in many tissues under the influence of tricresyl phosphate which is a neuropathy inducing organophosphate. Part of that is probably due to inefficient procaine esterase. In addition it also may be related to altered permeability; that is something that certainly should be looked at. Other areas that are in my own research interests involve environmental chemicals and drug metabolism. We are doing a study right now on the effects of polychlorinated biphenyls, etc. Of more interest, instead of cranking out another paper that says polychlorinated biphenyls induce microsomal enzyme, we are using them as tools to understand the importance of metabolism for the drug. It is easy to pump another agent through the mill and say here - here is another paper that shows it induces. It is much more important to find out the importance or relative importance of metabolism with certain of these agents because then you can start to understand interactions and predict them. I also look at inhibitors, and the classic one is SKF525A, the blocker of microsomal enzymes. I am more interested again from the pesticide background which makes me look to things like piperonyl butoxide which is the agent in Raid. The same way we make the pyrethrum more toxic, to insects those agents make many drugs more toxic to animals and humans. If a drug has to be activated to be therapeutically active a blockade of activation could neutralize them. If on the other hand, you are dealing with polychlorinated compounds which are all around us, you may increase levels of active metabolite if the drug has to go through an activation step. So there is really a lot to look at in terms of activation-inactivation pathways in drug metabolism and assessing their importance and of course their ability to affect different species even when you stay with mammals. Some of the work I did in the paper I published with Sheldon Murphy was on the comparative study of organophosphate interaction in frogs, two different species of fish, quail, compared to mice and subsequently to rats and that was an esterase dependent interaction. We found interestingly that one could increase the toxicity greatly of things like malathion, the common garden insecticide by pretreating with tricresyl phosphates and increased toxicity in mice, in birds and the species of fish we used and in the frogs. This has a lot of environment wildlife implications when you use a combination of insecticide, because insecticides are good if they kill insects and don't kill everything else in the environment. From the point

of view of selectivity if you increase effectiveness in killing insects and lose selectivity you have a very serious problem. What we found was very interesting, and that was that the relationship between esterase inhibition and increased toxicity of malathion, held for mammals and fish but not for frogs. We could not detect carboxyl esterases in frogs at all and yet the toxicity of malathion for frogs was increased more by the tricresyl phosphate than it was increased in any of the other species. So there is a whole world of interactions that still remain to be explored in terms of phosphate potentiation. We did things like assess in vitro sensitivity of their cholinesterases to malaoxon and we found no difference in in vitro sensitivity so it was not an alteration in the cholinesterase enzyme. We however have not studied penetrability, distribution, etc. in frogs. There are a lot of ways of enhancing organophosphate activity. Probably some of the earliest work on the interaction related to these non-specific binding sites, the distribution of Sarin changed greatly using EPN. Fleischer, etc. injected animals with EPN and challenged them with Sarin, by inhalation and showed that there was much less Sarin in the lung and much more in the brain with greater toxicity in the animals that had been exposed previously. That was probably some of the earliest work on interaction with insecticides which are in the same family as Sarin and in many respects they behave similarly. Two other areas interested me, one is the role of glutathione as an endogenous protective agent against endogenous chemical damage. Many agents react with glutathione and tie it up and in so doing they are themselves inactivated. Probably the most widely advertised example of that right now is Tylenol Acetaminophen. They say it's safer than aspirin and then say that anything in large doses is toxic even aspirin. The problem is that psychologists say that when people take Tylenol for suicide they do it because they don't really want to kill themselves; they want to ring a bell and let everyone know that they have a problem. So they take 100 Tylenol tablets because they know it's safe and then two days later their liver is gone. And this is all a reactive intermediate formed from the Tylenol and it's all related to excessive active metabolite which wipes out the glutathione stores in the body and the body's ability to inactivate the material.

The next step in acetaminophen pathway is to this reactive metabolite which normally would go to these nucleophiles. If they are not there it goes to these macromolecules in the liver then we have a great big black box. Inside that black box is a whole world that may some day be opened up. What I am interested in, in the environmental drug area is that many organic phosphates are inactivated by a glutathione dependent pathway. We are currently looking at organophosphate metabolism. Certain compounds more frequently undergo this glutathione dependent pathway and we are looking at that and its possible effect upon agents like acetaminophene. One would predict, and we have yet to bear this out, that organophosphate under certain conditions could potentiate or result in increased toxicity. The most immediate concern for this kind of interaction involves really a very descriptive part of the research which is to try and define whether any of these agents are capable of priming an individual for liver toxicity under conditions where there is no acute poisoning. That is the key. If an individual is severely poisoned with organophosphate you are not probably not going to load him up with Tylenol anyway. The first signs in many cases of exposure to the phosphates for an individual may only be a little headache, but maybe that comes from the solvent, some of the carriers and of course headache is Tylenol. That is an area that I see that should be explored.

I've got one student that is working on a cyanide project and it relates to something I came across with a former grad student. I came across something in the journals that didn't make any sense to me; it said that chlorpromazine (thorazine) protected animals against cyanide and it made no sense to me and it was a very descriptive piece of science. The cyanide antidotes which I am sure you are familiar with are the nitrite thiosulfates. Chlorpromazine was only effective in combination with thiosulfate or thiosulfate plus nitrite. It was not effective alone, hardly changing the LD50 of cyanide. It was not very effective with nitrite, but when you gave thiosulfate as a pretreatment and chlorpromazine as well, there was a dramatic increase. Let's say that the LD50 was around 7 to 11 milligrams per kilo, it went up to around 60. Now the thiosulfate alone increased the LD50 to around 20. So we're talking about a threefold advantage over thiosulfate alone. From your perspective the last thing you want is to have everyone flaked out on Thorazine so there has to be a lot more to this than that because they were fairly good doses of chlorpromazine sufficient to lower body temperature, but we have explored body temperature and find no correlation where the body temperature reaches its low point and protective nature. Because of the fact that interaction relates very closely to the thiosulfates, we have looked at blood thiocyanate in animals after chlorpromazine and thiosulfate challenged the cyanide versus no chlorpromazine. Then we see an elevated thiocyanate which suggests, what one would predict, that you are generating more thiocyanate which is a non-toxic or less toxic metabolite, and perhaps sparing target enzymes. One of my students right now has been working this past semester on the experimental protocol to measure rhodanase which is the enzyme that turns the cyanide thiocyanate. What we want to do is find a biochemical effect we can measure to see if we could come up with what is the mechanism. The people who published the earliest reports on this are going merrily along and trying other agents. I have never seen any reports on their probing mechanisms which is the way to go to design a better agent. We have some ideas in mind as to say is this peculiar to chlorpromazine or do all the phenothiazines do it. If they do, what is it about the phenothiazine molecule that makes it do that. Can we design one that doesn't have a depressant action. In my way of thinking the two are probably unrelated. Most of my work revolves around phosphates, but the cyanide project has been kind of dribbling along for a couple of years, with undergraduate students. When we saw the elevated thiocyanate levels I said now it is time to put a little more effort in the project.

Lindstrom: I will now call on Jim Henkel

Henkel: I am a medicinal chemist and I ought to take a minute or two to explain what medicinal chemistry is. We find that people outside schools of pharmacy don't always know what it is. My background, as you've probably seen on my CV is in organic chemistry. My undergraduate and graduate degrees are both in organic chemistry and following the organic chemistry degrees, I went on and did a post doc in the area of medicinal chemistry. Medicinal chemistry in essence is the use of organic chemistry to probe biological systems or drug effects or effects in some biological or physiological organism. It can mean a lot of things to a lot of people depending on one's research interests. Incorporated in medicinal chemistry are things like metabolism, from the point of view of the chemistry that goes on. Not so much the enzymes that are there, but rather what changes can we make in a molecule to either increase or decrease a particular pathway or a particular effect. Also in medicinal chemistry is the design of better ther-

peutic agents. We can look, with the aid of pharmacology, toxicology and pharmacokinetics, at the effects of a specific change in structure on a given therapeutic response or biological response. By looking at this in a quantitative way we can often predict what would be a better agent, one that might have better CNS penetrability, less toxicity, less side effects, etc. and we have done some of that. I don't have the extensive experience that most everyone else has, I haven't been in the business that long. Specifically the areas that I'm establishing at the moment are the areas of anti-cancer activity and also some other agents that are directed toward the central nervous system. Even in the cancer area we are looking specifically at the effects of some agents that are designed to penetrate the central nervous system. Looking at where it impinges on this area may be the fact that the agents that we are using are alkylating agents, the nitrogen mustards. We are building them into some rather complex frameworks that might give us some indication of particular modes of cross linking and different degrees of toxicity, cytotoxicity vs. specific anti-cancer activity.

There's a lot of parallel between alkylating agents in general and the agent that we don't understand what it is.

(Comment) - There is a tremendous hemorrhagic response that's like aerosolized Russell's viper venom.

As I say, our ultimate goal, where we get our money, is to design more and better effective agents. It might first give us some idea of how things are working and we can often draw inferences by looking at the structure activity relationships in a series of drugs and we can say that well, when you increase this portion of the molecule and make it bigger and change its electronic properties, change its steric properties, it either increases the therapeutic effect, decreases its side effect, has more or less potency. These are things we should look at. We can look at it both intuitively and quantitatively, specifically using some regression analysis programs, quantitative things like that. Some of the contributions we could make, or medicinal chemists could make to a conference like this might be to provide some chemical insight. I think that probably a medicinal chemist has a little bit of a unique perspective in that basically we are still chemists and we are still thinking of chemicals and pictures as opposed to code names. If we look at a structure of a molecule, we can often see things saying well, what happens if we change something over here, or this area looks like it might be very easily metabolized or easily converted and perhaps we can contribute in that way.

Lindstrom: I will now call on Dr. Giacobini

Dr. Giacobini: I would just like to divide my presentation in two parts. The first will just tell about what I am interested in and then I have some specific questions which will contribute to a discussion later. I am here as a neuropharmacologist. I have been working on cholinergic systems since 1953. My work started in Sweden at the Karolinska Institute at the time the Swedes were setting their chemical defense laboratory in Stockholm and I was collaborating with Dr. Holmstedt who is part of the chemical defense laboratory. I came to the United States to this University about eight years ago. I have collaborative work in a published paper that I am still keeping in contact with Dr. Heilbronn who is now the head of the neurochemistry section of the Chemical Defense Laboratory. Now, my work has been since 1953 when I published my first paper on cholinesterase.

Mainly I have been using anticholinesterase agents as tools to ask questions to the nervous system. In the beginning we were very interested to look at the selectivity of the anticholinesterases so as to be able to subdivide several types of cholinesterases. My actual work in the cholinergic system is centered around the cholinergic synapse and during the last ten years I have been working on how is the cholinergic synapse assembled during development and eventually how does a cholinergic synapse decay and evolve during the life of the animal. Now that brings me to four different specific mechanisms. One is how acetylcholine is manufactured and turns over in the synapse. The second is how it is released and how it acts on the receptors. Coming back to drugs again we have been interested to look at how cholinergic blocking agents when introduced into an organism at early stages of development, I am talking of an embryonic state or a very early embryonic state or even from day zero, how would they prevent or change the development of the cholinergic synapse itself. For instance, what kind of information do receptors store and what is the interaction of the receptors with the other part of the cholinergic synapse. Finally I have been interested in seeing whether by understanding more about the different types of molecular forms of cholinesterases we were able to detect particular forms which were more selectively related to the neural activity, that is the neurotransmission process. As to my personal particular curriculum, I would just like to add that I have been associated with the department of pharmacology at the Karolinska Institute for about ten years. I also had three years of experience in the drug industry and I came into a very lucky phase because I was a witness to the development of new and better B-adrenergic blocking agents. This was at a major northeastern European drug company, that is ASTRA and I was in one of their research groups. That is where I came in contact with medicinal chemistry which I did not know before and now I am very glad that we have medicinal chemistry.

One particular aspect which might be interesting to you is that I have been using as a model, during the last ten years, of the cholinergic system the eye and therefore I am particularly interested in cholinergic innervation of the eye. We have been using a system in which we have a group of cells which innervate the muscle of the eye. I am not saying the retina so I'm not interested in or working with the visual aspects of the eye, but rather in the cholinergic innervation of the main parts of the eye.

Well, then, let me just touch on how I could see my contribution to today's discussion. My major interest is in basic research and I am sure that you are all aware of the basic steps of what we have been talking about here today. The work that is most interesting to you now for instance the problem of inactivation of cholinesterases, was due to some very outstanding work by Wilson and others. One breakthrough is enough so that we can work for another 20 years. Now I have worked on the cholinergic system for more than 25 years and I'm almost historic like Rosenberg and Dettbarn. What really strikes me is that if I had to give a lecture today to students in an advanced course on anticholinesterases I would not be able to say exactly what the anticholinesterases do and the reason is that we are touching on mechanisms and some aspects of cholinergic metabolism that we don't know about. So there are some very specific questions to be asked which I think are of much importance, also to practical aspects because until we resolve these questions it will be very difficult to understand how things happen. One aspect is of course how much of the anti-

cholinesterase is due to the cholinergic mechanism and how much is due to mechanisms. Just a month ago I went to the fourth meeting on cholinergic systems and there I met several of my Russian colleagues with whom I have maintained a friendly cooperation and dialogue for many years and it appeared to me that for them and other people working on the cholinergic system there is a great deal of attention paid to the muscarinic aspects, which I think are those mostly related to the central nervous system. It seems that up to now we have known very little on the muscarinic system up to the last five years. I don't think that it is an accident that some of the major articles on the muscarinic receptor have as authors workers from the Unit of Biochemistry, National Defense Research Institute in Norway, by Defense Laboratories in Stockholm, and by Russian people. Now it is totally natural, the front is moving towards that line and major contributions are to be expected. Therefore in the afternoon if we have time I would like to entertain some discussions with regard to the new frontier in cholinergic mechanisms. Then I would like to address some specific questions about our knowledge of anticholinesterases in regard to what we know now of the cholinergic system.

Lindstrom: Phil Rosenberg, it's your turn.

Rosenberg: My training is really in the area of pharmacology but that's not a mistake by my name that is toxinologist, not toxicologist. I'm interested in animal toxins and venoms so I'm an editor of Toxicon, a Journal concerned with animal, plant and microbial toxins. To put things in perspective I might mention that there are animal toxins, 100-fold as potent as the most potent organophosphate. So just hope that the Russians don't develop an effective means of delivery for it. We might want to get back to some of these animal toxins also later on.

About the first eleven years of my scientific life anyway, we were concerned pretty much exclusively with organophosphates. I got my PhD under Coon at Jefferson Medical College. He had done some of the early secret work in Chicago. Tox Labs with Dubois and Doull so I was working there on the toxicity of some organophosphates looking for antidotes for organophosphates. I probably did some of the earliest studies on interaction between organophosphates showing that when you combine EPN and malathion the toxicity is much greater than you would expect from either agent alone and showing that this interaction is occurring at the level of the liver due to altered metabolism. From this work with Coon, I then went on my Career Development Award post-doc to Columbia University where I was working with Nachmansohn and who at that time of course was working on all that exciting work with Wilson on development of PAM and the oximes. During the next ten years I was working at Columbia with a group, in fact with Wolf Dettbarn. I guess the first study I did there was in fact the penetration of PAM into the brain and its ability to protect rabbits against paraoxone poisoning. We studied how to accurately measure brain cholinesterases activity. Although PAM does indeed penetrate the brain very poorly one can get sufficient amounts into the brain for it to have a protective effect. So it isn't that it zooms into the brain. It's lipid, insoluble and quaternary. Especially under the influence of an organophosphate you can get enough into the brain to exert protection of CNS cholinesterase. In addition to that work then I also worked on nerve conduction and essentiality of cholinesterase for axonal conduction, that

is what is the lowest level of cholinesterase activity which axons can continue functioning with. In the absence of cholinesterase activity there are other mechanisms to remove acetylcholine. Wolf and I did a lot of work concerned with the anticholinesterases effects on nerve conduction, axonal and junctional conduction. We also did some work with glaucoma, looking for blood levels in patients with glaucoma. We studied side effects with animals and studied effects and the penetration of different anticholinesterases following applications into the eyes in animals and relating this to blood levels which might be associated with human use. At that time we had learned some of the benefits that nature had created in animals. Nachmansohn always used to say that some animals are just created for the benefit of scientists, such things like the electric eel and squid giant axons. I may want to get back on the subject this afternoon of some of the advantages of using some of these types of preparations in working with organophosphates and to try to figure out some of the mechanisms of actions. Well at that time about when I came here my interests were somewhat changing toward trying to understand nerves and muscle membrane structure and trying to see what selective modifications into chemistry of the nerve would have on their sensitivity to drugs and permeability properties and functioning of the nerves. So then I became interested in animal toxins and venoms and especially when I found a phospholipase A which is present in all snake venoms and almost all animal venoms. I found that this had a marked effect on the permeability properties of nerves. I therefore thought that it would be interesting to use these enzymes, these phospholipases, to produce quantitatively known and specific changes in phospholipids of the nerve membrane to see what selective modification of phospholipids would have on nerve function. So some of the studies I've been involved in the past ten years or so, for example have been concerned with injecting phospholipases intraventricularly into the brain and observing then that at certain levels of phospholipid hydrolysis in certain areas of the brain one can obtain long lasting repetitive convulsive episodes, where the animal will go through convulsions, recover, go through convulsions again over a period of many hours. This may possibly be related to clinical conditions such as epilepsy or convulsive disorders where lysosomes might be ruptured within cells and lysosomes have phospholipase activity naturally. So there are endogenous phospholipases.

We're using the snake venom phospholipase as a tool to mimic what might be happening physiologically. Another study that we have been concerned about is trying to study what is the environment of the sodium channel site within a nerve or muscles membrane. Specifically we used muscles with either a sodium generated spike or calcium generated spike, that is muscles in which sodium is responsible for bio-electricity or calcium is. There has been a report for example that phospholipase C, which is another phospholipase, which acts at a specific point in the phospholipid molecule, only blocks conduction in muscles with a sodium generated spike. This is similar to tetrodotoxin, puffer fish poison, which only affects muscles and nerves that have sodium generated spikes. Basically what we were able to conclude from studies on measuring extent of hydrolysis is that two specific phospholipids, phosphatidyl serine and phosphatidyl inositol, seem to be very closely associated with the sodium and calcium pore in the membrane both in sodium and calcium muscles, however phospholipase C can only hydrolyze

these phospholipids in the sodium muscles and this explains why it cannot act on calcium ion muscles. Therefore we are trying to understand a little about muscle functioning and the muscle membrane. Some other studies have been concerned with gammaaminobutyric acid receptor within the brain. By isolating the pinched-off nerve endings, the so called synaptosomes, we've been able to show that GABA and a phospholipid phosphatidyl ethanolamine compete for binding to the GABA receptor. We can specifically then alter the potency of the neurotransmitter by incubation with the phospholipid for example and alter the ability to bind to its receptor protein. So phospholipids, although not often thought of as receptors for drugs, can drastically modify the functioning of receptors including possibly the cholinergic enzymes and receptor. Most recently I've been working on a comparison on toxic and non-toxic phospholipases. It's very dramatic that you can isolate a phospholipase from *Naja nigricollis* venom and it will be extremely toxic and isolate another phospholipase A, the same type of enzyme from *Hemachatus haemachatus* venom and it will be relatively non-toxic. Yet the non-toxic one has more enzymatic activity than the toxic one, if you measure activity in vitro. So we're doing a combined pharmacological and biochemical study to try to explain this difference. We are using phrenic nerve diaphragm, isolation heart and intraventricular injection to measure the extent of hydrolysis of each individual phospholipid. We also measure their activities on purified phospholipids singly and in combination to see if we then can explain why one is more toxic than the other one. We are also producing specific molecular modification of the enzyme, altering a histidine which is at the active site of the enzyme and seeing how that effects toxicity or enzyme activity. Trying then to relate the structure to the activity. the phospholipase from *N-nigricollis* venom is a very potent hemolytic agent and a very potent anticoagulant agent.

How does this all relate back to the organophosphate and I think that the point that it relates to is that I am very interested at this point in going back and combining my two interests - membrane structure and organophosphates, because I don't believe that all the effects of the organophosphates can be explained on the basis of their inhibition of cholinesterase enzymes.

<u>Effects - Expected</u>	<u>Expected Effects</u>	<u>Effects Expected or Different than Expected</u>
Subacute and chronic tolerance Receptor? Metabolism?	Acute effects	Delayed neuropathy Muscle necrosis Teratogenesis Metabolic effects Analgesic effects Visual disturbances EEG effects Behavioral changes Depression Memory Loss Decreased Vigilance Increased irritability, etc.

It's true for some years one was interested in the acute effects of these agents and many of the acute effects can be explained specifically by levels of inhibitions of cholinesterases. However, when you get away from the simple acute effects you come to areas which are not explained, so that for example, you have effects which are less than expected on the basis of cholinesterase inhibition mainly subacute and chronic tolerance and then you have effects greater than expected on the basis of cholinesterases inhibition.

With continued administration one finds that whatever effect your measuring, conditioned avoidance of response or whatever other physiological parameter, you find the effect disappears even though the cholinesterase level remains very low. This might be at the receptor level the receptor becomes less sensitive or it might be due to metabolism of the cholinergic transmitter. It is an effect that might be interesting to look at to see what's happening to the membrane. Effects greater than expected include many things which I think might come up this afternoon. We have this delayed neuropathy which many organophosphates cause after ten to twenty days. Suddenly the sciatic nerve starts to degenerate and it's not due to the inhibition of cholinesterase. Neurotoxic esterases might be involved. You also have what Dettbarn I'm sure will be discussing in a moment, muscle necrosis which would not necessarily be predicted. There have been reports of teratogenic effects and there have been reports of metabolic effects. I'm not going to say much about them, but it may serve as a basis for some discussion this afternoon. There is uncoupling of oxidative phosphorylation at levels which don't inhibit cholinesterase at all. At very low levels of certain agents which are not inhibitory for cholinesterases, there have been claims of long-lasting visual disturbances. I had an occasion to review the clinical literature because of consulting I was doing with a patient who was poisoned by a pesticide. I had this occasion to go back and find out that there is a lot in the literature on effects not correlated with cholinesterase inhibition. So we have visual disturbance and analgesic effects. There is interest lately in the ability of organophosphates to liberate enkephalins to produce analgesia. We have EEG effects including long-lasting EEG effects, for example a year after the last exposure, after a single exposure to organophosphates. This is to a great extent the work of Duffy at Harvard. What he did actually with persons poisoned with sarin at the Rocky Mountain Arsenal where an individual in disposing of sarin had gotten accidentally exposed. They found that EEG effects including increased beta activity, increased REM sleep which I find fascinating because there are very few things that increase REM sleep except LSD or growing older. There are also many long-lasting behavioral effects including decreased vigilance, depression, memory loss, learning disability, even a claim that Parkinsonism is induced by organophosphate.

Comment: Have you seen the work on the prolonged QT syndrome? Recently a lot of people, poisoned by parathion was the drug that they were using. Parathion has become a rage in suicides in Israel, I don't know why. You know that you treat a person and they get better, then they die and it turns out that if you pace them for a week or so that they will be all right. This is a prolonged QT syndrome associated with all kinds of really threatening arrhythmia. And this lasts for a long time after you might have expected the cholinesterases to return to normal. A person is declared well and discharged and dies. These long-lasting effects must be

associated with permanent or semi-permanent biochemical or structural changes. I would like to look at the membranes following the organophosphates and really see whether there are any structural changes in the phospholipids. I just might mention that there has been one report that organophosphates markedly increase the phospholipid content and decrease cholesterol contents way after cholinesterase is returned to normal. They found permanent structural alterations in the nerve membrane.

Dettbarn: My first association with organophosphates and acetylcholinesterase was a little later than each of yours. I was exposed to cholinesterase in Nachmansohn's lab where we studied the role of esterases in excitation, and the use of organophosphates as tools. It became very apparent in our studies that the organophosphates have additional effects; they don't only inhibit cholinesterases. They also affect ionophores or what is called the ionic conductance modulator, the pore in the membrane that is being controlled for instance by the acetylcholine receptor. They affect permeability changes in the axon membrane by a mechanism we don't know yet. We became aware of the fact that some organophosphates were detoxified rapidly in the animal, DFP for instance. We have done studies on squid with Dr. Hoskin where he injected a variety of organophosphates into the eyeball of squid and then measured LD50 and it turned out that you have to go up in this particular animal with DFP to 1500 mg in order to get an LD50. The squid has a specific enzyme that hydrolyzes phosphorus compounds and DFP is one of them. This enzyme is apparently also involved in sulphur metabolism such as taurin and some of the other sulphur containing compounds. Due to the work with glaucoma, we became interested in adaptation to prolonged subacute or small amounts of organophosphates. We used rats as models and looked at adaptation mechanism and the inhibitors we used were direct acting ones such as paraoxon or DFP, things that don't have to be activated. To make the story short there is no adaptation to the organophosphates, the animal apparently adapts to low cholinesterase levels and the mechanism by which it does that are two. One is presynaptic because the synthesis as well as the release of acetylcholine is modified and at the postsynaptic side the receptor becomes desensitized. Now one has to be careful because there is a difference between as you pointed out, the muscarinic receptor in the CNS and the nicotinic receptor in the neuromuscular junction. The nicotinic receptor apparently does not become desensitized because if you give over a long period of time the organophosphate at a concentration where it induces symptoms every time you give a shot the muscles become partially denervated. You lose a number of innervations and the muscle itself increases its sensitivity to acetylcholine. It suddenly has a spread of nicotinic receptor sites and you can show this by labelling them with a bungarotoxin which is very specific for nicotinic sites. So in the periphery there are apparently different mechanisms as compared to what is going on in the brain. But there are two mechanisms - one, presynaptic, change in synthesis, change in release, and the other one is postsynaptic - the muscarinic receptor developing a subsensitivity which has been measured with QNB binding. We looked at the recovery of acetylcholinesterases after organophosphates as a way of determining for instance half life and turnover time of the enzyme. It turns out that whatever organophosphate you are using whether it is the recovery time differs being the least for the diisopropyl as compared to the dimethyl or diethyl but the reaction with dimethyl occurs up to 50%

and then you see de novo synthesis the enzyme is restored spontaneously I would say up to 50% and then depending on the inhibitor the levels of spontaneous reactivation varies. You get de novo synthesis taking over. So it takes about 14 days to three weeks to get all the acetyl cholinesterases, depending on the preparations. Apparently in the retina are a few isozymes of acetylcholinesterase that have a very short half life. We are talking on that about the work of Agranoff and Davis who showed that these isozymes of cholinesterases have a half life of three hours. There is a very rapid de novo synthesis in the eye. The function of this isozyme is not known, whether it is the critically functional form of acetylcholinesterase or whether it is one of the other ones we don't know. The next endeavor arose when we became aware of a report in the literature which described necrosis in skeletal muscle.

We studied the mechanism and found that whichever organophosphate you used or whichever carbamylating inhibitor we used whether its physostigmine or neostigmine they all induced muscle fiber necrosis. We looked at the pictures and all the nerve terminals show changes, changes of hyperactivity, the vesicular population goes down, and you get mitochondrial changes. The nerve terminal membrane breaks up, you find an increased number of omega shaped vesicles that are vesicles fused with the membrane and you find vesicles in the synaptic cleft and you get greater changes on the post synaptic side. The secondary infoldings decrease, they become broader and you lose receptor sites on that membrane. And then within 30 minutes of your dose of paraoxon you see these changes already developing. If you wait longer than 6 hours you get muscle fiber necrosis which mainly is focal necrosis and begins in areas under the end plate. You can prevent all this by giving two 2-PAM within up to 60 minutes of the organophosphates in the nerve terminal as well as the muscle end plate. You can also prevent it by giving curare which blocks the nicotinic receptor at the end plate. You can prevent it by giving bungarotoxin which also is a specific inhibitor of the nicotinic receptor and you can also prevent it by pretreating the animal with bolulinum toxin, the toxin blocks the release of acetylcholine, the evoked release under normal conditions. If you pretreat an animal with local injections of bolulinum toxin into the muscles and then wait for a week or so, and then treat the animal with the organophosphates you will not get the lesion in the muscle fiber. You can also prevent the lesion in the muscle fiber by denervating the muscle prior to giving your organophosphate. That leads us to the fact that the organophosphate induces hyperactivity in the nerve fiber which we can record by measuring antidromic firing. We measure also the increased release of acetylcholine in the form of miniature end plate potentials which are focally recorded from the postsynaptic end plate with microelectrodes. You observe increased evoked potentials so that leads us to the conclusion that the organophosphate due to cholinesterase inhibition are inducing hyperactivity in the muscle. The muscle loses its metabolic reserves so that within two hours the muscle loses almost all of its glycogen, 50% of its ATP and has lost some creatine phosphate. We speculate that due to the loss of these metabolic energy supplies you get an accumulation of free calcium ion in the sarcoplasm which cannot be sequestered by the calcium pump into the sarcoplasmic reticulum. This calcium accumulates and is all being taken up by the mitochondria and you can show localization of calcium in mitochondria and that subsequently leads to uncoupling of oxidative phosphorylation.

The whole muscle is going to run down. The picture is almost the same as in malignant hyperthermia, the loss of energy the accumulation of calcium and the focal necrosis. You can get the same thing for instance in vitro if you give cholinergic agonists such as carbamylcholine, and they will induce similar lesions. You find the first changes in the Z band of the muscle fibers you get a "smearing". The hypothesis is that most of the calcium activated proteinases are localized and they are being activated due to the hyperactivity toxicity, and initiate breakdown of the muscle fiber.

COMMENTS: Some junctional effects sound similar to myasthenia gravis. Right. There is supposed to be less receptors widening of the gap, the infoldings become less. The initial steps are very much alike. You said that all the motor end plates are affected not 5% or something like that?

No, all motor end plates are affected but not all of the muscle fibers associated with the end plate show the necrosis and breakdown. It's unknown why. I think that because then denervation comes in, the organophosphates denervate the muscle fiber and therefore it does not progress. That's the same how the muscle fiber adapts to the necrotic agent because you can prolong for three or four days the organophosphate and you get an increase in the number of lesions per muscle, but if you continue to give the organophosphate no new lesions appear and even under the influence of the organophosphate the muscle will repair. Only after three weeks of intermission between the organophosphate you can induce again the lesions in the muscle fiber. I think that denervation is followed by reinnervation. We have a group of sensitive muscle fibers or motor units.

We have studied protective mechanisms; I mentioned a few of them. We have also given reversible inhibitors such as physostigmine and neostigmine which are carbamylating agents in a concentration so that they do not cause symptoms and they protect the acetyl cholinesterase against the organophosphates and we are not getting lesions induced by the organophosphates. It was described in the early 40's that the reversible inhibitors protect against the organophosphates. You cannot do it the other way around; if you give the organophosphate first it's over.

COMMENTS: Which is more effective physostigmine or neostigmine? This is very interesting; is this trick used at all?

Yes, in fact, the British use it all the time. The toxicity that you are talking about with the mechanism you are talking about doesn't look like a direct lytic action on the cell. It's just mediated by the enormous release of acetylcholine.

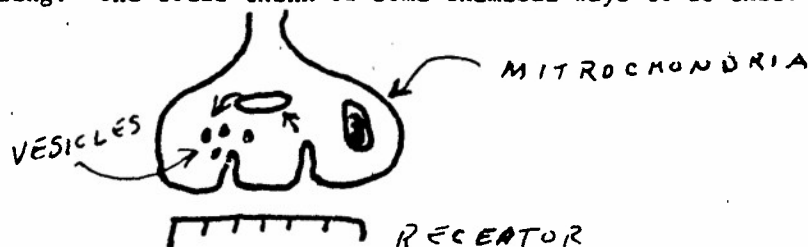
Then we have done some dietary manipulations of the animal. We have given choline which is a precursor of acetylcholine. This work was triggered by Wurtman's studies on precursors of transmitters. We couldn't repeat his initial work that a normal animal being fed a hypercholine diet increases acetylcholine levels in the brain. However, if you put your animal on a low choline diet to begin with and then

supplement with choline, you get your acetylcholine levels up. Atropine is known to lower acetylcholine levels in the brain, the mechanism of which is still under discussion, but if you have a choline rich diet the animal is protected against the effect of the atropine. Atropine action is modified by the presence of choline and it's not a direct offset of choline on the post synaptic side. The acetylcholine levels are not up but somehow there is a reserve of choline so that the acetylcholine that is being depleted or released by the atropine is rapidly replenished. You can induce REM sleep with physostigmine in doses which are very small. This early work done by Desmedt seems to imply that it's not the acetylcholinesterase but it's the butyryl cholinesterase which seems to have a central role in the arousal system. Since physostigmine is very unspecific and inhibits almost equally powerful butyryl and acetylcholinesterase, we used isompa which is very specific for butyrylcholinesterase. It does exactly what a physostigmine does. When the butyrylcholinesterase is inhibited to 70% and the acetylcholinesterase is not touched at all. You can get this arousal, you can induce the REM sleep, you can get them out of a diazepam coma, you can get them out of hexobarbital sleep, you can get them out of alcohol coma.

Mugnaini: My laboratory consists of four units - one is the electronmicroscopic unit, the other is the freeze fracture unit which we use for the splitting of membranes and looking at the distribution of the proteins at specialized sites and the unspecialized membrane regions - then we have a neurohistology and neurohistochemistry unit where we do various reactions. One of the enzyme descriptions we look at is acetylcholinesterase. Now we have entered also the field of immunocytochemistry. We are looking at GAD and its possible analogy with brain CST. The other unit is a tissue culture unit which has not been activated yet. In connection with the EM unit we have a stereometric unit where we can do very sophisticated quantitative analysis from light or electron micrographs. In terms of specific projects which may be related with people in this group we think that one of the best cholinesterase methods for looking at central neurons. It is essentially the classic method, but it is accompanied by a few tricks which we have learned during all these years. I think we get about the best pictures around and we can produce full results not only of neuropil arborization of cholinergic fibers but also of cell bodies. In this respect for instance we have seen that some of the aminergic cells in the brain stem which may be related to EEG activities and sleep are acetylcholinesterases positive. We have looked at the distribution of other acetylcholinesterase neurons in specific systems. We have done a lot of work on structure of myelin and structure of nerves and we are involved in the morphological studies of the same systems which Dr. Giacobini talked about; the accommodation system of the chick. We are looking at ciliary muscle, iris muscle, the ciliary ganglion and the motor neurons of the ciliary ganglion. So, in a sense we are looking all the time at the development and maintenance of nervous tissues and I emphasize the "s" because by looking at various systems we hope to have a better understanding for the range of the repertoire displayed by the brain and we have developed a specific technology for dealing with special circuits like the cerebellar cortex of olfactory bulbs or vestibular system or the acoustical system. So now we can attack various problems using the expertise which we have acquired. I think that we can detect very early changes in the myelin structure,

we can detect early changes in specific ganglion cells and synapses. We can detect very early changes in the CNS. We can offer expertise in the analysis of single circuits and single neurons so that our work could be used in two main lines; one in trying to develop animal model systems by dissecting out a piece of circuit in the brain and looking at what is the effect on that specific circuit of these agents or anti-agent. The other line is using these specific systems as a bioassay mechanism. I give you a very simple example, you are now developing an antibody against GAD which synthesizes GABA. Our knowledge of the cerebellum very quickly permits us to assess whether or not these antibodies are pure or not. That is we are using the cerebellar cortex as a bioassay system.

The acoustic system is one of the only elements in the brain which sends fibers to the periphery. This is the efferent vestibular and acoustic system. There is evidence that it is a cholinergic system. We can identify these cells. We know in at least six or seven animal species where they are, we know the number exactly on each side and where they project. We could use this system for looking at specific effects on individual neurons. The importance of this general research approach is not to a specific chemical but to the general area where these things are active. Now, by looking at what we know about synaptic mechanism one can foresee some development in the fields that you mentioned as needed. Here is a postsynaptic membrane with the receptor and here the synaptic vesicles which release transmitter systems. Paraoxin depletes the terminals, so it is an accelerator of this physiological mechanism. One could think of something which counteracts this effect as an anti-depletor. I know a thing which would be terrible to have is something which prevents the recycling. And actually I think Bungarotoxin seems to block the retrieval of the membrane, the recycling. One could think this process could be blocked at the level of the sarcoplasmic reticulum. And obviously another way of counteracting these depleting systems would be of accelerating the recycling mechanism. We know that temperature is one of the ways. If you cool a motor end plate preparation what happens is that you can still deplete but you don't get recycling. One could think of some chemical ways to do this.



Comment: You have this in the myasthenic patient, they show different symptoms depending on the season and also if you put the myasthenic in a bath you obtain an effect which is related to the temperature. You can increase the symptoms by using temperature.

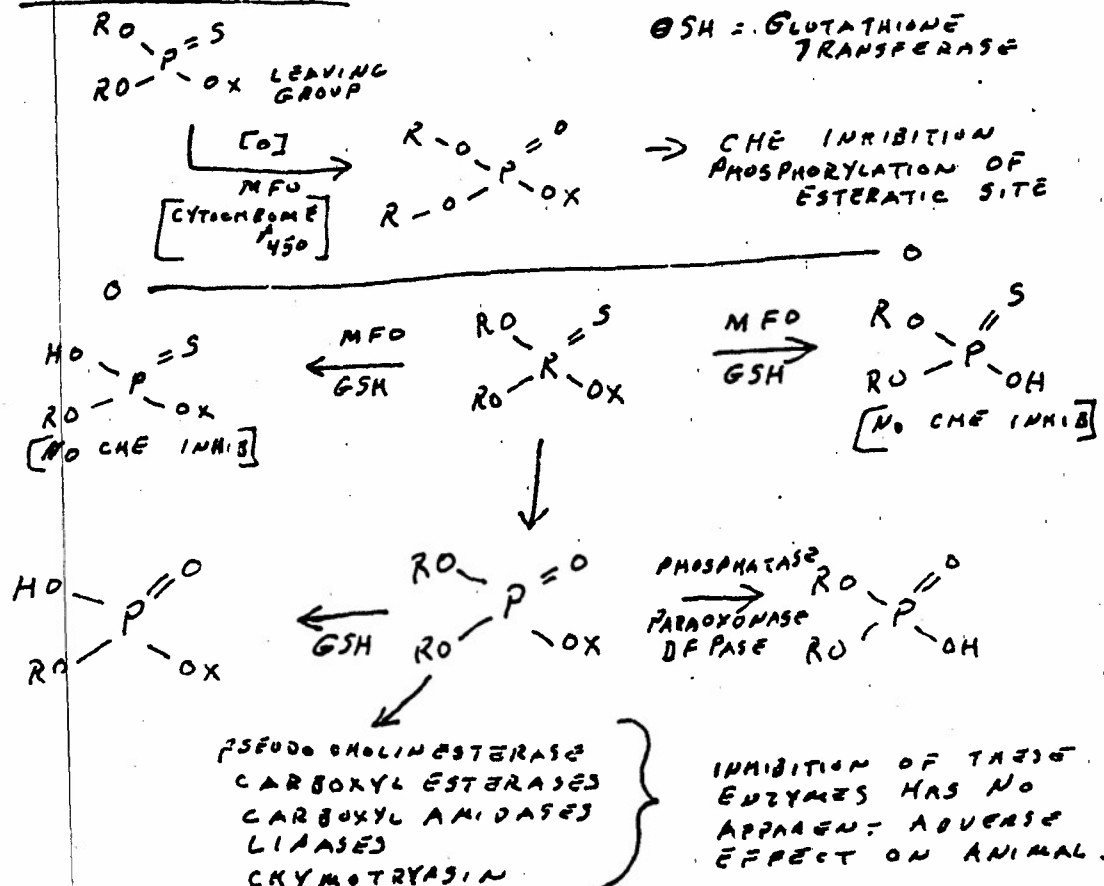
Yes, here we have like a small cell, an organelle assembly which is more or less independent of the cell body and you have all of these substances which are acting at different levels.

Comment - You know there has been a lot of work done with B bungarotoxin on synaptosomes showing inhibition of active transport of sodium and choline. B bungarotoxin seems to block the retrieval of the membrane. But it also causes release of acetylcholine prior to block.

Lindstrom: What I would like to see happen over the balance of the afternoon and certainly tomorrow morning is to get into a discussion as organized as possible. The thing that comes to mind now would be the organophosphates as a focal point to begin with. Certainly it's out of my field and I would ask that as the discussions progress, that those of you who are actively involved in the discussion keep some focus so that we keep heading in the right direction. There are obviously a multitude of problems that can be addressed. In this short time available I would suggest that as a beginning we look at organophosphates. Steve Cohen had put a diagram up the other day relating to the fate of organophosphates within the body which might serve as a jumping off point of some of the problems.

Cohen:

ORGANOPHOSPHATES



What I will try to do is go through very generalized schemes of organophosphates with emphasis on the ones I'm familiar with which are the insecticides. Now most of the insecticides fall into this general structure. Most are thiophosphates and the reason for the thiol group is to give them some stability so that when sprayed they don't break down as rapidly. The major thing that happens to them is they undergo an oxidative step. What would happen is you get the same molecule but in the oxone form. Just to look at the structures of these molecules what I've given as an example would be equivalent to the so-called leaving group of the phosphate molecule. And that is when the organophosphate interacts with cholinesterase, with the target enzyme, the target enzyme changes the organophosphate the way it changes the substrate acetylcholine. Normally you get acetate. Acetylcholine gives you the acetylated enzyme and choline coming off as the leaving group. Well in this case the X or leaving group if we're talking about parathion would be P-nitrophenol. The leaving group comes off and in case of parathion you can pick up p-nitrophenol in the urine of people that are exposed to parathion. These two alkyl groups can also be cleaved off but that reaction is not part of the cholinesterase interaction. There is a component of cholinesterase and phosphate interaction known as aging which perhaps we will get into in a few minutes and that is where one of these alkyl groups would come off. Generally, what happens is that an organism would be exposed to the thiophosphate. Under the influence of a family of enzymes known as mixed function oxidase systems, most of you have probably heard of cytochrome P450, the drug which oxidizes metabolizing enzyme system, this reaction occurs. We go from a parathion to a paraoxon molecule. This material interacts directly with the cholinesterase to cause inhibition of the cholinesterase plus phosphorylation of the esteratic site. It's at this step that we see the cholinergic symptoms as acetylcholine accumulate. If you administer paraoxon, if it can get to the enzyme, you will get poisoning sooner. and the LD₅₀ of paraoxon is going to be lower than that of parathion. Because it gets to the enzyme it doesn't have to undergo this activation step.

Now in addition to this route to toxicity and this is all of the toxicities related to cholinesterase inhibition, in addition to this there are other pathways that these molecules can go. One of them is this one that is also a mixed function oxidase pathway, involves an oxidative removal of this leaving group to give you this. There is some evidence and I confirmed it is not just in a chemist book, but I found it in the biochemistry of insecticide metabolism book that glutathione transferases can participate in removal of that group. Likewise we can have an oxidative metabolism or a glutathione dependent metabolism to give us a dealkylation so that we can have a molecule in which one of the alkyl groups is removed. Once you do this or once an organism does this it takes this molecule in either direction it is no longer capable of being converted to an active cholinesterase inhibitor. There is not another pathway that gives you a corresponding oxone.

Comment: But for all we know some of these metabolites could be responsible for other actions which are not related to cholinesterase.

That's very possible.

Is there any toxicity with these other metabolites?

I don't know of work in which animals have been dosed with these substances. I know that these materials in vitro don't react with cholinesterase. So these are inactivation pathways. In order to interact with cholinesterases you have to have the oxone and the leaving group and depending on the nature of those alkyl groups you can greatly change the reactivity of these cholinesterases.

Did you find anymore in terms of the substrate specificity of those pathways?

It has been studied but not that greatly. John Cassida is the medicinal chemist of organophosphates. He's done a lot of structure activity studies. In fact I think that he wrote one of the chapters in Wilkinson's Book.

Seems to me that the methyl groups are the most vulnerable to that.

That's right. Methyl substituents are more easily removed than longer chains substituents out here. What you get is something like this if that were methyl or ethyl you get a methyl glutathion. Those types of metabolites you can identify. The same kind of a thing occurs here but this one is a phosphatase or a hydrolase. With paraoxon it is called paraoxonase, DFP would be DFPase. This is a phosphatase enzyme whose importance in the intact animal varies greatly with the organophosphate. It seems to play a very important role with agents like vapona. Vapona is dichlorvos, the no-pest strip. Now vapona happens to exist in this form, it's an oxone already and is an active cholinesterase inhibitor. One of the reasons it is considered relatively safe is because it is a super substrate for "vaponaase". The leaving group is rapidly removed by plasma phosphatases. Now for paraoxon-parathion this thing probably is not very important. It exists. You can demonstrate it in vitro. In vitro the K_m 's are such that you will probably have to go to such high levels of paraoxon in the animals, that the animals will be long dead before the enzyme starts to turn over that substrate. So there is a demonstrable enzyme in the system but it probably doesn't play a very important role.

Question: Does it have a natural function?

I don't know.

It wasn't probably made for that kind of reaction.

It may have a natural function.

The other arrow here is for the things that I was talking about earlier this morning and that is in the category of other esterases. The other esterases I will talk about include the pseudo or butyryl cholinesterase, the carboxyl esterases and these are different enzymes. Biochemically you can describe them based on sensitivity to inhibitors based on optimum K_m 's etc. They are different enzymes the carboxylesterases and carboxylamidases which may be one and the same enzyme depending on what species or what tissues they come from. Certain lipases and you could even add on chymotrypsin and a number of other things that are capable of being inhibited by organophosphates. Now for the most part inhibition for these enzymes has very little if any biological effects that can be observed. It can be detected biochemically that the enzymes are inhibited. The animal

seems fine. Theoretically doses low enough to inhibit these but not inhibit critical nerve tissue cholinesterase can be tolerated by animals for fairly long periods of time with no apparent adverse effects. I have no idea whether any one has looked at some of the more subtle things.

Question: Can you inhibit 95% of butyrylcholinesterase without acute symptoms?

Sure, animals walk around and people walk around with it, that is why from the occupational health point of view what you monitor in pesticide work is principally the pseudo cholinesterase and also red cholinesterase cell. Red cell enzyme is tougher to measure and because it is so variable in the population, there is a real widespread variability. Normally, people that work with phosphates and every one of us that work with phosphates theoretically should have a normal background cholinesterase level determined and you should be monitored periodically. Occupational health requirements are that an individual whose cholinesterase plunges 25% down from its normal, whatever that is, that person should be put on another job and taken away from the phosphates. Some people say use whole blood and if you read the OSHA criteria document it varies from phosphate to phosphate. I don't think there is any rationale behind it. It is a conservative thing, if it feels you continue to monitor it, because it can be affected by other things.

Comment: The cholinesterases or butyrylcholinesterases is more interesting because it interacts with lots of other compounds. You also have some genetic defects where patients have no serum cholinesterase.

You are not talking about the atypicals are you? Yes, I am. About one in every 3,000 people. They have cholinesterase, but it is different. The k_ms are all different from the general population. Those are the people that if you give them succinylcholine when they go in for surgery, they get succinylcholine toxicity.

Red cell is a good monitor of nerve tissue cholinesterase. We have done this on rabbits just giving as eyedrops picrotoxin and diethyldithiocarbamate. Red blood cell enzyme is down to almost 20% before you even see changes in the tissue which is 40% - 50%. Okay, what I am saying is from a symptomatology point of view they are not good indicators. OSHA has them in there as conservative indicators that have been exposed, that is all. It is a biochemical indicator of exposures. The first help will be a study of the relationship between these and this we are talking about nerve tissue cholinesterases to show that that is a safe kind of market you are looking for.

Comment: The Army attempted to analyze all threat effects on the basis of blood cholinesterase. So they would take the number of missiles coming in and their fall, meteorological conditions, etc. and they would hang everything on plasma cholinesterase saying take that and do something with it.

Oh it is almost impossible to correlate even brain cholinesterase with symptomatology. If they wanted to use red blood cell is better at least it is the right esterase. The red blood cells at least have the acetyl cholinesterase which the plasma doesn't have.

In an experimental situation if you know what the individuals' cholinesterase levels are then you can determine how much has gotten into him. I am sure you can titrate an individual's cholinesterase given individual variations in fact before poisoning and since a lot of people don't have their levels. There is a very broad range that is used. If you look in the handbook for treating phosphate poisoning they say here is the range using so and so's method. This number of units to this number of units is a so-called normal range and if the individual is down below that initiate therapy. That is a wide range.

Dettbarn: Our rabbits were down to 20%, but they never showed any symptoms.

Rosenberg: I think everyone is agreed that you can have marked depression of cholinesterase even in the brain before you get any symptoms. I mean a 50% depression. You can even take them slowly down to 90% inhibition.

Dettbarn: You don't even get initial symptoms of tremor. It depends on the speed with which you take them down. Presynaptic adaptation occurs.

Cohen: I have seen it in our labs in rats and mice; I will go along very well with that. I have been working with phosphates since '67 and I can look at an animal and say that animal's got to be between 80 and 100% inhibited. But by then he's salivating, tremors, etc.

Replogle: One of our difficulties is that we deal with physicians, none of whom have ever heard of organophosphates except in passing somewhere at school. They know what a synapse is but most of most of them have been out of school long enough that they really don't understand modern views of what synapse means and they pass on our protocols. So for instance we have to draw blood every 15 or 20 minutes from every single subject to measure plasma cholinesterases levels.

Rosenberg: So you are saying, Steve, that if we increase all these other binding sites we could decrease toxicity.

Cohen: Yes, you are right. From the point of view of organophosphate it's important. The thing to be aware of is that there are agents which will stimulate or inhibit any of these pathways. The one thing I started on when we got into pseudo cholinesterases is that all of these enzymes over in that circle to your right represent places where the organophosphate can go. In many cases the organophosphates are capable of inhibiting these, the ISO's or at least equivalent to that for the cholinesterase and if you are talking about any kind of exposure other than direct into the nervous system, the opportunity for the organophosphate to bind to these even if their affinity is identical is going to be very high because the exposure is going to be there. It is going to be absorbed through the skin or by inhalation or ingestion. There are esterases in the skin, in the blood, in the liver and in all of the tissues in addition to the critical cholinesterases and every one of these enzyme molecules is capable of gobbing up a molecule of phosphate. We have been able to show in some of our research on interactions that if you block these you

greatly shift where the phosphate can go. The last study that we did was reported at the toxicology meetings in Washington and was a study in which we really got into looking at several tissues, titrating animals with doses of combinations of phosphates. We showed a progression of movement of organophosphates as indicated by inhibition of enzymes. If we give the compound intraperitoneally see what appears to be a saturation of liver esterases and as we go a little higher in the dose we see ever greater inhibition of plasma cholinesterases, then ultimately the cholinesterase of the red cell and brain and you get toxicity.

Replogle: What sort of agent would you use if to wipe out these other esterases if you want to double or triple organophosphates in the blood. So you could think of tying up these other esterases surreptitiously.

Cohen: If you really wanted subtly to wipe out a situation you could come in with a dose of a phosphate that causes no symptoms, wipe out all of these sites and then come in with a dose of another or same agent. These sites are now gone so that a much lesser or perhaps even non-detectable amount will have only here to go.

Rosenberg: What would be some of the most specific compounds in wiping out these enzymes? For pseudocholinesterase ISO-OMPA can be used.

Cohen: I don't know for every case, but TOCP is a beautiful inhibitor for carboxylesterases and amidases. In a rat doses are 10 mg/kg. You don't get any true cholinesterase inhibition until you reach a gram or more.

Rosenberg: Would that dose of TOCP cause delayed neuropathy?

Replogle: Who cares?

Cohen: Rats don't get delayed neuropathy. We know there are carboxylesterases in human liver and serum. Its importance in plasma is questionable.

Replogle: What about dose. Normal dose of savin is 40 mg min per m³. Would you expect it to come down to 4 or 0.4?

Cohen: I have seen work of Fleisher with EPN. Work I did with paraoxon we were able to increase toxicity with malathion about 30-fold.

Rosenberg: Same as I observed with EPN and malathion.

Replogle: Then two orders of magnitude would not be out of the question?

Cohen: No! The importance is selectivity in terms of priming. The other side of it is, is there a way of increasing these enzymes.

Replogle: One of the problems we have had over the years is disparate reports in terms of doses for toxicity.

Cohen: I've had the same thing in the course I teach. There were six people from pathobiology, a number of them were veterinarians and I got into this discussion of interaction between phosphates and anesthetic esters such as procaine. One of the fellows explained why all his cows died. The farmers would paint the backs of the cows with an organophosphate the day before this fellow would come to take the horns off. He administered procaine and the cow immediately died. That just fits because I can do that with a mouse, but the tie-in has never been made.

Replogle: We just had a flight surgeon who allowed us to take doses of echothiophate right after procaine.

Cohen: After is better than before because procaine is rapidly turned over. Procaine is so rapidly turned over that if you block those pathways it builds up. I can show you plasma blood curves in animals and it goes up incredibly fast. They reach levels that you can't get to even if you are trying to kill them with procaine without the treatment. The whole picture that I was trying to portray in terms of metabolism is that the metabolism is extremely complex. Every organophosphate will have a different metabolic profile and the importance of these pathways is going to vary for different compounds. The reason it doesn't kill people is because we turn it over so rapidly. There are agents that I alluded to, some of the enzyme inducers, and the inhibitors like Arochlors and so forth that will increase some of these pathways or block some of them and it is extremely difficult if not impossible to predict. Let's say that Arochlor increased mixed function oxidase activity. I have three places circled where mixed function oxidase activity has increased or occurs in the metabolism of this model compound. If I dosed an animal with Arochlor and turned on microsomal enzymes I couldn't tell you whether this pathway is going to predominate and make the stuff more toxic or whether these two pathways will and I would make it less toxic. Right now we are at the level of understanding things that we can say it varies with the compound, species, inducer. You talk about areas of basic research that have to be explored and that is one area that needs a lot of exploration.

Question: Could you push it by adding more glutathione transferase?

Cohen: I haven't been able to come up with anything that suggests that glutathione transferase reactions make anything more toxic. If you can get this system to function or provide more nucleophiles it may be that the enzyme is there and operating fine; it is just that glutathione may be rate limiting.

Rosenberg: You are saying that something like acetaminophen might make the compound more toxic.

Cohen: That is a double edged sword. Right now we are looking at now phenitrothion alters acetaminophen. Ultimately I want to turn it around and see if someone who works with phenitrothion and receives a dose of acetaminophen, if it works the other way. Some of the inducers like the PCB's will increase carboxyl esterases. Other agents that does it are aldrin and dieldrin, but those two are banned by the EPA because maybe they cause cancer. They are extremely toxic insecticides, they are the cyclodienes not phosphates at all, but they are good enzyme inducers. A single dose in live animals and four days later the activity is about 50% greater than what it was, and you can decrease the toxicity of an organophosphate by providing more sites for it to be bound. The other thing that increases in plasma and liver is

pseudocholinesterase which comes primarily from the liver.

Replogle: The analysis that we run every day takes the number of missiles involved or whatever delivery systems are going to be used, their targeting, filled weights, and the odds of hitting a certain place, meteorological conditions and all the rest of it and then computes dose.

Cohen: What's the population or troop dose or the individual persons dosage gradient?

Replogle: I can say if you are protected this way then you are going to get this dose and if not protected at all then you would be getting that dose. We can do sensitivity analysis quickly which would say a supposition is always a thousand times more than necessary. So, if you can help by a fact of 50% it isn't going to do anything, but that is one supposition. The other supposition says that they are always pretty close and that 50% means 50% more that we can save. So the sensitivity analysis ahead of time might tell us where the change would be effective. I can tell you for instance that shifting the LD50 by a certain amount would be effective in a war. I can give you that number and is that a help in thinking about what mechanism to try.

Cohen: Knowing the number is not so much. In terms of basic research if you are looking for ways to enhance inactivation and really this is probably the best inactivation because you are not looking at enzyme turnover because you have ready sites.

Dettbarn: You are speaking about unspecific binding?

Cohen: Yes, it is a form of unspecific binding.

Rosenberg: I would think that you could make an estimate on the basis of knowing how much you are able to increase these levels or how much you are able to alter the animal LD50. You should certainly be able to make estimates of the number of increased binding sites.

Dettbarn: Doesn't albumin bind to these organophosphates?

Cohen: It is not like the aspirin-warfarin sort of thing; they do not bind as readily. Carboxyl esterases are strange; they appear to have an anionic esteratic serine hydroxyl site but they are not reactivated by PAM. They are different but not a lot is known about them. There are only two or three groups who have done any work on this; there is Zerner out in Australia and a group in Germany and labs like mine, but we are not purifying carboxyl esterases and looking at them biochemically.

Dettbarn: How about the usual plasma albumin, don't they bind organophosphates?

Cohen: If they do, it's not very important in terms of toxicity. I have not come across it as an important area in the mechanism of intoxication.

Lindstrom: Do you know the relative affinity of other esterases?

Cohen: We are just starting to get into looking at that, in in vitro combinations and purchased pure cholinesterase and pure carboxyl esterase and comparing it to some of the homogenized tissue esterases that we have.

We are in the same ball park vis a vis cholinesterase. In other words, affinity is about the same. In other words, there is a lot that the phosphate is readily accessible to.

Rosenberg: That varies with the compound tremendously.

Cohen: Yes, but it is in the same order of magnitude that we are finding, with all the compounds that we have worked with.

Rosenberg: With esterases there are huge differences between true acetylcholinesterase and pseudo cholinesterases for some compounds.

Cohen: Sure. We have what we feel is a fairly specific inhibitor of carboxyl esterase that doesn't inhibit cholinesterases, BNPP. In that respect, yes. That is where the structure activity starts to be important. BNPP (bis p-nitrophenyl phosphate) at 10^{-4} molar will completely inhibit carboxyl esterase, but will not touch true cholinesterase or brain homogenate using acetylthiocholine as substrate. If we use alpha naphthyl acetate as a carboxyl esterase substrate in the same tissue preparation we get some inhibition.

Replogle: Can we discuss something about the aging of the Soman bond and tying up other esterases?

Cohen: Does everyone know what the aging phenomenon means? Basically it is the situation in which you have an esteratic site phosphorylated in the enzyme and the leaving group comes off; that is the first step. At this stage, the organophosphate enzyme complex is considered more readily reactivatable by the oximes. If it undergoes an aging in which there is a dealkylation that is you lose another alkyl group, this complex is considered pretty much irreversibly bound. PAM won't work. PAM's effectiveness also varies with what these chains are. I am not sure that that is totally related to aging. I know that PAM will more readily reactivate ethylparathion than methyl.

Replogle: Soman doesn't reactivate at all after a few seconds. When it ties up similarly, with some of these other esterases, would it also age?

Dettbarn: Well, we don't know. It is a different enzyme.

Cohen: They are different. They are not considered reactivatable from the other phosphates.

Replogle: Soman is really the drug of interest and hardly anything else is. If I had Soman running around and tying up with other esterases and being reactivated and then tying up with cholinesterase and aging and so forth, what I end up with is soman toxicity.

Cohen: The thing you are asking is how irreversible would soman inhibition be in order to be of any value.

Replogle: Sarin does not age nearly as fast. Soman ages in seconds; that is why there isn't any antidote. That is the drug which is our threat.

Cohen: That would be a fairly easy test because soman doesn't need any activation. You could get into a situation where in experimental animals you could test it just by inducing it.

Rosenberg: You could just measure duration of inhibition.

Dettbarn: Steve, in your experience, Buche is much faster re-activated spontaneously than acetylcholinesterase.

Cohen: I have not measured in any in vitro reactivation, but in doing time profiles of animals with butylcholinesterase. Carboxyl esterases with TCP in a dose that doesn't cause any true cholinesterase inhibition you can keep them down for 3 or 4 days and then by 10 days they recover. This is mouse data.

Replogle: There is no soman work going on in the free world on humans anywhere, and no soman work going on in this country that I know of.

Dettbarn: In the Army at Fort Dietrick they do some soman studies, I am refereeing a paper right now.

Replogle: Norway is doing a considerable amount of soman work and yet in the United Kingdom there is no soman work going on.

Dettbarn: Sweden probably does because I have plenty of the stuff; they give it away.

Cohen: Do you know Romachandran? He is from India but he was with Chemical Defense and he did some work with DFP inhibition of all these esterases; nothing on rates of reactivation or anything of that sort.

Dettbarn: The DFP hits the Buche first when you have about a thousand-fold affinity difference between ACHE and BUCHE towards DFP. What you could do is spike your stuff with one of these where you hit first, the other enzymes.

Cohen: BNPP would be perfect for that. It does not affect plasma, cholinesterase. When we measure plasma cholinesterase we are using acetylthiocholine as substrate.

Lindstrom: What would you anticipate about the effect of glutathione in addition to increased esterases?

Cohen: I don't know. I don't know about agents that really enhance this pathway so much, like the glutathione pathway. For some agents glutathione is more important for example, phenitrothion and sumithion. It's

Like methylparathion, the only difference is ethyl groups vs methyl groups. For this one glutathione pathways play a more important role. Phenitrothion has just a methyl group on the ring as well. For that one also glutathione pathways are more important. So that, if you block glutathione dependent metabolism by depleting glutathione, like we did fairly easily with certain chemical agents, you can greatly enhance toxicity with these types of agents. Depleting glutathione results very frequently in a rebound to glutathione levels much higher than normal. In fact, by depleting first and timing your

dosing right you can protect, but that is getting very tricky.

Henkel: Are soman and sarin both oxones and not thiones? Yes. It is incredible because structurally they are not that different.

Replogle: I went through all the World War II documents, they are all unclassified. All of the attempts that this country made to find a nerve gas during the Second World War, and we tried methyl-methyl-fluorophosphate and methyl-ethyl-fluorophosphate, ethyl-ethyl-fluorophosphate, ethyl-isopropyl fluorophosphate and we missed isopropyl methyl fluorophosphate. We stepped over it as if someone had planned to miss it. We missed soman completely. At the end of the document someone said that the fluorophosphates were not toxic enough to be used in a military sense, and that is the end of the document. It occurred to me that if we had not gotten it from Germany at the end of the war, we would never have had them. No one would have gone through the whole series again.

Lindstrom: You say that much is not known about the oxidative dealkylation?

Cohen: There has been much interest through the years of glutathione dependent metabolism of a number of agents. The thing is that they are not readily inducible.

Rosenberg: How about the glutathione down there in the already active compound? How important is that pathway?

Cohen: The difference is the importance pathways can be determined mainly when you can selectively block them. You can't selectively block one without affecting the other.

If you give paraoxone, deplete with diethylmalate or diethylformate, you will increase toxicity. Orders of magnitude, I am not sure, maybe 3- or 4-fold. Same thing if you give paraoxone and you pretreat with TCP. The problem is trying to put all the literature into some common format where you can try to draw some correlation. With paraoxone I have seen where they show the time course to cholinesterase depression with and without TCP treatment. You can easily double toxicity and depending on the compound probably triple it or even more.

Dettbarn: Do you think parathion is converted in the liver or also the brain, muscle, etc.?

Cohen: I think that there is some of each. Bob Neal has done some work on that. Activation in the CNS is more of an intuitive feeling and can't play that much of a role.

Dettbarn: He seems to think that it is very important because it works that fast as an inhibitor, and could not have gone through the liver.

Cohen: Parathion is not that readily detoxified. If it is activated

in the liver and gets out and if enough is given so that it quickly saturates these other places, then it goes into the CNS. When Mike Boyd was here he said that in some cases activation has to occur at the site because the reactive metabolite is just so short-lived. Paraoxon can move through and you would have to get very high concentrations before paraoxonase starts to do anything to it in the plasma. As long as you get inhibition of these other tissue esterases the theory is that eventually it would just go over to CNS. A lot of the older work says that the thiophosphates at 10^{-2} or 10^{-3} molar inhibit. A lot of these agents will isomerize. That is another problem. You find these as contaminants in the commercial materials and this is a potent cholinesterase inhibitor.

Replegle: People have been discussing recently of taking oximes really constantly before, during, and after an attack. What I worry about is phosphorylated oximes as a different source of toxicity; it might be more potent than soman.

Rosenberg: You brought up the question Clyde about problems of working with soman. What would the problem be as far as safety factors? How do you work with it? Do you use gas masks? Do you work within a laboratory environment?

Llewellyn: There are specific surety requirements that are stated by the Federal regulations. In order to receive soman you have to have an exclusion area. You have to have an inspection of your facilities. More controlled than "controlled" substances. In our laboratory we are able to use it in the lab outside of an exclusion area that has been secured, because we are using dilutions of it. It can be shipped in research amounts. If you have 200 ug/ml of a volume not greater than 20 ml then you can carry it through the lab, but still have to have a hood.

Dettbarn: What is the vapor pressure? Is it close to DFP? The danger is that you can't reactivate it, but I wonder whether the danger of becoming intoxicated by it is as great as with DFP.

Llewellyn: There are only two reported human exposures in the literature. One from Edgewood and one from Yugoslavia. We do not have any experience with DFP, paraoxon, etc. because it is quite clear that you can't use simulate to mimic the agent.

The hardest thing to deal with is the fact that there are two overwhelming problems that we are trying to work on right now. In trying to deal with organophosphate intoxication, specifically, nerve agents, especially GB, GD and VX and many other things that the intelligence community tells us that the bad guys have, the overwhelming problem is that we do not know how they work. Thirty years of empirical pharmacological work chasing the cholinergic system has provided at best marginal increments over the initial observation that atropine will deal with life-threatening signs and symptoms. There is a great deal of clinical experience that shows that even when someone has a huge ingestion of an organophosphate you can get to the individual and pay attention to the respiratory system, maintain other functions and keep him atropinized, he has a 80 - 90% chance of surviving. It does not mean that atropine does

anything about the metabolism of the chemical in his body. The data on the oximes is very neat in vitro and we were forced to do an in-depth review of oxime experience over a period of three months. This involved going to the laboratory which developed the oxime PZS and the labs which make it at the present time. In better than 75% of the clinical cases there is no documented evidence that a life-threatening exposure to any organophosphate had occurred. The indirect measurements that indicate that reactivation is important as far as clinical outcome is concerned are just exactly that, they are indirect measurements. This sounds terribly bleak; it is only bleak if one restricts himself to considering the cholinergic system and cholinergic effects and the almost sacrosanct bodies of knowledge that relate to in vitro experiments with inactivated cholinesterase. Having said that as one of the preparatory remarks, the second preparatory remark is an equally embarrassing and potentially disastrous situation. There is no model or series of models for demonstrating efficacy in animals. The animal models are so poorly characterized, the methodology has varied so much that there is virtually no control on what was being put in as the challenge agent. If anyone would like to spend 3 or 4 days in my laboratory, I could put you in touch with some of the people there who do not profess this, however, it is in fact the kinds of data they have obtained and put together that demonstrate this.

Rosenberg: Someone, possibly a pharmacologist or toxicologist might like to take you up on this. It is according to exactly what you mean by a good animal model for what purposes. Certainly, when Wilson at Columbia, for example, first discovered PAM, he said, we don't need any statistics since he injected 20 animals without PAM, they died; then injected 20 animals with PAM and they lived. He demonstrated the protection by PAM against DFP without using statistics. Certainly in a laboratory we can easily measure changes of LD₅₀ values under very carefully defined controlled conditions. Obviously, these are controlled conditions in the laboratory with certain animals and what relevance it bears to uncontrolled conditions in the field might be questionable. Certainly, we can control conditions a lot better than you can. We can control many different factors, genetic, we can use mice of the same strain and control a lot of variables to get the LD₅₀ values close. We can show huge differences in LD₅₀ by any route you want to name.

Llewellyn: That in fact is the basic problem. One of the ways it seems that people have gone about trying to show that one combination is more efficacious than another is to first find out what the tolerated or just barely subtoxic dose of the antidotal mixture is and then to challenge that with increasing levels of LD₅₀'s. If you look at what happens in the little bit of work that has been done to try to characterize the difference between an LD₅₀ or 2 LD₅₀'s which are roughly an LD₉₉, then go to 5, 10 and 100 you are talking about apples and oranges when you go from and LD₅₀ up to super toxic doses. In fact, the methodology that has been used in the past has relied upon showing that three drugs protected against 50 LD₅₀'s and 2 combinations of two of those drugs only protected against 10, therefore, the three are better. That is very shaky logic. For screening purposes that is not a bad way to go. The situation as I see it in a simplistic way right now is somewhat similar to what the situation was in trying to come up with better malarial chemotherapy. The only thing that we knew about the malaria parasite was that it parasitized red blood cells. Until it was possible to understand that there was also a phase that was in the liver, one could continually treat and clear the red blood cells but in the intact animal you would get relapses repeatedly.

Rosenberg: Sorry you were not here before when I mentioned all these various effects which do not appear related to cholinesterase inhibition; there is a lot more than only cholinesterase inhibition.

Llewellyn: Our headquarters is at Fort Dietrick. My lab is the lead lab for looking at prophylaxis and therapy. There are various kinds of disciplinary groupings throughout the rest of these laboratories that will be of assistance if we can ever clearly define what the most important problems are. Some of you here in the School of Pharmacy are probably aware of the antiparasitic drug program that is going on for 15 or 20 years, developing new anti-malarials. We have a fair amount of expertise in experimental therapeutics and a huge network of contractors who can synthesize, formulate, do the pharmacokinetics almost on demand. The Institute of Infectious Disease has a mission that is somewhat analogous to the one that we have for medical aspects of chemical defense; theirs is medical aspects of biological warfare defense. What is important about that tie-in is that they know a great deal about immunochemistry and they do a lot of toxin work. I am trying to orient you to the fact that we are a piece of an overall program, however, a very small piece of the Army research development program. I have 188 people with 40 new authorizations for next year which I hope that we will be able to fill. Our budget is 7.5 million dollars for this year and will be 8.5 million next year. I have a veterinary facility which is probably the size of half the School of Pharmacy here. I actually do research on about 12% of that 7.5 million and the rest is spent whether we do any work or not. The medical dept. was not involved until the fall of 1978. We do have the Department of Defense mission and are responsible for coordinating programs of medical aspects of chemical defense. We ran a variety of sessions late last summer and early fall to try and get a look at what the civilian technology base in the U.S. had to offer. We spent a considerable amount of time in the last 6 months looking at friendly foreign technology. From all these groups, we were asked what we thought our problems are and we were in no position to state those. To simply say that we need a better antidote or we need an antidote which causes no side effects is fairly easy to state. We tried to come up with a definition of what is an antidote. If you consider atropine to be an antidote, what is it doing? Is it curing, is it preventing death, is it supposed to prevent death for some period of time? Let us be clear then of what the top priority is and that is for an antidote that the individual can carry and administer to himself. As a physician, I am not sure if that is the best way to try and approach the problem. The surgeon general of the Medical Department only recommends options and the Chief of Staff of the Army and the Deputy Chief of Staff for operations will say whether or not they want to run the risk of having individuals carry around something which may be used inappropriately, as opposed to what benefits it might give. This is a very sticky point. When we are talking about compounds or mixtures for use as an antidote, we are not talking about doctors using them. If all we had to do was to get all the casualties within 15 to 30 minutes into an intensive care unit, we would have to do little research. The friendly foreign technology was a disappointment, mostly because we found out that the people who worked on this and NATO laboratories have been under tremendous pressure, not being in a life sciences research environment, but in a material-thing the research environment to develop new things P2S (an oxime) is a good case in point. If one were to look at the available data upon which the British put P2S into the hands of troops, I find it very difficult to believe that anyone would find

that data convincing. They are in the same type of predicament right now with their pyridostigmine pretreatment as a "potentiator of atropine P2S and valium mixture" given immediately after exposure. Again they are under a great deal of pressure to put something new in the field. The only combined antidote other than the so-called TAB which we have in the field at the present time, is the one produced by ASTRA, a combination of atropine and toxogonin. The Norwegians and Canadians bought that based on very little work in their own labs. The Dutch were about to buy it, but realized that there wasn't enough information to back it up, and entered into a deal with ASTRA to see if they could get either atropine and 2-PAM or atropine and P2S into an autoinjector. After three years of trying, ASTRA was in default of contract. They have subcontracted the work to various other people and now the Dutch are back looking at atropine and toxogonin. Toxogonin, as you are probably aware is not an approved drug in the U.S. and is only manufactured in one place in Europe - the Merck plant in Darmstadt. Presently they have 26 suits outstanding pending because of people who have either died from cancer or are still alive with cancer having been involved with the making of toxogonin. Until about a year ago there was a great deal of concern of what happens when toxogonin is metabolized in the body, and based on animal experiments, it looked like toxogonin combined with moieties of the agent to produce something more toxic than the agent itself. That was decided in a closed-door meeting a year ago in April in Paris with a number of experts sitting around and saying we think that it does and some saying we think it doesn't and the upshot was that nobody had good data to say it did; so they decided that it didn't. I am serious; that is about how the decision was made. As far as we can see right now there is no magic potion lurking in the labs or in the development process of some other country. We have established decent contact with the Yugoslavs and they are relatively open as long as one can speak about dealing with organophosphate, they can interpret their pesticide intoxications. As a matter of fact, we have an advisory group that is making the European swing right now. We are pursuing what is going on in the international arena. Things that we are hearing about turns out to be things that were worked on in Edgewood in the early and middle 60's. Talking about doctors who will have to take care of patients within a division. The farthest forward that you would find a doctor in the field of medical service is at the third echelon of medical care. The first echelon of medical care is the company medic from company A post to patients is roughly 1 to 2 kilometers back to the forward edge of the battle area. From there evacuation goes back to 6 kilometers (battalion aid station) where there are three medics and one physician assistant. There is very little in the way of capability to do resuscitation for patients in a mass casualty situation. In a mobile battlefield, you go back another 6 to 12 kilometers and you get to the beginning part of the division rear boundary. That is the first place you see a physician.

Rosenberg: Why don't they push the doctors forward; they don't want to go?

Llewellyn: We have been thinking about it. Either you provide detailed life support by taking it to the patient or the patient to it within a short period of time. There are a lot of reasons for having a physician at the battalion level, because taking care of casualties is one of the minor problems. What we are left with then is first aid, the sort of thing that is done by individuals or by medics. Put out of your minds rapid ambulance

or helicopter evacuation like there was in Vietnam. What we are talking about then is auto-injectors that people carry with them for nerve agents. The auto-injector in the field at present is the combopen, a little bit of atropine, a little bit more of benactyzine and not enough TNBY to make much difference. It is about one-tenth what the maximum tolerated dose would be. You are probably aware of the fact that the combopen is copied directly from captured Russian war supplies that the Israelis got from the Egyptians and Syrians. Unfortunately, when it came in, it was a highly classified project and four different groups went to work on it. Nobody thought to consider whether or not what they had was a breakdown product of having sat in the desert for a couple of years or in a warehouse. Even though it was admirably mimicked we really don't have any rationale for what the percentages of the components are.

Dettbarn: What is the dose of atropine?

Llewellyn: I am talking about combopens that have three things in them. Atropine is just a little over 1 mg in that combopen, while benactyzine I really do not remember. Don't worry about it because we are pulling it out of the field. The reason we are pulling it out of the field is that the data for toxicity and safety and for efficacy have been laughed at by the FDA three different times. There are a number of people now in the FDA hierarchy who have busied themselves with looking at what the state of the art is in providing cocktail antidotes to individuals and what you might need to do to demonstrate efficacy with those sorts of things. What we are going back to is atropine. Atropine is still produced in the States and Europe and we expect to put 2 mg in an auto-injector.

The question has come up three different times now how much are you willing to put in the hands of an individual. The reason being that if you look at the signs and symptoms of anxiety as manifested on the battlefield and the sorts of things that lead people to become psychiatric casualties in the forward area, that is kind of similar to the chest tightness and that type of thing that one expects to see with the onset of symptoms with nerve gases. Secondly, everyone is supposed to wear an overgarment; this builds up the heat load, but is supposed to protect you from the agent. Some of the early symptoms of heat exhaustion also caused the sensation of running nose, tightness in the chest and that sort of thing. If you used atropine when you are on the verge of heat exhaustion it would push you over the edge.

Dettbarn: How much do you give?

Llewellyn: You can't say how much you give because we are talking about three autoinjectors, 2 mg in each one. How do you come up with a safe set of guidelines so that people can decide when to use them on themselves?

Rosenberg: This is what is so important because in the presence of organophosphate poisoning, people can tolerate, without getting into problems, much greater amounts of atropine so that if a person is trigger happy and injects himself when he shouldn't, he may get into trouble using the amount of atropine which may be safe at other times.

Llewellyn: By the time someone comes for medical aid you know whether or not those symptoms were from nerve agents because of the progression over time. If you consider what concentration of organophosphate agent Soviet weaponry is able to put on the battle field, for example, to expose a battalion size unit, then you get some very strange sorts of impressions. The maximum amount that they could put down on a battalion without using strategic delivery means, without using big rockets, like they use on an air base, is 5 LD₅₀'s and that is something that would really only cover a very small part of the battle field. That is modeling estimates say that 50% of the people in that area would be exposed to 1 LD₅₀ or less, another 25% for 2 LD₅₀'s or less and another 12 1/2% for 3 LD₅₀'s or less. Now we are getting close to 90% of the people there not being exposed to anything more than 3 LD₅₀'s. We are trying to turn ourselves in the direction of not looking for overkill as far as antidotes are concerned. What other people are concerned about naturally is the massive dose on a person who by intent or by accident has had a very large exposure. Our concern is to try to put in the hands of individuals an amount of drug which will not incapacitate them. If we incapacitate troops in the forward area even a little bit with these things there is a good chance that they will become casualties from other kinds of things; either being run over by tanks or standing up when they should have been lying down, etc. In thinking about which hand of the spectrum of challenge one wants to look at and what one wants to characterize in the animal models, it changes things considerably. We have spent a fair amount of time going through the literature trying to see who spent much time paying attention to one LD₅₀ challenges, 1/10 of an LD₅₀ challenges. Trying to find out what markers one can use to identify the challenging effect and then titrate with atropine, with cholinolytics or with whatever else, to the point where you lose the effect. We have good information from our behavioral toxicologists in a variety of cognitive paradigms that allow us to tailor a sign free dose. Only by pulling hair and kicking shins for three months could I get the pharmacologists in the lab to do business with the behavioral toxicologists so that we could tailor a sign free dose, put it into animals and then challenge. Everyone knew that that small amount of those drugs was not going to protect at all. In the middle 60's there were 4 or 5 publications from Edgewood that dealt with cocktails for pretreatment, atropine, mecamylamine and a carbamate (pro-stigmine or physostigmine) in a huge amount. We essentially got a sign free dose of both mixtures and challenged rats with 2 and 3 LD₅₀'s and then looked to see how fast they could recover as measured by some unlearned behavior, rotorods and that sort of thing. Atropine, mecamylamine and phosostigmine mixture prevented full recovery within the tests that we were using within 30 minutes. The pyridostigmine pretreatment provided the same amount of protection but they couldn't move for 24 hours.

Tests included balancing on a rotorod, being able to make choices and being able to respond to the variety of cognitive tests. Let us think about the lower challenge level as the target that we are trying to get at, and then think about something that might be used as a pretreatment or as an antidote that might be injected by everyone on command or at the first suspicion of any kind of attack. One way of doing that would be to tie it to military operational procedure. When guys have to go from having gloves, boots and an overgarment on and also having a mask on and then they stick

themselves with something like this, that gets you away from talking about chronic dosing people with carbamates, oximes and that kind of business. So, it is something that we are thinking about from the standpoint of specific indications for putting something into yourself on knowing that there is something else that you use if you develop symptoms later on. The planning of things so that it is a package, is what we would like to do and the data base doesn't exist to do that right now. Pacing problems is a phrase that highlights major problems. One such pacing problem is an incomplete understanding of mechanisms of action. No one has really looked at anything other than the cholinergic system. With the advances made in the neurosciences in the last ten years you have to look in other kinds of things. Even the tidbit of information that are around about what the organophosphates do to high energy phosphate metabolism, for example, is an important thing to look at. If you can damage high energy phosphate metabolism all bets are off for whatever happens to membrane permeability, etc. In looking at mechanisms of action there has been a lot of reverse reasoning. Looking at the effect of a non-agent drug in a non-challenged animal then putting it into a challenged animal and whatever effect one sees one explains on the basis of what was seen on the non-challenged animal. The poisons, in fact, may open up areas which are otherwise more or less closed whether you believe in the blood brain barrier or not. There are those kinds of issues that have to be dealt with and they haven't been. Another problem is that there is no standard methodology for evaluation of new compounds or mixtures. Tied to that is the inadequate characterization of animal models and lest the extrapolation from animal to man. What does delayed death mean? What are the delayed electrophysiological effects on the heart? Why is it that in the largest series with good documentation from Europe of organophosphate intoxications they can't demonstrate any clinical improvement at all with oximes? I have already talked about what you need to know of what the military constraints are. Some of those are of overwhelming importance to us, because of the kinds of things that may be useful in clinical toxicology, handling agricultural and industrial accidents and that sort of thing.

New thrusts.

1. Data base development; analysis
2. Research on mechanism of action
3. Development of screening systems to develop new prophylactic and therapeutic candidate compounds
4. Development of standard, scientifically valid evaluation methodology for promising candidates
5. Development of triage criteria, diagnostic methodology for chemical casualty care.

Just trying to get together a complete data base on GD looks like it will take about 9 months at a minimum and run half a million dollars. That is an interactive process and involves hard and soft sciences, information management experts, getting into a treasure trove of classified documents and also laboratory notebooks which are scattered all over where people did bits and pieces but never really put it together. Some of it is good and some bad. In a number of cases we find that we have very different results depending on the source of the GD, which part of the chemical systems lab made it and in which year. You get very different results based on the age

of the rats. All of those are things that we hope to get at by looking at GD first. Not that we believe that GD is a primary threat, we are told that it is. The Intelligence community says that it is. We are aware that there are a number of things that are much hairier, dirtier and so forth. There is a compound which has been synthesized by the good guys and the bad guys which in fact seems to be between ten and 15 times as toxic as GD. The nasty thing about it is that you do not get any symptoms until about a minute before death occurs. All of our research starting last July is unclassified. In some ways that may be cutting off our noses to spite our faces but medical R & D has never done classified research and we cannot afford to be cut off from the academic community. We have to be able to discuss these things. That is we have some research on the mechanisms of action going. Screening systems; not too much going on there, a little on *Aplysia* (sea snail). What we are spending more time on actually than on the screening systems is looking at the very low dose effects. If you use sub-human primates, duplicate this in cats and rats, very low dose exposures in a short period of time, you can pick up behavioral defects, loss of ability to perform various kinds of cognitive functions for six weeks to two months. In sleep lab experiments, we have cats with chronically implanted electrodes. After a third of an LD₅₀ exposure to GD and five months later there is still electrophysiological disturbances, disturbances in the sleep pattern that you can't just ignore. After getting a base line on some of these things, we hope to be able to go through the various interventions to see if it makes any difference. Actually we have looked at atropine and benactyzine and TMBY and it does not make any difference. That concerns us.

Dettbarn: The TMB₄ you wouldn't expect to work anyway.

Llewellyn: That depends on whose data you look at. If you believe that acetylcholinesterase activity has any importance in recovery from poisoning, then there are even some data that would indicate that 2 PAM chloride has some beneficial effects in humans challenged with soman.

Rosenberg: How does that correlate with the fact that the soman is supposed to be aged so rapidly and become irreversible?

Llewellyn: It is worth pulling out the data from which people make the statement that soman has a very rapid half time to becoming an irreversible agent. It is all done in a test tube. A few people who have gone to animal models to try and confirm that are very itchy about the fact that maybe the half time in the rat is 20 minutes, or maybe it is as long as 40 minutes. Very funny things happen if you set up an animal model that you intend to observe for a while not just to see if it lives or dies. Work by the Dutch out of TNO lab, in the late fall, was in a rat model where they intubated and pretreated with atropine and gave 5 LD₅₀'s of soman and within about 5 minutes spontaneous respiration comes back. It will not worsen even if you increase the dose of soman. They don't predose with soman, but what they have done is to take purified eel acetylcholinesterase and on an hourly basis, inject large amounts of it and then measure the inactivation by soman. Hour after hour the slope of inactivation is the same. If in fact, soman binds irreversibly with the cholinesterase then what is happening? They have some other data which would indicate that there are probably something like depots.

Replogle: There are all kinds of mechanisms that could be true and still have it combine irreversibly.

Llewellyn: When you get to a 100-fold excess of molecules of cholinesterase it should be bound by the soman, and they are still being bound as far as the activity measurements are concerned. Maybe you have to consider some kind of inactivation other than binding.

Dettbarn: These animals are always on the respirator or otherwise they would be dead.

Llewellyn: Yes, otherwise they would be dead. The whole purpose is to see whether or not this small amount of soman can continually, hour after hour inactivate large excesses of injected purified ACHE; it appears to be. The Yugoslavs are working along the same lines and you have probably seen some of the work that they have done, where they potentiate the toxicity of GD by 20 times, GA by 10 times, GB by 5 to 7 times by pretreating with TOCP.

Rosenberg: This is very interesting because this is exactly what we were discussing earlier.

Llewellyn: They increased the toxicity of the three G compound and decreased the toxicity of VX with that pretreatment. Then they treated with atropine and HI6 and this pretreatment potentiated the therapy. We just ran experiments last week with 4 LD₅₀ challenges of cyanide i.v. It is interesting that if you use the LD₅₀'s of cyanide from the literature you don't kill many of the animals either dogs or monkeys. We went back to look at that in some detail and finally got to a point where we thought that we had an honest LD₅₀ and every morning we tried it and we did 2 animals a day and it didn't work in the afternoon. We have put DMAP (dimethylaminophenol) into these beasts and got a faster response than from i.v. sodium nitrite.

Research plans.

- Assessment of acute and chronic neurophysiologic effects of chemical warfare agents
- Effects of mustard on skin - the athymic mouse and other human skin models
- Efficacy of centrally and peripherally active pretreatment and treatment compounds against nerve agent intoxication
- Selection and validation of models for evaluating efficacy of antidotes against nerve agents
- Assessment of existing NATO and TTCP models for determining efficacy of cutaneous and systemic protection
- Behavioral toxicology of CW agents and treatment with prophylactic and therapeutic compounds
- Analysis for potential toxic material(s) in aged atropine injector
- Effects of CW agents on fine structure and function of neuromuscular junction: BBB
- Neurotransmitter systems interaction: effects of antiACHE and treatment compounds
- Effects of sulfur mustards on lysosomes

Research plans (continued)

- Mechanism of action of antiCHE and antiCHE antidotes
- Efficacy of phosphinates as prophylactic agents in OP poisoning
- Compilation of current research documents addressing the prophylaxis and therapy of mustard-induced injury
- To compare the efficacy of current US therapy for cyanide poisoning with current therapy from foreign countries and investigation of possible novel therapies
- Assessment of direct neuronal effects of CW agents and efficacy of P & T compounds.

If I were running our Institute for Infectious Diseases and I wanted to consider a research program for a virus vaccine, I wouldn't have any problem in identifying 10 or 15 of the leading virologists in the world and put them around a table and they would cover 90 - 95% of the pertinent literature. Try to do that with organophosphates and it doesn't work, particularly you are talking about agents and particularly if you are not talking about therapy in a clinical setting, but about antidotes.

Giacobini: Are those projects presently progressing? Are they all experimental?

Llewellyn: Yes, many of these are experimental. For example, we looked in the literature to see how mustard causes lesions, not what mustard does in the body. This is a project that relates to using phosphonates as prophylaxis as opposed to carbamates. Phosphonates are more manipulable because you can reactivate the phosphate bound esterase with an oxime. There is someone in the lab who is running about 80 of those through right now. The mechanism of action work, we have a group of about 8 working on that, sulphur mustards on lysosomes relates to again what the mechanism of action would be on skin, on bronchi, etc.

Rosenberg: Naloxone antagonizes the analgesic effect of these organophosphates. These organophosphates have good analgesic activity, naloxone as well as atropine inhibiting it.

Llewellyn: Another thing that we are looking at right now if the finding that the toxicity of THC is potentiated by organophosphates and also by carbamates. Some people thought that maybe you could treat THC hallucinations and atropine-like effect using carbamates. In using the carbamates they actually caused more deaths. They they used DFP and also found increased toxicity. The practical importance is because of the tremendous usage of hashish by U.S. troops in Europe. It might be one of the few well-documented hazards of hashish used in a combat zone. With nerve agent at the same time, you would really be in trouble.

Dettbarn: We have done this with students who were smoking pot and we gave them an infusion of physostigmine and they were ready to kill themselves. They had never been in such a deep depression. There seems to be some agonistic effect on the postsynaptic receptors or somewhere else. You also got muscle contraction potentiation if you take diaphragm for instance from the treated rabbits.

Llewellyn: I would have to send you a copy of the full research plan so it would indicate what is going on in-house and what is going on in-contract and what goes on in some of the other labs. We have some direct ties also with what has been happening with the armed forces radiobiology institute. They probably have the only other cluster of neurobiologists in the Department of Defense. In the past there was a lot of concern about CNS effects. Until we have exhausted the potential of a variety of animal models there is no way that I as a physician can recommend that we put agent in the people. We are not set up to do that at all. The way doses of humans have been arrived at in the past was based on inhibition of peripheral acetylcholinesterases activities. If you are going to do that, you are dealing with very small amounts and you don't know whether it has any effect other than decreasing that biological activity. Since that is how you monitor the dosage you have to be sure that there is nothing leaking in from anywhere else in your lab. That would be a real problem.

Replegle: It has been very well shown that even in humans you can't take the abstract behavioral tests and then extrapolate to flying an airplane for instance or for a person to do anything else. You can't go from human to human. That is why the selection of pilots is so imperfect. I don't mean man to man. You can't go from an abstracted performance test to the judgment of performance in a weapons system. You can't go from an animal performance test to how well you can fly an airplane.

Llewellyn: With 90-degree confidence limits, absolutely not. If anyone can come up with what kinds of confidence limits are required you can make a hard run. A simple-minded approach is let's do this in humans, given the levels at which people have in fact been exposed, has caused an incredible diverse array of behavior at the same dosage level. The only people that are doing human challenge at the present time are the British and if anybody from their National Research Council is looking closely at those, I think that they would shut them down, because of the way they are doing it. Not so much that it is hazardous to the individuals; the design isn't going to tell you anything.

Rosenberg: Well, you have a very nice research program there. In basic research this could be carried on in academia.

Llewellyn: To be perfectly frank with you, a lot of that is paper. Those are things that we tried to start having finished a research inventory in the lab. There are a number of people there who have not tilted a test tube or poisoned a mouse in the last 5 or 7 years. It is an aging work force; that is not necessarily bad. We do expect to have a considerable infusion of new blood. We have to try and make some starts and we have to try and make some people think in different ways than they have been in the last 25 or 30 years. If decent studies are done that indicate that everything that happens is in the cholinergic system; that is all that we have to worry about, that's great. If one accepted just for the sake of argument that the only important effects are in the cholinergic system, do you think that there is an equal predominance of effect centrally versus peripherally?

Rosenberg: Subacute and chronic, it looks like there are long-lasting central effects which are not related apparently to the cholinergic system. Peripherally, I don't know of any long-lasting effects, except delayed neuropathy.

Llewellyn: One of the guys in my lab, a neurophysiologist, is about to publish some work that he finished last summer on cats, challenging them i.v. with soman, I think it was an LD₅₀. He was able to monitor what happened in the brain stem in the respiratory center and it shows a phase shift so that when respiration stops it is because of the shift in the inspiratory, expiratory cycle and at the same time he was able to stimulate the phrenic directly, the diaphragm directly and get in just before the neuromuscular junction. All three of those were intact. When respiration had stopped after GD challenge, the peripheral system is still functioning. That is the only thing that makes any sense when you see in certain kinds of animals, for instance rats, that is if they don't get killed in the initial onset of signs and symptoms respiration comes back spontaneously. There is no indication of adaptation at either the ganglionic area or the neuromuscular junction. Our assumptions that atropine exerts primarily peripheral effects; I don't know if anyone has looked at where atropine goes in the face of agent challenge. We know it produces central effects too and it may very well be that most of the beneficial effects from atropine are central. On the other hand, if what is going to really threaten your life is that you will drown in your own juices then it is the peripheral effects. We can tailor cholinolytics for predominantly central or peripheral effects.

Rosenberg: Atropine is a better antidote than atropine methylnitrate, which would suggest that some of its beneficial action is due to its central effects; atropine methylnitrate cannot penetrate the CNS.

Llewellyn: If you look at oximes from the same standpoint then people would say that pro-PAM should be better than PAM and in fact more pro-PAM does get into the CNS and it doesn't make a damn bit of difference as far as survival or decreasing the symptoms.

Rosenberg: As discussed before, I have shown a long time ago that enough PAM can be pushed into the CNS for protection, even if it is a small amount.

Lindstrom: Let's continue with the discussion we were having before, led by Steve Cohen.

Cohen: The question I just heard was, would it be logical to use for antidotal treatment adrenergic agents rather than anticholinergic.

Rosenberg: I think the point that Wolf made before is the problem that when you are stimulating ganglia of course you are stimulating adrenergic nerves as well as cholinergic nerves so therefore when you use these organophosphates you are also causing a release of norepinephrine. Theoretically, of course, on isolated organs you expect the adrenergic system to be opposite of the cholinergic in many cases, but I don't think it is a very effective way to go.

Cohen: It seems really what you are after is preventing acetylcholines effect rather than trying to counter it by stimulating another system.

Dettbarn: The other thing would be to use ganglionic blockers, then you would prevent the stimulation.

Rosenberg: This is what you mentioned in the combination of mecamylamine. There you have a good ganglionic blocker.

Llewellyn: A number of the bispyridinium series that don't have an oxime group people theorize have some beneficial effect because they are ganglionic blockers.

Cohen: From what Craig said and going back to Fleisher's work that I started with we know that we can greatly increase the toxicity. I know that Romachandran when he was at Sweden in the chemical defense group, he did a run-down on DFP and probably on some other fluorophosphates for their interaction with carboxylic ester hydrolases, (carboxylesterases) which are not cholinesterases. They bind and inhibit carboxylesterases and this evidence, with the EPN from Fleisher and now this report that TCP does enhance suggests very strongly that that might be the way to go. If you can boost this activity and binding you might have a prophylactic protection. The question is from how many fold. You said soman was one of the ones that was potentiated. There is no way that I could hypothesize for that potentiation to have occurred with TCP or CVDP, because CVDP is a cyclized derivative of that TCP. TCP is the one that causes the peripheral neuropathy, the axonal degeneration. CVDP is thought to be the active neurotoxic metabolite of this material. There is some work that goes back about 8 years where they injected this material into cats. It is a question if that evidence is correct; if TCP which is a selective inhibitor of these things can increase the toxicity of soman that tells me that there is a good possibility that soman is binding to this and that this mechanism that we were talking about of detoxification is working. It should be easy to test as long as you have the facilities to do it with soman. I have the doses that are necessary to boost carboxylesterase level.

Dettbarn: If you boost carboxylesterase and all the other enzymes over long periods of time which you would have to do, would you expect that they would interfere with normal physiological functions?

Cohen: We do not know what carboxylesterase's normal physiological function is, maybe that is a different way of finding out. Inhibiting them doesn't tell us what their function is; maybe if we increase them for a prolonged period it will. The problem with enzyme induction and that is another side of things is that it is very difficult to get a prolonged enzyme induction. By prolonged, I mean weeks or months. If you are concerned with a few days there is no question. Some of the classical work was done with DDT as an inducer to turn on mixed function oxidase. These enzymes by the way from the liver are in the microsomal endoplasmic reticulum preparation. They are readily inducible but are not mixed function oxidases, they seem to be associated with mixed function oxidase fraction with subcellular fraction.

DDT experiments, when they did them to see how long they could be induced and how long the induction lasted and so forth; they started to go to repeated administration of DDT to see if they could keep the response up. What they found was that in addition to turning on microsomal enzyme it turned on DDT metabolism, there the DDT as a trigger to keep induction going was being turned over faster. Eventually it was a law of diminishing return situation where you got into toxicity and other effects. Prolonged enzyme induction in an experimental situation cannot be that easy. Everyone is concerned from the drug interaction point of view. I think that it is the short-term dosage that will turn things on and it will come up to a level and then dissipate given the turnover of the enzyme system. Someone who is on a chronic exposure may be induced to a slight level, but that comes back to work that needs to be done, knowing how much you can kick this enzyme activity up and how long you can keep it up there for and what its effects might be. We have three different procedures that we have done for induction. Only one of them am I comfortable with and that was a four-day single injection and four days later it has peaked staying up for another couple of days. It comes up to about 2 1/2 to 3 times and plateaus.

Replogle: Most of the desirable scenarios are not those where you are totally surprised by the use of chemicals. The analysis just shows that you aren't going to make it. If you assume for a moment that you know within 3 or 4 days of about when you are going to get them and you are not going to fight for more than 6 to 12 days afterwards, you probably will be all right.

Cohen: You can take it to the extreme and say let's do all of this and what do you do about the first wave of cyanide. Cyanide is real nice, but only in certain weather conditions.

Llewellyn: You get a divergence of opinion depending on what service you are talking to as to the importance of agents. It is a reasonable thing. The way the Air Force would expect to be attacked and participate in combat operations is a lot different than the way the Army does.

Replogle: We expect Air Force bases to be attacked by soman and we don't expect cyanide but mustard lewisite is a fantastic thing for an Air Force base. The kinds of agents that are thought to be not as good as thickened soman for example, not as good because their effects dissipate too rapidly are ideal if you figure that you are going to have to go into the area where you just used the agent. That is what happens with troops.

Henkel: How do they thicken soman?

Replogle: Polyacrylate gel.

Rosenberg: If they would want to do that wouldn't VX be simpler to use. That is already pretty thick stuff.

Replogle: This goo is very difficult to get off, it's not just a matter of viscosity. You not only have a longer term resistance problem, but you also have a decontamination problem.

Cohen: Some work that I have done, now just generalizing for enzyme inducers, probably the most potent inducers are the polychlorinated biphenyls. You are running into a real toxicology problem.

Replogle: A lot of the difficulty is not really in the war, it is testing. I have to have human performance data in a cockpit, I have to have people flying in simulators at 150 feet off the deck and dressed in their garb and dosed or with an antidote.

Cohen: You can induce with phenobarb and not bring on phenobarb behavior.

Rosenberg: You would even have worse problems than that because you don't want these pilots to be out of commission for the next year with behavioral problems. The problem is that you haven't even considered that if you change all of this you may be altering other pathways of metabolism and we only look for acute LD₅₀ differences. You may be missing all non-cholinergic long term effects, which might influence a pilot for the next year to be depressed or whatever.

Replogle: I don't have a lot of fear in using DFP on pilots in running a study. We have a lot of data on this.

Dettbarn: If you only instill it into the eye in small amounts you don't have to worry. The main thing that you have to worry about is probably demyelination of nerves.

Llewellyn: I have serious doubts that in time of peace and until the balloon goes up and war begins that anybody is going to be willing to bite the bullet and say, yes we will use some drug prophylactically or for pretreatment that doesn't have the blessing of the regulatory agencies. I only raise that to suggest that instead of thinking about prophylaxis as something that goes on over time, that you consider the specific indication pretreatment on prophylaxis that might be done over time, but which would be done in response to some specific indication. That may be a cheap way of getting around the problem but you could foresee a situation where a war goes on 16 to 30 days. Instead of having someone on pills all through that and maybe during the two weeks beforehand, if you had good intelligence, they might just be carrying the stuff around waiting for alarms to go off, the command to put the mask on the whatever. Instead of being restricted to thinking about the maintenance dosing, short-term coverage may be important.

Replogle: Actually, we are more worried about continuous low doses than about people dying from an attack.

Cohen: I am still not sure I understand what you are saying.

Replogle: If you had a continuous low dose situation of phosphate, the person is going to take a consequential dose every day, perhaps 2, 3 and 4 times a day, how many days do you have? Nobody knows, but if he were to start at that point potentiating enzyme systems that could start sequestering these molecules one by one. Then the long-term effects of low doses would really be a lot less. It would be a continuous prophylactic.

Cohen: What you were just talking about, soman and shooting in cholinesterase doesn't make any sense. Where is that soman sitting that every time they can get to the new cholinesterase level.

Llewellyn: You mentioned what CDVP is thought to do. In that paper they postulated that CDVP is displacing soman from the nonspecific binding sites; places where it probably binds but without high affinity. It is being pushed to maintain higher levels.

Cohen: These sites are very often called nonspecific even though the affinity might be high. They are often referred to as nonspecific because they have no biological effect. The in vitro affinity, as far as we can tell for some of the oxones is very close to true cholinesterase as it is for these carboxylesterase. I don't think that it is that nonspecific. The sites are biologically tailored to attract organophosphates just like cholinesterases. I am not that comfortable discussing the lipases, I just know that the oxones inhibit them and some work has been done and people are concerned about malnourished individuals. The effect that phosphates may have on fat mobilization under times of diet restriction may be significant.

Rosenberg: It might be of significance since they found with organophosphates a huge increase of phospholipids in nerve tissue indicating that, I don't know if anyone has checked this, maybe the organophosphates are inhibiting phospholipase. Therefore, you would have a build-up of phospholipids in the nerve.

Cohen: The other side of attacking it, is to go to what we were talking about, antidotes and things like that. When you said that there was no good model, I just kind of sat and felt very uneasy and responded. I remember that when Eva Bartels was here, she spoke about some preliminary work with antihistamines and so forth as protectants. She was talking about clinical treatment down in South America that they were giving kids, diphenhydramine to treat them for poisoning in a combination with atropine. We thought that that would be a pretty easy thing to repeat, but like you had said in the morning and in the afternoon the variability was terrible. Trying to show any dramatic effect over atropine was not possible. We could show an atropine effect, there is no question about that, but it is very touchy with the dose and so on. Let me ask the other side of the question. I know that you were talking about tanks sucking in stones and things like that. The aircraft system, the same problem?

Replogle: In aircrafts it is even worse. All the Russian aircraft you can shut off the outside air to the cockpit. No American aircraft can shut off the air to the cockpit, because it is the coolant for the Avionic system. You literally suck in tons of air through the cockpit. We are not really worried however about picking up any agent airborne, since Air Force bases on the ground are the main target.

Cohen: So you are saying that the personnel are going to get it before they get into the craft.

Rosenberg: What is the half like of cholinesterase in the plasma? It would be hard for them to make i.v. injections. I was really thinking of something way out; if you could inject huge amounts of cholinesterase in the plasma to tie up organophosphate directly.

Llewellyn: Then you better hope that he gets exposed.

Rosenberg: I don't know what the protease activity would be and what the rate of breakdown would be, but it would certainly be nontoxic; it would have to be human cholinesterase. You could get human red blood cell cholinesterase.

Cohen: It is still going to be antigenic even it is human red cells, unless it is your own.

Lindstrom: What is the working end of the oxime?

Cohen: The nitrogen binds to the anionic site of the cholinesterase. You get an oxime attack on the phosphorylated group.

Rosenberg: You end up with aphosphorylated oxime.

Replogle: The phosphorylated oxime itself is toxic.

Cohen: The oxime alone will inhibit cholinesterase.

Lindstrom: How does it inhibit the cholinesterase?

Dettbarn: By blocking the anionic site and then preventing access of the substrate.

Cohen: Cholinesterase is depicted as simplistic sense as anionic and esteratic site:



This is the one with the serine hydroxyl and the phosphorylation occurs here. The oxime has a charged nitrogen that is attracted here and then it interacts with the phosphorus. Of course with acetylcholine you get the quaternary nitrogen here and the ester group here. When the thing comes off cholinesterase it comes off as a phosphorylated oxime. Oxime will be attracted to the anionic site even if there is no phosphate at the esteratic site. That is one of the reasons that it is not recommended with the carbamates because they reverse so readily that you just add insult to injury by throwing in something that is going to tie up the anionic site.

Lindstrom: Is there enough known about the reactions involved here to synthesize polymeric polyfunctional materials?

Cohen: There is one that I mentioned earlier, supposedly very specific - p-Nitro phenylphosphate, which is categorized as a specific selective carboxylesterase inhibitor. What you are speaking about may bring us back to injecting cholinesterase. There are just countless sources of carboxylesterase enzyme that could be made up into tremendous batches if you are not talking of putting it in the body. I am not sure of what you are thinking of, are you thinking in terms of a mask?

Comment: I am just thinking, if you could avoid the problem of injecting it in the body and at the same time trapping all this stuff before it gets into the body. Clothing coated with this material could be porous,

so that it could breathe, and anything that hits it could just be deactivated before it goes through.

Cohen: Carboxylesterase is very abundant and considered more stable than a lot of enzymes that I have worked with. You could leave tissue out sitting on a bench for a week and still detect carboxylesterase. In the freezer it keeps; cholinesterase in the pure form keeps a very long time.

Rosenberg: That would make some sort of thing almost possible. It is a characteristic of these esterases including phospholipases that they are extremely stable.

Comment: Has anyone looked at that possibility? Developing something that you don't have to inject, that will trap the nerve gas before it gets in?

Rosenberg: I don't know of anything. If you people aren't doing anything, then it probably isn't getting done. It would seem to be an interesting idea. Whether you use cholinesterase or carboxylesterase because it could really tie up large amounts and could even like an affinity column be reutilized and reactivated.

Llewellyn: I think one of the problems there is the aging, how long is it going to be tied up. If it is released two seconds after it binds what good is it?

Rosenberg: No, aging isn't the same as being released.

Cohen: Carboxylesterases in vivo stay inhibited for many days. The recovery pattern varies with the phosphate from let us say if you use different phosphates and take them down to almost zero percent, 95% inhibition and then you try to monitor recovery with time and killing animals at different times and sampling different phosphates; the rates of recovery are different. There can't just be regeneration of new enzyme. It has to be some release or reversibility as well. If it were just regeneration in the case of the phosphates they would all come back at the same time because the new enzyme would be regenerated at the same rate.

Rosenberg: It might be something worthwhile because you know with the affinity columns one of the advantages is that oftentimes the kinetics of an enzyme are better when attached to an inert support than when in solution. Therefore you might have some of these materials acting a lot more effectively on some sort of a support matrix than if they were just in solution.

Replogle: A better filter is a nice thing. I don't think it would make a big difference in a war. Analysis on an Air Force base amounts to how long it would take a person to put a mask on.

Rosenberg: Of course we are thinking of civilian population.

Replogle: No, we are not.

Rosenberg: I am.

Replogle: It is against the law to protect foreign nationals.

Rosenberg: There is no scenario of chemical warfare agents being dropped in the U.S. then.

Replogle: We are not worried about the possibility of missiles that get this far carrying chemicals; it doesn't make any sense to use them this way. A new mask helps, but in a very minor logistic fashion. What would help is if you could for instance develop some sort of cream to rub on the skin underneath a garment because you are going to contaminate yourself getting these garments on and off. You have to get a pilot hydrated because when he goes out on a mission and loses 2% of his body weight then you are in a lot of trouble. He comes back and doesn't drink anything and then goes on another mission and if it's a hot day, he is through. A pilot has to be able to do four missions a day.

You have to get this mask off, get him cooled down and you have to get him drinking. With all of this he is taking on and off contaminated equipment that we have no test, no dosimeter.

Dettbarn: High alkalinity might be useful because organophosphates are not very stable. Maybe a soap lotion one should put on the body or something like that.

Replogle: You sweat a lot too; that is a problem.

Dettbarn: Air condition the suits.

Cohen: That would be the same problem with the cream.

Comment: You just put the enzyme in as a substitute for the charcoal in the suit.

Cohen: What is the resorption problem; is it coming off the charcoal, is that what you are saying?

Replogle: Decontaminating an airplane is a very difficult problem. It turns out that human skin will take more than an airplane will.

One of the other enzyme uses is that it would be very nice to have indicator underwear that would be cheap enough to produce. Our big problem is that we don't know what is going to be decontaminated and what is not. There is no way of finding out. If you knew that somebody was contaminated you would really be in great shape. The physicians won't touch anyone who they think might be contaminated which means everybody in the world. It would be good if you would turn bright orange if you were contaminated.

Cohen: There are so many chromogenic substrates in carboxylesterases. There is some work where thin layer plates are sprayed with carboxylesterase after being spotted with phosphates. He uses these as an indicator on TLC to find out where the phosphates are and where they have moved in the different solvents. He then comes back and sprays the plates with chromium diethyl

acetate or something like that, which colors the plate except where the inhibitor is.

Replegle: That system would probably save more people than anything else that I know of. Even if you could tell the troops that they would turn bright orange if they were doaed, you would convince everyone to think they are all right as long as they are not colored.

Rosenberg: There are of course dyes that are used for phosphorus containing compounds. The question I think might be for specificity of what else you might come in contact with false positive. There are molybdc acid etc. which would react very nicely with phosphorus.

Llewelyn: There are about five volumes of technical reports from the Chem Systems Lab looking at a variety of things like that for detectors and alarm systems. Once you get down to the kinds of sensitivity that you would like to have, the false positives are just overwhelming, carbon monoxide and all kinds of other things.

Cohen: It would be better with enzymes.

Lindstrom: I thought I would ask on this the second day of our meeting that the focus be shifted a little bit today from the organophosphates. I have asked Dr. Giacobini to get into some areas relating to mechanisms of action and the central nervous system. Then I would ask Dr. Rosenberg to give us a little bit on toxina. As we reach the end of the morning maybe we could get into a discussion relative to the types of followup meetings, etc.

Giacobini: I would like to carry on the discussion, focusing on the mechanism of action. I have seen in some of the slides that you showed the words "mechanism of action" occurred, and that made me feel that I was not alone in thinking that we are still far from understanding what is the mechanism of action of these organophosphates. In order to have a basis for this discussion, I would like to make two postulates. One is that the evidence that we have today is that many symptoms due to the organophosphate action are indeed dependent on their action on the cholinergic synapse. The second is that in spite of the fact that we know there are now so-called synaptic modulators which could interfere. Yesterday it was mentioned that enkephalin might interfere in acetylcholine metabolism in organophosphates. It is very likely that there are other neurotransmitters involved in the mechanism of action of organophosphate, however, we know very little about it. As a matter of fact, we know very little about the modulation of the cholinergic synapse by other neurotransmitters. The third assumption that I would like to make is that the evidence that we have is that at least part of the action of the organophosphate is based on one event in the synapse and that is that the neurotransmitter which is finely regulated to have a physiological concentration all the time is suddenly increased tremendously. That is precisely the point that I would like to discuss. Number one is to see what is the evidence for that, what happens, what are the consequences of this and, third whether this might be the only mechanism of action of the organophosphate on the whole cycle of acetylcholine. Finally, I would like to close with a comment on muscarinic receptors which is another aspect of the cholinergic system. One thing we know is that acetylcholine as opposed to other

neurotransmitters, for instance, norepinephrine or 5-HT is very finely regulated, having an upper and lower limit of concentration within the synapse. That seems to be a characteristic for the system. There is a lot of evidence that acetylcholine is indeed increased when we block hydrolysis. I have taken data from a 1979 paper by Drs. Dettbarn and Wecker. The other aspect, which is a little more complicated and perhaps we could pass on this for the moment and maybe Dr. Dettbarn would like to make a comment, is what happens to the precursor during this action of the organophosphate. Depending on the region of the brain you might have a decreased and in other parts an increase of choline. I would like to point out just one thing - it is that we have just now started to understand a little bit of the mechanics of the regulation of acetylcholine. One important step here is the connection between the different mechanisms. One aspect is the acetylcholine content and I have to distinguish between the intra- and extracellular. What is called the intracellular or the cytoplasmatic acetylcholine content seem to regulate the synthesis of the transmitter. The other problem is what is the link between the uptake of the precursors and the synthesis. Finally, how is the intracellular level of the acetylcholine produced in the synapse connected to the synthesis and how is the uptake connected to the synthesis? There are different theories and an explanation of physical coupling and a kinetic coupling. We can also think in terms of an enzyme, choline acetyltransferase. There are a few things that we know; one is that the rate of synthesis of acetylcholine varies inversely with the tissue concentration of acetylcholine as was demonstrated both in vivo and in vitro. The concentration of acetylcholine is therefore regulated very carefully. There is another aspect here and that is how much of the acetylcholine is leaving the synapse. If there is an increased release due to an activation by a pharmacological agent or an increased release due to an activation by a pharmacological agent or an increased release through stimulation, we have also increased synthesis. So release and synthesis seem to be comparable. The other coupling is between the level, the increased accumulation and the synthesis, so there is a mechanism of regulation so that whenever the synthesis and storage achieves a certain limit intracellularly, there is a decrease into the synthesis. This brings up several questions which are directly related to the mechanism of action of the organophosphate. While going through the literature and particularly concentrating on the behavioral aspects of the organophosphate action there are two points that I would like to bring up. One, which was already emphasized yesterday, is that behavioral symptoms in animals and also in humans appear only when the brain acetylcholine is reduced to 50 - 59%, that is you must have a very extreme reduction. The question is, what is the critical level of enzyme activity? If you go through the literature, there is no universal agreement; these figures vary a great deal. The animal can perform a wide variety of tests including those that we know or think are using cholinergic pathways with impressively low levels of cholinesterase in the brain. We can ask two questions: first, can we discard acetylcholinesterase as being an important enzyme for the regulation of this mechanism? The second is that can we think of some mechanism which is capable of lowering the brain acetylcholine following anticholinesterase? So that, in spite of the persisting and prolonged depression of the cholinesterase we have some mechanism which is activated which is getting rid of the acetylcholine which starts to accumulate. We know that high levels of acetylcholine in the tissue inhibits the synthesis of acetylcholine and it has been discussed as to whether there is a kinetic coupling or a physical coupling. The other

possibility is that there is not only a synaptic mechanism for disposal of acetylcholine or an inactivation of acetylcholine which has been accumulated but there is also another mechanism, which was also considered yesterday. This is one which really gets acetylcholine out of the brain. Such a mechanism is not at all theoretical since we know the action of the organophosphate on the blood brain barrier. However, this is another point that should be considered and studied. Another aspect of this problem is that acetylcholine release is geared to the synthesis and is under the control of the cholinergic receptor which sits in the presynaptic nerve ending, the so-called presynaptic cholinergic receptor. We have learned that this presynaptic receptor apparently has to do with the regulation of release. There are already agents, toxins which are preferentially binding to these receptors. Yesterday, it was mentioned that beta-bungarotoxin is one of the agents which binds to the presynaptic receptor. We have another one which has an excellent presynaptic action and that is the venom which is released by the black widow spider.

There is some evidence that there are actually some drugs such as neostigmine that also have an inaction on the acetylcholine receptor by activating the receptor and they also have an effect on release. The interesting thing is that for instance physostigmine decreases the release. Physostigmine has a pronounced action on the release. Recent papers pointed out the difference of action between neostigmine and the physostigmine which depends on their access to the brain through the blood brain barrier. Thus, we have another mechanism which is the action of the organophosphate on the release mechanism and the action perhaps on these acetylcholine receptors. I would like to just conclude this by stressing once more that it is very important that we go back and use organophosphates as tools and therefore try to understand more about these different links which I think are the crucial parts of the cholinergic mechanism that is the relation to acetylcholine metabolism, synthesis, uptake of choline, and the release as well as going to permeability studies at the level of the synapse and permeability studies at the level of the brain. There is an interesting curiosity here which was in part studied in our laboratory. We studied different isozymes of ACHE in the brain of dogs and we found that one of these molecular forms is released, that is the enzyme appears in the cerebrospinal fluid. We did not do any experiments with organophosphates. Is acetylcholine released during organophosphate action? It is known that it is released.

Dettbarn: You always find acetylcholine released.

Giacobini: It is released in ganglia, which is very difficult to prove. It is released in the adrenal which is easier to prove and you can find it in the blood, in the cerebrospinal fluid. Only one of the isoenzymes was preferentially released.

Dettbarn: It is mainly the 16S form, which is the one localized in the end plate region and it is the one which is transported at the fastest rate in the axon. Whether the 16S form of the axon is actually being released in the cleft and incorporated in the postsynaptic site is not known.

Giacobini: Also some of these enzymes that are formed have a very short half-life; in 2 to 3 hours they can be restored. Again, there is the interesting question about what is the relationship of the organophosphates and some of these isoenzymes which are formed? I would like to now ask questions. Are you really persuaded that the accumulation of acetylcholine is an important factor in regulating the action of the organophosphate and perhaps I would like to ask Dr. Dettbarn, if he would like to make some comment.

Dettbarn: One factor which triggers the accumulation of acetylcholine is probably not the degree of inhibition initially, it is the rate with which you inhibit the enzyme. I think we all agree that there is a certain critical level and I think that that is about 70% reduction, that is 30% activity remaining when you see changes.

Giacobini: Is this critical level based upon neurophysiological experiments?

Dettbarn: On neurophysiological experiments and on measuring ACHE levels. However, if you very slowly raise your level of inhibition by giving successive shots of the inhibitor at low concentrations, then you can reduce your enzyme to a very low level, levels like 15% or so before you see symptoms. You really can't get tolerance to acetylcholinesterase inhibitor. What the animal does is it sort of adjusts to the low level of cholinesterase. If you pretreat the animal with low dosage of organophosphates over 14 days to three weeks which we have done and then look at acetylcholine parameters at the neuromuscular junction, one will find very low levels. If however you test the animal with an LD₅₀ when the animal is supposedly tolerant to the organophosphate, there is no tolerance. There are two adaptation mechanisms, one is a presynaptic one and the other postsynaptic. There seems to be a distinct difference to the brain versus the periphery (neuromuscular junction). In the brain all these behavioral studies seem to indicate (whatever parameter they are measuring whether temperature control, drinking, weight gain, etc.) that there is a desensitization of the muscarinic receptor. After even one shot of DFP they get a drop in temperature. If they follow that up with pilocarpine which initially gave a drop in temperature, the effect of the pilocarpine is reduced. They have to increase the pilocarpine dose in order to get the same effect. In the neuromuscular junction it is somewhat different. There is evidence for instance that if you give neostigmine over a period of 5 to 7 days (small dosage) that you lose 50% of your receptor sites. That is measured by a bungarotoxin binding. That by the way is seen in myasthenia gravis patients that have been treated with neostigmine. If you give organophosphates in such a way as you give the neostigmine for instance, let us say only once a day so that it causes slight symptoms but is not killing the animal, you can do that by reducing the daily dose so that you come to the same level of enzyme inhibition. You get denervation and that you get whether you use DFP, paraoxon or whether you use so-called neurotoxic inhibitors or non-neurotoxic inhibitors. You increase the binding sites of a bungarotoxin in these muscles which means that on denervation you have the very well-known effect that there is an increase in acetylcholine receptors away from the end plate.

The muscle fibers all of a sudden become very sensitive to acetylcholine in areas where they had not been sensitive before. That occurs with denervation and also occurs after bolulinum toxin and you see it also after repeated doses of DFP and paraoxon. With DFP you get three effects - a very acute effect with shaking. Within a day after one shot of DFP you have nerve terminal denervation. It looks like an axotomized axon; the nerve terminal is lost and then 2-3 weeks after one single dose of DFP you get the well-known delayed neurotoxic changes of demyelination. This DFP delayed effect is shown on the cat, chicken, etc. On the rat you get the acute denervation effect. As I pointed out yesterday, when we induce muscle necrosis with the organophosphate we also find that there is a certain optimum and then the amount of necrosis that is induced with prolonged medication goes down. Only if the organophosphate is withdrawn and you wait for a few weeks can you induce necrosis again in the muscle. The initial injections have denervated some of the muscle fibers, not all. You can show with iontophoretic application of acetylcholine you get sudden responses to acetylcholine away from the end plate in a number of fibers. You get a tripling of alpha bungarotoxin binding. Then you get the repair mechanism operating within 3 weeks and the muscle is reinnervated and now the organophosphate can do the same thing again. There are certain motor units that are affected differently than other motor units. We do not know yet what the characteristics of these motor units are. All muscle fibers or end plates are affected, but not all progress to necrosis; about 10 - 15% of the muscle fibers become necrotic. By the way you can get that with neostigmine and physostigmine. You have to dose them over very short periods of time. If you assume that paraoxon and DFP cause symptoms that last 2 to 3 hours, you give neostigmine and physostigmine 3 times as often. If you get the animal to show symptoms for 2 hours then they show the increase of receptors the denervation of motor end plate and the necrosis.

Giacobini: Do you have any evidence for this denervation effect on the central nervous system?

Dettbarn: No, that is what I would like to see. Phil is going to talk about the long-delayed effects on EEG and that sort of thing. One should look whether there aren't changes in certain neurons. They may be very sensitive and they either become necrotic and since the repair mechanism in the brain is not as good as in the periphery, we lose neurons and therefore we get these effects. The only time that I have seen anything like that is a paper by a veterinarian. He used carbaryl, a carbamylating insecticide, in pigs. He also showed the muscle necrosis and he found lesions in the brain stem. I don't think that anyone has really followed that up. There may be the basis for your respiratory failure and that sort of thing. If you overstimulate certain neurons, they run out of energy, ADP and phosphocreatine and you get calcium accumulation and neuronal breakdown. Initially you get this peripheral hyper-activity, constriction of the diaphragm, high frequencies of phrenic nerve firings, constriction of the respiratory muscle of the rib cage of the thorax. By doing that you get hypoxia in the brain which may induce seizures and cramping and that in turn may reduce the oxygen tension further where you may get loss of some critical neurons. That could explain some of these delayed changes. These may be indirectly induced by the organophosphates, not as a direct action but as a consequence of the initial effects.

Replogle: Do you think the selective metabolic changes in the brain after organophosphate poisoning follow the patterns of selective metabolic protection in the brain or are different?

Dettbarn: These organophosphates are not very selective, they inhibit anything that has a serine active site and they could be phosphorylating kinases, etc.

Replogle: I didn't go through the data but I have seen brain sections; work on deoxyglucose following poisoning and there were some very active sites and apparently had no metabolic activity at all.

Dettbarn: That could be a subsequent consequence due to reduced oxygen tension.

Replogle: Do you expect it to follow the normal pattern of reduced oxygen tension in the brain or do they seem different?

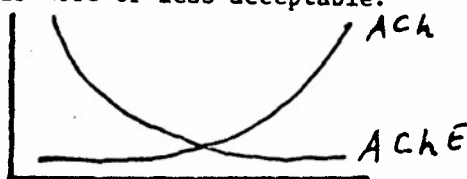
Dettbarn: Let's put it this way, that could be one of the mechanisms it may not be the only one. It is known that we can inhibit certain enzymes involved in glycogen metabolism that binds to organophosphates.

Llewellyn: So actually you get an increase in metabolism at a certain point in time.

Dettbarn: Yes, that is why I think you are losing neurons because the animal or the cell can't keep up with replenishing energy supplies. I have seen it in the muscle where you lose glycogen within 2 hours.

Giacobini: How good is this correlation when you mention the esterase activity.

Dettbarn: I think that the Holmstead curve that you see in biochemical, pharmacology is more or less acceptable.



Giacobini: How do you explain variations in changes of choline?

Dettbarn: There is one problem. We know that almost 50% of the choline resupplied for the synthesis of acetylcholine come from the periphery. The acetylcholine is normally hydrolyzed in the synaptic cleft into acetic acid and choline and 50% of that is being taken up again by different rates of transfer, high affinity, low affinity transfer, and is being reutilized for the synthesis of acetylcholine. If you inhibit acetylcholinesterase, this supply of choline for the resynthesis is not available because you have a fairly stable acetylcholine in the cleft which is slowly removed by diffusion. Where does the remainder of the choline come from or replace the choline that is not being retaken up with the synthesis?

Rosenberg: I recently read a paper where it said that everyone is concentrating on the high affinity transports systems and they were making the point that the low affinity have more significance. In total amount, more is taken up by the low affinity even though the high affinity will show nice enzyme kinetics.

Dettbarn: I agree, but there is no choline there that could be retaken up under conditions of inhibited enzyme. The supply has to come from other sources. That would explain why all of a sudden in spite of the low inhibition or the low activity of the enzyme which persists you get sort of a normal-looking animal because now there is not enough acetylcholine there to do the job. It cannot be synthesized at such a high rate under the conditions of inhibition.

Giacobini: Do you think that it is possible that acetylcholine itself is taken up?

Dettbarn: If you look at nerve terminals after organophosphates, it could very well be because there are holes in the nerve terminals. It is a possibility, I don't know, no one has looked at that. We know that horseradish peroxidase can be taken up into the nerve terminal after prolonged activity and that is a much larger molecule than acetylcholine. You have to explain why if you give a second shot of paraoxon or other cholinesterase inhibitor, you again can set up all the symptoms. Either it is not due to acetylcholine or the store has been refilled either by reuptake of acetylcholine or by nerve terminals that haven't been damaged by the first dose. You can give these rats shots every day and they show symptoms. Then you kill them and measure acetylcholine levels and it is up to 150 - 180% depending on the area that you are looking at. If this is done for two weeks as we and others (Stovinichu, DuBois), have done you always get your initial response. Acetylcholine goes up and comes down again. If you look at the distribution you find that the change is only in the free and the unbound acetylcholine. That remains almost the same with every shot. If you look at what is called the critical acetylcholine, the bound acetylcholine, which is in the vesicles and which is the one which is being released, then after 3-4 days you don't see that change anymore in this storage site of acetylcholine. There is an adaptation which I would call a presynaptic mechanism that somehow relates to the release of acetylcholine. The point that hasn't been brought up yet but I think that is looked at by the Dutch people is calcium. The organophosphates interfere with the calcium uptake and storage in the nerve terminal and I think that they have played around with the veratridine and some derivatives of that. The other point is something called calmodulin which is a protein that is involved in the control and release of neurotransmitters and probably also involved in the release of neurotransmitters and probably also involved in the release of acetylcholine from the vesicles. Again there is a protein kinase involved and a phosphorylation and if the organophosphates phosphorylate because calmodulin has a serine site, that could trigger off the release of acetylcholine.

Llewellyn: Does everyone think that there is conclusive evidence that the organophosphates are in fact working at the serine sites and anywhere there is an active serine site they act? Are you concerned about secondary sites, tertiary sites?

Dettbarn: Yes, as we discussed yesterday, we get effects on other proteins. You get effects on the protein which is now called the ionic conductance modulator that is sort of controlled by the acetylcholine receptor and sits on top of the pore that allows the ions to go through. Albuquerque and others and Nachmansohn have shown a long time ago that if you give higher concentrations than needed to inhibit cholinesterase, you get binding on these proteins and they block axonal conduction, they interfere with permeability changes in postsynaptic membrane and you get prolonged ionic currents. This effect is reversible, you can wash that out very rapidly, while the effect on the enzyme is irreversible. The second effect you only get when you use much higher concentrations.

Rosenberg: I would add, however, what I am going to discuss later, the fact that maybe we have spent all of the time looking where the light is. It is very easy to measure cholinesterase and we have learned a great deal about it and there is no doubt that it is very important to the mechanism of action. As I mentioned yesterday that when I was going through the literature I found a pile of reprints describing long-lasting effects apparently not related to cholinesterase inhibition.

Replegle: If you are without warning and a certain percentage of your troops are out in the rain we don't really care what the mechanism is at that point.

Dettbarn: I don't know what has been going on before but there are lots of reports in the literature that workers in the area of insecticide production who work with storage and movement, never had acute symptoms until they go to the hospital having weight loss, etc.

Replegle: From what sort of exposure?

Dettbarn: Insecticidal powders.

Cohen: It is probably a prolonged exposure to the material as opposed to test animals exposed to something like soman where they would not live very long anyway in most cases. Then again, it is a single exposure so you may not get it.

Dettbarn: There is the work by Riker with DFP on cats and they induced mild symptoms and within 3 weeks the cats developed lesions.

Replegle: Again, you are going to expect a very large vapor hazard. Most of the buildings have air exchanges of 4 to 10 times an hour.

Dettbarn: We never know how these industrial accidents happen; don't they have protective garb or respirators or something like that?

Llewellyn: In most industrial exposures you can't assume that the exposure is only by one route.

Replegle: I suppose we are going to get a lot of that on an Air Force base after an attack also.

Llewellyn: I would think so. The vapor hazards are the first hazard even with an overgarment. If you got wind speeds of over 5 miles an hour it would go right through.

Dettbarn: Even in my lab it happens once in a while where a fellow is preparing DFP under the hood and another chap has his test tubes with the tissues that he wants to test on the next bench and he is surprised that he doesn't have any cholinesterase in his assay system. It goes right through the hood and over there. The other point that Dr. Giacobini brought out is that you can modify some of these effects with dietary choline. You can inject it into the animal in advance of your organophosphate inhibitor. In the brain only certain areas seems to be responsive.

Giacobini: What certain areas?

Dettbarn: The striatum for instance. It has a very high affinity.

Llewellyn: Do you know if anyone has done really crude kinds of ablation experiments and looked at the response to organophosphates?

Dettbarn: I can only draw a parallel to the denervation experiments on muscle where when you denervate you don't get the necrotic lesions on the muscle. If you have superior centers in the brain that control lower central activity or something like that, it might be relevant.

Replegle: There has been some other work in terms of hypoxia and in terms of circulatory changes in the brain and behavior where they did find sizes of microspheres under acceleration getting about the same distribution in terms of blood flow in the brain, as you are getting in terms of deoxyglucose metabolism after organophosphorus poisoning. You could take reduced blood flow, virtually zero under whole body acceleration and assume you don't get any blood to that part of the brain that isn't working.

Dettbarn: We have certain interneurons that let us say are innervated by DOPamine or GABA or adrenergic fibers. They then in turn are cholinergic and release acetylcholine on another center which is DOPaminergic or adrenergic.

Rosenberg: It is important to realize that if you try acetylcholine on most isolated CNS neuronal junctions you find it is stimulatory but behaviorally we know that the cholinergic system and cholinergic compounds will cause depression that will convert manics to depressives and so forth. Obviously you are getting stimulation of inhibitory pathways and therefore it is a very complex thing, so when acetylcholine levels are changing in the CNS you are also affecting GABA neurons etc.

Dettbarn: That is not always true.

Giacobini: Could I just for a moment get into the muscarinic receptor. I am going to be throughout this conference a strong partisan of the cholinergic system as being involved with organophosphates. The muscarinic receptor as mentioned yesterday has been very underdeveloped in its knowledge as compared to cholinergic nicotinic receptors. The reason being

that there has been some very fortunate discoveries during the research on the nicotinic receptor such as specific toxins (alpha-bungarotoxin). Number two, you can actually find brains of invertebrate that have an accumulation of cholinergic synapses with nicotinic receptors that you can isolate and fractionate so that it can be studied. The combination of these two plus the fact that there was a great deal of pharmacology known has greatly increased our knowledge of the nicotinic receptor. However, during the last ten years and mostly during the last three years, there has been a strong revival of interest in the muscarinic receptor. And we assume that most of the receptors in the central nervous system are muscarinic although there are also nicotinic receptors. There is also the possibility that there are different types of nicotinic receptors. These two receptors, the muscarinic and nicotinic have very different properties which I think are worth discussing because by interfering with one or the other we would have completely different effects. One is just physiological, the nicotinic is a very rapid response and the muscarinic is very slow. The muscarinic has a longer physiological response that is measured in the 100's of milliseconds. The nicotinic in synapses functions in the msec range. There are also some very distinct basic mechanisms, one is the relationship to calcium, there being a preference for calcium and calcium involvement in the action of the muscarinic receptor. As a matter of fact, it is understood that when a muscarinic drug attaches to the receptor as for instance carbachol, there is an influx of calcium and this calcium in the synapse triggers a series of events. It seems that the nicotinic receptor is calcium independent so the ionic effect for the nicotinic is obviously sodium and potassium channels so there are two different types of ionic preference. Finally, there is one peculiarity which makes the muscarinic receptor in a way quite exciting for biochemical responses and that is that the action of the nicotinic receptor is on the membrane and we have not so far discovered a system of perturbations within the synapse, a second messenger. The muscarinic receptor has apparently a second messenger. We do not know how many steps there are between the muscarinic receptor and the enzymes, mainly the guanylate because it is mainly cyclic GMP which seems to be increased as a consequence of the binding to the cholinergic muscarinic receptor. We can monitor our effect pharmacologically on the muscarinic receptor as we can monitor for the cyclic nucleotides for norepinephrine and DOPamine. This is important for the biochemists because you can measure the enzyme effect and you can measure the second messenger accumulation. For more practical purposes, there are more and more indications that the muscarinic receptor is mapped very extensively. You can hardly open an issue of Brain Research without seeing someone who has done a careful map of the muscarinic receptor in the brain. QNB is one of the preferential binders used and there are others which have been synthesized which can be used. As pointed out by Kuhar there is a method of autoradiography which allows very precise if not quantitative measurement of the distribution of the muscarinic receptor in the central nervous system. There has been an advance just as the cholinesterase method of Koelle made possible very rapidly to study the distribution of this enzyme, we are exactly in the same phase now in regard to the muscarinic receptor, that we now have a chemical method that allows us to study not only the distribution but even the density of those receptors in the brain. I would also like to point out that there is more and more evidence that as for the nicotinic receptor the muscarinic receptor constitutes a family of different receptors.

One of the most interesting lectures at the meeting in Florence in April, 1980 was the one by a group of distinguished Soviet colleagues who have been working in this field for many years, that is Prof. Karcovitch from the First Medical Institute in Moscow, who pointed out that you can distinguish with specific drugs different types of muscarinic receptors. The sensitivity of muscarinic and nicotinic receptors of different localizations was studied by this group. The sensitivity of the cholinoreceptor has been evaluated according to changes in evoked potentials using different muscles. In their first group of experiments they were using antidepolarizing and depolarizing curare-like substances. They have been using not only the classical tubocurarine, but also compounds such as anactrosonium, cyclobutonium, pavulum, diadonium, decadonium, succinylcholine and decamethonium. They have shown that these compounds induce a preferential paralysis which is interesting. They show a paralysis which goes in different succession and my question to Dr. Dettbarn is how can you explain this different succession of paralysis?

Dettbarn: Do you mean the typical curare effect that you start with small muscles first and then the respiratory muscles last?

Giacobini: Right. What is the basis for that?

Dettbarn: I have taught that so often to my pharmacology and medical students that I have never asked myself the question.

Giacobini: This was so precise by these Russians that they could interfere with just one group of muscle with a certain dosage.

Dettbarn: The nicotinic receptors even in the periphery are not all the same. You can clearly demonstrate that if you denervate a muscle you have one set of muscles at the endplate and another set are the ones away from the endplate. They clearly show a different sensitivity to curare. It is possible that there are different nicotinic receptor populations. The other thing is if you look at the small muscles, they are much more highly innervated than the large muscles. For instance the intraorbital muscles have an innervation ratio of probably one nerve fiber to three muscle fibers. While in the periphery in long leg muscles you may have one nerve fiber innervating 100 muscle fibers. The different types of muscles have different types of endplates. Fast muscle has a very large endplate and slow muscle has a much smaller, compact endplate.

Giacobini: Working with the same philosophy they looked at agents which are more specific blockers for the muscarinic receptor. They have synthesized a very large group of compounds; some of which blocked and some did not. These different new antimuscarinic agents were tested on the following organs - the heart, blood vessel arterial pressure, bronchi, small intestine, urinary bladder, and salivary glands. They could again and perhaps with more precision block the muscarinic receptor of one or the other organ. For instance derivatives of troxilic acid as well as adamantane containing compounds. They emphasize that the introduction of an adamantane group into these structures, by making the complex more hydrophilic, can change the penetration. They were able to obtain antimuscarinic agents with these adamantane groups that penetrate the blood brain barrier very easily. They showed very selective effects on muscarinic receptor of the heart and no effect at all of the same compound on the bronchi. Some only affected the

bronchi and not the heart. The work which was very interesting was the one with respect to the CNS. There was a great deal of interest and we wanted to know more from our Russian colleagues. We were told that this was just the beginning and that they have a lot of new compounds with this adamantyl radical. However, their data which were largely physiological, emphasized the difference in different types of muscarinic receptor and the possibility of attacking these receptors with different compounds.

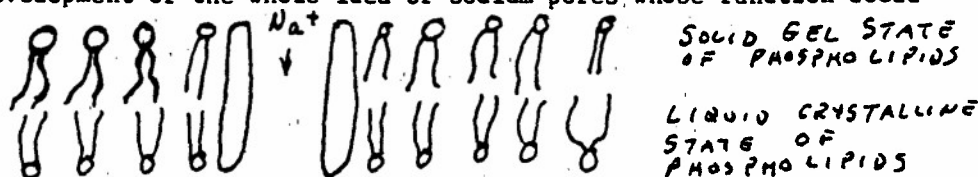
Dettbarn: I would like to come back to this other observation about striated muscle. Most of the junctions operate with a high safety margin that are about seven-fold. You really have to knock out a large number of receptors to get block of transmission by curare, and that safety factor may vary from muscle to muscle. The other problem of course is that they also react differently to depolarizing agents.

Rosenberg: I want to change the focus a little bit at my own peril, considering all the interest everyone has on the cholinergic system and acetylcholine and cholinesterase. I just want to point out again that there are many effects which have not been associated with cholinesterase inhibition. That does not mean that they are not due indirectly and post long periods of time to cholinesterase inhibition, but no one has really shown this. I am speaking of effects in the literature, metabolic effects and whether these metabolic effects might lead to structural changes in membrane. I am speaking of the well-documented EEG effects of Duffy, increased REM sleep, increased beta activity. Now, I am not saying that these effects were not triggered by cholinesterase inhibition, they may or may not have been, but not necessarily. Likewise, I am speaking of analgesic effects; cholinesterase inhibitors have potent analgesic effects which are overcome by naloxone. Whether the release of enkephalin has also been triggered by inhibition of cholinesterase is very likely in this particular case. Nevertheless, we have here secondary effects which are not directly related to nor necessarily predicted by the cholinesterase inhibition and all of these behavior changes which I had mentioned, learning disability, schizophrenic reactions, increased irritability, decreased vigilance, memory loss, depression and so forth, all may not be true if they have been triggered by cholinesterase, then you have to ask the question, how are they so long-lasting. Karczmar for example has done work recently published showing effects on the CNS which he claims has no relationship to cholinesterase inhibition, effects on EEG and behavior of animals. Therefore, we are left with the point of where shall we look. If we are not going to look where the light has been for the last forty years where else should we look in order to try and get at some of these other changes not directly associated with cholinesterase inhibition. I would suggest that we look at membrane structure and membrane biochemistry. I think that this has not been done adequately. Obviously, we can look for structural changes through electron-microscopy, freeze fracture and things like that. I make the point that I think we can really look at the membrane itself in order to find out. For example, you know the classical membrane structure, we have the phospholipids, the polar head groups, the two fatty acid tails and we have then proteins interdigitating in some cases all through the membrane and in some cases only on the surface.

We know that the individual phospholipids on the inside and outside of membranes may be quite different in different membranes. There has been one very interesting report recently saying that organophosphates markedly increase the phospholipid composition of membranes and decrease the cholesterol composition of membranes. This can have dramatic effects on long-term function of membranes. How would you go about finding out if for example under the influence of organophosphates if this is the exterior and this is the interior and we know through studies on red blood cell and other membranes that in fact on the exterior surface there will be a high concentration of phosphatylcholine and sphingomyelin; on the interior surface, a high concentration of phosphatylethylamine and phosphatylserine. Not absolute differences, but the concentrations of these particular phospholipids vary markedly on the interior and exterior surfaces of the membrane.

Dettbarn: What was the concentration of these organophosphates?

Rosenberg: These were non-lethal amounts to the animals with repeated administration. There are ways of determining then if there has been a long-lasting change. I am suggesting that after either this single dose in the case of the work by Duffy or in animals a single dose of sarin or chronic exposure produces these EEG changes. You can take a nerve membrane or take a brain and isolate synaptosomes, the isolated nerve endings and you can test for the composition of the membrane. You could apply specific probes. There are specific probes which will covalently bind to an amine grouping or will specifically bind to protein. Then you could isolate phospholipids, you could isolate proteins, do polyacrylamide gel electrophoresis for proteins or phospholipid mapping. You can actually determine where these probes have gone, whether enzymatic probes or chemical probes. You can actually find when applied from the outside what do they bind to after organophosphates. Do they bind to different things now that they didn't bind to before? We can really get at these structural changes in the membrane. By now we know that there are whole series of chemical probes that can be used to study membranes. If there is a change in the biochemistry of the membrane there will be a marked change in its function. There has been the development of the whole idea of sodium pores whose function could



be controlled by acetylcholine. Surrounding these proteins which control the function of this sodium or potassium channel pores in the membrane, there is an annulus of phospholipid which is in the crystalline gel state. You can indicate this by putting these tails, these fatty acid changes being straight. It is very rigid, it maintains and keeps a barrier to the pore so the pore can open and close very readily without being interfered by the phospholipid. If you just get this critical transition state between the solid gel and the liquid crystalline, it would look something like that with fatty acid tails which are waving around in the fluid in the membrane. They would exert a much greater lateral pressure on these protein pores on these proteins which

are controlling the function of the ionophore. If you just change the transition temperature between the solid gel and the liquid crystalline state just a couple of degrees or a fraction of a degree, you could have marked changes in the function of the sodium pore. It has been suggested for example that anesthetics act by removing this annulus of phospholipid in the solid gel state, converting it to the liquid crystalline state, that this exerts lateral pressure on this pore, closes it up, and then you get block of nerve conduction. This has also been suggested for transmission of acetylcholine across the synapse. For example, if the proportion of saturated and unsaturated fatty acid were changed in the phospholipids of the membrane that could very easily change this critical transition temperature, that could very easily change the functioning of the sodium pore and that could, for example, be responsible for behavioral effects. These are things which are very possible to measure which as far as I know have never been done with organophosphates because I think in some cases there has been a tunnel vision looking exclusively at the cholinergic system. I think that we have the means now to look at membrane structure to see if there are permanent long-lasting changes induced. They may very well be induced by cholinesterase inhibition but remaining well after the cholinesterase inhibition has been lost. A suggestion that I would like to bring up to see what you people think of it.

Llewellyn: I have two people looking at membranes. The point that they have right now is that they think they have solid negative data relating to effects on lipids in the membrane system. We have a group of four people who have some capabilities to do this sort of thing. It appears to me that stating the questions that you would like to answer based on a physiological rationale and then trying to get to the physiochemical sort of approach to answer a physiological question is something that is missing in the work that we have been doing.

Rosenberg: I think that if a structural change is found you certainly would have to have a lot of other evidence to show the structural changes are the cause of any physiological changes, but the obtaining of a clear-cut, long-lasting structural change would be of tremendous interest in and of itself, and would possibly relate to behavior.

Dettbarn: Most of these insecticides have a sort of surface activity and work like detergents at high concentrations.

Rosenberg: It is very critical as to what system you are using. Ideally you would like a closed vesicle sort of system like the red blood cell where you can selectively expose from the inside or the outside and therefore you can see which phospholipids, which proteins are oriented for the internal aspects of the plasma membrane and which toward the external aspects. Red blood cells are nice because you can produce everted inside-out vesicles, right-side out vesicles, leaky ghosts, tight ghosts and all sorts of things can be done with red blood cells. With the synaptosome you can only do it to a certain extent. It is very difficult to selectively apply material from the inside. People have attempted to rupture and reseal synaptosomes. There is always the question if you have to 100% been able

to get them right-side out and so forth. Synaptosomes can be easily exposed from the outside assuming that they are not leaking and that the stuff won't get in and act from the inside. The ideal preparation is the electric eel cell. You have a single cell which you isolate from the electric eel and you have a conducting membrane and a non-conducting membrane and you can split the cell in half and get a conducting surface and a non-conducting surface and mount these in a special chamber and expose the internal aspect of the conducting membrane to any material you want, the external aspect to any material you want and vice versa with the non-conducting surface. Here you can really get at what is the structure. You apply selective proteases and see which proteins they act on from each side. Apply selective phospholipases, DTNB and fluorescent probe and whatever you want and you have in effect all the advantages of the red blood cell in such a system. So, I think that it depends very much on the system you are using in order to get at the structural changes because it is not enough to take a membrane and fling it in solution and you have the stuff acting all over. The overall membrane may look like the lipids have not been changed but if it has gone from 50% phosphatylcholine outside to 20% on the outside, that may be very significant even though the total amount of phospholipid may not have been altered. Or, if the fatty acid changed exclusively on the inside have had their degrees of unsaturation modified relative to those on the outside, you would never know this by analyzing the whole membrane.

Llewellyn: My impression is that if a lot of people are looking at things like this, they are using the easily manipulated system without thinking through very much whether or not the results in that system can be related to the sort of hypothesis that you have made up there. I know of two other groups who are trying to work on exactly the same sort of thing. They are much more interested in the membrane for its own sake than what it might do. For example, with the little model that you drew, the crystalline gel is the normal resting state, right.

Rosenberg: Well no, the membranes usually have domains. The membranes will normally have domains of solid gel. This is the annulus surrounding the proteins. Usually it is found that surrounding proteins there is an annulus of phospholipid in the solid gel.

Llewellyn: Does it require active metabolism for that state to be maintained or is that a resting state. If it is the resting state, what turns on the metabolism that allows it to change form?

Rosenberg: It seems to be that the interactions with the protein are very essentially involved in maintenance. The transition from the solid gel to the liquid crystalline is influenced by bonds apparently formed with the adjacent proteins and it is maintained energetically in the solid gel state for a considerable extent because the proteins are next to it. Anesthetics seem to squeeze their way in and somehow break these bonds and therefore it is converted to the liquid crystalline state which exerts a much greater lateral pressure on the membrane which just closes up these pores.

Llewellyn: I heard before that there was an intimate relationship with the proteins. If whether it is an anesthetic or whatever, it may be that initially breaking the influence of the bond or whatever the protein has with the phospholipids that are in the annulus, maybe that is what kicks off the reaction.

Rosenberg: It seems to me that one can easily test these membranes following organophosphate exposure. For example, the isolated electoplax is exactly equivalent to a peripheral neuromuscular junction. Acetylcholine is the transmitter and you have everything identical. If you want to study the properties of peripheral neuromuscular nicotinic junctions you could use the isolated electoplax. If you want to study central properties of membranes, you would have to settle with something like synaptosomes from specific areas in the brain in order to try to get some specificity.

Dettbarn: How does TTX work in this scheme of things?

Rosenberg: I was going to show a slide of TTX. What I found, for example, with muscle has been that TTX will protect PE, phosphatidylethanolamine, against hydrolysis. What is usually said of course is that if this is the sodium channel, TTX comes in and the guanidinium group comes in to the sodium channel and substitutes for sodium. Then the big bulky tail of the TTX stops up the sodium channel and it can't go through. What I found in muscle is that if we took the sodium channel muscles and measured hydrolysis of phospholipid by phospholipase C alone and plus tetrodotoxin and had all sorts of phospholipids. Phosphatidylethanolamine (PE), phosphatylserine (PS), phosphatylinositol (PI), phosphatylcholine (PC), we found that in the sodium channel muscles, all of these phospholipids were hydrolyzed. Under the conditions that we were using, phospholipase C would block conduction of the sodium channel muscles and we would get extensive hydrolysis of the sarcolemma. This wasn't the whole muscle because we didn't want all the other phospholipids of mitochondria and so forth, just the isolated sarcolemma membrane on the surface of the membrane got extensive hydrolysis. In the calcium channel muscles PS and PI were not hydrolyzed at all. That is the reason we think that phospholipase C has no effect on the calcium channel muscle suggesting that PS and PI are probably the phospholipids which are in the annulus most close to the sodium channel. If we added TTX to this we found normal hydrolysis of PE and PC, but now almost zero hydrolysis of PS and PI. Somehow TTX when it goes into the sodium channel is interfering with the access of phospholipase C to what we think are the annular phospholipids because phospholipase C only acts on muscles whose action potentials are generated by sodium activation. Only on three sodium channel muscles were PS and PI hydrolyzed. On the three calcium channel muscles PS and PI were not hydrolyzed at all by phospholipase C and the enzyme had no effect at all. You see this competition between TTX and phospholipase C. We think in this particular case of these muscles anyway. It is interesting that the phospholipase C has the exact same specificity as tetrodotoxin only acting on the sodium channel muscle. No one has ever looked at how organophosphates might alter these membrane properties.

Dettbarn: There is some controversial data on the red blood cell.

Rosenberg: Yes, some real old work as I remember about effects. Of course, there has been the idea that cholinesterase might be involved in permeability because it is in a lot of other membranes, non-bioelectrically excitable membranes, so people have asked what is it doing in the placenta or red blood cell and other places. The whole theory has developed that it is essential for control of permeability properties of membranes so cholinesterase might be intimately involved in the membrane functioning per se outside of bioelectricity in nerve and muscle.

Dettbarn: Where is your cholinesterase localized in for instance nerve membrane?

Rosenberg: In the nerve membrane I guess it would depend on the old argument of whether Nachmansohn was right or wrong. Certainly at the acetylcholine activated sites for sodium say at the acetylcholine receptor - nicotinic postsynaptic sites where you get increased permeability to all ions (non-specifically increased permeability), you would of course have to say that the receptor, the cholinesterase must be very close to this ionophore, to this acetylcholine ionophore. The protein must be very close to the channel protein.

Dettbarn: The fact is that you have acetylcholinesterase in the nerve membrane.

Rosenberg: Right, no doubt about that. If as some of us might believe that acetylcholine is involved in axonal conduction then I would expect it to be very close still to these sodium channels in the nerve membrane. What do you think, Wolf?

Dettbarn: I know that acetylcholinesterase is there, but what it is doing there, I don't know.

Rosenberg: We know that people have claimed that they have isolated a receptor-like material, an acetylcholin receptor-like material from axons. It doesn't have the exact same properties certainly as nicotinic receptor, but it displays binding to cholinergic materials at reasonable levels. There is something resembling acetylcholine receptor in axonal membrane.

Dettbarn: There is of course very good evidence that there is some metabolic exchange between the Schwann cells around the axon and the axon proper, since the axon has little protein synthesis. The Schwann cell itself has cholinesterase to the enzyme may be involved in the transport across the membranes of nutrients.

Rosenberg: That of course brings up another important complication on these sorts of studies depending on what system you indicated they may have been using. They may have been measuring changes in the Schwann cell because this plasma membrane is only about 100 or 150 Angstroms. This is another advantage of the electroplax for example; there is no Schwann cell so you get right at the plasma membrane, likewise synaptosomes might be isolated nerve endings. You avoid some of the problems associated with using a myelinated or even just a nerve with the Schwann cell.

I have a few slides that I thought that I might show on animal toxins if anyone is interested. One slide especially gives the relative toxicity of some of these animal toxins.

Cause	Cases Worldwide	U.S.A.	Deaths Worldwide	U.S.A.
Reptiles	1,000,000	8,000	30,000	10-15
Arthropods		3,000,000		50-150
Bees				50-150
Spiders				1-5
Scorpions		1,000		0-1
Marine - Venomous	400,000	5,000	20-50	3-7
Poisonous	20,000		100-200	

This is just to show that envenomation is still very much of a worldwide problem. There are about 30,000 deaths worldwide due to snakes alone, for example. Most of these, however, do not occur in the United States; most occur in India, at least 15,000 which are due to snake envenomation. Of course, with bees, the main problem is an allergic reaction. You need at least 100 bee stings to die from the venom itself.

Snake	Length (cm)	Venom Yield (mg)	LD50 Mice (mg/kg)	Danger Index (LD50/Yield)
<u>U.S.A.</u>				
Timber Rattlesnake (CT)	90-140	100-200	2.6	58
Copperhead Moccasin (CT)	60-90	40-70	10.9	5
Eastern Diamondback Rattlesnake	110-165	250-500	1.7	220
Cottonmouth Moccasin	75-115	100-150	4.0	31
Mojave Rattlesnake	75-100	50-90	0.21	333
North American Coral	60-80	3-5	0.38	11
<u>Foreign</u>				
Australian Brown Snake	150-175	5-10	0.01	750
Tiger Snake	50-150	30-70	0.03	1667
Many-banded Krait	90-120	8-12	0.07	143
Indian Cobra	120-155	170-250	0.24	875
King Cobra	210-360	350-750	1.6	344
Beaked Sea Snake	90-125	10-15	0.03	417

This slide indicates the potency of crude snake venoms. We have some crude venoms here which on a mg per kg basis are very potent; for example, the Australian brown snake, .01 mg/kg of venom. This, I think is as potent or more potent than any organophosphate. This is in mice intravenously. Australia is very rich with snakes that have very potent venom; the tiger, the Australian brown snake.

Components of Snake Venom

Water (80-90%)	Proteins
Non-Proteins	Anticoagulants
Amino Acids	Cardiotoxins
Biogenic Amines	Coagulants
Carbohydrates	Cobra Venom Factor (anticomplement)
Inorganic Constituents	Cytotoxins
(Metals)	Enzymes (20)
Lipids	Hemorrhagins
Nucleotides	Myotoxins
Peptides	Nerve Growth Factor
	Neurotoxins (Pre- and post-synaptic)
	Releasing Factors (Release Bradykinin and histamine)

Some of the components of snake venoms are shown here. There may be 20 different enzymes. Myotoxin which specifically destroys muscle membrane;

so-called cardiotoxins which have effects on heart but have effects on all sorts of membranes. In some cases they are called cardiotoxins because the effects on the heart were first observed, other cases they are called direct lytic factor because of their effect on lysis of red blood cell was first observed. All sorts of neurotoxins acting pre- and postsynaptically at nicotinic junctions as well as many which will act at other junctions, GABA junctions and so forth. This is just to give an indication of the 20 or so enzymes in snake venom.

Enzymes in Snake Venoms

Acetylcholinesterase (<u>Elapidae</u>)	Lactate Dehydrogenase
L-Amino Acid Oxidase	NAD Nucleosidase
Amylase	Phosphodiesterase
Arginine Ester Hydrolase (Not <u>Elapidae</u>)	Phospholipase A ₂
Catalase	Phospholipase B
Deoxyribonuclease	Phosphomonoesterase
Endoproteidase (Not <u>Elapidae</u>)	Protease
Factor X Activator (Not <u>Elapidae</u>)	Prothrombin Activator (Not <u>Elapidae</u>)
Glycerophosphatase (<u>Elapidae</u>)	Ribonuclease
Hyaluronidase	Thrombin-like Enzyme (Not <u>Elapidae</u>)
Kininogenase (Not <u>Elapidae</u>)	Transaminase

Some of these enzymes like phospholipase A₂ seem to be associated very intimately with the presynaptic toxicity of some of these toxins. On the next slide we see some toxins but there are many many more. Maybe there are 50 toxins whose complete amino acid sequences have been worked out.

Toxin	M.W.	AMINO ACID RESIDUES	LD50 (mg/kg)
<u>Presynaptic</u> (have PLA ₂ Activity)			
B Bungarotoxin	25,000	179 (2 subunits)	0.09
Crotoxin	30,000	200 (2 subunits)	0.06
Notexin	13,574	119 (1 subunit)	0.02
Taipoxin	46,800	374 (3 subunits)	0.002
<u>Postsynaptic</u>			
<u>Short Chain</u> (1)		60-62 (4S-S)	
Cobrotoxin	6,949	62	0.05
<u>Long Chain</u> (11)			
Bungarotoxin	7,983	74	0.30
<u>Membrane Active</u>			
Cardiotoxins (Direct Lytic Factor)	7,000	57-60 (4S-S)	0.05-2
Cytotoxins	7,000	57-63 (4S-S)	- -
Basic Phospholipases	13,000	115-125 (6-7S-S)	0.05-2

Basically they fall into these three major groupings and they are not identical in their amino acid sequences but nevertheless they are quite similar. For example, presynaptically acting toxins block transmission at pre-synaptic nicotinic junctions. Beta-Bungarotoxin, for example, will bind to the pre-synaptic site and there is good evidence that this binding is not an enzymatic action. Its binding is associated with inhibition of acetylcholine release.

Once it binds specifically then phospholipase acts. Beta-bungarotoxin has phospholipase activity, indeed all of these presynaptic acting toxins have phospholipase activity and seems to be essential for their actions. We are finding out now in our studies, any molecular modification which destroys phospholipase activity destroys toxicity also. Once it is bound to the nerve endings then phospholipase seems to exert hydrolytic effects on the membrane causing the release of acetylcholine. You get a marked release of acetylcholine and then subsequent to that a block of transmission. These materials are in some cases slower acting than the direct postsynaptic ones and could take an hour or two. Notice the extreme potency, for example, of taipoxin 2 ug/kg the most potent animal toxin known, more potent than tetrodotoxin or black widow spider venom or anything as far as lethality is concerned. Postsynaptic toxins have a typical curare-like toxin, alpha-bungarotoxin, cobrotoxin, etc.. which block the acetylcholine receptor just like curare does and prevent acetylcholine from getting there. Then we have what I call before the cardiotoxins, cytotoxins or myotoxins and certain phospholipases which do not specifically act presynaptically, but which are quite potent. These are so-called basic phospholipases and we are extensively studying now, their properties and the differences between the basic and the acidic. The acidic phospholipases are relatively much less lethal whereas the basic ones are more toxic. Some things about the active sites of these materials are known, but not completely. Their complete amino acid sequences are known. Barbara Low, at Columbia has done a lot of work on the x-ray crystallographic structure of these toxins. Something is known about the active site which groupings might mimic the effect of curare, for example, this is a postsynaptic acting one. Perhaps, one could envisage that if you could synthesize or obtain part of the active site you might be able to get a much smaller molecular weight compound which might have the toxicity of some of these materials.

Giacobini: What is the major difference between cobrotoxin and the alpha-bungarotoxin?

Rosenberg: The major difference is relative reversibility. Alpha-Bungarotoxin is much more irreversible, so for example, with the isolated acetylcholine receptor some people tried to use alpha-bungarotoxin on affinity column and couldn't use it because there was nothing that could take it off. If they used cobrotoxin it works very nicely you could then take it off. But the mechanism seems to be identical. The reversibility implies different kinetics certainly of leaving. There are some differences of affinity also. I think the alpha-bungarotoxin has a greater affinity than the cobrotoxin. By now there may be at least 30 others that might have certain advantages if they were tried but they are not readily available, you almost have to prepare your own. Tremendous advances have been made with these toxins now. The number is large that have been completely sequenced and they are studying effects of selective modification of the amino acids.

Giacobini: Sometimes these tools were so popular that they didn't pay attention to phospholipases which were present in the extract of alpha-bungarotoxin.

Rosenberg: Very, very stable, that has been part of the problem. Initially people were saying that all phospholipases are extremely toxic and responsible for all of the venom's actions because what they did was to heat venom at an acid pH and it was known that phospholipases are the only enzyme resistant to boiling at an acid pH. What they didn't know was that all of these cardiotoxins and all of these neurotoxins have the exact same heat stability characteristics. What they were getting in their preparation of acid boiled-venom was a mixture of phospholipase plus neurotoxins, cardiotoxins, etc. These are very resistant to boiling at acid pH, destroyed by boiling at alkaline pH. They have a lot of disulfide bridges which increase stability.

Lindstrom: How are they inactivated?

Rosenberg: You mean in the body? Proteases I presume. This just gives you an idea that there are many toxic things in other animals besides snakes.

VENOMOUS ARTHROPODS

HYMENOPTERA

Bees
Wasps
Yellow Jackets
Hornets
Ants

SPIDERS

Black Widow
Brown Recluse
Tarantula

MISC

Scorpions
Ticks
Mites
Caterpillars
Centipedes
Assassin Bug

Pain Producers:

Serotonin, Histamine, Acetylcholine

Spreading Factors:

Hyaluronidase, Phospholipases

Paralyzing Factors:

Mellitin, Apamine, Latrotoxin, Tityustoxin

We have venomous arthropods with just some of the materials in them. Mellitin is in arthropod venoms especially bee, wasp, yellow jacket and hornet venom. Mellitin is about 50% of the venom and has effects identical to the cardiotoxins and direct lytic factors in snake venoms. Apamine, I think is something that is going to be extremely interesting for neurochemists in the future because no one has been able to figure out what apamine is doing. It has a powerful stimulant action on the central nervous system and they tried to test so many different neurotransmitters to see what it is doing in the central nervous system. There has been a recent paper indicating that in fact it is acting to decrease potassium activation. It is present in bee and hornet venom. Latrotoxin is in black widow spider venom; it seems to be the active constituent of black widow spider venom. Tityustoxin is in certain scorpion venoms and is the main acting constituent there. Almost all of the venoms once again have phospholipase; they all have high hyaluronidase. They all have these materials which are called pain producers. Certain hornet venoms are very very high in acetylcholine. Some of these toxins will

Rosenberg: You shouldn't have any phospholipase with alpha-bungarotoxin. Alpha-bungarotoxin has no phospholipase activity at all; beta-bungarotoxin does.

Giacobini: This was an extract from the venom.

Rosenberg: Yes, if they didn't do a good separation, right. It is known that pure beta-bungarotoxin itself has phospholipase activity whereas pure alpha-bungarotoxin doesn't. There was a big argument with the beta-bungarotoxin of whether this was an evolutionary hangover, that is venom glands are derived from salivary glands, all salivary glands have phospholipase and that maybe this protein, this beta-bungarotoxin evolved from a primitive phospholipase and you have vestigial enzymatic activity. This doesn't seem to be the case at all, because whatever you do to modify the phospholipase activity modifies the toxicity. Phospholipase activity seems to be an intimate part of the mechanism of action of beta-bungarotoxin. It seems to be part of the molecule responsible for the specificity of binding. You say why are these acidic phospholipases no good; it seems to be to a great extent a pharmacokinetic problem. The acidic phospholipases go all over and are diluted in the body. They will attack phospholipids all over, but by the time they do, they are in very low concentrations. Whereas, these basic phospholipases or especially the presynaptic acting toxins they are protected from binding to other sites, they will not act at the surface tension of most membranes. There is some beautiful work by Eaker in Sweden on the ability of monolayers to affect phospholipids. Notexin, for example, the presynaptically acting neurotoxin will not act at the usual surface pressure, 35 dynes or something like that. Notexin will only act at much lower surface pressures which suggests that the surface tension at the nerve ending may be much less than the surface tension of general cellular membranes.

Giacobini: What about the penetration to the central nervous system?

Rosenberg: It is surprising that in some cases these big molecules can penetrate. They eat their way through, especially phospholipases. I found that in the squid giant axon you can actually measure enzymatically active phospholipase in the axoplasm of the squid giant axon following external exposure. You can observe hydrolysis of phospholipids in the axoplasm as well as active enzyme in the axoplasm. So, it is able to eat its way through the nerve membrane. I would say that in these cases very little will get into the central nervous system following peripheral injection. We have been doing a lot of this now with the basic and the acidic phospholipase, the ones from Naja nigricollis and Hemachatus haemachatus or Naja naja. We find that very little gets into the central nervous system. Some gets out, that is the permeability is not identical both ways. If you inject high amounts intraventricularly, you can observe a small amount of hydrolysis in peripheral tissues. If you inject in peripheral tissue we could never detect anything inside the brain. So there is a differential permeability.

Comment: Can you comment on the chemical stability of these compounds.

penetrate the blood brain barrier and have marked effects on transmission.

Here we see the structure of batrachotoxin from the skin secretions of certain poisonous frogs. Once again a very, very potent material acts exactly the opposite of tetrodotoxin. Instead of blocking the sodium channels it opens up the sodium channels. So you are converting a normal appearing action potential to a cardiac appearing action potential. This shows histrionicotoxin another very useful toxin in biological studies. This toxin will not act at the acetylcholine receptor but specifically acts on the acetylcholine ionophore. Acetylcholine will bind normally but won't do a thing. It will bind to its receptor, this histrionicotoxin will not compete with acetylcholine for binding the receptor but nothing happens subsequent to the acetylcholine binding. Sodium permeability is not increased and Changeux and others claim that there is evidence that specifically it is acting on the ionophore. This is a very useful tool for separating the two major components of acetylcholine actions, binding to receptors and subsequent transduction of binding to a physiological effect. This slide shows that there are many venomous marine animals also. Some of these have extremely potent toxins, from the stonefishes and toad fishes.

A FISHES

Venomous sharks
Sting-Rays
Catfishes
Weeverfish
Stargazers

Scorpion fishes
Zebrafishes
Stonefish
Toadfishes

B COELENTERATES (CNIDARIANS)

Fire Coral
Portuguese Man of War

Sea Wasps
Anemones

C ECHINODERMS

Starfishes

Sea Urchins

D MOLLUSCS

Cones

Octopus

The problem is that they are much more difficult to work with because of their extreme instability, it is hard to do any long-time studies on them. This slide shows what was said before about tetrodotoxin and saxitoxin which seem to act the same way basically, that the guanadinium group inserts itself into the sodium channel and then the rest of the molecule plugs up the sodium channel and you get block.

Replogle: I have never seen the raw data, but cobra toxin has been used as a pretreatment for organophosphates and in some primates and in some rodents according to a variety of people it protected against from 2 to 5 LD₅₀.

Dettbarn: That could be because it protects acetylcholine, etc. That is what I have done with my preparation. I have injected bungarotoxin in the thorax and you chase it with DFP or paraoxon and the animal survives, and the lesions of the muscles do not develop.

Rosenberg: Of course this is well known for curare although clinically it is not useful in cases where they are trying to overcome depolarizing or non-depolarizing block. Experimentally it can be observed, but clinically it is not a useful thing to do.

Dettbarn: If you can develop something that protects the critical areas, then we might have something they have done the same thing with bolulinum toxin which prevents release of acetylcholine and then you don't get effects from organophosphates.

Rosenberg: I was on the FDA panel and was down in Washington for the Proven material that the Miami serpentarium has been supplying for treating multiple sclerosis.

Henkel: That was one thing that struck me when you were speaking of soman being so highly specific and the fact that it ages or apparently is non-reversible. The structure doesn't look as though it should be that different from sarin or anything else. If you could administer something of the same shape, that would sit on the membrane and protect in that same way and be fully reversible with acetylcholine and maybe cut down the toxicity of soman.

Rosenberg: Is anything being done to attempt to obtain smaller molecular weight derivatives of animal toxins, volatilization of material?

Replogle: The Air Force is doing nothing. There are only three programs in the Air Force that have anything to do with chemical defense. Two of them are in exploratory development and one is virtually in the planning stage. The exploratory development program is not basic research, it is applied. By applied, it means that someplace on a sheet of paper, there is an end product and that is the one that I described to you yesterday. Then there is an engineering development program that is a haberdashery; it is producing protective ensembles. That is all there is and there is no basic research occurring right now and I don't know personally that there should be any in the Air Force. Perhaps the army is more suited because they have a laboratory. The Air Force will probably never have a laboratory that will actually do the kind of basic research that the army is doing. It is silly, to me, to sponsor work that you don't understand and that you have no one who understands it. We don't have the technical staff and I suppose we never will have the technical staff to do that. The Air Force is definitely interested on your conversations on molecules that protect a site. This is something that the Air Force should shift its concentration on rather than symptomatic relief which itself is perhaps worse than the dose to begin with.

Llewellyn: The animal toxins are viewed by the U.S. as being biological and from 1969 and on, the biological agents were destroyed and there are

not supposed to be any stock pile. Any toxin which is formed in a biological process is viewed by the U.S. as coming under the proscription against biological warfare.

Rosenberg: That of course gets very hairy at the border line. You can synthesize a material with almost identical or the same effects and is no longer a biological compound.

Llewellyn: Miselson made this one of his major coups to be sure that in treaty that those sorts of materials were not labelled as chemicals. There isn't any work going on other than trying to redo some of the anti-toxins. Few people that I know are paying real attention to the potential threat of the toxins whether they are synthetic analogs of the biological process or the biological thing itself.

Replogle: If you had an animal toxin possibility for an antidote pretreatment certainly it wouldn't be out of the question to use it for that.

Llewellyn: Not at all. There are research quantities owned and reported and measured on a month-by-month basis. That is very strictly controlled and there is a list of all that stuff at NIH. We are hoping to put together in the fall a seminar getting people who work on the antitoxins and see how some of what they do might be redirected to pay attention to some of the problems of organophosphate intoxication.

Cohen: I have been involved for a couple of years now on reviewing a couple of anticholinesterases perspective of their broad spectrum use in forests up in New Brunswick. When I go to these panel meetings we hear more stories, nothing really documented, on the guys flying these planes. They have to be inhaling phosphates, and many planes go down each year.

Replogle: It is very difficult because the pilots don't follow regulations but they won't tell you that they don't follow regulation. So very often they will fly in very poor protection and not report it.

Comment: In Israel they pay when they get the material on the ground and in this country we pay our pilots by the hour or by the acre covered. So in this country you could be a little high and still get paid but an Israeli spray pilot has to really get on the deck. They very often get exposed. There are histories of them titrating themselves in the air with atropine with one hand and landing their aircraft.

Replogle: We can't perform any kind of experiment on anyone that is ill for any reason. The classic example I think is some work that was going on to measure some performance factors. That is with permission from the patient non-invasive, and safe by anybody's standards but we are prohibited. We were prohibited from going to Canada to measure the performance of their people who had received whole body irradiation.

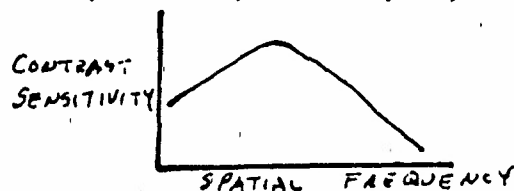
Cohen: You mentioned that there are enzyme kits to measure contamination. Are there such kits to monitor dosimetry like radiation badges and things like that.

Replogle: There is also a lot of reasons to suspect in chemical warfare that you are not going to get one agent at a time. Much of the scenario has to do with putting a mask on, if you can get a mask on pretty quickly then you can do a lot of other things. If you have troops trained well, and you have notification, it makes a tremendous difference to the outcome of the chemical situation. There are a lot of things that change that whole picture. One breath and then put your mask on and then a couple of minutes later you are taking your mask off; then of course the rest of the nerve agent will finish the job for you. So why not mix phosgene, sarin, etc. Cyanide works the same way, and in 30 seconds or so will break a mask down so that you have to take it off or die with it on or however way you like it. There are all kinds of other things; I suppose BZ would be a marvelous mixture with the organophosphate.

Lindstrom: Could someone speak to the problem of contrast sensitivity that Clyde raised yesterday.

Rosenberg: I don't know if necessarily it is work with the eye. To a great extent on contrast it is determined in the CNS and not in the retina.

Replogle: Consider the lateral inhibition and consider potentiated lateral cells so that you had a very deep profound lateral inhibition. What this would do to contrast sensitivity was shown to be fantastic. The contrast sensitivity versus spacial frequency looks like this.



Henkel: Does the retinal blood flow inside the blood brain barrier? If it is inside the CNS that in order to do anything the fact is that you are going to have to penetrate the barrier or else go in through the front of the eye.

Replogle: That is not true, DFP penetrates just like that. We are putting this right in the eye and on the eye and it penetrates very rapidly. Our problem is that we used echothiophate because it was currently in clinical use and not because we thought it was a good drug. It is a charged molecule so we have to wait around a little while longer as it penetrates much more slowly. The other problem is of course that you have a lot of melanin. We are getting something like 60 times the dose for a black through the eye than for a caucasian. I haven't looked at specific transmitters in the neuroretina and don't know anything about them.

Rosenberg: It may not be at least in some layers of the retina that there are any transmitters. I think that not until the ganglion cell do you get action potential. There may not be specific transmitters between some of these, it might be electrical transmission.

Replegle: We have to know why it is that we are getting 2-fold contrast sensitivities in the eye. This means seeing an airplane or not seeing an airplane. We not only measure contrast sensitivity but also measure target detection. You get the same results with benactyzine. Benactyzine is injected i.v. and produces the exact same low frequency dropoff of contrast sensitivity.

Llewellyn: We got a request to comment upon a standard being prepared for maximum tolerated ocular exposure for pilots. No data base for the value at all. To know even how you would measure it so that there would be no exposure overtime, and what you would do when if you could measure it, somebody had exceeded it, now what do you do. It is fairly clear that you could get a significant amount of absorption just through exposure of the eye. As best as one can determine you could have in the old group models almost no effects on a number of visual functions when you have severe symptoms and looking at that matter is a problem and worthwhile to exploit. It seems obvious that if you are getting expiratory exposure you are also going to get it in the eye.

Replegle: That individual dose isn't quite as silly as it seems. From a flight surgeon's point of view with individual patients it is extremely silly because you will never know how much he was exposed to. From an analyst's point of view it is not silly at all because I can compute the remaining percentage of pilots available for duty on an Air Force base given a certain strike if I know what dose it is at which they stopped flying. So I could take a very large Air Force base or a series of bases and I could make some estimate of that. When I am asked by USAF to predict the viability of an Air Force base given a certain set of strike parameters, those numbers are the basis of my answer.

Giacobini: I just want to emphasize that out of the many models that you can use, brain, muscle, endplates, etc., actually the preparation of the iris is very nice for studying correlation of anticholinesterase agent and function because you have the physiological model. You have a possibility of isolating in the iris both sympathetic and cholinergic innervation; then you can study the effect on the muscle by both chemicals and physiological stimulation on the eye. The Norwegian defense laboratory studied the topic administration of organophosphate anticholinesterase to the eye and they compared the anticholinesterase effect on pupillary contraction and they found that there is a very nice correlation between the degree of inhibition and the pupillary diameter. This pupillary contraction was relieved after 24 hours (the type of organophosphate used was not specified), when acetylcholinesterase activity had only covered by 10-20% so we have the same problem.

Replegle: They have used GD on the eye and they also have measured cholinesterase activity on the retina and that is the only data that I know of in the free world on retinal cholinesterase.

Giacobini: They have used soman and administered it daily to study the effect on pupillary function and the rate of pupillary contraction, pupillary diameters, etc. were changed and could not be described as due to lessening inhibition of acetylcholinesterase nor to reduction of muscarinic receptors. The Israelis have done extensive studies on the eye and I have a paper here from Tel Aviv Univ. where they are testing myotic activity of anticholinesterase agents.

I just want to emphasize that this is a very convenient model for doing physiological and biochemical studies at the same time. Since you seem to be very expert in this, I wonder, what is it that is a good system to measure pupillary contraction in either animals or human. Is there any simple method that you can titrate very finely? Is it classified?

Replogle: No, there are machines produced that measure pupil size at a distance; they work very well.

Lindstrom: - As a last item I might ask if we could discuss some possibilities on furthering this sort of exchange and what sort of steps could be taken to follow up on this.

Llewellyn: Now that the orientation group has gone by the board, you got the wrong group. You have to get the people not like Clyde and myself. It takes time for people to unburden themselves with various biases they have and there are also semantics problems. People say things in different ways and you may think that someone is agreeing with you when in fact they mean something different. What I am saying is that I would strongly support a redo of what happened here in the last two days with a totally different group of players and in several different places. I know that it is taking very valuable time. As you noticed there were not many solid comments; you were not getting much feedback from us, we can't give it to you.

Rosenberg: At least we are aware of your problems and things that we never think about. We got an insight into a whole different world. I think this was useful.

Llewellyn: I am not so sure that it is necessary to have all the members of this group in one place at the same time, but I think it is healthy to have at least two people from outside visiting an inside group because otherwise the darts are all thrown at one dot. It is a very useful sort of thing and should be pursued.

Replogle: I think I would agree with you on that. I also think that you probably should do something less broad. On the other hand, having one manager there is going to be very helpful because it is very difficult for the managers in their own laboratories to very often control the work to an end point that is militarily useful and to send out their people to work at a university setting might diverge the procedure even further.

Rosenberg: From my point of view it certainly has been very worthwhile and interesting and I really think amongst the civilian community there are a lot of people who are doing work which would be of interest to all of you and they wouldn't have to change the focus of their studies one iota; it is just a matter of communication and funding. It is the interpretation of the uses to which the work is put and so forth. There are a lot of people in academia who could contribute greatly to the problems we have been discussing. Provided the work is not classified and publishable we will have no difficulty in collaboration.

Replogle: I have spent 20 years in military research and hardly ever have funded a research endeavor of any kind until I knew what the next series of steps were assuming that they were causing the results of the intended study. I can sit here and watch examples of work that can be done and I think suppose you really found the answer, then what. I really don't work very well as a basic research director. I think about how many years before you get to this point and how many steps are required to do so.

Lindstrom: Basically I am the organizer and my job starting in September is to identify areas of basic research, and OSR is one agency and Army has their own which would actually fund the research projects to the type that you would be thinking of and the people of DOD are in here to give a perspective on problems. They are not the ones who have the money; this is called 61 money, basic research.

Llewellyn: Close to a third of our budget is 61 in medical army research.

Lindstrom: They were here to give a perspective on the problem and hopefully in turn to demonstrate some other ability that they might have forgotten in their own work but all is not lost. The funding would be from OSR and my plan is to this time next year to pinpoint areas of basic research in the Air Force which we might work at.

Rosenberg: Today you may not see what the next step is going to be and next week you might see it. I don't think anyone wants to be that short-sighted and say I will not fund any basic research unless I immediately know where it is going to lead to.

Llewellyn: Are you going to have the option or the capability, Dick, of going in and putting together different groups of people like this once you get into the new job?

Lindstrom: That's one of the things that I am hoping to do. This is kind of experimental and certainly there are a lot of things that I have learned. I hope that the next one will be run with the modifications included perhaps getting more people from the trenches. I think that I will have the option of setting up some of these things. We have a new military commander coming on board and I don't know if he is inclined to the CW problem as the former one.

Replogle: A continuing process would be beautiful and furthermore even setting up a very formal review process quite separate from how life sciences normally operates.

Lindstrom: I am not quite convinced that the Air Force needs are all biological. There are certain biological aspects, however we have problems with decontamination. My feeling now and it may change is that we are at least equally and perhaps more chemical. I would like to have a nice review process all set up and a number of people involved. I'll be back to all of you for your comments.

Amen.

APPENDIX 1

POST MEETING COMMENTS

DOD PARTICIPANTS

DEPARTMENT OF THE AIR FORCE
AIR FORCE AEROSPACE MEDICAL RESEARCH LABORATORY (AFSC)
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433



REPLY TO
ATTN OF: HE

10 July 1980

SUBJECT: Air Force Office of Scientific Research Conference

TO: Dr Richard E Lindstrom
School of Pharmacy
University of Connecticut
Storrs CT 06268

Dear Dick,

1. The conference you arranged between DoD participants in Chemical Defense and academic expertise in related fields was an overwhelming success. Air Force R&D programs have been concentrating on problems rather than broad technical areas. Contact in a number of technically collateral fields will help broaden our outlook and add a needed spark to the creative process. Many technical approaches which we had not considered are now possibilities. Just as important, is a source for consultation in difficult technical areas. The whole Air Force is new at Chemical Defense R&D and needs consultation in many fields.

2. I believe a series of such conferences is in order; perhaps with more technical representation by DoD. After my return to the Laboratory, following your conference, a number of officers expressed interest. The open discussion was provocative and emphasized possibilities for Basic Research in the Air Force.

A handwritten signature in cursive script, reading "Clyde R Replogle", is positioned above the typed name.

CLYDE R REPLOGLE, PhD
Asst Chief for Special Projects
Human Engineering Division

DEPARTMENT OF THE AIR FORCE
HEADQUARTERS AEROSPACE MEDICAL DIVISION (AFSC)
BROOKS AIR FORCE BASE, TEXAS 78235



23 JUN 1968

Dr. Richard E. Lindstrom
Associate Professor and Chairman
Pharmaceutics
The University of Connecticut
Storrs, Connecticut 06268

Dear Dick

Thanks for inviting me to the University of Connecticut last week. I had never been to your state so was very interested in seeing it. I really appreciate your hospitality while visiting your home on the two nights. You have a home to be very proud of.

As far as the conference is concerned, I offer the following comments:

- The organization and planning were excellent, i. e., preliminary information, housing, meals, etc.
- The selection of university scientists was also excellent. They are obviously authorities in their respective fields and were enthusiastic about our problems. Their participation was good. I was very impressed with them and it gives me some hope for solving our problems.
- I agree with Craig that while it was good for us managers to present the overview and our problems, it would have been better to have in addition to us a scientist or two from our labs. Maybe next time. I think you are on the right track, though.

Thanks again for inviting me. Hope to see you more during the coming year.

Sincerely

A handwritten signature in dark ink, appearing to read "Tom", is written above the typed name.

THOMAS M. BUTLER, Lt Colonel, USAF, BSC
Chief, Manned Weapon Systems Effectiveness Div
Directorate of Research & Development



DEPARTMENT OF THE ARMY
OFFICE OF THE SURGEON GENERAL
WASHINGTON, D.C. 20310

REPLY TO
ATTENTION OF

DASG-MIR

Dr. Richard E. Lindstrom
School of Pharmacy
The University of Connecticut
Storrs, CN 06268

Dear Doctor Lindstrom:

The conference on "Problems in Chemical Toxicology" was an unqualified success, both in organization and content. It was a unique opportunity to exchange information with civilian scientists on an informal basis. It changed my perspective on some very critical problems and stimulated further thought on others.

I hope that this concept of small informal groups will be repeated and extended to the "bench" scientists in the future. The possibilities for significant advances are enormous.

One remark made at the conference was particularly significant, a scientist said, "until this meeting, I did not realize that our separate fields of expertise had such significant commonality in the chemical antidote area." That statement reflected the cross-fertilization of diverse fields that characterized the entire meeting.

I want to thank you again for including me in your meeting and express the continued interest of myself and my Agency in the continuation and expansion of this program.

Sincerely,

WALLACE A. DEEN, DVM
MAJ, USA
Liaison, Medical Intelligence and
Information Agency



REPLY TO
ATTENTION OF

SGRD-UV-ZA

DEPARTMENT OF THE ARMY
US ARMY BIOMEDICAL LABORATORY
ABERDEEN PROVING GROUND, MARYLAND 21010

13 SEP 1980

12 SEP 1980

Richard E. Lindstrom, Ph.D.
Associate Professor
Pharmaceutics Section
University of Connecticut
School of Pharmacy
Storrs, Connecticut 06268

Dear Dr. Lindstrom,

I was chagrined by your reminder that I had not provided you my thoughts and comments concerning the June 1980 conference on "Problems in Chemical Toxicology" which you hosted at the University of Connecticut. My embarrassment is even greater since I have touted both the meeting and the concept to others as being highly productive.

The selection of participants from academia was excellent. I strongly indorse a mixture of disciplinary experts including life scientists in the neurosciences, toxicologists traditional and behavioral, and chemists of various types.

The more difficult problem is to obtain Department of Defense personnel who can provide the proper orientation for the academic experts. This I think we did not fully accomplish in June and will require more preparation. Defining what is not known as precisely as possible for a variety of chemical agents is essential. Then defining what the chemical battlefield would look like and some high priority needs for troops in such an environment should follow. Next, some description of research thrusts currently under way and where they seem to be going should be presented.

With this as background it should be possible for the academic group to either 1) critique or spin-off what they have heard or 2) decide to begin again from ground zero.

In your first meeting there was a mixture of both these approaches, and I feel that if we, the people from DOD had been better prepared to provide orientation and state what we saw our problem to be, much more could have been gained from the group you had assembled.

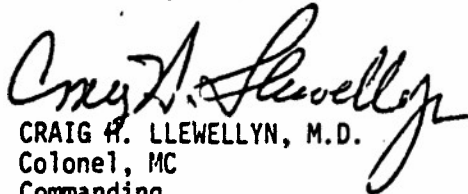
I hope you continue these exercises. I would be willing to participate again. But I also think you need selected DOD researchers not the managers and staff people.

12 SEP 1980

SGRD-UV-ZA

Hope this is what you wanted. My apologies for tardiness, I owe you two (2) drinks!

Warmest regards,


CRAIG H. LLEWELLYN, M.D.
Colonel, MC
Commanding

APPENDIX 2
POST MEETING COMMENTS
CIVILIAN SCIENTISTS

MEMO

To: R. E. Lindstrom

From: P. Rosenberg

Re: Problems in Chemical Toxicology Conference

Date: June 27, 1980

A. Areas of new research which were discussed at the conference.

1. Effects of organophosphates on membrane structure.

Most research studies using organophosphates have concentrated on the acute effects which can be related, to a great extent, to the level of cholinesterase inhibition. In contrast, I proposed an entirely new area of research; to study the long term effects of anticholinesterases on protein and phospholipid organization within nerve and muscle membranes. If changes in the membranes are found they might help explain the long-term EEG, behavioral and other organophosphate effects which cannot be correlated with cholinesterase inhibition, and indeed remains after the enzyme levels have returned to normal.

2. As pointed out, especially by Ezio Giacobini, Steve Cohen and myself, a rapid development of tolerance to the acute effects of organophosphates (salivation, fasciculations, diarrhea, etc.) often develops following repeated administration even though the levels of cholinesterase in the body tissues remains depressed. The cause of this tolerance could be decreased acetylcholine receptor sensitivity, decreased acetylcholine synthesis, compensatory activation of an antagonistic neurotransmitter system, etc. The importance of investigating this topic was discussed at the meeting.

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3. The possibility of developing clothing which could monitor exposure to anticholinesterases or more adequately protect against exposure was discussed. The possible use of immobilized cholinesterase bonded to the cloth fabric was mentioned as was the use of phosphorus sensitive sprays which might detect exposure to organophosphates.

B. Areas of new research needed, but not discussed at the conference:

1. The design of better, safer and more effective antidotes to organophosphate poisoning. These could be in the nature of cholinergic antagonists, oxime reacti-vators, etc. Although this need was mentioned at the conference, no real proposals or in depth discussion ensued.

2. The possible effects of organophosphates on the immune system may have been mentioned at the conference (I am not certain), but if so, it was not discussed to any extent. This is a special interest of Steve Cohen and indeed he and Dr. DiCapua have an NIH grant application currently being considered for funding. Effects on the immune system could be responsible for certain long-term effects of the organophosphorus inhibitors.

3. Except for a brief period of time on Thursday morning, the meeting was exclusively concerned with anticholinesterase agents. Therefore, many areas of research related to other toxic chemicals (mustard gas, Lewisite, cyanide, etc.) were not even considered. These are probably of great importance for future conferences.

PR:jd

July 8, 1980

To: R.E. Lindstrom

From: S.D. Cohen

Re: "Problems in Chemical Toxicology"

1. [REDACTED]

Address: 70 Robbie Road
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2. (a) Means of protection against organophosphates.

i) Can we selectively stimulate the development of organophosphate - detoxifying binding sites in the body without altering the metabolism and action of other drugs and chemicals.

ii) Can we develop a means of rapidly detecting exposure or contamination by phosphates, e.g. enzyme based color indicators.

(b) Cyanide treatment or prophylaxis.

i) Identify the mechanism by which chlorpromazine enhances thiosulfate antagonism of cyanide toxicity.

ii) Develop new agents which have such action but lack the CNS effects of chlorpromazine.

SDC/sk

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SCHOOL OF PHARMACY

TO: Dr. R. E. Lindstrom

FROM: James G. Henkel *JGH*

DATE: July 10, 1980

The areas of research addressed at the conference seemed mostly to be directed toward "plugging the gaps", rather than extending knowledge. This is particularly apparent in the area of organophosphates. However, it is likely that several potential research projects will evolve from the conference, particularly among those more closely involved with the biological sciences. One possible area in which I could be involved is an investigation of the SAR of the Soman and Sarin type organophosphates. It is not clear to me why Soman has such high activity.

It's difficult to state the areas of research that weren't discussed, except in generalities. Mostly, I would like to see some divergence from organophosphates to address other potential threats, including those agents designed to incapacitate the individual. Adequate defenses against these may be needed. It is, of course, difficult to know what is needed without more information on what the Soviets have. The topic of simulant design and use could also merit further discussion.

I hope these topics will be of some help.

JGH/eem

June 25, 1980

SUBJECT: Wrap-up for "Problems in Chemical Toxicology" Conference

TO: R. E. Lindstrom, School of Pharmacy

From: Enrico Mugnaini [REDACTED]

I found the meeting very informative and interesting. It seems to me that neurotoxicity in general is a field on which we are learning more and more. Neurotoxic agents not only are of interest per se, but also since they can be used as tools to understand mechanisms of neural action. Thus, there are well defined applied and basic aspects of the problems and the meeting served greatly to highlight the issues now at hand.

Metabolism of organophosphates and their relations to "other esterases" struck me as two immediate pieces of new research worthwhile pursuing. Studies on neurotoxicity related to the glutamate receptor (in view of its distribution in crucial areas of the brain) and the use of specific AChE positive central neuronal networks for the elucidation of mechanisms of action of organophosphates represent for me very intriguing issues which could have been discussed more incisively at the conference.

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THE COLLEGE OF
LIBERAL ARTS AND SCIENCES
Department of Biobehavioral Sciences

June 21, 1980

Dr. R. Lindstrom
School of Pharmacy U-92
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Storrs, CT 06268

Dear Dick,

Following our meeting of June 19-20, 1980 and the very fruitful exchange of information with regard to the field of organophosphates, here is a list of studies which I would like to suggest as a high priority in this field:

1. Mechanism of action of organophosphate related to acetylcholine metabolism, particularly to synthesis, uptake and release of acetylcholine. This is mainly to find out whether there are mechanisms in the brain capable of lowering acetylcholine following its increase by anticholinesterase agents.
2. Studies on permeability of the blood brain barrier to selective regions of the brain with regard to organophosphates.
3. Studies in the field of molecular forms of acetylcholinesterase.
4. The problem of the cholinergic receptor desensitization and activation following anticholinesterases.
5. Titration of acetylcholinesterase inhibition in vivo in the intact mammalian eye, biochemical and physiological correlation in the same preparation.
6. Muscarinic receptors in the brain and new antimuscarinic agents.

In general I perceived an agreement by all members of the panel that much basic research is needed in this field in order to understand the mechanism of action of anticholinesterase agents and effectively protect the organism from its toxic effects.

I enjoyed very much the meeting and I would appreciate receiving a copy of the report. [REDACTED] [REDACTED].

Sincerely,

Ezio Giacobini, M.D., Ph.D.
Professor